Determination of the tyrosine phosphorylation sites in the T cell transmembrane glycoprotein CD5

Kevin M. Dennehy1,4, William F. Ferris2, Hanne Veenstra1, Linda A. Zuckerman3, Nigel Killeen3 and Albert D. Beyers1

1Department of Medical Biochemistry and MRC Centre for Molecular and Cellular Biology, University of Stellenbosch, Tygerberg, 7505, South Africa
2Experimental Biology Programme, Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa
3Department of Microbiology and Immunology, University of California, San Francisco, CA 94143-0414, USA
4Current address: Institute for Virology and Immunobiology, University of Würzburg, 97078 Würzburg, Germany

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Abstract
Studies of CD5-deficient mice indicate that the transmembrane glycoprotein CD5 negatively regulates antigen receptor-mediated signals in thymocytes, lymph node T cells and B1a cells. CD5 contains four tyrosine residues in its cytoplasmic domain and is phosphorylated on tyrosine residues following antigen receptor ligation. Recently it has been proposed that CD5 function is dependent on the recruitment of the tyrosine phosphatase SHP-1 to tyrosine-phosphorylated CD5 and subsequent dephosphorylation of signaling molecules. In this study we investigated the requirements for, and sites of, CD5 tyrosine phosphorylation. Using a T cell line deficient in the tyrosine kinase p56<sub>Lck</sub> and the same cell line reconstituted with this kinase, we show that p56<sub>Lck</sub> expression is required for efficient CD5 tyrosine phosphorylation. Using tyrosine-phosphorylated peptides corresponding to CD5 cytoplasmic sequences we also show that the Src homology 2 (SH2) domain of p56<sub>Lck</sub> binds prominently to pY429SQP, with 30-fold less affinity to pY463DLQ and not to pY441PAL. A number of murine CD5 Y<sub>→F</sub> and deletion mutants were expressed in Jurkat T cells. The Y441F mutant was tyrosine phosphorylated at levels comparable to wild-type, but the Y429F and Y463F mutants were phosphorylated at lower levels. Two deletion mutants, which contain only one tyrosine residue (Y378) located at the interface of the transmembrane and cytoplasmic domains, were not tyrosine phosphorylated, suggesting that Y378 is not readily available for phosphorylation. Taken together these results suggest that both Y429 and Y463 can recruit p56<sub>Lck</sub>, and that these residues are the only prominent sites for CD5 tyrosine phosphorylation.

Introduction
The destructive potential of the immune system necessitates tight control of cellular effector functions by means of positive and negative regulatory receptors. One such receptor is the glycoprotein CD5, which is expressed on thymocytes, T cells and a B cell subset. Initial experiments using antibody-mediated cross-linking of surface receptors suggested that CD5 enhances TCR–CD3-mediated responses (1–5). Treatment of purified peripheral T cells with solid-phase-bound anti-CD3 antibody together with anti-CD5 antibody led to the production of IL-2 and proliferation (2). However, subsequent studies of CD5-deficient mice indicated that TCR-mediated proliferative responses were enhanced in thymocytes, suggesting a negative regulatory function for CD5 (6). Lymph node T cells and B1a cells from CD5<sup>–/–</sup> mice similarly
CD5 tyrosine phosphorylation sites

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**Fig. 1.** Comparison of the cytoplasmic domain of human and mouse CD5 with ITAM and ITIM sequences. Residues identical for human and mouse CD5 are indicated by an asterisk. The C-termini of the transmembrane sequences are boxed and residues 382–424 are not shown. ITIM sequences are from erythropoietin receptor and killer cell lectin-like receptor G1 (KLRG1). Sequences of tyrosine-phosphorylated peptides used in this study are underlined.

show enhanced antigen receptor-mediated proliferative responses, again suggesting a negative regulatory function for CD5 in these cells (7,8).

CD5 associates with the TCR (9) and BCR (10), and is phosphorylated on serine, threonine and tyrosine residues following TCR stimulation (11,12). One of the kinases responsible for phosphorylating CD5 is the tyrosine kinase p56<sup>lck</sup>, which can also bind to tyrosine phosphorylated CD5 by means of its Src homology 2 (SH2) domain (13). The transmembrane/cytoplasmic domain of CD5 contains four tyrosine residues, and shows some similarity to both immuno-receptor tyrosine-based activation motif (ITAM) and inhibition (ITIM) sequences (Fig. 1) (14–16). We have previously shown using tyrosine-phosphorylated peptides corresponding to CD5 cytoplasmic domain sequences that the ITAM-like sequence of CD5 binds to the TCR ζ-associated protein ZAP-70 with low affinity and is unlikely to be an ITAM (14). A number of negative regulatory receptors contain ITIM sequences, which once phosphorylated recruit SH2 domain-containing phosphatases (17). Recently CD5 has been proposed to negatively regulate TCR-mediated signaling by recruiting the tyrosine phosphatase SHP-1 (15). Such negative regulatory function was dependent on the phosphorylation of Y378, which was identified as the site of SHP-1 binding to CD5 (15).

The aim of this study was to determine the requirements for, and sites of, tyrosine phosphorylation in the CD5 cytoplasmic domain. To determine how important the tyrosine kinase p56<sup>lck</sup> is for tyrosine phosphorylation of CD5, a comparison was made between a cell line lacking this kinase and the same cell line reconstituted with p56<sup>lck</sup>. Using tyrosine-phosphorylated peptides corresponding to CD5 cytoplasmic domain sequences, the potential sites of p56<sup>lck</sup> binding to CD5 were determined. Lastly, Y → F and deletion mutants of the murine CD5 cytoplasmic domain were generated and expressed in the human CD5<sup>−</sup> T cell line Jurkat 1.15 M46 (3) in order to determine the sites of tyrosine phosphorylation in the cytoplasmic domain of CD5, and eventually to address the functions of the four cytoplasmic tyrosine residues in CD5.

**Methods**

Reagents, vectors and antibodies

The pGEX-2T vector (18) was a gift from Dr A. Cowman (Walter and Eliza Hall Institute, Victoria, Australia). Murine anti-human CD5 (UCHT12, IgG1) hybridoma was from Dr P. Beverley (University College, London), rat anti-mouse CD5 (YTS 121.5.2, IgG2b) (19) hybridoma was from the ECCC (Salisbury, UK), murine anti-human CD3 (OKT3, IgG2a) (20) hybridoma was from the ATCC (Rockville, MD), and murine anti-human C3b inactivator (OX-21, IgG1) (21) hybridoma was from Dr D. Mason (MRC Cellular Immunology Unit, Oxford, UK). mAb against phosphotyrosine (PY20) was from Transduction Laboratories (Lexington, KY) and polyclonal antibody to p59<sup>fyn</sup> was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum directed against the p56<sup>lck</sup> sequence RNGSEVRDPLVTYEGSNPPA (22) was from Dr J. Borst (The Netherlands Cancer Institute, Amsterdam) and rabbit antiserum directed against the human CD5 cytoplasmic sequence SSMQPDNNSDSYDYLHQAQR (23) was from Dr D. Mason (John Radcliffe Hospital, Oxford, UK).

Peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Caltag (San Francisco, CA). Glutathione-agarose, streptavidin-agarose, Protein A–Sepharose and Protein G–Sepharose were from Sigma (St Louis, MO), and rabbit anti-mouse Ig, sheep anti-rat Ig, sheep anti-rat–FITC and sheep anti-mouse–FITC were from Serotec (Oxford, UK). The ECL Western blotting detection system and [γ<sup>32P</sup>]<sub>ATP</sub> (3000 Ci/mmol) were from Amersham (Little Chalfont, UK).

Cell culture and flow cytofluorometry

Cells were routinely grown in RPMI 1640 medium (ICN, Aurora, OH) supplemented with 50 µM β-mercaptoethanol,
0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine and 10% FBS (Delta Bioproducts, Johannesburg, South Africa) in a humidified incubator at 37°C gassed with 5% CO₂. Flow cytometric analysis was performed using a Becton Dickinson (San Jose, CA) FACScan apparatus. Cells (10⁶) were stained at 4°C for 30 min with mAb [100 µl (1–2 µg antibody) hybridoma culture supernatant containing 1 mM Na₃H₂PO₄, washed twice with PBS (0.25% BSA and 1 mM NaCl), stained with a 1:100 dilution of appropriate secondary antibody–FITC conjugate (30 min, 4°C) and washed with PBS containing 1 mM Na₃. Analysis was performed using Lysys II or CellQuest software.

**Phosphopeptides**

N-terminal biotinylated tyrosine-phosphorylated peptides corresponding to the CD5 sequences VDNEYSQPPR and pSV-(ψ-)-(pYPAL) denoted pYD (24) were synthesized by Genosys (Cambridge, UK). A tyrosine-phosphorylated peptide corresponding to the optimal binding phosphopeptide for p56⁶ck SH2 (EPFYEEPI (Fig. 1) was also synthesized by Genosys. Peptides were immobilized at 0.3–10 nmol on 25 µl packed streptavidin–agarose or Affigel-15 (BioRad, Hercules, CA) beads to give a final concentration of 0.3–10 µm peptide in 1 ml lysate/solution used for each precipitation.

**Synthesis of p56⁶ck SH2 fusion protein construct and preparation of fusion protein**

RNA was prepared from phytohemagglutinin (PHA)-stimulated T blasts. cDNA was made using the Amersham cDNA synthesis PLUS kit and PCR was performed with oligonucleotides, containing flanking EcoRI and BamHI sites, designed to generate the SH2 domain of p56⁶ck (amino acids 118–235). Products were run on 2% agarose gels, correctly sized fragments were retrieved from the agarose and ligated into the pGEX-2T vector, cut with EcoRI and BamHI, for generation of glutathione S-transferase fusion products. The sequence of the insert was verified, and fusion protein was expressed in Escherichia coli MC1061 cells and purified by affinity chromatography using glutathione–agarose (18). Protein concentrations were estimated using the Bradford assay as modified by Read and Northcote (25).

**Generation and expression of mutant CD5 in Jurkat cells**

Mismatch primer mutagenesis (26) was used to produce mutations within murine CD5 cDNA. A 3’ fragment of CD5 cDNA (nucleotides 525–1657), containing the region coding for the intracellular domain, was excised from the plasmid pCD CD5 (27) using the unique EcoRI and Apal restriction sites. The fragment was ligated, using a DNA linker containing flanking regions complimentary to Apal and KpnI restriction sites, into RF M13 mp18 (28). Single-stranded, uracil-containing phage DNA was extracted from infected E. coli CJ236 (dut ung) cells, and mutagenesis and selection performed as described by Kunkel et al. (26).

The 5’ coding region of CD5 (nucleotides 1–524) was excised from pCD CD5 using the unique Ncol and EcoRI restriction sites. The fragment was ligated, using linker DNA-containing flanking regions complimentary to Ncol and SalI restriction sites, into RF M13 mp18 and then excised using the flanking HindIII and EcoRI sites. The 3’ mutated region of CD5 cDNA was excised from mutant RF M13 mp18 CD5 Δ 1–524 using the EcoRI and Apal restriction sites, and ligated with the 5’ CD5 fragment into the HindIII and Apal sites in the eukaryotic expression plasmid pcDNA 3.0 (Invitrogen, Carlsbad, CA). The full length of the final wild-type and mutant CD5 constructs were verified by sequencing.

Initially pcDNA 3.0 CD5 constructs were introduced into eukaryotic cells by electroporation. Levels of CD5 expression varied, so a retroviral infection system was used to achieve high and comparable levels of CD5 expression. A sequence encoding the cytomegalovirus promoter and CD5 was excised from pcDNA 3.0 CD5 constructs using NruI and Apal restriction sites, and ligated into the eukaryotic expression vector pBabe Puro (29) cut with SnaBI. pBabe Puro CD5 constructs and pSV-(ψ-)-(A)-MLV (30) (20 µg each in 10 mM Tris–HCl, pH 8.0), were co-electroporated into 293T cells (31) (250 µl/well, every 24 h, filter sterilized and incubated with Jurkat cells in the presence of 8 µg/ml polybrene for 24 h. After 3–4 days cells were electronically sorted for CD5 expression using a FACS Vantage cytometer (Becton Dickinson). Selected cells were expanded, labeled with rat anti-mouse CD5 antibody followed by anti-rat µ chain-magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and magnetically sorted for a second time.

**Cell stimulation, lysis and affinity precipitation of protein**

Cells were washed with HBSS, pH 7.3, and 100 µl aliquots containing 2×10⁷ Jurkat cells were used per sample. Cells were preheated to 37°C for 1 min and stimulated by the addition of 5 µl Na-pervanadate (final concentration 3 mM H₂O₂, 0.1 mM Na-pervanadate), a tyrosine kinase activator and phosphatase inhibitor (32,33), followed by incubation at 37°C for 1–5 min. For studies involving receptor cross-linking, cells were treated with 10 µg mAb premixed with 2 µg anti-lg secondary antibody for 2 min at 37°C (12). Cells were lysed by the addition of 1 ml 1.1×NP-40 or Brij-96 lysis buffer (final concentration 1% NP-40 or Brij-96, 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM Na-venadate, 1 mM EDTA, 1 mM PMSF and 5 mM iodoacetamide) and incubated on ice for 20–30 min. Nuclei were pelleted at 14,000 g for 10 min at 4°C. Supernatants were added to 25 µl glutathione–agarose prebound to 5 µg fusion protein, or to 25 µl Protein A– or Protein G–Sepharose beads prebound to 2–5 µg antibody. Precipitations using synthetic peptides were performed using 0.3–10 nmol (µM) peptide coupled to 25 µl Affigel-15 (BioRad, Hercules, CA) or streptavidin–agarose beads. Cell lysates were incubated with the beads for 1.5–2 h at 4°C.

**Western blotting**

For detection of proteins by Western blotting, beads were washed 4 times with NP-40 lysis buffer. Bound proteins were denatured by boiling for 5 min in 50 µl reducing sample buffer. Aliquots (20 µl) were subjected to SDS–10% PAGE (34) and proteins were transferred to Hybond-C extra (Amersham, Little Chalfont, UK) membranes for 1 h at 100 V. Proteins were probed with primary antibody in TBS–Tween (10 mM Tris–HCl, pH 7.4, 140 mM NaCl and 0.1% Tween 20) for 1 h at room temperature, followed by peroxidase-conjugated
showed that CD5 is a substrate for the tyrosine kinase tyrosine phosphorylated, but a low level of Y429F CD5 tyrosine phosphorylation in Jurkat T cells. These proteins was enhanced following cross-linking of CD3. Requirement of p56\(^{\text{lck}}\) expression for efficient CD5 tyrosine phosphorylation in Jurkat T cells

Using co-transfection experiments in COS cells Raab et al. (13) showed that CD5 is a substrate for the tyrosine kinase p56\(^{\text{lck}}\). In order to complement this study, the p56\(^{\text{lck}}\), deficient (JaCaM1) cell line and the same cell line reconstituted with p56\(^{\text{lck}}\) (JaCaM1/lck) (35) were used to determine the importance of p56\(^{\text{lck}}\) expression for CD5 tyrosine phosphorylation in Jurkat T cells.

CD5 is phosphorylated on tyrosine, serine and threonine residues \textit{in vivo} (11,36) and \textit{in vitro} (9,12,37). To detect CD5 tyrosine phosphorylation, Western blotting was used following cell lysis in NP-40 lysis buffer (Fig. 2). Pervanadate, a non-specific tyrosine kinase activator and phosphatase inhibitor (32,33), was used to induce maximal tyrosine phosphorylation of CD5. There was no detectable CD5 tyrosine phosphorylation in unstimulated or pervanadate-stimulated lck\(^{+}\) cells. However, in unstimulated lck\(^{+}\) cells there was a low level of constitutive CD5 tyrosine phosphorylation and this was increased following pervanadate stimulation (Fig. 2). Tyrosine phosphorylation of CD5 could also be induced, although not efficiently, following cross-linking of CD3 in lck\(^{+}\) cells. Given such poor induction of CD5 tyrosine phosphorylation in JCaM1/lck cells, a comparison of CD5 tyrosine phosphorylation was not made following cross-linking of CD3 in lck\(^{-}\) and lck\(^{+}\) cells.

Sites of tyrosine phosphorylation in the CD5 cytoplasmic domain

To determine the importance of each tyrosine residue in CD5 tyrosine phosphorylation, Y → F as well as deletion mutants of murine CD5 were generated. There are four tyrosine residues in the CD5 transmembrane/cytoplasmic domain. Y378 occurs at the junction of the transmembrane and cytoplasmic domains, N-terminal to three lysine doublet anchoring sequences (Fig. 3). To remove as much of the cytoplasmic domain as possible without interfering with the anchoring in the membrane, the deletion mutant 389\(\Delta\) was constructed. The deletion mutant 429\(\Delta\) was also constructed to remove the C-terminal three tyrosine residues. Each of these tyrosine residues was mutated to phenylalanine to generate Y429F, Y441F and Y463F CD5 point mutants.

Murine wild-type CD5, and 389\(\Delta\), 429\(\Delta\), Y429F, Y441F and Y463F CD5 mutants were expressed in the human CD5\(^{-}\), CD3\(^{+}\) cell line Jurkat 1.15 M46 (3). Assessed by flow cytometric analysis, levels of CD5 expression were high and comparable (results not shown), and concanavalin A-peroxidase blotting of CD5 precipitates from infectants lysed in NP-40 lysis buffer demonstrated the expression of all CD5 constructs (Fig. 4A).

Pervanadate stimulation of Jurkat 1.15 M46 CD5 mutants induced the comparable tyrosine phosphorylation of wild-type and Y441F CD5, and a lower level of Y429F CD5 tyrosine phosphorylation (Fig. 4A). There were very low levels of Y463F CD5 tyrosine phosphorylation, whereas the deletion mutants 389\(\Delta\) and 429\(\Delta\) were not tyrosine phosphorylated following pervanadate stimulation (Fig. 4A).

Differential tyrosine phosphorylation of CD5 mutants was also induced following cross-linking of CD3 (Fig. 4B). There were low levels of constitutive tyrosine phosphorylation of wild-type and Y441F CD5, and tyrosine phosphorylation of these proteins was enhanced following cross-linking of CD3. By contrast, Y429F and Y463F CD5 were not constitutively tyrosine phosphorylated, but a low level of Y429F CD5 tyrosine phosphorylation could be induced following cross-linking of CD3 (Fig. 4B). The deletion mutants 389\(\Delta\) and 429\(\Delta\) were not tyrosine phosphorylated following cross-linking of CD3 (results not shown).

Association of the p56\(^{\text{lck}}\) SH2 domain with CD5 cytoplasmic domain phosphopeptides and CD5 mutants

Three tyrosine-phosphorylated peptides corresponding to CD5 cytoplasmic sequences (Fig. 1) were used to determine the potential sites at which p56\(^{\text{lck}}\) interacts with CD5. Although cellular p56\(^{\text{lck}}\) could not be detected in CD5 phosphopeptide precipitates (14), the recombinantly expressed SH2 domain of p56\(^{\text{lck}}\) bound strongly to pY429SQP, with ~30-fold lower affinity to pY463DLQ and not to pY441PAL (Fig. 5A). The binding of the p56\(^{\text{lck}}\) SH2 domain to CD5 thus appears to be mediated predominantly by the pY429SQP sequence with a possible minor contribution by the pY463DLQ sequence.
In order to confirm the sites of p56<sub>lk</sub> association with CD5, the recombinant SH2 domain of p56<sub>lk</sub> was used to precipitate CD5 Y→F point mutants from pervanadate-stimulated Jurkat 1.15 M46 cells. Immediately after protein transfer the nitrocellulose membrane was stained with Ponceau S to ensure equal loading of precipitating p56<sub>lk</sub> SH2 domain (result not shown), following which the membrane was blocked and probed with a polyclonal antibody to CD5. Wild-type and Y441F CD5 bound equally well to the p56<sub>lk</sub> SH2 domain. However, neither Y429F nor Y463F CD5 mutants were

**Fig. 3.** Murine CD5 deletion and Y→F mutants. The N-termini of the transmembrane sequences are boxed, and residues 390–424 and 447–458 are not shown. The four tyrosine residues and three lysine doublets in the wild-type CD5 sequence, as well as mutated residues are underlined.

**Fig. 4.** Tyrosine phosphorylation of murine CD5 and CD5 mutants expressed by Jurkat 1.15 M46 cells. (A) Jurkat 1.15 M46 parental or CD5 infectant cells (2×10<sup>6</sup>/H<sub>11003</sub>10<sup>7</sup>) were stimulated with pervanadate (V), lysed in NP-40 and cellular proteins were precipitated with anti-CD5 mAb. Samples were resolved by SDS–10% PAGE, Western blotted and probed with anti-phosphotyrosine mAb or concanavalin A–peroxidase as indicated. (B) Jurkat 1.15 M46 parental or CD5 infectant cells were treated with anti-human C3b inactivator (Φ) or anti-CD3 mAb premixed with anti-mouse Ig secondary cross-linker (C) (2 min, 37°C). Cells were lysed in NP-40 and cellular proteins were precipitated with anti-CD5 mAb. Samples were resolved and blotted as in (A), and probed with anti-phosphotyrosine mAb or anti-CD5 polyclonal antibody. The position of wild-type CD5 and Y→F CD5 mutants is indicated.
CD5 tyrosine phosphorylation sites

**Fig. 5.** Binding of the p56<sup>lo</sup> SH2 domain to CD5 cytoplasmic domain phosphopeptides and to tyrosine-phosphorylated CD5 mutants from Jurkat 1.15 M46 infectants. (A) Fusion protein (5 µg) of the p56<sup>lo</sup> SH2 domain was incubated with the indicated concentrations of immobilized CD5 phosphopeptides or p56<sup>lo</sup> target peptide pYEEI. Precipitated proteins were resolved by SDS–10% PAGE and stained with Coomassie blue. (B) Jurkat 1.15 M46 CD5 infectants (2 x 10<sup>7</sup>) were stimulated with pervanadate (V), lysed in NP-40 and cellular proteins were precipitated with 5 µg of the p56<sup>lo</sup> SH2 domain. Samples were resolved by SDS–10% PAGE, Western blotted and probed with an anti-CD5 polyclonal antibody.

precipitated by the p56<sup>lo</sup> SH2 domain (Fig. 5B), despite the clearly detectable tyrosine phosphorylation of the Y429F mutant (Fig. 4A).

**Discussion**

The previous demonstration that CD5 is tyrosine phosphorylated by p56<sup>lo</sup> used co-transfection experiments in COS cells (13). Using p56<sup>lo</sup>-deficient and reconstituted cell lines we show here that CD5 is tyrosine phosphorylated in <i>lck</i><sup>−</sup> cells but not in <i>lck</i><sup>+</sup> cells following pervanadate stimulation (Fig. 2). Although both <i>lck</i><sup>−</sup> and <i>lck</i><sup>+</sup> cells express the tyrosine kinase p59<sup>fyn</sup>, as detected by Western blotting and <i>in vitro</i> kinase assays (results not shown and 38), the levels of tyrosine phosphorylation in whole-cell lysates from <i>lck</i><sup>−</sup> cells induced after pervanadate stimulation or cross-linking of CD3 were <10-fold lower than that from <i>lck</i><sup>+</sup> cells (results not shown). Even though such low levels of tyrosine phosphorylation occur in <i>lck</i><sup>−</sup> cells, the results presented here do not necessarily exclude a role for p59<sup>fyn</sup> in CD5 tyrosine phosphorylation. Nevertheless, these results complement the study by Raab et al. (13) in demonstrating that p56<sup>lo</sup>, and potentially other kinases whose activity depends on p56<sup>lo</sup>, plays a prominent role in CD5 tyrosine phosphorylation.

The comparable tyrosine phosphorylation of wild-type and Y441F CD5 following pervanadate stimulation or cross-linking of CD3 (Fig. 4) indicates that Y441 is not phosphorylated or phosphorylated at low stoichiometry. Additionally, the comparable binding of the p56<sup>lo</sup> SH2 domain to tyrosine-phosphorylated wild-type and Y441F CD5 (Fig. 5B) and lack of binding of this domain to the pY441PAL peptide (Fig. 5A) suggest that the Y441 sequence is not a binding site for p56<sup>lo</sup>. The functional significance of Y441 remains unresolved since the pY441PAL peptide did not bind to a panel of recombinant fusion proteins or a number of signal transducing proteins in thymocyte lysates (39 and results not shown).

Neither of the 389Δ or 429Δ CD5 deletion mutants were tyrosine phosphorylated following pervanadate stimulation or cross-linking of CD3 (Fig. 4A, result not shown). Since both deletion mutants contain only one tyrosine residue (Y378), which potentially could lie in the cytoplasmic domain, this residue is unlikely to be a site for CD5 tyrosine phosphorylation. These results differ from those of Perez-Villar et al. (15). These authors generated chimeric molecules encoding the extracellular domain of murine CD6 and the intracellular domain of human CD5 to analyze the function of CD5. In direct contrast to the results presented in the present study, the 429Δ mutant CD5–CD6 chimera was shown by Perez-Villar et al. (15) to be tyrosine phosphorylated, suggesting that Y378 lies within the CD5 cytoplasmic domain and is available for phosphorylation. The reason for the differences between the study of Perez-Villar et al. (15) and the present study is not clear. One possibility is that the architecture of the chimeric transmembrane domains, and therefore the localization of Y378, differs from that of native murine CD5. Secondly, Perez-Villar et al. expressed the chimeras in CD5<sup>−</sup> cells as opposed to CD5<sup>+</sup> Jurkat cells. It is possible that the tyrosine phosphorylation of the 429Δ CD5–CD6 chimera is due to transphosphorylation by p56<sup>lo</sup> associated with full-length CD5 in the cells used by Perez-Villar et al. (15). To address this possibility, we expressed the panel of murine CD5 mutants in the human CD5<sup>−</sup> cell line Jurkat J5. Again the 429Δ mutant was not tyrosine phosphorylated following pervanadate stimulation or co-cross-linking of CD3 with murine and human CD5, despite the tyrosine phosphorylation of Y429F and Y441F mutants as well as low levels of Y463F tyrosine phosphorylation (result not shown). Taken together, the location of Y378 N-terminal to three lysine doublets suggests that this residue is at the interface of the transmembrane and
cytoplasmic domains, and the lack of phosphorylation of Y378 in this study suggests that it is not readily available for phosphorylation. The diminished tyrosine phosphorylation of Y429F and Y463F CD5 mutants (Fig. 4) indicates that these residues are the two prominent sites for CD5 tyrosine phosphorylation. Given that the residues N-terminal to Y429 are identical to those in the autophosphorylation site of Src-like kinases (Fig. 1) (9), this sequence was predicted to be the most prominent site for CD5 tyrosine phosphorylation. However, contrary to expectation, the Y463F mutant is phosphorylated at lower levels than the Y429F mutant. The inability of the p56\(\text{lck}\) SH2 domain to precipitate the tyrosine-phosphorylated Y429F mutant (Fig. 5B), together with the prominent binding of this domain to the pY429SQP peptide (Fig. 5A), suggest that Y429 is the major site for p56\(\text{lck}\) recruitment. However, the fact that the Y429F mutant is tyrosine phosphorylated indicates that p56\(\text{lck}\) can be recruited in the absence of Y429 phosphorylation. The weak binding of the p56\(\text{lck}\) SH2 domain to the pY463DLQ peptide suggests that Y463 can recruit p56\(\text{lck}\). Although we cannot explain the lack of prominent Y463F mutant tyrosine phosphorylation, such weak phosphorylation may imply that Y463 is phosphorylated at greater stoichiometry than Y429 or that phosphorylation of Y463 is required for subsequent phosphorylation of Y429. The latter possibility would account for the inability of the p56\(\text{lck}\) SH2 domain to precipitate the Y463F CD5 mutant from pervanadate-stimulated cells (Fig. 5B) as a consequence of the weak tyrosine phosphorylation of this mutant (Fig. 4A).

A number of groups have demonstrated the recruitment of SH2 domain-containing molecules to tyrosine-phosphorylated CD5. Such molecules include the p85 subunit of phosphatidylinositol 3-kinase (14, 40), the proto-oncogene product c-Cbl and the RAS GTPase-activating protein (39), as well as the tyrosine phosphatase SHP-1 (7, 15, 16, 41). Consistent with the tyrosine phosphorylation of Y429 and Y463 shown in this study, we were able to detect the p85 subunit of phosphatidylinositol 3-kinase associated with wild-type CD5 and the Y441F CD5 mutant, but not with either Y429F CD5 or Y463F CD5 mutants (result not shown). We were not able to detect c-Cbl, Ras GTPase-activating protein or the tyrosine phosphatase SHP-1 in association with wild-type CD5 or any CD5 mutants in this Jurkat system. Tarakhovsky et al. (6) have observed decreases in tyrosine phosphorylation of TCR–CD3 components from CD5\(^{\text{–}}\) compared to CD5\(^{+\text{+}}\) mice, and Perez-Villar et al. (15) observed decreased TCR \(\zeta\) chain tyrosine phosphorylation following co-cross-linking of CD3 and CD5 compared to cross-linking of CD3 alone. These studies suggest that the tyrosine phosphatase SHP-1 or other phosphatases account for at least a part of the negative regulatory function of CD5. However, a recent study comparing the molecular interactions of CD5 and a killer cell inhibitory receptor has shown that association of SHP-1 with CD5 is not required for CD5\(^{\text{–}}\) regulatory function in a B cell line (42). Additionally, deletion of the pseudo-ITAM sequence abrogated CD5\(^{\text{–}}\) regulation, suggesting that CD5 tyrosine phosphorylation is required for its function in B cells (42). Previous observations by Perez-Villar et al. (15) indicated a necessary role for Y378 in the interaction between CD5 and SHP-1 in Jurkat T cells. We have been unable to detect phosphorylation of Y378, suggesting that if phosphorylation of this residue is important for SHP-1 interaction, the association is unlikely to occur at high stoichiometry. Clearly more extensive mutagenesis studies are required to clarify the role of CD5 tyrosine phosphorylation as well as the tyrosine phosphatase SHP-1 in the negative regulatory function of CD5.

Jurkat 1.15 M46 cells were chosen to express CD5 and CD5 mutants because these cells produce IL-2 following stimulation through the TCR–CD3 complex (3). Although Jurkat 1.15 M46 CD5 infectants did produce detectable levels of IL-2 in isolated experiments, these results were not reproducible (results not shown). The panel of CD5 mutants will be expressed in other cell lines in order to compare IL-2 secretion following stimulation through the TCR–CD3 complex as well as following co-cross-linking of TCR–CD3 and CD5. In this study the Y378F CD5 mutant was not produced because of the location of Y378 N-terminal to three lysine doublet sequences and hence the likely location of this residue in the transmembrane region. In light of the results of Perez-Villar et al. (15) that Y378 is necessary for the inhibitory function of CD5, it will be necessary to compare cellular effector functions of the Y378F mutant in murine CD5 with other CD5 mutants used in this study. Such experiments are currently underway and will form the basis of a further communication.

**Acknowledgements**

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**Abbreviations**

ITAM immunoreceptor tyrosine-based activation motif
ITIM immunoreceptor tyrosine-based inhibition motif
PHA phytohemagglutinin
SH2 Src homology 2

**References**

CD5 tyrosine phosphorylation sites


