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Immunology

CD5 acts as a tyrosine kinase substrate within a receptor complex comprising T-cell receptor ζ chain/CD3 and protein-tyrosine kinases $p56^{lck}$ and $p59^{fyn}$

(signal transduction/CD5)

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ABSTRACT **T-cell antigens including CD2, CD4, CD6, CD8, and CD28 serve as coreceptors with the T-cell receptor (TCR)/CD3 complex in control of T-cell growth. The molecular basis by which these antigens fulfill this role has remained a major issue. An initial clue to this question came with our finding that the sensitivity of *in vitro* kinase labeling (specifically using protein-tyrosine kinase $p56^{lck}$) allowed detection of a physical association between CD4–$p56^{lck}$ and the TCR/CD3 complexes. Another T-cell antigen, CD5, is structurally related to the macrophage scavenger receptor family and, as such, can directly stimulate and/or potentiate T-cell proliferation. In this study, we reveal that in Brij 96-based cell lysates, anti-CD5 antibodies coprecipitated TCR ζ chain (TCRζ)/CD3 subunits as well as the protein-tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. Conversely, anti-CD3 antibody coprecipitated CD5, $p56^{lck}$, and $p59^{fyn}$. Indeed, anti-CD5 and anti-CD3 gel patterns were virtually identical, except for a difference in relative intensity of polypeptides. Anti-CD4 coprecipitated $p56^{lck}$, p32, and CD3/TCRζ subunits but precipitated less CD5, suggesting the existence of CD4–TCRζ/CD3 complexes distinct from the CD5–TCRζ/CD3 complexes. Consistent with the formation of a multimeric CD5–TCRζ/CD3 complex, anti-CD5 crosslinking induced tyrosine phosphorylation of numerous T-cell substrates, similar to those phosphorylated by TCRζ/CD3 ligation. Significantly, as for TCRζ, CD5 was found to act as a tyrosine kinase substrate induced by TCR/CD3 ligation. The kinetics of phosphorylation of CD5 ($t_{1/2}$ = 20 sec) was among the earliest of activation events, more rapid than seen for TCRζ ($t_{1/2}$ = 1 min). CD5 represents a likely TCR/CD3-associated substrate for protein-tyrosine kinases ($p56^{lck}$ or $p59^{fyn}$) and an alternative signaling pathway within a multimeric TCR complex.**

The CD5/Ly-1 antigen is a 69-kDa monomeric differentiation antigen expressed on the surface of thymocytes, mature T cells, and a small proportion of B lymphocytes (1–3). Structurally, CD5 belongs to a family of receptors typified by the scavenger receptor cysteine-rich (SRCR) family of extracellular domain-like structures (4, 5). This family includes the type I macrophage scavenger receptor, the human complement factor 1, the sea urchin speract receptor, and the lymphoid antigen CD6 (6, 7). Scavenger receptor cysteine-rich domains include a 100-amino acid stretch with six positionally conserved cysteine residues (6, 7). Particularly close homology exists between the CD5 and CD6 antigens that share a further stretch of 30 conserved residues as well as two additional cysteine residues not found in other members of the scavenger receptor cysteine-rich family (7). Both CD5 and CD6 possess large cytoplasmic tails and act as substrates for protein kinase C (8, 9).

Although the function of CD5 on T cells is unclear, it has been reported to deliver either direct activation signals or costimulatory signals, similar to CD2, CD4, CD6, and CD28 (10–16). Mitogenic antibodies to CD5 stimulate in a monocyte-dependent manner (10). Anti-CD5 may provide a second signal, as shown by its ability to "synergize" with submitogenic concentrations of anti-CD3 (11–14). The antigen increases intracellular Ca^{2+} and cGMP levels (13), interleukin 2 secretion, and interleukin 2 receptor expression (11, 14). Significantly, signaling via CD5 requires the coexpression of the T-cell receptor (TCR)/CD3 complex (14). Anti-CD5 augments T-cell help for *in vitro* B-cell immunoglobulin production (16). CD5 may bind to the B-cell antigen CD72 (17) and, therefore, play a signaling role in the collaboration between T and B lymphocytes.

A variety of T-cell antigens including CD4, CD8, TCR/CD3, interleukin 2 receptor, Thy-1, and Ly-6 have been found to be complexed to intracellular protein-tyrosine kinases, such as $p56^{lck}$ and $p59^{fyn}$ (refs. 18–24; for review, see ref. 21). We have reported that CD5 can coprecipitate kinase activity, although the kinase was unidentified (24). In an effort to uncover the molecular basis of CD5 signaling in T cells, we have demonstrated that CD5 forms a physical aggregate with the TCR ζ chain (TCRζ)/CD3 complex and the protein-tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. Consistent with this, antibody-induced crosslinking of CD5 induced tyrosine phosphorylation of numerous substrates, similar to those induced by anti-TCR/CD3. Importantly, anti-TCR/CD3 could induce the rapid tyrosine phosphorylation of CD5. The combined observation that CD5 associates with the TCR/CD3 complex and acts as a tyrosine kinase substrate supports a model in which CD5 constitutes a potential signaling component within a TCR/CD3 oligomeric complex.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs), Antisera, and Cells. mAbs used included antibodies to CD5 [24T6G12 (IgG2); UCHT2 (IgG) (from Peter Beverley, University College, London); OKT1 (IgG2a)], to CD4 [19Thy5D7 (IgG2)], to CD3 [RW2-8C8 (IgG1)], or to TCRζ [TIA-2 (IgG1); from Paul Anderson (Dana–Farber Cancer Institute)]. Anti-$p56^{lck}$ and $p59^{fyn}$ sera were generated against synthetic peptides corresponding to the N-terminal residues 39–64 and 22–35, respectively. Anti-

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Abbreviations: PBL, peripheral blood lymphocyte; TCR, T-cell receptor; TCRζ, TCR ζ chain; mAb, monoclonal antibody.

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phosphotyrosine mAbs included PY20mAb (ICN) or 4G10 provided by B. Druker (Dana–Farber Cancer Institute). T cells (HPB-ALL, Jurkat) were grown in RPMI 1640 medium/10% (vol/vol) fetal calf serum/1% (wt/vol) penicillin-steptomycin at 37°C. T cells were purified from the peripheral blood lymphocyte (PBL) population by conventional sheep erythrocyte rosetting techniques. Thymocytes were obtained from children having undergone cardiac surgery (Children's Hospital, Boston).

Immunoprecipitation, *In Vitro* Kinase Assays, and Membrane-Purification Techniques. Cells at 50×10^6 cells per ml were solubilized in either 1% (vol/vol) Nonidet P-40 or Brij 96 lysis buffer in 20 mM Tris·HCl buffer, pH 8.0, containing 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C, as described (18, 20). Membrane vesicles were purified and labeled with [γ-^{32}P]ATP, as described (25). Membranes were solubilized in ice-cold Nonidet P-40 (1%) lysis buffer/20 mM Tris·HCl, pH 8.0/150 mM NaCl/0.4 mM sodium vanadate/10 mM sodium fluoride/10 mM sodium pyrophosphate/0.5 mM EDTA/1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C. Immunoprecipitates were washed with ice-cold lysis buffer and subjected to SDS/PAGE or two-dimensional nonequilibrium gel electrophoresis, as described (18).

Antibody-Induced Crosslinking and Immunoblotting. Antibody-induced crosslinking was conducted in two ways: (*i*) 5×10^6 resting peripheral blood T cells were initially incubated with 5–10 μg of anti-CD5 mAb (UCHT2 and 24T6G12), anti-CD4 mAb, or anti-CD3 mAb for 1/2 hr at 4°C, washed several times, incubated with 1 μg of rabbit anti-mouse antibody (DAKO, Carpinteria, CA) for 1/2 hr at 4°C, followed by an incubation for various times at 37°C. Alternatively, (*ii*) for kinetic experiments, Jurkat cells were preincubated at 37°C in RPMI 1640 medium/2% fetal calf serum, incubated with a mixture of anti-CD3 mAb and rabbit anti-mouse antibody (5 μg/1 μg, respectively), and harvested by touch-spin centrifugation in an Eppendorf microcentrifuge before solubilization in 1% Nonidet P-40 lysis buffer containing phosphatase inhibitors (1 mM sodium vanadate, 10 mM sodium pyrophosphate, 1 mM sodium fluoride, and 0.1 mM EGTA). Cell lysates and anti-CD5 mAb (OKT1; 5 μg/ml) or anti-TCRζ mAb (5 μl/ml) precipitates were immunoblotted with an anti-phosphotyrosine antibody. Reactivity was determined by using either iodinated antibody or rabbit anti-mouse alkaline phosphatase and an alkaline phosphatase detection system (Promega) (26).

RESULTS

Initially, microsomal membrane vesicles from a variety of T cells were labeled with [^{32}P]ATP to assess whether the CD5 antigen could act as a substrate for membrane-associated kinases. CD5 possesses several potential sites of tyrosine phosphorylation (4, 5). Anti-CD5 precipitates from Brij 96-based lysates of labeled membranes showed the presence of a labeled doublet at 67 kDa and 69 kDa from PBLs (Fig. 1, lane 3), thymocytes (lanes 6 and 7), and HPB-ALL cells (lane 11). Two different mAbs to CD5, 24T6G12 and UCHT2, precipitated labeled 67/69-kDa (lanes 6 and 7, respectively). For comparison, mAbs to CD4 coprecipitated labeled 56- to 62-kDa bands (lanes 2, 5, and 10), which correspond to bands precipitated by anti-p56^{lck} serum (lanes 4, 8, and 12). An antiserum to p59^{fyn} precipitated a band at 59 kDa (lane 13). CD5 is, therefore, readily phosphorylated by kinase(s) associated with membranes from a variety of T-cell types.

The above data suggested that CD5 might be associated with an intracellular kinase. To determine the nature of the associated molecules, anti-CD5 (24T6G12) precipitates from Brij 96 lysates of peripheral blood T cells (Fig. 2*A*, lanes 1–5), T-cell lines Jurkat (lanes 6–9), and HPB-ALL (lanes 10–14)

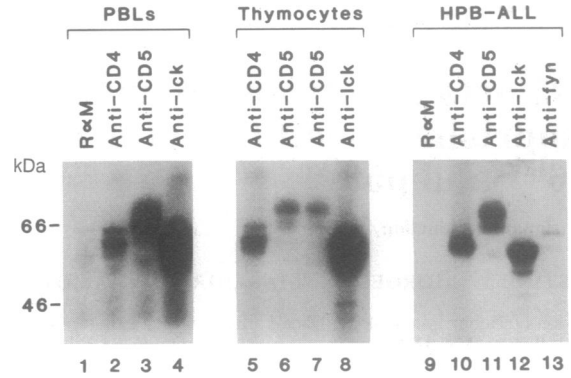

FIG. 1. CD5 antigen is phospholabeled in membrane vesicles from a variety of T cells. Membrane vesicles were purified and labeled with [^{32}P]ATP from various T cells, as described (25): resting PBLs (lanes 1–4), thymocytes (lanes 5–8), and the T-cell line HPB-ALL (lanes 9–13) treated with rabbit anti-mouse mAb (lanes 1, 9), anti-CD4 mAb (lanes 2, 5, and 10), anti-CD5 mAb (12T6G12) (lanes 3, 6, and 11), anti-CD5 mAb (UCHT2) (lane 7), anti-p56^{lck} mAb (lanes 4, 8, and 12), and anti-p59^{fyn} mAb (lane 13).

were labeled in an *in vitro* kinase assay with [γ-^{32}P]ATP. From PBLs, anti-CD5 precipitated a major labeled band at 69 kDa (CD5) together with bands at 55–60 kDa, 32–34 kDa, 28 kDa, 20 kDa, and 16–18 kDa (lane 2). Little if any of the 28-kDa bands were observed in Jurkat cells, although the 32/34-kDa, 20-kDa, and 16- to 20-kDa bands were visible (lane 7). These patterns strikingly resembled those precipitated by anti-CD3 antibody. For example, from peripheral T cells, anti-CD3 precipitated the 69-kDa band, a 55- to 60-kDa band, CD3 subunits at 28-kDa, 20-kDa, and TCRζ chains at 16- to 20-kDa (lane 5). The only detectable difference in the patterns was a CD5-associated 32/34-kDa band absent in anti-CD3 precipitates. In HPB-ALL cells, anti-CD5 precipitated 28-kDa, 20-kDa, and 16- to 20-kDa bands that were also found in anti-CD4 precipitates (lanes 11 vs. 13). We had previously shown that these bands correspond to CD3/TCRζ (25). As a positive control, anti-p59^{fyn} precipitated a 59- to 62-kDa band (lanes 4 and 12), whereas anti-CD4/p56^{lck} precipitated p56^{lck} at 56 to 60 kDa (lanes 3, 8, and 13). Rabbit anti-mouse failed to precipitate detectable amounts of antigen (lanes 1, 6, and 10). Anti-CD6 precipitated a single band at $\approx$130 kDa (lane 14).

Previously, using Nonidet P40 as a detergent, we showed a physical association between the CD3 ε chain, TCRζ and CD4–p56^{lck}, but only from HPB-ALL cells (25). Using Brij 96 as a detergent, we also coprecipitated faint, but reproducible, 20-kDa CD3 ε chains from peripheral T cells and Jurkat cells (lanes 3 vs. 5; 8 vs. 9). Their identities were verified by two-dimensional isoelectric focusing (data not shown), indicating that the CD4–p56^{lck} and CD3 association is more widespread than previously thought, being found in peripheral blood T cells and other cell lines.

Beside the shared CD3/TCRζ subunits, the anti-CD5 pattern from peripheral T cells shared an additional subunit at 32/34 kDa with anti-CD4, which was not found in the anti-CD3 patterns (lanes 2 and 3 vs. 5). In contrast to the anti-CD3 pattern, anti-CD4/lck precipitated little, if any, of the 69-kDa band (CD5) material (Fig. 2*A*, lanes 2 vs. 3; 7 vs. 8; and 11 vs. 13).

Positive identification of the 69-kDa band in the anti-CD5 precipitate as CD5 was made by eluting the 69-kDa polypeptide from SDS/PAGE and reprecipitating with an anti-CD5 antibody. Fig. 2*B* shows that anti-CD5 mAb recognized the 69-kDa protein (lane 2), whereas irrelevant antibody failed to precipitate the antigen (lane 1).

Detection of CD5-associated CD3 chain and TCRζ required use of Brij 96 detergent because they were not

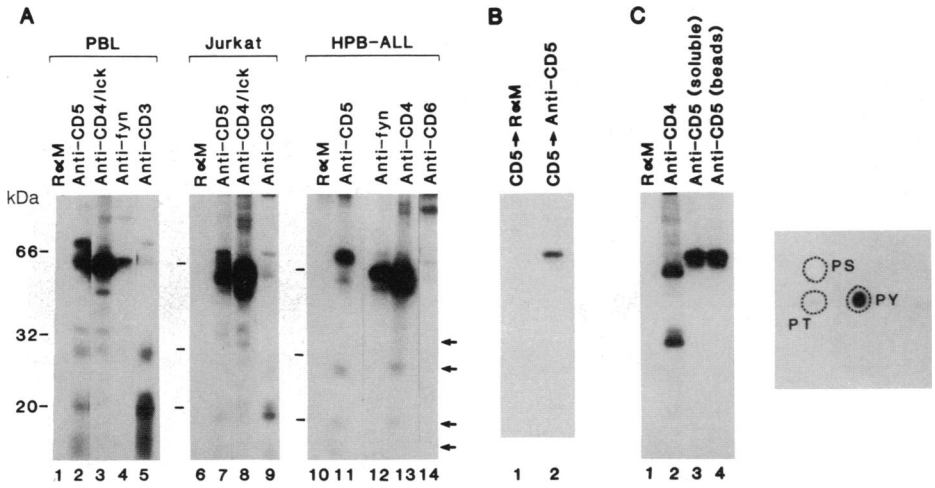

FIG. 2. Anti-CD5 copurifies associated kinase activity, CD3 subunits, and TCRζ. Peripheral T cells (lanes 1–5), Jurkat (lanes 6–9), and HPB-ALL cells (lanes 10–14) were immunoprecipitated in Brij 96-based lysis buffer and labeled by *in vitro* kinase assay, as described (25). (*A*) Rabbit anti-mouse antibody (RαM) (lanes 1, 6, and 10), anti-CD5 mAb (lanes 2, 7, and 11), anti-CD4, anti-p56^{lck} mAb (lanes 3 and 8), anti-CD4 mAb (lane 13), anti-p59^{fyn} mAb (lanes 4 and 12), anti-CD3 mAb (lanes 5 and 9), and anti-CD6 mAb (lane 14). Arrows indicate positions of comigrating bands. (*B*) Labeled 69-kDa protein corresponds to the CD5 antigen, as detected by reprecipitation analysis. Anti-CD5 immunoprecipitates from HPB-ALL cells were denatured by boiling in SDS and reprecipitated, as described (18, 20): Reprecipitation was with rabbit anti-mouse antibody (lane 1) and anti-CD5 mAb (lane 2). (*C*) Anti-CD5 copurifies associated kinase activity from Nonidet P-40 based lysates. Peripheral T cells (lanes 1–4) were immunoprecipitated in Nonidet P-40-based lysis buffer and labeled by *in vitro* kinase assay: RαM (lane 1), anti-CD4 mAb (lane 2), soluble anti-CD5 mAb (lane 3), and anti-CD5-linked Sepharose (lane 4). CD5 is a phosphotyrosine-labeled polypeptide (*Right*). Phosphoamino acid analysis of the phospholabeled CD5 antigen was conducted as described (18). PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

detected in Nonidet P-40 lysates (Fig. 2*C*, lanes 1–4). In Nonidet P-40 based lysates, anti-CD4 precipitated p56^{lck} and associated p32 (lane 2). 32-kDa protein has been shown to bind GTP and to be recognized by an anti-G protein antisera (28). In Nonidet P-40, soluble and Sepharose-coupled anti-CD5 mAb only precipitated heavily labeled bands at 67 kDa and 69 kDa (lanes 3 and 4). It is noteworthy that anti-CD5 precipitated kinase activity even in the absence of TCRζ/CD3 chain. Confirmation of phosphotyrosine labeling was made by two-dimensional phosphoamino acid analysis (Fig. 2*C*, *Right*).

Two-dimensional isoelectric focusing further revealed a similarity in the patterns precipitated by anti-CD5 and anti-CD3 mAbs from peripheral blood T cells (Fig. 3). Anti-CD5 precipitated a CD5 doublet at 67/69 kDa, p56^{lck}, p59^{fyn}, and a well-labeled series of lower-molecular-mass spots that comigrated exactly with those precipitated by anti-CD3 mAb (*A* vs. *B*). These spots included the CD3 γ,ε chains as well as a series of TCRζ spots. Conversely, anti-CD3 precipitated the CD5 antigen, in addition to p56^{lck}, p59^{fyn}, CD3/TCRζ chain (*B*). Anti-CD5 and anti-CD3 patterns were almost identical, except for a difference in relative intensity of spots, as well as the precipitation of a faint CD5-associated 34-kDa spot not seen in the anti-CD3 pattern (*A* vs. *B*, unmarked spot to left of CD3). Anti-CD5 mAb precipitated CD5, p56^{lck}, and p59^{fyn} with greater intensity, whereas anti-CD3 mAb preferentially precipitated CD3. Identities of the kinases were shown by their comigration of spots precipitated by anti-p56^{lck} (*C*) and anti-p59^{fyn} (*D*) mAbs. This result was verified by reprecipitation and peptide-map analysis (data not shown).

The physical interaction between CD5 and protein-tyrosine kinases correlated with an ability of CD5 to induce a tyrosine-phosphorylation cascade within peripheral T cells. Cells were preincubated at 4°C with either anti-CD5, anti-CD3, or anti-CD4 mAb followed by secondary antibody (rabbit anti-mouse) and then incubated for various times at 37°C. Under this regime, anti-CD5 crosslinking resulted in the rapid phosphorylation of a variety of intracellular substrates (Fig. 4, lanes 7 and 8 vs. 1 and 2). These included substrates at 130 kDa, 115 kDa, 110 kDa, 105 kDa, 95 kDa, 92 kDa, 87 kDa, 82 kDa, 69 kDa, 42 kDa, and 14–21 kDa. Significantly, anti-CD5 mAbs induced a pattern of tyrosine phosphorylation identical to that seen with anti-CD3 mAbs (lanes 10–13). Most substrates appeared to undergo maximal phosphorylation by 1 min. The anti-CD4 pattern was similar but was not identical to the anti-CD5 pattern (lanes 3–6). This antibody induced the phosphorylation of substrates at 115 kDa, 110 kDa, 105 kDa, 95 kDa, 87 kDa, 82 kDa, and occasionally at 42 kDa. Less consistent was the phosphorylation of the 130-kDa and the 14- to 21-kDa (TCRζ) substrate by anti-CD4 mAb. Secondary antibody alone had no effect on cells (lane 2). These data were consistent with the hypothesis that the CD5 and TCRζ/CD3 antigens form a functional aggregate unit that includes p56^{lck} and p59^{fyn}.

An important extension of this hypothesis was the finding that anti-TCRζ/CD3 ligation induced the rapid phosphorylation of CD5 on tyrosine residues. In this approach, Jurkat cells were preincubated at 37°C and incubated for various times with a mixture of anti-CD3 and rabbit anti-mouse antibodies; this allowed measurement of the kinetics of receptor-induced tyrosine phosphorylation. Anti-CD3 crosslinking followed by immunoblotting with ^{125}I-labeled anti-phosphotyrosine antibody revealed a spectrum of phosphorylated proteins (Fig. 5, *Left*). As previously noted for peripheral blood T cells, anti-CD3 induced the rapid phosphorylation of two substrates of 69–70 kDa and 14–21 kDa. To identify them, anti-CD3-treated cells were lysed and immunoprecipitated with anti-CD5 (Fig. 5, *Upper right*) or anti-TCRζ (Fig. 5, *Lower right*) and subjected to anti-phosphotyrosine immunoblotting. Importantly, anti-CD3 crosslinking induced the dramatic and rapid tyrosine phosphorylation of CD5 at 69 kDa (Fig. 5, *Upper right*). During the time course of crosslinking, a slight shift in molecular mass of CD5 was noted in cell lysates (Fig. 5, Left) and precipitates (Fig. 5, *Upper right*). As a positive control, TCRζ was also found to undergo an increase tyrosine phosphorylation in cell lysates (Fig. 5, *Left*) and anti-TCRζ precipitations (Fig. 5, *Lower right*). Intriguingly, comparison of the kinetics of CD5 labeling over three experiments showed CD5 to undergo rapid phosphorylation ($t_{1/2}$ = 20 sec), slightly more rapidly

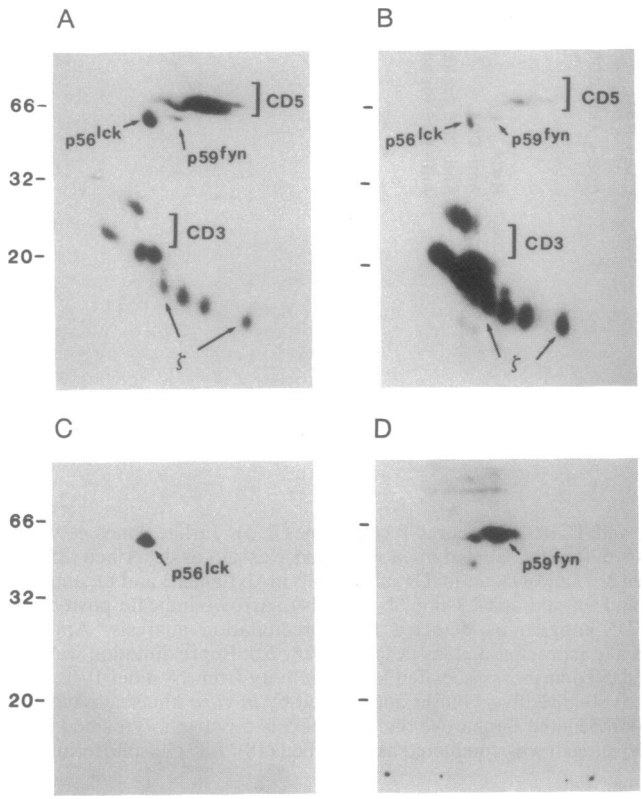

FIG. 3. Two-dimensional gel electrophoresis demonstrates the presence of p56[lck], p59[fyn], CD3 subunits, and TCRζ in complex formation with CD5 antigen. Immunoprecipitates from Brij 96-based detergent lysates from peripheral blood T cells were conducted by using the designated antibody, subjected to *in vitro* kinase labeling and two-dimensional isoelectric focusing, as described (18). (*A*) Anti-CD5. (*B*) Anti-CD3. (*C*) Anti-CD4 and anti-p56[lck]. (*D*) Anti-p59[fyn]. Anti-CD4 and anti-p56[lck] were intentionally underexposed to pin-point position of the p56[lck] spot.

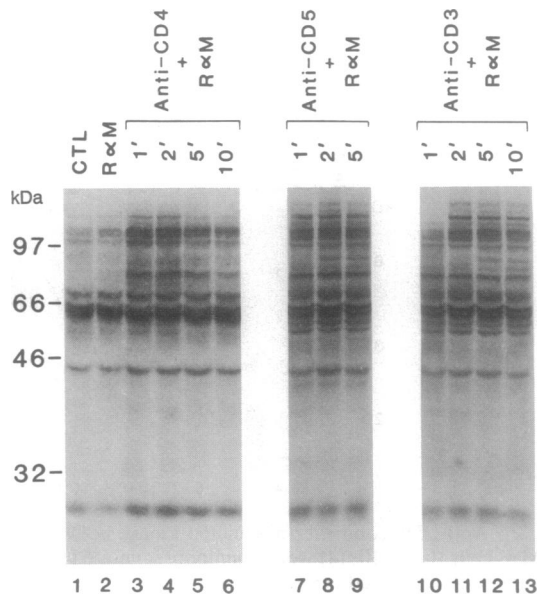

FIG. 4. Anti-CD5 and anti-CD3 crosslinking induces tyrosine phosphorylation of a similar spectrum of intracellular substrates. Resting peripheral T cells were equilibrated at 4°C and exposed for various times to either anti-CD4 plus rabbit anti-mouse antibody (RαM; lanes 3–6), anti-CD5 plus RαM (lanes 7–9), or anti-CD3 plus RαM (lanes 10–13) at 37°C and then subjected to anti-phosphotyrosine immunoblotting. Lanes: 1, untreated control; 2, RαM control; 3, 1-min anti-CD4 treatment; 4, 2-min anti-CD4 treatment; 5, 5-min anti-CD4 treatment; 6, 10-min anti-CD4 treatment; 7, 1-min anti-CD5 treatment; 8, 2-min anti-CD5 treatment; 9, 5-min anti-CD5 treatment; 10, 1-min anti-CD3 treatment; 11, 2-min anti-CD3 treatment; 12, 5-min anti-CD3 treatment; 13, 10-min anti-CD3 treatment.

than TCRζ ($t_{1/2}$ = 60 sec). Furthermore, CD5 phosphorylation was transient, having undergone appreciable dephosphorylation (i.e., 50–60%) within 10 min of TCR/CD3 ligation.

DISCUSSION

CD5 is well-established as a costimulatory antigen that functions in conjunction with the TCR/CD3 complex; however, little information exists on the molecular basis of CD5 function in signal transduction (10–16). In this study, we demonstrated that CD5 associates as a multimeric complex with TCRζ/CD3 subunits and the protein tyrosine kinases p56[lck] and p59[fyn]. Brij 96 has been found efficient in extracting p56[lck] activity and associated proteins (29). The physical association provides a basis for the dependency of CD5 function on the TCR/CD3 complex. CD5, therefore, shares characteristics with TCRζ—namely, the detection of an association with TCR/CD3 in mild detergents and the fact that both serve as substrates for protein-tyrosine kinases during TCR/CD3 ligation. In contrast to TCRζ, CD5 does not appear necessary for surface expression of the TCR/CD3 complex (12). Anti-CD5 mAb precipitated a complete assortment of CD3/TCRζ proteins, thereby making it unlikely that CD5 displaces individual subunits of the TCRζ/CD3 complex. The CD5–TCRζ/CD3 association may represent a second-order interaction on the T-cell surface. Murine and human CD5 transmembrane regions possess several conserved basic lysines as well as a single aspartic acid (4, 5). Theoretically, CD5 could interact with negatively charged residues within the CD3/TCRζ complexes or positively charged residues within the TCR α,β-chain transmembrane regions. Alternatively, CD5 and TCR/CD3 could interact by virtue of associated intracellular kinases or via extracellular domains.

Importantly, both anti-CD5 and anti-CD3 coprecipitated the src-family members p56[lck] and p59[fyn] (Figs. 2 and 3). Previous reports have shown that p59[fyn] coprecipitates with the TCR/CD3 complex (30). Our data indicate that on peripheral T cells TCR/CD3 also coprecipitates p56[lck] and, generally, to a greater extent than p59[fyn] (Fig. 3). An important issue, therefore, concerns the subunit to which the kinases bind. For example, p56[lck] binds directly to CD4, which, in turn, can physically associate with TCR/CD3, thereby contributing p56[lck] to the receptor complex (21, 25). Fastidious detergent requirements may reflect the susceptibility of the receptor–kinase interaction to dissociation or the dissociation of the preformed receptor–kinase complex from the TCR/CD3 complex. Beyers and coworkers (27) recently reported similar data showing the coprecipitation of CD2, CD4, and CD5 with the TCR/CD3 complex from rat cells. Our data differ slightly from this study in that anti-human CD5 precipitated both p56[lck] and p59[fyn] (Fig. 3), whereas anti-rat CD5 coprecipitated only p56[lck] (27). In human TCRζ/CD3$^-$ cells, anti-CD5 was restricted in coprecipitating p56[lck], thereby suggesting that p59[fyn] associates with another component within the complex (C.E.R. and M.Y., unpublished data). Finally, only a small amount of CD5 was coprecipitated with anti-CD4, suggesting that a portion of CD5–TCRζ/CD3 complexes may exist distinct from the CD4–TCRζ/CD3 complexes. Anti-CD4 coprecipitation of TCRζ/CD3 was also generally less prominent than CD5-TCR/CD3 coprecipitation. Different molecular aggregates may allow for the generation of distinct sets of intracellular signals via the TCR/CD3 complex.

Consistent with the formation of the TCRζ/CD3–CD5 complex, anti-CD5 crosslinking was found to induce the tyrosine

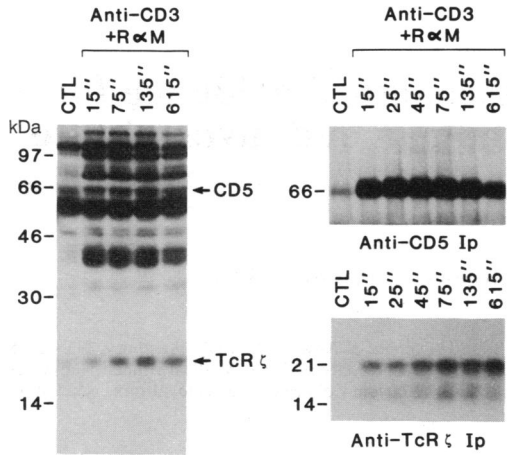

FIG. 5. Anti-CD3 crosslinking induces tyrosine phosphorylation of CD5 antigen. Lysates from Jurkat cells exposed for various times to anti-CD3 mAb plus rabbit anti-mouse antibody (RαM) were subjected either to anti-phosphotyrosine immunoblotting (*Left*) or to precipitation by anti-CD5 mAbs (*Upper right*) or anti-TCRζ (*Lower right*) followed by anti-phosphotyrosine blotting. Cell lysates (*Left*); anti-CD5 mAb (*Upper right*); anti-TCRζ mAb (*Lower right*). Ip, immunoprecipitation.

phosphorylation of a spectrum of substrates identical to anti-CD3. CD5, therefore, may act to regulate $p56^{lck}$ and/or $p59^{fyn}$ activity. Further, anti-CD3 caused the rapid phosphorylation of CD5 itself (Fig. 5*A* and *B*). CD5 thus represents a second TCR/CD3-associated protein that undergoes tyrosine phosphorylation in response to TCR/CD3 ligation. TCRζ had previously been identified as a tyrosine substrate within the complex (31). CD5 underwent rapid phosphorylation ($t_{1/2}$ = 15–20 sec), more rapid than observed for the TCRζ ($t_{1/2}$ = 60 sec) (Fig. 5). Even slower rates of TCRζ phosphorylation have been observed, an event that may vary with the growth conditions of cells (31, 37). The rapid kinetics of CD5 phosphorylation is roughly comparable with other substrates (37) and with the activation of phospholipase C, a correlation compatible with a linkage between these events. CD5 phosphorylation is also transient, suggesting regulation by phosphatases, possibly CD45. Particularly intriguing is the presence of a Y-$(X)_{11}$-Y motif that is found in other substrates such as TCRζ (32) and immunoglobulin-associated B29 and MB1 (33, 34) proteins. The first tyrosine within the CD5 motif possesses adjacent residues similar to the autophosphorylation site of src-related kinases (DNEYSQP vs. DNEYTAR, respectively). CD5 appears especially well-tailored to serve as a substrate for src kinases such as $p56^{lck}$ and $p59^{fyn}$ within the receptor complex. Nevertheless, the involvement of other kinases cannot be excluded. CD5 phosphorylation could have various functions, such as altering the conformation of the antigen, its association with the TCR/CD3 complex, binding of src homology region 2-carrying proteins, or negatively regulating $p56^{lck}$ or $p59^{fyn}$ activity by competing with the ability of the kinases to autophosphorylate.

Distinct signals can be generated by TCRζ and CD3 subunits within the complex (35, 36). The presence of CD5, $p56^{lck}$, and $p59^{fyn}$ adds yet another branch to this multifarious signaling unit. Unlike TCRζ, CD5 possesses an extracellular region that can bind to ligand, the B-cell-surface protein CD72 (17). The interaction between CD5 and CD72 may use $p56^{lck}$, $p59^{fyn}$, and TCR/CD3 in the generation of signals required specifically for T-cell–B-cell collaboration.

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