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Signaling Through CD5 Involves Acidic Sphingomyelinase, Protein Kinase C-ζ, Mitogen-Activated Protein Kinase Kinase, and c-Jun NH₂-Terminal Kinase

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The CD5 lymphocyte surface glycoprotein is a coreceptor involved in the modulation of Ag-specific receptor-mediated activation and differentiation signals. The molecular basis for its modulatory properties is not yet well understood. In the present study we describe early biochemical events triggered by CD5 stimulation, which include the phosphatidylinositol-specific phospholipase C (PC-PLC)-dependent activation of acidic sphingomyelinase (A-SMase) in normal and lymphoblastoid T and B cells. The functional coupling of PC-PLC and A-SMase is demonstrated by the abrogation of A-SMase activation by 1) xanthogenate tricyclodecan-9-yl (D609), a selective inhibitor of PC-PLC, and 2) replacement of several C-terminal serine residues (S458, S459, and S461) present in the cytoplasmic tail of CD5 that are known to be critical for PC-PLC activation. Additionally, we demonstrate that activation of protein kinase C-ζ (PKC-ζ) and members of the mitogen-activated protein kinase (MAPK) cascade (MAPK kinase and c-Jun NH₂-terminal kinase), but not the NF-xB, are downstream events of the CD5 signaling pathway. A-SMase, PKC-ζ, and MAPK family members are key mediators of cell responses as diverse as proliferation, differentiation, and growth arrest and may contribute to CD5-mediated modulation of TCR or BCR signaling. The Journal of Immunology, 1999, 162: 5149–5155.

The CD5 Ag is a monomeric 67-kDa glycoprotein that appears early during thymocyte development and is expressed on all mature T lymphocytes and a small subset of peripheral B lymphocytes (B1a) (1). CD5 belongs to the scavenger receptor cysteine-rich family of extracellular domain-like structures (2) and possesses a large cytoplasmic domain that contains potential tyrosine and serine/threonine kinase phosphorylation motifs compatible with a function in signal transduction (3). The physical association between CD5 and the Ag receptor on both T and B1a cells has been demonstrated (4, 5). Although the ultimate function of the CD5 Ag remains unknown, earlier studies with anti-CD5 mAb pointed to a costimulatory role in TCR-mediated signal transduction (6–9). Recent studies with CD5−/− mice suggest that CD5 may negatively regulate Ag receptor-mediated signaling in thymocytes and B1a cells (10, 11).

It has been shown that CD5 acts as a receptor and/or substrate for the protein tyrosine kinases p59Fyn, p56lck (12), and ZAP-70 (13) and for the protein serine/threonine kinases casein kinase II (CKII) (14, 15) and Ca²⁺/calmodulin-dependent kinase (CaMK) IIδ (16). Early reports showed that anti-CD5 mAb augment the intracellular Ca²⁺, inositol triphosphate, IL-2 secretion, and IL-2R expression induced by anti-CD3 mAbs (6–8). Recently, phosphatidylinositol 3-kinase (PI 3-K), Vav, Rac1, Ras GTPase-activating protein, c-Cbl, and CaMK type IV have been reported to be involved in the CD5 signaling pathway in human T lymphocytes (17–20). The relevance of these associations for the physiological function of CD5 is not well defined. Interestingly, data from CD5−/− mice have shown hyper-reactivity of thymocytes associated with increased tyrosine phosphorylation of various proteins (Vav, ZAP-70, PLC-1γ, and CD3ζ) in response to CD3 stimulation (10). Consistently, a preliminary study indicates that CD5 could associate with the protein tyrosine phosphatase 1C in thymocytes (21).

We have previously shown that some anti-CD5 mAbs induce protein tyrosine phosphorylation and phosphoinositide turnover and Ca²⁺-independent protein kinase C (PKC) activation (9). The latter is achieved through diacylglycerol (DAG), which is itself the result of both phosphatidylinositol-specific phospholipase C (PC-PLC) activation and de novo synthesis (22). This indicates that CD5 is an accessory molecule with its own signaling pathway, which evokes that used by some cytokine and cell surface receptors (TNF receptor I (TNFRI), CD95/Apo-1/Flas ligand, CD28, and IL-1R) to modulate lymphocyte activation (22). The DAG generated by PC-PLC has been implicated in the activation of acidic sphingomyelinase (A-SMase) (23, 24). The resulting sphingomyelin (SM) metabolites (ceramide) have been implicated in the activation of other downstream signaling elements (PKC-ζ, NF-κB,

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3 Abbreviations used in this paper: CKII, casein kinase II; CaMK, Ca²⁺/calmodulin-dependent kinase; PI 3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; DAG, 1,2-diacylglycerol; PC-PLC, phosphatidylinositol-specific phospholipase C; TNFRI, TNF receptor I; A-SMase, acidic sphingomyelinase; SM, sphingomyelin; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; INK, c-Jun NH₂-terminal kinase; D609, xanthogenate tricyclodecan-9-yl; GAMMe, goat antiserum to mouse Ig; ERK, extracellular signal-regulated kinase.
CD5.H449 stop ) are named according to the amino acid where a premature stop codon was introduced and its position in the wild-type sequence. CD5 mutants carrying single-amino acid substitutions (CD5.S459A, S461G, and CD5.Y463A) are named by the inserted amino in single-letter code.

... etc.) and the modulation of the pleiotropic cellular effects (from cell proliferation and differentiation to growth arrest and apoptosis) induced by a number of extracellular inducers (25). In this study we demonstrate that the CD5 signaling pathway involves PC-PLC-dependent A-SMase activation as well as the activation of PKC-ζ and members of the mitogen-activated protein kinase (MAPK) cascade, namely MAPK kinase (MEK) and c-Jun NH2-terminal kinase (JNK), in the absence of NF-κB transcriptional activity. The relevance of these findings in the context of the modulatory effects of CD5 on lymphocyte activation is discussed.

Materials and Methods

Chemicals

PMA and wortmannin were obtained from Sigma (St. Louis, MO). The xanthogenate tricyclodecan-9-yl (D609) and the calcium ionophore A23187 were purchased from Calbiochem (La Jolla, CA).

Antibodies

All mAbs were of mouse origin and were clustered in the International Workshops on Human Leukocyte Differentiation Antigens. The F145-6F3 (CD5, IgG1) and A50 (CD5, IgG2a) mAbs were gifts from D. Carrière (Sanofi Recherche, Montpellier, France) and A. Bernard (Institut National de la Santé et de la Recherche Médicale, U343, Nice, France), respectively. Cris-1 (CD5, IgG2a) and 152-2E10 (CD28, IgG1) were produced in our laboratory by R. Vilella. The OKT3 (CD3) and Leu 1 (CD5) mAbs were obtained from American Type Culture Collection (Manassas, VA). The rabbit polyclonal anti-PKC-ζ was raised against the last 16 amino acids. The Leu 19 (CD56) mAb was purchased from Becton Dickinson (Mountain View, CA). Goat antiserum to mouse Ig (GAM Ig) was obtained from Dako (Copenhagen, Denmark). Noncommercial mAbs were produced as ascites fluid and affinity purified on protein A-Sepharose. The preparations were >90% pure as assessed by SDS-PAGE.

Cells and transfectants

Jurkat J6.1 cells were obtained from American Type Culture Collection. The isolation and the phenotypic characteristics of the CD5+ Jurkat 2G5 clone have been previously reported (22). Thymocytes were obtained by teasing apart thymus specimens that had been removed from children undergoing cardiac surgery. Human PBMC were obtained by Ficoll-Hypaque density gradient centrifugation. B-CLL cells with >95% purity were obtained after two rounds of complement-mediated cell lysis of PBMC with anti-CD3, -CD56, and -CD14 mAbs.

Construction of cytoplasmic tail mutant CD5 molecules and transfection conditions

The procedure for the generation of constructs coding for wild-type and cytoplasmic tail mutant CD5 molecules (see Fig. 1) has been previously reported (15). All plasmid constructions were checked by dsDNA sequencing in an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Foster City, CA) and were purified by cesium chloride density gradients. The constructs were stably expressed in 2G5 Jurkat cells by cell electroporation and selection for CD5 surface expression of neomycin-resistant cell clones (22).

Assays for neutral and acidic SMases

At the indicated times, stimulation of the cells was stopped by immersion in a methanol-dry ice bath. Cells were centrifuged for 5 min at 4°C and washed with ice-cold PBS. Neutral and acidic SMase activities were measured and solubilized by monitoring the amount of phosphotidylcholine produced from exogenous [14C]phosphatidylcholine in a tricine micellar assay system (23).

Assay of PKC-ζ activity

Cell samples (20 × 10^6 cells) were incubated for different times with anti-CD5 mAb (20 μg/ml) and then lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM orthovanadate, and 1 mM PMSF. Equivalent lysate samples (200 μg of total protein) were immunoprecipitated for 2 h with an affinity-purified rabbit polyclonal Ab against PKC-ζ plus protein A-Sepharose. Immune complexes were extensively washed with lysate buffer containing 0.5 M NaCl and then incubated (20 min at 30°C) in 20 μl of kinase buffer (35 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.5 mM EDTA, 0.1 mM CaCl2, and 0.1 mM phenylphosphate) containing 2 μg of myelin basic protein and 5–10 μCi (100 μM) of [γ-32P]ATP. Reactions were stopped by the addition of SDS sample buffer and were separated by SDS-PAGE. The gels were autoradiographed, and the relative densities of the bands were quantitated with a FluorS-MultiImager (Bio-Rad, Hercules, CA) and MultiAnalyst software.

NF-κB-dependent promoter activity assay

Cell samples (10^7 cells) were transfected by electroporation with 100 ng of 3EconAluc plasmid (26). The power settings were 960 V and 220 V. After transfection, cells were left in culture (at 1 × 10^6 cells/ml) for 16 h with RPMI medium supplemented with 10% FCS. Transfected cells were then incubated with different stimuli for 6 h. Total cell extracts were prepared in a lysis buffer, and samples were analyzed in a luminometer. Data are expressed in terms of relative luciferase activity units.

Assay of MEK and JNK activities

Extracts from 10^7 stimulated Jurkat cells were immunoprecipitated with polyclonal anti-MEK-1 or anti-JNK Abs (Santa Cruz Biotechnology, Santa Cruz, CA) plus protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitates were incubated for 20 min at 30°C with 2 μg of recombinant ERK2-GST or c-Jun-GST proteins in kinase assay buffer containing 5–10 μCi of [γ-32P]ATP. The assay buffer for MEK was 75 mM β-glycerolphosphate (pH 7.3), 3.75 mM EGTA, 0.15 mM orthovanadate, 1.5 mM DTT, 30 μM calmidazolium, 30 mM MgCl2, and 0.3 mM ATP. The assay buffer for JNK was 20 mM MOPS (pH 7.2), 2 mM EGTA, 10

FIGURE 1. Schematic representation of wild-type and cytoplasmic tail-mutated CD5 molecules used in this study. CD5 truncation mutants (CD5.K384<sup>stop</sup> and CD5.H449<sup>stop</sup>) are named according to the amino acid where a premature stop codon was introduced and its position in the wild-type sequence. CD5 mutants carrying single-amino acid substitutions (CD5.S459A, S461G, and CD5.Y463A) are named by the inserted amino in single-letter code.
mM MgCl₂, 1 mM DTT, and 0.1% Triton X-100. The reaction was stopped with SDS sample buffer and then run on SDS-PAGE. The gels were autoradiographed, and the relative densities of the bands were quantitated as indicated above.

Results

Activation of A-SMase following CD5 cross-linking

Various studies have shown that DAG generated via PC-PLC activation serves as an important factor for A-SMase activation (23, 24) and the subsequent generation of ceramide, a relevant lipid messenger intermediate. Since CD5 engagement mediates PC-PLC activation in Jurkat cells (22), we explored whether CD5 triggering couples to increased A-SMase activity. For this purpose, Jurkat cells were stimulated for different times with identical amounts (10 μg/ml) of mAbs directed against CD5 and other surface receptors (CD3 and CD28). The A-SMase activity was measured in cell solubilizes following a previously reported micellar assay with exogenous radiolabeled SM (23). CD5 stimulation of Jurkat cells resulted in a rapid and transient increase in A-SMase activity regardless of the anti-CD5 mAb (Cris-1, A50, or F145-GF3) used (data not shown). As illustrated in Fig. 2A, this increase reached maximal values 1–2 min after stimulation and declined to basal levels by 5 min. This kinetics closely paralleled those of CD5-mediated DAG production (22). As previously reported, CD28 stimulation resulted in A-SMase activation, and CD3 triggering failed to induce the activation of A-SMase (27). Human PBMC and thymocytes were also assayed for CD5-mediated A-SMase activity. In PBMC the CD5-mediated A-SMase activity was relatively low, but was greatly improved by the addition of secondary cross-linking Abs (Fig. 2B). In thymocytes, the CD5-induced A-SMase activity was detectable only under cross-linking conditions with GAM Ig (Fig. 2B). Similar results were obtained when purified B-CLL cells were subjected to CD5 cross-linking (Fig. 2C). These results indicate that CD5-mediated A-SMase activity is a property not only of lymphoblastoid cells but also of normal cells. Notably, the activation of neutral SMase was explored on CD5 cross-linked normal and leukemic cells, and we could not detect products of SM breakdown (data not shown).

Functional coupling of CD5-mediated PC-PLC and A-SMase activation

The need for PC-PLC activation for CD5-mediated A-SMase activity was explored by using the xanthogenc tricyclodecan-9-yl (D609), which is an specific inhibitor of PC-PLC (28). As shown in Fig. 3A, pretreatment of Jurkat cells with D609 (50 μg/ml) resulted in a complete blockade of CD5-mediated A-SMase activation. A-SMase activity was unaffected by pretreatment of Jurkat cells with wortmannin, a PI 3-kinase inhibitor. These findings indicate that CD5-induced activation of PC-PLC probably couples to the production of the second messenger ceramide by activation of A-SMase. To further explore this functional coupling we mapped the CD5 cytoplasmic region involved in A-SMase activation by using Jurkat 2G5 (CD5⁺) cell transfectants stably expressing cytoplasmic tail mutant CD5 molecules (Fig. 1). As expected, the analysis of transfectants expressing wild-type CD5 (CD5.WT) showed that they display the ability to activate A-SMase (Fig. 3B), with kinetics and magnitude similar to those of Jurkat JE6.1 cells (see Fig. 2A). In contrast, 2G5 transfectants expressing CD5 molecules devoid of either most of their cytoplasmic domain (CD5.K384mut) or only a C-terminal 23-amino acid region (CD5.H449mut) failed to induce A-SMase activity. This is in line with our previous findings on PC-PLC activation (22) and indicates that the C-terminal 23-amino acid region is not only necessary but is also sufficient to activate the A-SMase pathway. To further narrow this region we analyzed CD5 mutant molecules carrying either single (Y463→A) or double (S459→A and S461→G) amino acid substitutions (CD5.Y463A and CD5.S459A,S461G, respectively). The Y463 residue is phosphorylated in response to CD3 and CD5 cross-linking (our unpublished observations), and this may allow src homology domain 2-containing proteins to bind to and subsequently activate the PC-PLC/A-SMase pathway. The S459 and S461 residues are the sites for CKII-mediated constitutive phosphorylation of CD5 and are critical for CD5-mediated PC-PLC activation (15). As shown in Fig. 3B, the CD5.Y463A transfectant retained its ability to signal CD5-dependent A-SMase activity, although it was reduced compared with that of CD5.WT. In contrast, the CD5.S459A,S461G transfectant

![FIGURE 2.](http://www.jimmunol.org/DownloadedFrom/)
CD5-MEDIATED ACTIVATION OF A-SMase, PKC-ζ, MEK, AND JNK

A-SMase pathway (see above). Consistently, the presence of D609 did not abolish the CD5-induced PKC-ζ activation. These findings indicate that other upstream biochemical events are involved in CD5-induced PKC-ζ activation.

CD5 signaling involves activation of MEK and JNK, but not of NF-κB

Ceramide and PKC-ζ have been shown to be critically involved in the activation of NF-κB and some members of the MAPK family (25). To determine whether CD5 triggering leads to activation of NF-κB, we stimulated Jurkat JE6.1 cells transfected with a luciferase reporter plasmid harboring three copies of the κB enhancer from the long terminal repeat of HIV (26). Our results show that CD5 stimulation, either alone or in combination with CD3 and CD28 stimulation, does not result in κB-dependent promoter activity (Fig. 5). Similarly, CD5 stimulation does not increase the κB-dependent promoter activity induced by PMA plus the calcium ionophore A23187 (Fig. 5). Identical results were obtained regardless of the anti-CD5 mAb used (data not shown). As expected from previous reports, CD28 stimulation of Jurkat cells did not result in NF-κB activation, either alone or in combination with CD3 stimulation, but clearly increased the NF-κB activation induced by PMA plus A23187 (27).

The involvement of members of the MAPK cascades in CD5 signaling was investigated by analyzing the activities of MEK and

was completely defective in signaling CD5-mediated A-SMase activation. These results indicate that S459 and S461, but not Y463, are critical residues for CD5-mediated A-SMase activation.

**CD5 signaling involves PKC-ζ activation**

Ceramide generated by SMase activation has been reported to be a positive regulator of PKC-ζ activity (29, 30). This fact prompted us to analyze whether PKC-ζ is also involved in CD5 signaling. Jurkat cells were stimulated for different times with the anti-CD5 Cris-1 mAb, and PKC-ζ was immunoprecipitated from the extracts and assayed for in vitro kinase activity. As shown in Fig. 4A, CD5 triggering increased the basal activity of PKC-ζ in Jurkat JE6.1 cells. The activity peaked at 5 min and declined by 10 min. Stimulation of transfectants carrying CD5.WT induced a CD5-dependent activation of PKC-ζ, with similar kinetics (not shown). These kinetics are compatible with a model in which the kinase assays on different Jurkat 2G5 cell transfectants. The analysis of transfectants expressing CD5.H449stop, which is missing the C-terminal region critical for A-SMase activation, also showed PKC-ζ activation similar to that found in JE6.1 cells and CD5.WT transfectants (Fig. 4B). This indicates that the C-terminal cytoplasmic region of CD5 involved in PC-PLC/A-SMase activation is dispensable to CD5-induced activation of PKC-ζ. This was further supported by the analysis of CD5.WT transfectants pretreated with D609 at doses (50 μg/ml) that completely inhibited the PC-PLC/A-SMase pathway (peaks at 2 min). These kinetics are compatible with a model in which the PKC-ζ triggered by Cris-1 mAb, and PKC-ζ was immunoprecipitated. Immune complexes were assayed for kinase activity over 21-kDa myelin basic protein and then separated by SDS/12% PAGE. Autoradiographs and band densitometric analysis are shown.

**FIGURE 3.** Functional coupling of PC-PLC and A-SMase activation following CD5 stimulation. A, Jurkat cells were either untreated or were preincubated with D609 (50 μg/ml) or wortmannin (100 nM) before the exposure to Cris-1 (at 10 μg/ml). B, Jurkat 2G5 cell transfectants stably expressing CD5.WT, CD5.K384stop, CD5.H449stop, CD5.S459A/S461G, and CD5.Y463A molecules were stimulated with 10 μg/ml Cris-1, and A-SMase activity was measured at the indicated times. Results are expressed as the percent change from control cells. Each point is the mean ± SD of a representative experiment of at least three others performed.

**FIGURE 4.** Activation of PKC-ζ following CD5 stimulation. A, JE6.1 cells were incubated at 37°C in the presence 10 μg/ml of Cris-1 mAb (+) or GAM Ig (−). At the indicated times, cells were lysed, and PKC-ζ was immunoprecipitated. Immune complexes were assayed for kinase activity over 21-kDa myelin basic protein and then separated by SDS/12% PAGE. Autoradiographs and band densitometric analysis are shown. B, Assay of PKC-ζ activity in lysates from CD5.WT, CD5.K384stop, and CD5.H449stop transfectants stimulated with 10 μg/ml Cris-1 mAb for the indicated times. Before CD5 stimulation, the CD5.WT transfectants were pretreated for 1 h with 50 μg/ml D609 (CD5.WT+D609). Results are representative of three experiments performed.

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To further address the role played by the PC-PLC/A-SMase pathway in the activation of PKC-ζ, we performed kinase assays on different Jurkat 2G5 cell transfectants. The analysis of transfectants expressing CD5.H449stop, which is missing the C-terminal region critical for A-SMase activation, also showed PKC-ζ activation similar to that found in JE6.1 cells and CD5.WT transfectants (Fig. 4B). This indicates that the C-terminal cytoplasmic region of CD5 involved in PC-PLC/A-SMase activation is dispensable to CD5-induced activation of PKC-ζ. This was further supported by the analysis of CD5.WT transfectants pretreated with D609 at doses (50 μg/ml) that completely inhibited the PC-PLC/A-SMase pathway (peaks at 2 min). These kinetics are compatible with a model in which the PKC-ζ triggered by Cris-1 mAb, and PKC-ζ was immunoprecipitated. Immune complexes were assayed for kinase activity over 21-kDa myelin basic protein and then separated by SDS/12% PAGE. Autoradiographs and band densitometric analysis are shown.

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Before CD5 stimulation, the CD5.WT transfectants were pretreated for 1 h with 10 μg/ml D609 (CD5.WT+D609). Results are representative of three experiments performed.

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With this aim, in vitro kinase assays over recombinant extracellular signal-regulated kinase-2 (ERK2) and c-Jun were performed on anti-MEK-1 and anti-JNK-1 immunoprecipitates from lysates of stimulated Jurkat cells. As shown in Fig. 6A, CD5 stimulation resulted in ERK2 phosphorylation, which was maximal at 5 min and declined at 15 min. Stimulation of Jurkat cells with PMA (10 ng/ml) for 5 min served as a positive control. Similarly, increased JNK activity was observed following CD5 stimulation, either alone or in combination with simultaneous CD3 stimulation (Fig. 6B). Taken together, these results indicate that different MAPK cascades are involved in the CD5-induced signaling pathway.

**Discussion**

In this report we have examined early biochemical events induced by ligation of the CD5 coreceptor. We show that CD5 stimulation causes the activation of a signaling cascade that includes PC-PLC/A-SMase, PKC-\(\zeta\), and members of the MAPK family, namely MEK and JNK. This represents the first reported evidence for the involvement of these enzymatic activities in the CD5 signaling pathway. The CD5-mediated activation of A-SMase and PKC-\(\zeta\) reported here is consistent with our earlier finding that CD5 triggering induces phosphoinositide turnover- and \(Ca^{2+}\)-independent PKC activation (9), as well as DAG release via PC-PLC and de novo synthesis (22). DAG generated through PC-PLC is known to trigger activation of A-SMase (23, 24). In turn, ceramide generated by A-SMase contributes to the activation of both PKC-\(\zeta\) (29, 30), a \(Ca^{2+}\)-, DAG-, and phorbol ester-independent atypical PKC isoform, and members of the MAPK cascades (25). Because TCR/CD3 triggering does not induce PC-PLC and A-SMase activation, our data confirm CD5 as a signal transducing molecule with its own second messenger cascade that collaborates with the TCR/CD3 complex in eliciting optimal T cell responses.

Ceramide accumulation has been reported for a number of extracellular inducers, including 1,25-dihydroxyvitamin D\(_3\), TNF-\(\alpha\), endotoxin, IFN-\(\gamma\), IL-1, Fas ligands, CD28, dexamethasone, retinoic acid, progesterone, ionizing irradiation, chemotherapeutic agents, heat, and nerve growth factor (25). From now on, CD5 should be added to this list. Ceramide appears to be involved in the diverse outcomes (from cell proliferation and differentiation to apoptosis and growth arrest) reported for some of these inducers (25). Ceramide can be generated by the activation of two forms of sphingomyelinases, a membrane-bound neutral SMase and a DAG-dependent endo/lysosomal A-SMase (23). These two forms are triggered independently from each other and lead to different signaling pathways (23). The simultaneous, albeit independent, activation of both A-SMase and N-SMase has been reported following engagement of CD95, TNF receptor I (TNFRI), and IL-1R (23, 24, 31). The activation of A-SMase in the absence of neutral SMase activation has been reported for CD28 (27) and is also shown here for CD5. This indicates that signaling through the CD5 and CD28 receptors differ from that of other inducers of ceramide release.
The coupling of PC-PLC and A-SMase activation has been reported for the signaling pathways of TNFRI, CD95, and IL-1R (23, 24, 31), but not other receptors that also induce PC-PLC activation, such as IL-4R (32). The data presented here point to the functional coupling of these two phospholipases in the CD5 signaling pathway: 1) the kinetics of CD5-mediated A-SMase activation closely parallel those of DAG production (22); 2) D609, a specific inhibitor of PC-PLC, completely abrogates CD5-mediated A-SMase activation; and 3) the CD5 cytoplasmic residues critical for A-SMase activation are the same as those previously reported for PC-PLC activation (15, 22). In this regard, it is surprising that only partial effects in abrogating PC-PLC/A-SMase activation induced by Y463 replacement are found compared with the dramatic effects seen after replacement of the C-terminal serine cluster (S458, S459, and S461). It can be hypothesized that constitutive phosphorylation of serine residues by CKII (14, 15) provides the C-terminal region of CD5 with the optimal conformation for PC-PLC/A-SMase activation to proceed. The inducible phosphorylation of Y463 may also assist in optimizing this activation.

Our data indicate that PKC-ζ activation following CD5 engagement is not fully dependent on PC-PLC/A-SMase activation. PKC-ζ activation can still be achieved either in the presence of D609 or by CD5 molecules lacking the C-terminal region critical for PC-PLC/A-SMase activation. This is not surprising, since the remarkable complexity of PKC-ζ regulation is achieved through a delicate balance involving different modulators: 1) ceramide (29, 30), PI 3-K (33), and phosphatidic acid (34), which activate PKC-ζ; and 2) arachidonic acid (29) and the product of the par-4 gene (35), which inhibit PKC-ζ. Therefore, the identification of upstream activators of PKC-ζ different from ceramide should be investigated in the CD5 signaling pathway. Although controversial, the recently reported involvement of PI 3-K in signaling through CD5 (18, 20, 36) could be claimed as an alternative mechanism for PKC-ζ activation. This would involve a CD5 cytoplasmic region different from the C-terminal and, very likely, encompassing the immunoreceptor tyrosine-based activation motif-like motif (18). Based on its bifunctional modulation by lipid second messengers, it has been suggested that PKC-ζ acts as a molecular switch between mitogenic and apoptotic signals (29). However, the role of PKC-ζ in T cell activation remains controversial. PKC-ζ has been reported to control IL-2-mediated T cell proliferation and cytoskeleton organization (37). Experiments in oocytes and fibroblasts have suggested a role for PKC-ζ in NF-κB induction and mitogenic signals (26, 38), but the expression of constitutively active PKC-ζ or the overexpression of PKC-ζwt was not sufficient for NF-κB, AP-1, and NF-AT-1 trans-activation in T cells (39). This could reflect the fact that the mechanisms to regulate the activity of these transcriptional factors in T cells and fibroblast differ. In the present study we could not detect NF-κB-dependent transcriptional activity following CD5 stimulation of Jurkat cells. However, we detected MEK activity, another event downstream of PKC-ζ (26).

It has been reported that the CD5 signaling pathway up-regulates the DNA binding activity of AP-1 but not that of NF-κB (17). AP-1 is a heterodimer composed of different Fos and Jun family members that can bind to the IL-2 promoter alone or complexed with NF-AT. The AP-1 activity is regulated at both transcriptional and post-translational levels by members of the MAPK family (JNK, ERK, and p38), the Janus kinase family, CaMK, and protein kinase A. Based on results obtained with specific kinase inhibitors, the above-mentioned report claims that CaMK IV, but not the MAP kinases ERK, JNK, and p38, play a role in the CD5-mediated induction of AP-1 activity (17). This contradicts our results and the previously reported ability of CaMK IV to activate JNK, ERK, and p38 (40). Furthermore, JNK and p38 are downstream effectors of Rac1, which have been recently reported to be essential to the CD5 costimulatory signal pathway leading to up-regulation of the IL-2 promoter activity (20). In fact, we show that JNK is activated during CD5 stimulation of Jurkat cells. Ceramide, either directly or indirectly, is known to activate JNK, thereby affecting the activity of c-Jun (25), and its possible contribution should be taken into consideration. The reported conflicting results may reflect the use of different cell systems (purified peripheral T cells vs Jurkat T cells), stimulation conditions (PHA, anti-CD28, and anti-CD5 vs anti-CD5 alone), and parameters to evaluate kinase activity (IL-2 secretion or IL-2 promoter activity vs in vitro kinase assays).

The lymphocyte surface receptor CD5, first considered as a costimulatory molecule in mature T cells, is now known to also be a negative regulator of Ag receptor-mediated signals in thymocytes and B1a cells (10, 11). Therefore, CD5 seems to behave as a dual receptor, providing either positive or negative signals depending on the cell type and the maturational stage. The CD5 signaling pathway is only beginning to be understood, and the molecular basis for its ability to modulate TCR and BCR signals has not yet been defined. The work reported here shows that activation of A-SMase, PKC-ζ, and MAPK cascade members are components of the intracellular signaling pathway initiated by CD5 ligation. These enzymatic activities are triggered by a number of cell receptors and have been largely implicated as key elements in the control of cellular processes as diverse as differentiation, proliferation, and apoptosis (25). The final outcome of these enzyme activities depends on factors such as the cell type and their integration with other signals originating from the same receptor or other receptors concurrently engaged. Thus, the involvement of A-SMase, PKC-ζ, and MAPK family members in the CD5 signaling pathway could also be on the basis of the modulatory effects of CD5 on lymphocyte activation and differentiation. The dynamic balance between the opposing effects attributed to the ERK and JNK pathways (41) may be of special relevance.

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