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Role of Two Conserved Cytoplasmic Threonine Residues (T410 and T412) in CD5 Signaling

Josep M. Vilà,* Javier Calvo,** Lourdes Places,* Olga Padilla,* Mònica Arman,* Idoia Gimferrer,* Claude Aussel,† Jordi Vives,* and Francisco Lozano*‡

CD5 is a transmembrane coreceptor that modulates activation and differentiation signals mediated by the Ag-specific receptor present on both T and B1a lymphocytes. CD5 lacks intrinsic catalytic activity, and its immunomodulatory properties result from intracellular interactions mediated by the CD5 cytoplasmic tail. The nature of these interactions is currently a matter of investigation. Here, we present a selective mutagenesis analysis of two conserved threonine residues (T410 and T412) located at the membrane-proximal cytoplasmic region of CD5. These residues are contained within consensus phosphorylation motifs for protein kinase C and are shown here to be critical for in vivo protein kinase C-mediated phosphorylation of CD5. Functional studies revealed that the integrity of T410 and T412 is also critical for CD5-mediated phosphatidylcholine-specific phospholipase C (PC-PLC) activation and phorbol ester-mediated inhibition of Ab-induced internalization of CD5. These results strongly argue in favor of a role for T410 and T412 in the signaling mediated by CD5.


Human CD5 is a 67-kDa type-I transmembrane glycoprotein expressed on thymocytes, mature peripheral T cells, and a subpopulation of B cells (B1a) (1, 2). Structurally, it belongs to the family of receptors bearing extracellular domains of the scavenger receptor cysteine-rich type (3, 4). CD5 is physically associated with the Ag-receptor complex present on both T (TCR/CD3) and B1a (B cell receptor (BCR)) cells (5–7), and it promotes the association of TCR/CD3 with rafts (8). The physiological function of CD5 in lymphocytes remains unclear. There is current agreement that CD5 behaves as a dual receptor by modulating either positively or negatively the activation and differentiation signals mediated by the Ag-specific receptor complex (2). The type of modulatory signal depends on the cell type and the maturation stage. On peripheral T lymphocytes, CD5 acts as a costimulatory molecule by sustaining TCR/CD3-mediated proliferative responses (9–11). Contrary to this, recent studies of CD5−/− mice show that CD5 negatively regulates Ag-receptor complex-mediated signals in thymocytes and B1a cells (12, 13). It has been proposed that the Src homology 2 domain-bearing protein tyrosine phosphatase-1 (14, 15), the proto-oncoprotein c-cbl (16), or casein kinase II (CKII) (17) are key elements for the negative regulation of TCR/CD3 and BCR signaling by CD5. However, the CD5 signaling pathway and the molecular basis for its ability to modulate TCR/CD3 and BCR signals are not completely understood.

CD5 is an accessory molecule with its own signaling pathway (18–21) but its dependency on the TCR/CD3 complex has also been reported (22–24). Mitogenic anti-CD5 mAbs activate protein kinase C (PKC) in the absence of intracellular Ca2+ mobilization and phosphoinositide turnover (19). This activation is achieved through diacylglycerol (DAG) release after phosphatidylcholine-specific phospholipase C (PC-PLC) activation and de novo phospholipid synthesis (21). PC-PLC-generated DAG can in turn mediate the activation of acidic sphingomyelinase (A-SMase) (25). Other signaling elements involved in the CD5 pathway include the Ca2+/calmodulin-dependent kinase type IV (CaMK IV) (26), the phosphatidylinositol 3-kinase (PI 3-K) (27), the guanine exchange factor Vav (27), the small Ras-related GTPase Rac 1 (27), PKC-ζ (25), mitogen-activated protein kinase kinase (MEK) (25), and c-Jun NH2-terminal kinase (JNK) (25).

The cytoplasmic tail of CD5 is highly conserved and lacks intrinsic catalytic activity. It contains potential targets of intracellular protein kinases and sites of interaction with signaling mediators. In this respect, the association of the CD5 cytoplasmic tail has been shown for p56lck (28), Src homology 2 domain-bearing protein tyrosine phosphatase-1 (15), CKII (29, 30), ras GTPro-activating protein (ras GAP) (16), p116cbl (16), Tctex-1 (31), CaMK II (31), PI 3-K (32), and an as yet uncharacterized inducible kinase (33). CD5 is constitutively phosphorylated by CKII at a cluster of three C-terminal serine residues (S458, S459, and S461) (29) and undergoes rapid hyperphosphorylation on serine, threonine, and/or tyrosine residues following cell stimulation by phorbol esters (34), anti-CD3, or anti-CD5 mAbs (33, 35). The exact nature of both the kinases and the residues involved in the inducible hyperphosphorylation of CD5 remains to be fully deciphered. Previous data indicate that CD5 is hyperphosphorylated by the protein tyrosine kinases p59fyn (24), p56lck (24, 28), and Zap-70 (36), and by the protein serine/threonine kinases PKC (37) and CKII (17). The relevance of the membrane-proximal region of the CD5 cytoplasmic tail for CaMK IIβ association and PKC targeting has been shown...
Here we demonstrate that two threonine residues (T410 and T412) within this region are critical for PKC-mediated CD5 hyperphosphorylation in vivo. Furthermore, we show that substitution of T410 and T412 by alanine residues blocks the CD5-mediated DAG release as well as the inhibitory effects of phorbol esters on the Ab-induced internalization of CD5. This demonstrates that residues T410 and T412 are critical in lipid second-messenger generation and cytoskeletal interactions.

Materials and Methods

Chemicals and Abs

PMA and bisindolylmaleimide I (GF109203X) were purchased from Calbiochem (La Jolla, CA). Purified rat brain PKC (mixture of α, β, and γ isoforms) and human CKII (recombinant Sf9 cell product) enzymes were obtained from Upstate Biotechnology (Lake Placid, NY). Staurosporine and 3,3′-diaminobenzidine (DAB) were purchased from Sigma (St. Louis, MO). The mouse anti-CD5 mAb Cris-1 (IgG2a) was produced in our laboratory by R. Villela using PBMC as immunizing cells. Rabbit polyclonal antiserum against the extracellular and the intracytoplasmic domains of human CD5 were produced in our laboratory as previously described (38, 39).

Construction and expression of wild-type (WT) and cytoplasmic tail-mutant CD5 molecules

The constructs coding for WT, H449E, E418A, and K384E CD5 molecules were generated and cloned into the pEF-BP1-neo expression vector as previously reported (21, 29). The T410A-T412A and E418A/T410A-T412A constructions were similarly cloned and obtained by introducing T410A or T412A substitutions by inverse PCR mutagenesis with the sense/antisense oligonucleotide pairs 5′-CAACCGTCGGATCCCATGCT-3′ / 5′-CGGCGTGGTTGCGATGGAAAGACA-3′ and 5′-CCATGCT-3′ / 5′-CGGTGTTCCGGATGAGAACAG-3′, respectively. All oligonucleotide-directed changes were checked by double-stranded DNA sequencing (ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit; PE Applied Biosystems, Warrington, U.K.). Plasmid constructions were purified by cesium-chloride density gradients. The constructions were stably expressed in the CD5-deficient 2G5 Jurkat subclone by cell electroporation and selection for CD5 surface expression of neomycin-resistant cell clones (21).

Metabolic 32P labeling and PMA stimulation of CD5 transfectants

Cell labeling with 32P]orthophosphate was performed as previously reported (37). Briefly, cell transfectants were washed with phosphate-free medium (20 mM HEPES, pH 7.2, 10 mM dextrose, 1 mM CaCl2, 2 mM KCl, 150 mM NaCl, 1 mM MgCl2, 7 mM H2O2, 20 mM NaHCO3, 10% dialyzed FCS) and resuspended to a final density of 3 × 105 cells/well. Cells were preincubated for 30 min at 37°C and labeled with 0.5 mCi of [32P]orthophosphate for 3 h at 37°C. For phorbol ester stimulation, 100 ng/ml of PMA were added 30 min before the end of the 32P-labeling period. When needed, PKC-inhibitors GF109203X (5 μM) and staurosporine (250 mM) were added just prior stimulation with PMA. The radiolabeling was stopped by washing the cells with ice-cold PBS.

Immunoprecipitation and Western blot analysis

Immunoprecipitation was performed as previously described (37). Briefly, cells were disrupted with lysis buffer (1% Nonidet P-40, 10 mM Tris pH 7.6, 140 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.4 mM sodium orthovanadate, 10 mM iodoacetamide, 5 mM sodium pyrophosphate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml chymostatin, and 1 μg/ml of ε-antitrypsin) for 15 min on ice, and the insoluble fraction was discarded after 15 min of microcentrifugation at 4°C. The lysates were precleared with protein A-Sepharose and then immunoprecipitated with 1 μg/ml Cris-1 mAb plus Protein A-Sepharose for 1–2 h at 4°C. Beads were washed twice with lysis buffer, twice with lysis buffer plus 0.5 M NaCl, twice with lysis buffer plus 0.1% SDS, and twice with lysis buffer. Bound immune complexes were denatured by boiling in 2X reducing sample buffer and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA), which were next blocked for 30 min at 37°C with 5% nonfat dry milk powder in wash solution (PBS plus 0.1% Tween 20). Membranes were incubated at room temperature for 30 min with anti-CD5 polyclonal antiserum and, after extensive washing with wash solution, incubated at room temperature for 15 min with 1:800 peroxidase-conjugated protein G (Sigma). Colorimetric detection was performed by using DAB and H2O2 as peroxidase substrates.

In vitro PKC and CKII assays

For direct in vitro protein kinase assays, CD5 immunoprecipitates from 10 × 106 cells were suspended with 25 μl of a kinase buffer containing 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium vanadate, 1 mM DTT, 1 mM CaCl2, 15 mM MgCl2, 100 mM ATP, 2.5 μCi [γ-32P]ATP (Nuclear Iberica, Madrid, Spain). The in vitro kinase assays were conducted at 30°C for 10 min in the presence of purified PKC (10 ng) or CKII (50 ng) enzymes, with and without PKC lipid activators (25 μg phosphatidylserine and 0.25 μg diglyceride, respectively). The reaction was stopped by adding 500 μl of lysis buffer. Beads were washed twice with lysis buffer and suspended in 30 μl of 2X reducing sample buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose. 32P incorporation was detected by autoradiography of the membranes.

CD5 modulation assays

Monoclonal Ab-induced modulation and immunofluorescence assays were performed as previously reported (40). Cell transfectants were suspended for 30 min at 4°C in 100 μl of culture medium (RPMI 1640 plus 10% FCS) containing saturating amounts of Cris-1 mAb (1–10 μg/ml cells). After three washes with ice-cold PBS, cells were adjusted to 1 × 106 cells/ml with culture medium, distributed in a 24-well plate (1 ml/well), and incubated at 37°C in a humidified 5% CO2 atmosphere in the presence or absence of 100 ng/ml PMA. At the indicated times (0, 3, and 6 h), 200 μl of cell cultures were collected and washed in cold washing solution (PBS pH 7.2 containing 2% FCS and 0.1% sodium azide). Next, cells were stained for 30 min with 100 μl of a 1:100 dilution of FITC-conjugated anti-mouse polyvalent IgGs (Sigma). Cells were washed, fixed in washing solution containing 0.3% formaldehyde, and subjected to flow cytometry analysis in a FACScan (Becton Dickinson, San Jose, CA). Mean fluorescence intensity (MFI) was recorded and used in the calculation of percentage of anti-CD5 binding: (MFI of treated cells)/MFI of untreated cells) × 100%.

TLC analysis of membrane lipids

The analysis of DAG production was performed as previously described (21). Briefly, Jurkat cells were isotypically labeled by overnight incubation with [3H]palmitic acid and then stimulated for the indicated times (0, 1, 2, 5, and 10 min). Lipids were extracted from the cell pellets, and DAG was separated from triglycerides, cholesterol esters, and phospholipids on silica gel plates. Radioactive measurements were performed on an automatic linear thin layer radiochromatography scanner.

Results

PKC-dependent phosphorylation of CD5 involves the membrane-proximal region

Protein phosphorylation plays a key role in the regulation of both enzymatic activity and protein-protein interactions. The relevance of constitutive phosphorylation of C-terminal serine residues (S458, S459, and S461) on the signaling function of human CD5 has been recently reported (29). However, the residues responsible for the inducible phosphorylation of CD5 had not been determined to date. We have analyzed the phosphorylation levels of a series of CD5 cytoplasmic tail mutants (Fig. 1) following cell stimulation with the potent PKC activator PMA, a CD5 hyperphosphorylation inducer (34, 37). All the constructions were stably expressed on a previously reported CD5-deficient 2G5 Jurkat T cell subclone (21). Metabolically 32P-labeled 2G5 cell transfectants were incubated with or without PMA (100 ng/ml) for 30 min, and then CD5 molecules were immunoprecipitated from detergent-soluble cell fractions. Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and autoradiographed to show relative phosphorylation levels. Membranes were probed with a rabbit anti-CD5 polyclonal antiserum as a loading control. As shown in Fig. 2A, the analysis of WT and mutant human CD5 molecules lacking either 23 aa (H449mutp), 54 aa (E418mutp), or 88 aa (K384mutp)
cytoplasmic residues (Fig. 1) confirmed a major constitutive phosphorylation site at the C terminus of CD5 (29) and revealed PMA-inducible phosphorylation sites elsewhere. The most membrane-proximal CD5 cytoplasmic region (from K384 to E418) was the shortest region to undergo PMA-induced phosphorylation (Fig. 2A). This region encompasses two serines and three threonines, of which only two (T410 and T412) are within PKC phosphorylation consensus (41) (Fig. 1). Results in Fig. 2B show the successful inhibition of the PMA-induced phosphorylation of WT, H449stop, and E418stop molecules by the potent PKC inhibitor GF109203X. Similar results were obtained by using the PKC inhibitor staurosporine (data not shown). These observations involve PKC in the PMA-induced phosphorylation of the membrane-proximal CD5 cytoplasmic region.

Residues T410 and T412 are phosphorylated in vitro by PKC

To explore whether the membrane-proximal region is a direct substrate of PKC, we conducted in vitro kinase assays with purified enzyme preparations. In these experiments, CD5 immunoprecipitates from cell transfectants expressing WT and mutant CD5 molecules were incubated with purified PKC or CKII in the presence of \( \gamma-\)32P-ATP. The resulting products were separated by SDS-PAGE, transferred to nitrocellulose, and autoradiographed. Fig. 3A shows that both WT and truncated CD5 molecules (H449stop, and E418stop) can be phosphorylated in vitro by PKC. This is in agreement with the presence of consensus PKC phosphorylation sites on the most membrane-proximal cytoplasmic region of CD5 (Fig. 1). The lack of CKII phosphorylation of H449stop and E418stop indicates that the C-terminal region of CD5 is the only CKII substrate. It seems that residues S415 and S423, which are within CKII phosphorylation consensus motifs (Fig. 1), are not accessible residues to CKII-mediated phosphorylation.

To further narrow the region responsible for PKC phosphorylation we analyzed a series of CD5 mutants carrying either single or double amino acid substitutions (T410→A, T412→A) central to the two PKC motifs (Fig. 1). CD5 immunoprecipitates from cell transfectants expressing E418stop/T410A, E418stop/T412A, and E418stop/T410A-T412A were subjected to direct in vitro PKC phosphorylation. As shown in Fig. 3B, either of the two single amino acid substitutions (T410→A or T412→A) enable only partial phosphorylation of the membrane-proximal cytoplasmic region. The phosphorylation of this region was completely abolished in the mutant carrying double amino acid substitutions (Fig. 3B). These results indicate that both T410 and T412 are the only PKC targets within the membrane-proximal region of CD5.

T410A and T412A substitutions completely abrogate in vivo PMA-induced phosphorylation of full-length CD5 molecules

The sequence analysis of the CD5 cytoplasmic tail reveals three additional putative PKC phosphorylation motifs at S436, S439, and S452 (Fig. 1). Their contribution on PMA-induced phosphorylation was explored in vivo on cell transfectants expressing full-length and truncated molecules carrying double T410→A and T412→A substitutions (T410A-T412A, and E418stop/T410A-T412A, respectively) (Fig. 1). As shown in Fig. 4, the introduction of double T410→A and T412→A substitutions completely abrogated the inducible hyperphosphorylation of both full-length and truncated CD5 molecules. These results parallel those obtained with PKC inhibitors (see Fig. 2B). This demonstrates that T410 and T412 are critical for in vivo PMA-induced phosphorylation in addition to good in vitro PKC substrates. Interestingly, the mutant T410A-T412A molecules are efficiently phosphorylated in direct in vitro PKC assays (data not shown). These apparently conflicting in vivo and in vitro results indicate either that S436, S439, and S452 are not targets of PKC-dependent PMA-induced phosphorylation in vivo or that T410 and T412 phosphorylation must precede that of the serines.

T410 and T412 are relevant to CD5-mediated DAG release

CD5 signaling involves hydrolysis of PC to DAG and phosphocholine by activation of PC-PLC (21). Therefore, we explored the contribution of T410 and T412 to CD5 signaling by analyzing the generation of DAG on cell transfectants expressing WT or T410A-T412A molecules. We used three independent stable clones (21, 25, and 29) expressing T410A-T412A and CD3 molecules at surface levels similar to those of the WT transfectant (data not shown). As seen in Fig. 5, cell stimulation with the anti-CD5 Cris-I mAb do not induce significant DAG release in any of the T410A-T412A transfectant clones analyzed. Positive CD3 expression of these clones excluded CD3 deficiency as a possible cause. Stimulation of WT transfectants resulted in rapid and transient
DAG production, as previously reported (21). These results indicate that the membrane-proximal residues T410 and T412 are required for early biochemical events induced by ligation of the CD5 receptor, such as lipid second messenger generation.

T410 and T412 are potential regulatory sites of mAb-induced CD5 internalization

Ab-induced internalization of CD5 is regulated by mechanisms involving actin microfilament polymerization and PKC activation (40, 42). Given that T410 and T412 are PKC phosphorylated we investigated their involvement on the PMA-mediated inhibition of CD5 modulation. WT or mutant CD5 cell transfectants were incubated at 4°C with saturating concentrations of anti-CD5 mAb, washed, and then left at 37°C for different periods of time, either in the presence or absence of PMA. A secondary FITC-conjugated anti-mouse serum was used to monitor mAb-bound CD5 levels at each time point (0, 3, and 6 h). As shown in Fig. 6, PMA inhibited mAb-induced CD5 modulation of transfectants expressing WT and cytoplasmic tail-truncated (H449stop, E418stop, and K384stop) CD5 molecules. PMA had no inhibitory effects on the modulation of “almost tailless” CD5 molecules (K384stop) (Fig. 6). These results confirmed the membrane-proximal cytoplasmic segment as a regulatory region for CD5 internalization. Our results also describe the first nine cytoplasmic residues (K384stop) as sufficient for mAb-induced internalization of CD5. Interestingly, this region contains a tyrosine-based motif (Y378XXL) that has been involved in endocytosis and sorting of some membrane receptors (43–45).

Proof of T410 and T412 as regulatory sites for mAb-induced CD5 down-modulation came from the analysis of E418stop/T410A-T412A molecules. As shown in Fig. 6, the introduction of T410A and T412A substitutions rendered the CD5.E418stop molecule insensitive to the inhibitory effects of PMA on CD5 down-modulation. This indicates that the cytoskeleton reorganization induced by PMA interferes with CD5 down-modulation through residues T410 and T412 at the CD5 cytoplasmic tail.

Discussion

Lymphocyte activation is controlled by symbiotic interactions between the cytoskeleton and signaling pathways initiated by Ag receptors and costimulatory molecules (46). One such molecule is CD5, which has its own signaling pathway and modulates the activation and differentiation signals mediated by the Ag-specific receptor complex (2). The molecular basis of the immunomodulatory
properties of CD5 remains ill defined. The present work illustrates the functional relevance of the membrane-proximal cytoplasmic region of CD5, particularly residues T410 and T412, in intracellular signaling. Our results show that the integrity of these two residues is critical in at least three CD5-related signaling events: PKC-dependent hyperphosphorylation, lipid second messenger generation, and cytoskeletal interactions.

The whole cytoplasmic domain of CD5 is evolutionarily highly conserved among mammalian species (78% aa sequence homology), and this is specially true for its membrane-proximal region (82%). The functional relevance of this region is further supported by the recent identification of two intracellular proteins, namely, Tctex-1 and CaMK II\(^6\), which interact with a 33-aa peptide region containing T410 and T412. Tctex-1 is a dynein motor complex component (47), and CaMK II\(^6\) is a serine/threonine kinase involved in IL-2 down-regulation (48), implying a role in CD5 internalization and negative signaling, respectively. These interactions be confirmed experimentally, it would be interesting to explore the role of the two threonines in the binding to Tctex-1 and CaMK II\(^6\). Indeed, T410 is itself in a CaMK II consensus phosphorylation site (Fig. 1).

The PKC family of serine-threonine kinases are important signal transducers participating in many different agonist-induced signaling cascades (46, 49). One such cascade is that induced by Ag receptor triggering (50). Most PKC isoforms are activated by DAG increases upon translocation to the plasma membrane, where they modify various cell functions through phosphorylation of target substrates (49). The association with, and/or phosphorylation of, a wide range of cytoskeletal components has supported the idea that PKC is an important regulator of cytoskeletal function (49). Previous reports have shown that CD5 is a good PKC substrate (34, 37, 51) and that PKC-dependent mechanisms regulate CD5 internalization (40, 42). Here we have mapped these events to T410 and T412. Interestingly, the fact that double T410A and T412A substitutions completely abolish in vivo PMA-induced phosphorylation suggests that T410 and T412 are the only PKC substrates in vivo. This would mean that other serine residues present in PKC consensus motifs are not functional (Fig. 1). However, mutant CD5 molecules containing double threonine substitutions (T410A-T412A) are efficiently phosphorylated in vitro by PKC (data not shown). The latter is in agreement with reported serine phosphorylation following PMA stimulation by phosphoamino acid analyses (37, 51). Therefore, the possibility exists that early phosphorylation of T410 and T412 could induce conformational changes on CD5, making serine residues accessible to PKC phosphorylation. In fact, phosphorylation-dependent changes in the electrophoretic mobility of CD5 appear following PMA stimulation (37).

We previously reported that CD5 triggering activates signaling including the generation of lipid second messengers, namely DAG and ceramide (21, 25). This is achieved through the coordinated activation of PC-PLC and A-SMase, respectively (21, 25). In this work, we show that T410→A and T412→A double substitution prevents CD5-mediated DAG release. Similar deleterious effects on DAG production have been reported for CD5 mutants carrying alanine substitutions at the C-terminal S459 and S461 residues (29). Contrary to this, an alanine substitution of the C-terminal Y463 was neutral to PC-PLC activation (29). Therefore, the integrity of residues responsible for the constitutive (S458, S459, and S461) and the inducible (T410 and T412) phosphorylation of CD5 is necessary for CD5-mediated lipid second messenger generation. This indicates that phosphorylation-related events may play an important role in coupling CD5 to the PC-PLC/A-SMase signaling pathway. Additionally, binding of Tctex-1 to CD5 may also assist in this pathway. A-SMase hydrolyzes sphingomyelin exclusively in acidic environments such as the endo/lysosomal compartment. Thus, attachment to the dynein complex through Tctex-1 may result in the rapid intracellular transport of CD5-containing vesicles to lysosomes.

In addition to Tctex-1 binding, current evidence for the interaction between CD5 and the cytoskeleton is only indirect. First, CD5 resides, at least in part, in the detergent-insoluble glycolipid-enriched raft membrane fraction, and its coligation with CD3 enhances the association of the Ag receptor with rafts (8). Rafts serve as platforms for signal transduction and membrane trafficking as they are enriched in signal transduction molecules, actin, and actin-binding proteins (52). Second, signaling through CD5 activates a pathway involving PI 3-K, vav, and Rac1 (27). The products of

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**FIGURE 4.** In vivo PMA-induced phosphorylation of CD5 is abolished by T410→A and T412→A double substitutions. Equivalent cell samples of \(^3\)P-labeled 2G5 unmutated or doubly mutated full-length (WT and T410A-T412A, respectively) and cytoplasmic tail-truncated (E418stop and E418stop/T410A-T412A, respectively) CD5 transfectants were stimulated for 30 min in the presence (+) or absence (−) of 100 ng/ml PMA. Detergent-soluble cell lysates were subjected to CD5 immunoprecipitation with Cris-1 mAb adsorbed to protein A-Sepharose beads. Immune complexes were resolved by 8% SDS-PAGE and transferred to nitrocellulose. The filter was autoradiographed (top) and developed with anti-CD5 polyclonal Ab plus peroxidase-labeled protein G and a DAB/metal solution (bottom).

**FIGURE 5.** Relevance of T410 and T412 in CD5-mediated DAG release. \(\text{[^{3}H]Palmic acid-labeled Jurkat 2G5 cell unmutated or doubly mutated full-length CD5 transfectants (WT and T410A-T412A, respectively) were stimulated with 10 \mu\text{g/ml of the anti-CD5 Cris-1 mAb for the indicated times.}

Following cell extraction, phospholipids were analyzed by TLC, and the radioactivity incorporated into DAG was analyzed. Values are expressed as percent change from unstimulated control cells. Each point is the mean ± SD of triplicate determinations.
expression.

PMA (thick solid line). The dotted line represents basal CD5 surface expression of PMA (100 ng/ml). At the indicated times, cells were stained with FITC-labeled anti-CD5 Cris-1 mAb. After incubation in ice with saturating amounts of the anti-CD5 Cris-1 mAb. After the indicated time, cells were stained with FITC-labeled anti-CD5 Ig and analyzed by flow cytometry. A. Fluorescence histograms showing CD5 cell surface expression following a 6-h incubation with anti-CD5 mAb, either alone (thick solid line) or in the presence of PMA (thick solid line). The dotted line represents basal CD5 surface expression. B. Time course experiments on anti-CD5 Ab-modulated cell transfectants. The experiment shown is representative of three performed.

PI 3-K, together with tyrosine phosphorylation, activate vav, resulting in activation of Rho family GTPases such as Rac1, Rho, and Cdc42 (46). These GTPases control the dynamic organization of the actin cytoskeleton (46). Third, cytoskeleton associations exist between some leukocyte surface Ags after specific ligand binding or cross-linking by bivalent Abs (53). Accordingly, Ab-induced internalization of the TCR/CD3-dependent and -independent transduction pathway: involvement of SH2-containing phosphotyrosine phosphatase SHP-1. Mol. Cell. Biol. 19:2903.

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References

CD5 SIGNALING VIA T410 AND T412


