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A New Pathway of CD5 Glycoprotein-mediated T Cell Inhibition Dependent on Inhibitory Phosphorylation of Fyn Kinase*[S]

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Triggering of the T cell receptor initiates a signaling cascade resulting in the activation of the T cell. These signals are integrated alongside those resulting from the triggering of other receptors whose function is to modulate the overall response. CD5 is an immunotyrosine-based inhibition motif-bearing receptor that antagonizes the overt T cell receptor activation response by recruiting inhibitory intracellular mediators such as SHP-1, RasGAP, or Cbl. We now propose that the inhibitory effects of CD5 are also mediated by a parallel pathway that functions at the level of inhibition of Fyn, a kinase generally associated with T cell receptor-mediated activation. After CD5 ligation, phosphorylation of the negative regulatory tyrosine (Tyr^{531}) of Fyn increases, and this correlates with a substantial reduction in the kinase activity of Fyn and a profound inhibition of ZAP-70 activation. The effect requires the last 23 amino acids of the cytoplasmic domain of the receptor, strongly implying the involvement of a new CD5-interacting signaling or adaptor protein. Furthermore, we show that upon CD5 ligation there is a profound shift in its distribution from the bulk fluid phase to the lipid raft environment, where it associates with Fyn, Lck, and PAG. We suggest that the relocation of CD5, which we also show is capable of forming homodimers, to the proximity of raft-resident molecules enables CD5 to inhibit membrane proximal signaling by controlling the phosphorylation and activity of Fyn, possibly by interfering with the disassembly of C-terminal Src kinase (Csk)-PAG-Fyn complexes during T cell activation.

CD5 is a surface receptor that participates in the fine-tuning of T cell responses (1). CD5 is expressed on thymocytes, mature peripheral T cells, and also on B-1a cells (2, 3). CD5 has been shown to establish homotypic interactions (4) and is reported to interact with different surface molecules expressed by antigen-presenting cells, including CD72 (5), gp40–80 (6), gp150 (7), and IgV(H) framework region sequences (8). However, it is still debatable which, if any, of these molecules are true physiological CD5 ligands. CD5 is a 67-kDa type I glycoprotein composed of three extracellular scavenger receptor cysteine-rich domains in the extracellular region and a highly conserved cytoplasmic domain containing multiple Ser/Thr and Tyr phosphorylation sites, including two potential immunotyrosine-based inhibition motifs (9). Although initial studies of antibody-mediated cross-linking had characterized CD5 as a co-stimulatory molecule in T cells, capable of enhancing TCR[6]-mediated cell proliferation (10, 11), the current view is that CD5 is a major attenuator of signals arising from the engagement of the TCR with cognate antigen-MHC complexes expressed on antigen-presenting cells.

The production and analysis of CD5-knock-out mice established an inhibitory role for CD5 on TCR signaling (12). Thymocytes from mice with a disrupted CD5 gene and bearing an $\alpha\beta$TCR transgene exhibit increased proliferative responses and enhanced phosphorylation of signaling effectors such as LAT, phospholipase Cγ1, or Vav in response to TCR stimulation. Similarly, CD5-deficient B-1a cells exhibited increased Ca^{2+} mobilization and proliferative responses (13). However, the function of CD5 in development, particularly during thymocyte selection, remains unclear. Although double-positive thymocytes from CD5-deficient mice are hyperresponsive to TCR antigen stimulation, CD4 or CD8 single positive thymocytes or mature T cells are not measurably different from cells of wild-type animals, suggesting that the inhibitory role of CD5 may be selective for different types of cell or be dependent on the maturation stage (12, 14). The dynamic regulation of CD5 expres-

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[6] The abbreviations used are: TCR, T cell receptor; Csk, C-terminal Src kinase; LAT, linker of activated T cells; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; WB, Western blot; IP, immunoprecipitation; PBMC, peripheral blood mononuclear cells; BRET, bioluminescence resonance energy transfer; Ab, antibody.

sion during thymocyte selection, which correlates with the avidity of the TCR, may be involved in fine-tuning TCR signaling during thymocyte selection (15), in this way perhaps maximizing positive selection.

Upon TCR/CD3 stimulation, the cytoplasmic domain of CD5 is phosphorylated with very fast kinetics (16), with residues Tyr^{429} and Tyr^{463} of the cytoplasmic domain of CD5 being the prime targets of phosphorylation (17, 18). The protein-tyrosine kinase Lck appears to be responsible for most of the phosphorylation, although Fyn and Itk may complement or regulate this aspect of Lck function (17–19). The induction of tyrosine phosphorylation of CD5 may in turn allow Lck itself to dock onto the cytoplasmic tail of CD5, with the Lck SH2 domain interacting primarily with phosphorylated Tyr^{429}, reportedly resulting in an increased Lck activity (17, 20). However, these coupled effects were obtained by co-expressing Lck and CD5 in insect cells or using short peptides or fusion proteins in cells stimulated with pervanadate. The docking of Lck to CD5 upon cell activation through the physiological receptor has yet to be demonstrated, and it has been shown that direct ligation of CD5 with mAb has little effect on Lck phosphorylation (21).

It has been shown that the inhibitory function of CD5 on TCR signaling during thymocyte development is dependent on the functional integrity of the CD5 cytoplasmic domain, as a transgene encoding a mutant form of the protein lacking the double-tyrosine motif and distal sequences failed to rescue the $CD5^{-/-}$ phenotype (22). One hypothesis raised to explain the inhibitory effects of CD5 involves the SH2 domain-containing phosphotyrosine phosphatase, SHP-1. CD5 has been reported to associate with SHP-1 in Jurkat T cells, murine thymocytes, and B-1a cells (21, 23–25). The recruitment of SH2 domain-containing phosphatases to phosphorylated tyrosine residues positioned within immunotyrosine-based inhibition motif sequences is a common feature among negative regulatory receptors, and a tyrosine residue contained within the immunotyrosine-based inhibition motif (LAY378KKL) that straddles the CD5 transmembrane/cytoplasmic junction was proposed to be the binding site of SHP-1 in Jurkat T cells (21). In addition to SHP-1, several other signaling molecules may associate with the cytoplasmic tail of CD5, potentially conferring a negative regulatory function to CD5, including RasGAP and Cbl (26, 27).

Although the inhibitory role of CD5 is generally attributed to its association with signaling attenuators upon TCR ligation, the targets of these inhibitory molecules have not been fully identified. On the other hand, little attention has been given to the possibility that signal modulation by CD5 results from the active down-regulation of activatory enzymes. We have examined the signaling effects of CD5, with an initial focus on T cell-activating kinases, considering the possibility that CD5 triggering represses the activity of these enzymes. We find that whereas Lck is largely unaffected by CD5 triggering, Fyn is rapidly phosphorylated, and its activity is down-regulated. We propose that this is responsible for the CD5-dependent inhibition of at least some of its downstream effectors, including ZAP-70 and PAG.

EXPERIMENTAL PROCEDURES

Cells and Cell Lines—Human PBMC from normal healthy adult volunteers were isolated by Ficoll-Hypaque density gradient centrifugation. Jurkat cell lines used were JKHM (23), donated by D. A. Cantrell (Imperial Cancer Research Fund, London), and the CD5-deficient 2G5 subclone (28). 2G5 lines stably transfected with wild-type and cytoplasmic mutant human CD5 molecules were also used (30). Cell lines were maintained at 37 °C in a 5% CO_2 humidified incubator in RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 units/ml), and streptomycin (50 μg/ml). Human embryonic kidney HEK293T cells (29) were grown in Dulbecco's modified Eagle's medium containing 10% FCS 5 mg/ml glucose, penicillin (100 units/ml), streptomycin (50 μg/ml), and 200 mM L-glutamine.

Antibodies and Reagents—Monoclonal antibodies used were: anti-phosphotyrosine mAb 4G10 conjugated with HRP (Upstate Biotechnology); CD5, Y-2/178 (30), a kind gift from J. Cordell and D. Y. Mason (John Radcliffe Hospital, University of Oxford); CD3, OKT3 (31); PAG, C1 (32), a kind gift from B. Schraven and J. Lindquist (Institute of Immunology, Otto-von-Guericke University, Magdeburg, Germany); anti-phospho-Fyn (Tyr^{531}), from Santa Cruz Biotechnology (the antibody has the commercial name of p-c-Src(pY530) but was originally raised against the inhibitory tyrosine residue of Fyn; in the text we use the nomenclature pFyn(Y531)); the isotype control antibody IgG1 from BD Biosciences. Polyclonal Abs were: anti-CD5, a gift from D. Y. Mason; anti-Lck (DA3), anti-Fyn (BL90), and anti-ZAP-70, given by J. B. Bolen and M. G. Tomlinson (DNAX Research Institute, Palo Alto); anti-PAG and anti-phospho-PAG (Tyr^{317}), gifts from B. Schraven and J. Lindquist; anti-phospho-ZAP-70 (Tyr^{493}), a gift from S. Valitutti (Institut Claude de Préval, Toulouse); anti-phospho-Src Family Negative Regulatory Site (BIOSOURCE), anti-phospho-Src Family (Tyr^{416}) and anti-phospho-Lck (Tyr^{505}) from Cell Signaling; anti-C-terminal Src kinase (Csk; Santa Cruz Biotechnology); anti-LAT (Upstate Biotechnology); goat anti-mouse peroxidase conjugate (Molecular Probes Europe); goat anti-rabbit peroxidase conjugate (Sigma); rabbit anti-mouse FITC-labeled from Dako; donkey anti-rabbit FITC-labeled (Jackson ImmunoResearch). Biotinylated proteins were detected in Western blotting with ExtrAvidin peroxidase (Sigma). [γ-^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham Biosciences.

Cell Surface Biotinylation, Immunoprecipitations, and Western Blotting—Cell surface biotinylation, immunoprecipitations, Western blotting, and detection of biotinylated antigens were performed as described previously (23).

In Vitro Kinase Assays and Reprecipitations of Phosphorylated Proteins—Between 1 and 5×10^7 cells were lysed for 30 min on ice in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1% (v/v) Nonidet P-40 or Triton X-100). The nuclear pellet was removed by centrifugation at 11,000 $\times$ *g* for 10 min at 4 °C, and the supernatants were mixed with 100 μl of a 10% protein A-Sepharose CL-4B (Amersham Biosciences) slurry and with mAb (1–10 μg) or antisera (1–3 μl). Samples were incubated for 90 min at 4 °C. The beads containing the immune complexes were washed 3 times in 1 ml

of lysis buffer and washed for 2 more rounds in kinase assay buffer (25 mM HEPES and 0.1% detergent). Nonidet P-40 or Triton X-100 assay buffer (30 μl) containing 10 mM $MnCl_2$, 1 mM sodium vanadate, 1 mM NaF, and 50 μCi of (185 KBq) [γ-^{32}P]ATP was added to the immune complexes, and *in vitro* kinase reactions were allowed to occur for 15 min at 25 °C. Reactions were stopped by the addition of 30 μl of 2 × SDS buffer after which the samples were boiled for 5 min. Products were separated on SDS-PAGE gels, and autoradiography of the dried gels was done with BioMax MR films (Kodak). For reprecipitations, the beads containing the immune complexes were boiled for 5 min in 2% SDS and diluted 8-fold with lysis buffer. After centrifugation, supernatants were recovered and precleared for 30 min with 100 μl of protein A-Sepharose beads. Proteins were reprecipitated with antibodies and protein A-Sepharose beads for 90 min as above. Reprecipitates were washed three times with 1 ml of lysis buffer. Samples were boiled for 5 min and subjected to SDS-PAGE. When indicated, a biotinylated peptide containing the rat CD5 pseudo-immunoreceptor tyrosine-based activation motif sequence (Biotin-AASHVDNEYSQPPRNSRLSAYPALE-OH, purchased from New England Peptide) was also included as a Fyn substrate in the reaction mix at a final concentration of 0.5 μg/μl, and in this case the kinase reaction was at 30 °C for 10 min. The biotin-labeled CD5 peptide was recovered using avidin beads (Pierce), and the incorporated [γ-^{32}P]ATP measured in a Beckman liquid scintillation counter.

Cellular Activation—Cells were maintained in RPMI 1640 medium or serum-deprived for 18 h before stimulation. For activation, cells were washed and resuspended in RPMI 1640 (without FCS) containing Y-2/178 at 10 μg/ml, OKT3 at 2 μg/ml, or isotype-matched negative control antibody at 10 μg/ml. Stimulation was induced without the use of cross-linking secondary Abs. Cells were maintained at 4 °C for 15 min and subsequently incubated at 37 °C for the indicated time points. Cells were then pelleted and lysed for 30 min in ice-cold 1% Nonidet P-40 lysis buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% (v/v) Igepal CA-630, and 1 mM sodium orthovanadate). The nuclear pellet was removed by centrifugation at 11,000 × *g* for 10 min at 4 °C, and the supernatants were subjected to immunoprecipitation or analyzed by immunoblotting. In a typical experiment, 5×10^7 cells were activated per condition. For lipid raft analysis of activated cells, $\sim 1.7 \times 10^8$ cells were used per sample. Cells were washed and resuspended in 1 ml of RPMI medium containing Y-2/178 at a 1:5 dilution of hybridoma supernatant. After 5 min of incubation on ice, cells were activated at 37 °C for 15 min, collected, and prepared for sucrose gradient centrifugation as described below.

Sucrose Gradient Centrifugation—Sucrose gradient centrifugation was performed as described (33). Briefly, activated cells were washed twice with ice-cold PBS and lysed for 30 min on ice in 1 ml of MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% Triton X-100, 1 mM PMSF, and protease inhibitors (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin A; Calbiochem). Lysates were homogenized by a brief sonication for 10 pulses on ice using a Heat Systems/Ultrasonics sonicator (model W-375) equipped with a microtip and set to 50% duty cycle, output 3. To obtain the rafts fractions, cell lysates were mixed with an equal volume of 85% sucrose in MBS buffer and transferred to the bottom of Sorvall ultracentrifuge tubes. Samples were overlaid with 6 ml of 35% sucrose followed by 2 ml of 5% sucrose. After centrifugation at 200,000 × *g* for 17 h at 4 °C in a TST41.14 swing-out rotor (Sorvall), 10 fractions of 1 ml were collected from the bottom of the tube and analyzed by Western blotting. Fractions are labeled from the top of the gradient.

Flow Cytometry—For the detection of surface receptors, cells were washed and resuspended in PBS containing 0.2% BSA and 0.1% NaN_3 (PBS/BSA/NaN_3) at a concentration of 5×10^6 cells/ml. Staining was performed by incubation of 5×10^5 cells/well with mAb (20 μg/ml) for 15 min on ice followed by rabbit anti-mouse FITC-labeled in 96-well round-bottom plates (Greiner). For measurement of intracellular phosphotyrosine proteins, activated cells (5×10^6 cells per sample) were collected and fixed for 20 min at 4 °C with 4% paraformaldehyde, washed twice with ice-cold PBS, and permeabilized for 20 min at 4 °C with PBS/BSA/NaN_3 containing 0.4% saponin. Intracellular staining was performed in 96-well round-bottom plates by incubation of 1×10^6 cells/well with polyclonal phosphorylation site-specific antibodies for 15 min on ice followed by FITC-labeled donkey anti-rabbit antibody. Cytometric analysis was as previously described (23) using a FACSCalibur flow cytometer (BD Biosciences).

Densitometry—Densitometric analysis was performed on a GS-800 densitometer (Bio-Rad) using Quantity One quantitation software (Bio-Rad). Signals obtained from the volume analysis of densitometric data are expressed in arbitrary units. All densitometric values were calculated from non-saturated signals.

cDNA Cloning and BRET Assay—Human CD5 cDNA in pGFP-N1 (a gift from G. Bismuth, Institute Cochin, Paris) was subcloned into pGFP-N3 (PerkinElmer) using the EcoRI restriction site and into prLuc-N3 (PerkinElmer) using BglII and BamHI. CD2 was amplified from the Jurkat cell line E6.1 using as primers 5′-TAGTAGCTGCAGCCCCTAAGATGAGCTTTCC-3′ and 5′-ATCATCGGATCCTTAGAGGAAGGGGACAATGAG-3′. The PCR product was cloned into pGFP-N3 and prLuc-N3 using PstI and BamHI sites. For the CD5Ext/CD2Int chimera, two rounds of PCR amplification were performed. Oligonucleotide pairs for the overlapping 5′ and 3′ products were 5′-TAGTAGGATATCGCCAGAAACCATGCCCATG-3′ and 5′-CGACTCCTCTGTTTTTTCCTTTTGGCAAGGGGGCCGCACACGA-3′ using CD5 pGFP-N3 as template and 5′-TCGTGTGCGGCCCCCTTGCCAAAAGGAAAAAACAGAGGAGTCG-3′ and 5′-CTACTACCGCGGCAGCCTCTGAGCCCCATGC-3′ using CD2 pGFP-N3 as template, respectively. The amplified products were then used as templates in a chimeric PCR reaction scheme. The resulting genes were digested using the appropriate restriction sites and cloned into the BRET vectors. All constructs produced were sequenced to check reading frame and integrity. The BRET experiments were carried out as described previously using the type-I assay (34). Effective BRET ($BRET_{eff}$) values were assayed by adding DeepBlueC substrate and measuring luminescence immediately.

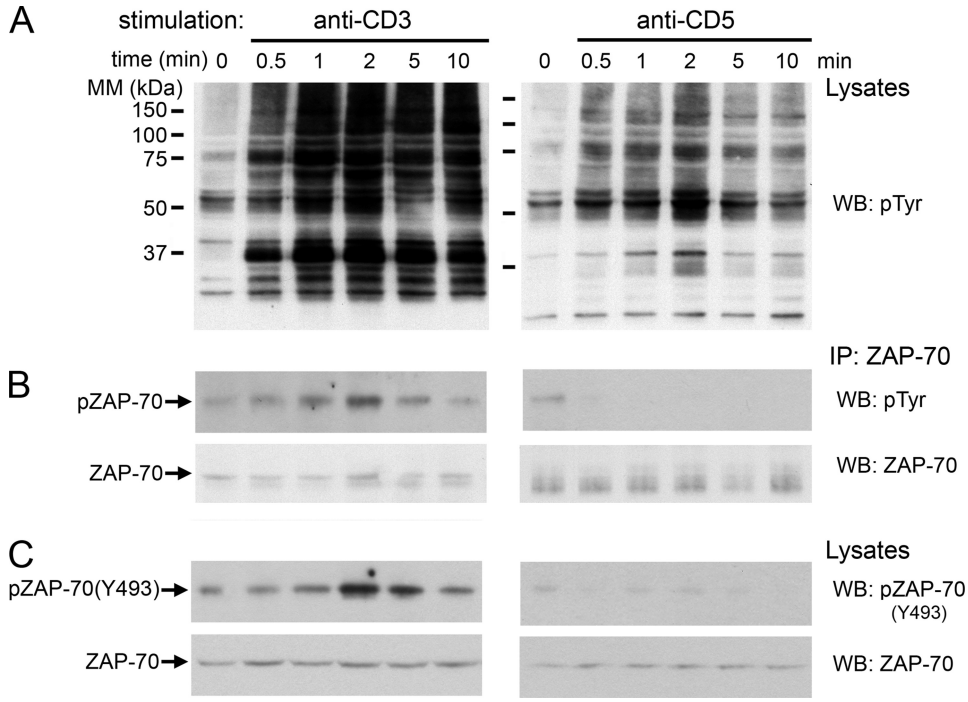

FIGURE 1. **CD5 ligation induces low phosphorylation of intracellular substrates and down-modulates ZAP-70 phosphorylation.** *A*, JKHM cells were incubated with anti-CD3 mAb OKT3 (2 μg/ml) or anti-CD5 mAb Y-2/178 (10 μg/ml) for the indicated time points. All stimulations described were induced without the use of cross-linking secondary Abs. Cells were lysed, and global phosphotyrosine content was revealed by Western blotting with the phosphotyrosine-specific mAb 4G10. *B*, JKHM cells were activated with CD3 or CD5 mAbs for the indicated time points. Subsequently, cells were rapidly collected and resuspended in lysis buffer. Aliquots (90%) of the lysates were subjected to anti-ZAP-70 immunoprecipitation, resolved by 10% SDS-PAGE, and immunoblotted with HRP-labeled anti-phosphotyrosine mAb 410G (*B*, *top panels*). *C*, the rest (10%) of the lysates was directly immunoblotted with anti-phospho-ZAP-70(Tyr^{493}) antibody followed by HRP-conjugated goat anti-rabbit antibody. For loading control, membranes were probed with anti-ZAP-70 antibody (*lower panels*).

RESULTS

CD5 Ligation Induces Weak Phosphorylation of Intracellular Substrates and Down-regulation of ZAP-70 Phosphorylation—We examined the early response of JKHM T cells to CD5 stimulation, namely the appearance of tyrosine phosphorylated intracellular substrates. As a true physiological ligand for CD5 has not been unequivocally demonstrated, we stimulated cells via CD5 using the CD5 mAb Y-2/178 to mimic ligand binding. CD5 triggering induced a slight increase in the phosphorylation of a number of cellular proteins within 30 s of activation, peaking at 2 min (Fig. 1*A*), consistent with previous observations (21). Despite being much reduced compared with the activation induced via CD3, this rapid response to CD5 stimulation indicated that CD5 is not only involved in down-modulating signals transduced through the physiological receptor for antigens but is instead capable of autonomous signaling.

We, therefore, asked whether the CD5-mediated signals reflected simply a quantitative difference from the more potent TCR-induced activation or whether there would be qualitative effects, and for that purpose we initially assessed tyrosine phosphorylation of ZAP-70, a key step in TCR-mediated signal transduction. Upon conventional TCR/CD3 stimulation of JKHM cells using OKT3 antibody, ZAP-70 is rapidly phosphorylated, as can be seen when total cellular ZAP-70 is immunoprecipitated at different time points of activation, and phosphorylation was measured using anti-phosphotyrosine antibodies (Fig. 1*B*). Additionally, using a phosphospecific antibody recognizing phosphorylated Tyr^{493} of ZAP-70, which correlates with positive signaling by ZAP-70 (35), we detected a clear increase in the phosphorylation of this residue in total cellular lysates collected at the different time points of activation (Fig. 1*C*). By contrast, JKHM cells treated with anti-CD5 Y-2/178 antibody displayed a decrease in ZAP-70 tyrosine phosphorylation both at the global level as well as at the specific phosphorylation of Tyr^{493} (Fig. 1, *B* and *C*).

CD5 Stimulation Induces Fyn Phosphorylation in the C-terminal Inhibitory Tyrosine Residue and Down-regulates the Kinase Activity of Fyn—The small burst of tyrosine phosphorylation induced by CD5 triggering together with the blockade of signaling at the level of ZAP-70 activation prompted us to examine the activity of the protein-tyrosine kinases that may phosphorylate ZAP-70, *i.e.* the Src-family kinases Lck and Fyn. JKHM cells were stimulated for different times with the Y-2/178 CD5 antibody, and after cell lysis Lck and Fyn were immunoprecipitated and analyzed by Western blotting. We were unable to detect any significant changes in the overall tyrosine phosphorylation of Lck (Fig. 2*A*), in accordance to what was previously reported (21). Strikingly, Fyn was strongly phosphorylated upon CD5 stimulation of JKHM cells (Fig. 2*A*).

As CD5 ligation induced the tyrosine phosphorylation of Fyn while also resulting in the inhibition of ZAP-70 phosphorylation, we asked whether phosphorylation occurred at the inhibitory C-terminal Tyr^{531} residue of Fyn. Lysates of JKHM cells stimulated for different times with Y-2/178 were run by SDS-PAGE and incubated with a specific monoclonal antibody recognizing phopho-Tyr^{531} of Fyn. As can be seen in Fig. 2*B*, there

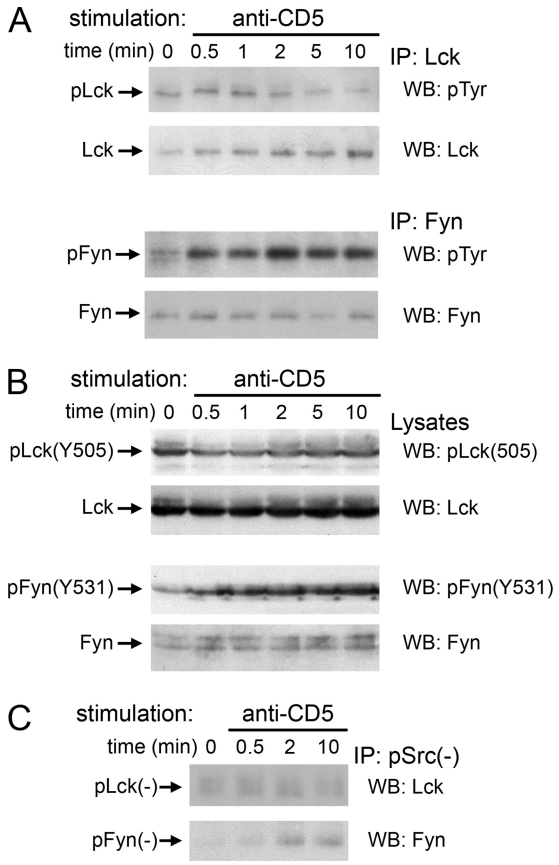

FIGURE 2. **CD5 triggering induces tyrosine phosphorylation of the protein-tyrosine kinase Fyn at the C-terminal inhibitory residue and downmodulates the activity of Fyn.** *A*, JKHM cells were incubated at 37 °C with the anti-CD5 mAb Y-2/178 (10 μg/ml) for the indicated time points or with the isotype control antibody IgG1 (10 μg/ml) for 2 min (corresponding to time point 0 of activation). Subsequently, cells were rapidly collected and resuspended in lysis buffer. Lysates were immunoprecipitated (*IP*) with polyclonal anti-Lck or anti-Fyn, resolved by 7.5% SDS-PAGE, and blotted onto PVDF filters. Membranes were incubated with HRP-conjugated anti-phosphotyrosine mAb 4G10 (*top panels*) or with polyclonal anti-Lck or anti-Fyn followed by HRP-labeled goat anti-rabbit antibody (*bottom panels*). Proteins were visualized by enhanced chemiluminescence. *B*, JKHM cells were activated with Y-2/178 for the times indicated or incubated with isotype-control antibody (time 0) for 2 min at 37 °C. Subsequently, cells were collected and lysed. Lysates were run in SDS-PAGE and blotted with polyclonal antibodies reacting with phosphorylated Tyr(P)^{505} of Lck or Tyr(P)^{531} of Fyn. Lysates were also blotted with anti-Fyn and anti-Lck polyclonal antibodies, as controls. *C*, lysates of CD5-stimulated cells for the times indicated were precipitated with anti-pSrc(−) antibodies. After Western blotting, membranes were incubated with either Lck (*top*) or Fyn (*bottom*) polyclonal antibodies followed by goat anti-rabbit-HRP incubation and enhanced chemiluminescence.

was a marked increase in the phosphorylation of Tyr^{531} upon CD5 activation. By contrast, no major changes were detected in the phosphorylation of the correspondent inhibitory tyrosine residue in Lck, Tyr^{505} (Fig. 2*B*). Given that phosphospecific antibodies have the potential to cross-react, we obtained additional confirmation that Fyn, but not Lck, was phosphorylated in the inhibitory tyrosine residue. We stimulated JKHM for different times, lysed the cells, and performed immunoprecipitations using the polyclonal Abs reactive with phosphorylated C-terminal tyrosine residues of Src-family kinases. Immunoprecipitates were transferred to nitrocellulose membranes and probed with Lck- and Fyn-specific Abs. Whereas Lck was not phosphorylated at the inhibitory tyrosine residue, phosphorylation of the corresponding residue in Fyn increased significantly within 2 min compared with $t = 0$, where it was virtually undetected (Fig. 2*C*).

On the other hand we excluded that Fyn was significantly phosphorylated at the activatory tyrosine residue. Using a cytometry-based method for intracellular tyrosine detection and a polyclonal Ab, pSrc(+), which recognizes phosphorylation of Tyr^{394} of Lck and Tyr^{417} of Fyn, we could not observe any increase in the overall phosphorylation of the activatory tyrosine residues after CD5 activation for 2 min (supplemental Fig. 1, *A* and *B*). By contrast, using a pSrc(−) serum we noted a small but reliable increase in the phosphorylation of C-terminal inhibitory tyrosine residues of the Src-type kinases, consistent with the observed phosphorylation of Tyr^{531} of Fyn. Moreover, assessing the phosphorylation of Src positive and negative residues of CD5-activated JKHM by immunoblotting, we confirmed that the status of the activatory tyrosine residues did not change significantly, whereas using the pSrc(−) serum we could detect a marked increase in the phosphorylation (supplemental Fig. 1*C*).

Having established that CD5 triggering resulted in the phosphorylation of the inhibitory tyrosine residue of Fyn, we promptly tested whether the kinase activity of Fyn decreased as a consequence of Tyr^{531} phosphorylation. We assessed the kinase activity of total cellular Fyn in *in vitro* kinase assays in resting cells and after CD5 activation. JKHM cells were stimulated for different times with Y-2/178, after which the cells were lysed and Fyn was immunoprecipitated with a polyclonal antibody and subjected to a kinase reaction in the presence of [γ-^{32}P]ATP. As we had hypothesized, the autophosphorylation activity of Fyn decreased approximately to half of its initial value, whereas the activity of Lck remained constant in the same period (Fig. 3*A*). We also measured the kinase activity toward an exogenous substrate. For this purpose we added in the kinase reaction a 25-amino acid-long synthetic peptide corresponding to a cytoplasmic sequence of rat CD5 that contains the pseudo-immunoreceptor tyrosine-based activation motif and that we had previously shown to be a substrate for both Lck and Fyn (19). After CD5 activation, Lck and Fyn were collected from cell lysates and allowed to phosphorylate the CD5 peptide in the presence of radioactive ATP. The phosphorylation of the peptide induced by Fyn markedly decreased over time, whereas the levels of Lck-catalyzed phosphorylation varied only slightly, confirming our previous results (Fig. 3*B*). Collectively, the data indicate that the regulation of Fyn during CD5-mediated signaling is a specific effect. Interestingly, phosphorylation of CD5 itself, detected by kinase reactions as well as by immunoblotting using a phosphotyrosine-reactive mAb, increased markedly within 2 min, confirming the functional coupling of CD5 with tyrosine kinases during CD5-mediated signaling (Fig. 3*C*).

Tyrosine Kinases Responsible for CD5 Phosphorylation Interact with Sequence Lys^{384}—Glu^{418} of the Cytoplasmic Tail of CD5—CD5 does not have intrinsic enzymatic activity, so it is likely that CD5 phosphorylation as well as the CD5-induced phosphorylation of Fyn is catalyzed by tyrosine kinases that associate with CD5. We used a set of Jurkat cell line variants, *i.e.* the CD5-negative cell line 2G5, reconstituted 2G5 cells expressing the 471-amino acid-long wild-type CD5 protein (2G5/CD5.WT), and also 2G5 transfectants expressing the trunca-

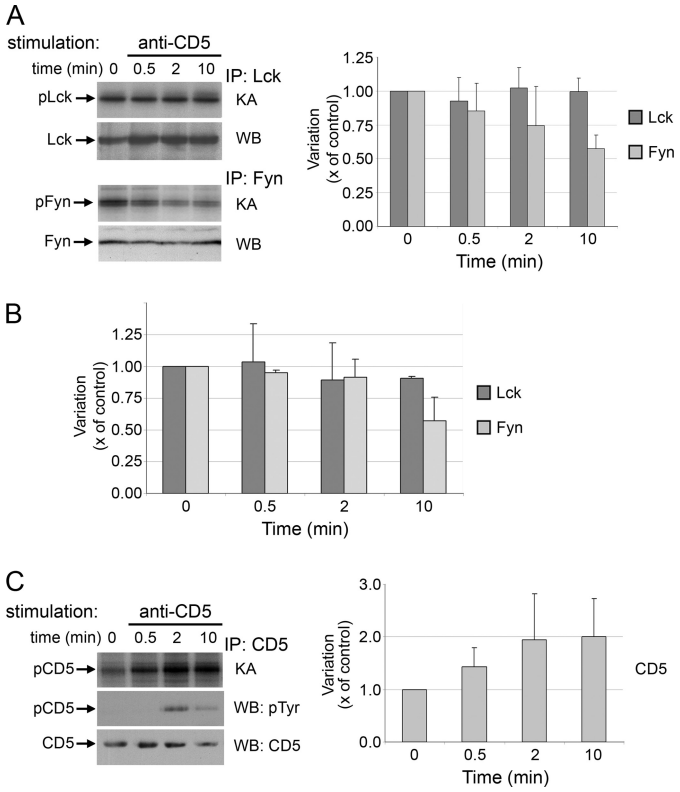

FIGURE 3. **The kinase activity of Fyn decreases significantly after CD5 stimulation.** JKHM cells were activated with Y-2/178 for the indicated time points at 37 °C and lysed in lysis buffer. *A*, *in vitro* kinase assays (*KA*) of Lck and Fyn immunoprecipitates were performed in the presence of [γ-^{32}P]ATP. Phosphorylated products were separated by 11% SDS-PAGE and visualized by exposure of the dried gels to Eastman Kodak Co. BioMax MR films. IPs were also run in parallel gels and immunoblotted with Lck and Fyn polyclonal antibodies. Changes in the levels of phosphorylation of Lck and Fyn were determined by densitometry. Gels are from one of three independent experiments, and graphs show the mean values and S.D. of Lck and Fyn activities, relative to time 0, from the three experiments. *B*, kinase assays of Lck and Fyn immunoprecipitates were performed in the presence of [γ-^{32}P]ATP and an exogenous substrate, a 25-amino acid-long synthetic biotinylated peptide containing the pseudo-immunoreceptor tyrosine-based activation motif of rat CD5. After the kinase reaction, the CD5 peptide was precipitated by streptavidin beads, and radioactive counts associated with the peptide were measured in a Beckman liquid scintillation counter. *C*, after CD5 stimulation, kinase assays on CD5 immunoprecipitates were also performed. The kinase activity associated with CD5 was detected by autoradiography and measured by densitometry. Values shown in the graph are from three separate experiments. Immunodetection of phosphorylated CD5 in CD5 immunoprecipitates was performed using anti-phosphotyrosine antibodies, confirming that CD5 is tyrosine phosphorylated upon activation. Immunoblotting of total CD5 in the immunoprecipitates is also shown.

tion mutants CD5.K384^{stop}, CD5.E418^{stop}, and CD5.H449^{stop}, to investigate the association of tyrosine kinases with the cytoplasmic domain of CD5 (Fig. 4*A*). As assessed by flow cytometry, the different cells expressed the various forms of CD5 at comparable levels, except the 2G5 untransfected cells, which were completely CD5-deficient (Fig. 4*B*). SDS-PAGE analysis of CD5 immunoprecipitated from cell surface-biotinylated cells confirmed that wild-type CD5 and CD5 mutants were of the expected sizes (Fig. 4*C*, *top panel*). Using Y-2/178 antibody, CD5 was immunoprecipitated from lysates and subjected to *in vitro* kinase assays. As expected, the most prominent phosphoprotein in each lane corresponded to the CD5 polypeptides at their respective weights (Fig. 4*C*, *second panel*), except (phospho)CD5.K384^{stop}, which was not at all visible. Although slight variations in band intensity result from the number of tyrosine residues present in each mutant (*i.e.* four tyrosine residues in the full-length molecule, three in the H449^{stop} mutant, and one tyrosine in E418^{stop}), the lack of detection of the CD5.K384^{stop} mutant (one tyrosine) suggests that the stretch of amino acids between 384 and 418 is required for the association of protein-tyrosine kinases that, at least partially, phosphorylate CD5.

Lck is thought to be the tyrosine kinase that most efficiently phosphorylates CD5 (17–19); therefore, we used the CD5 immune complexes obtained from the kinase reactions of the different cell lines and attempted to re-precipitate Lck. As can be seen in Fig. 4*C* (*third panel*), phosphorylated Lck was recovered from the immune complexes from 2G5 cells reconstituted with full-length CD5 and the truncation mutants E418^{stop} and H449^{stop} but was not detected in 2G5 or 2G5/CD5.K384^{stop} cells. This suggests that Lck binds to the cytoplasmic tail of CD5 after Lys^{384} and, confirming most previous reports (17–20), is likely to be a major kinase phosphorylating CD5 tyrosine residues, including the SHP-1 binding site, Tyr^{378}. This residue, the only tyrosine present in mutants CD5.E418^{stop} and K384^{stop}, is phosphorylated when Lck is present (former case) and not phosphorylated when Lck is not coupled to CD5 (latter case) (Fig. 4*C*). In these experiments the time required for detecting reprecipitated Lck in the autoradiographies was significantly longer than that needed to detect phosphorylated CD5 (5–10 times), indicating that in resting cells the Lck-CD5 interaction is considerably week, albeit specific. By contrast, we were unable to reprecipitate Fyn from the immune complexes of CD5.WT or mutants (Fig. 4*C*, *fourth panel*). We confirmed that the lack of detection of Fyn was due to the lack of association between CD5 and Fyn and not to a technical difficulty to reprecipitate Fyn in the denaturing conditions used, as from Fyn immunoprecipitates subjected to kinase reactions we were successful in recovering Fyn by reprecipitation (Fig. 4*C*, *lower panel*).

The Tyrosine Kinase Responsible for the CD5-induced Fyn Phosphorylation Functionally Interacts with the C-terminal End of CD5—We tested which of the truncated forms of CD5 could induce Fyn phosphorylation after anti-CD5 antibody stimulation. Initially, we used the cell line expressing full-length CD5, 2G5/CD5.WT, and activation of these cells with the Y-2/178 antibody recapitulated the CD5-induced phosphorylation of Fyn seen in JKHM cells (Fig. 5*A*, *top*). Moreover, using lysates from 2G5/CD5.WT cells and analyzing the phosphorylation of the C-terminal inhibitory tyrosine residue of Fyn, we were able to confirm that this specific residue was phosphorylated after CD5 stimulation, with an increase of >10-fold within 2 min (Fig. 5*A*, *middle panels*). By contrast, the phosphorylation of the C-terminal tyrosine of Lck did show a slight increase but with a much lower amplitude (2.5 times on 2 min; Fig. 5*A*, *lower panels*). We went on to determine whether the CD5 truncation mutants could also induce Fyn phosphorylation. Unexpectedly, not even the longest of the truncated isoforms of CD5, *i.e.* 2G5/CD5.H449^{stop}, was able to induce Fyn phosphorylation (Fig. 5*B*). Similarly, Fyn was not phosphorylated in 2G5 cells expressing either CD5.H384^{stop} (Fig. 5*B*) or E418^{stop} (not shown).

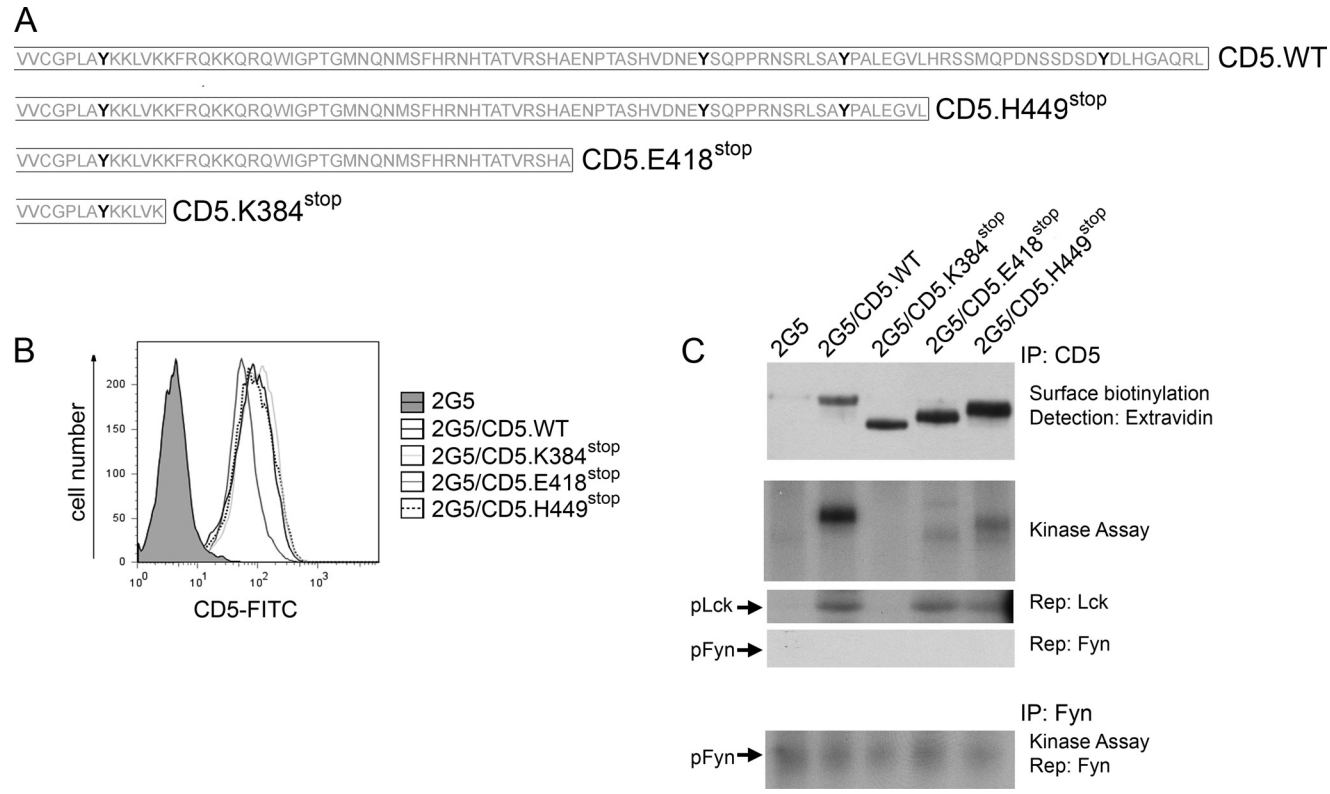

FIGURE 4. **Lck association and CD5 tyrosine phosphorylation require sequences of the cytoplasmic tail of CD5.** *A*, shown is a scheme containing the amino acid sequences of the CD5 wild-type molecule and truncation mutants used in this study. Shown in CD5.WT is the amino acid sequence between residues 371 and 471 of the mature protein, with tyrosine residues Tyr^{378}, Tyr^{429}, Tyr^{441}, and Tyr^{463} in *bold*. CD5 truncation mutants (CD5.K384^{stop}, CD5.E418^{stop}, and CD5.H449^{stop}) are named according to the amino acid in which a premature stop codon was introduced. Mutant CD5.H449^{stop} contains 448 amino acids, 23 less than the full-length protein, and misses only the membrane distal tyrosine residue. Mutants CD5.E418^{stop} and CD5.K384^{stop} contain 417 and 383 amino acids, respectively, and both include just the membrane proximal Tyr^{378}. *B*, expression of CD5 in the indicated cell lines was measured by flow cytometry of cells stained with anti-CD5 mAb followed by FITC-labeled rabbit anti-mouse antibody. Non-transfected 2G5 cells were used as control (*gray filling*). *C*, cell lines were surface-biotinylated, lysed in lysis buffer, and CD5 immunoprecipitated with Y-2/178. Immunoprecipitates were either run in SDS-PAGE/Western and subjected to immunoblotting with HRP-conjugated ExtrAvidin (*top panel*) or subjected to *in vitro* kinase assays. Half of the CD5 immunoprecipitates in kinase assays were separated by 11% SDS-PAGE and visualized by exposure of Kodak BioMax MR films to the dried gel (*2nd panel*). The rest of the CD5 immunoprecipitates were denatured in 2% SDS and reprecipitated (*Rep*) with polyclonal anti-Lck or anti-Fyn antibodies. Lck and Fyn reprecipitates were separated by SDS-PAGE, and signals in dried gels were detected on films (*lower panel*). To confirm that Fyn can be reprecipitated in the denaturing conditions used, Fyn immunoprecipitated from the different cell lines was subjected to kinase assays, denatured, and reprecipitated using specific anti-Fyn polyclonal antibodies.

Csk is the only protein kinase known to phosphorylate the C-terminal inhibitory tyrosine residues of Src-family kinases (36), so we investigated whether Csk associates with the cytoplasmic tail of CD5. In Western blot analyses we could find no evidence for a direct association of Csk with CD5, as Csk was not detected in CD5 immunoprecipitates or CD5 in Csk precipitates (Fig. 5*C*). Thus, although the C-terminal end of CD5 is mandatory for Fyn phosphorylation after antibody ligation, we were not able to establish a direct interaction between CD5 and Csk.

CD5 Associates with Signaling Effectors within Lipid Rafts upon Antibody Ligation—CD5 binding partners and effectors such as Lck and Fyn, among others, are reported to associate with plasma membrane lipid rafts. Therefore, we examined whether CD5 also segregated to these membrane microdomains after antibody ligation and if that translocation would favor CD5 interactions with the effector molecules. JKHM cells were activated with anti-CD5 antibody for 10 min or left undisturbed, after which they were lysed in Triton X-100 and subjected to sucrose gradient centrifugation. Fractions were collected and assayed by immunoblotting for the presence of CD5, Lck, Fyn, and Csk, and LAT was used as a rafts marker. Most of the CD5 translocated en masse from the soluble to the lipid raft fraction upon antibody ligation, whereas there was no net movement of Lck, Fyn, or Csk upon CD5 triggering (Fig. 6*A*). We then pooled the soluble and raft fractions from Y-2/178-treated cells, immunoprecipitated CD5 from these fractions, and tested for CD5-associated kinases using *in vitro* kinase assays. This showed that phosphorylated Lck and Fyn associated almost exclusively with the CD5 fraction localized in the rafts of stimulated cells (Fig. 6*B*). As previously, we were not able to detect any CD5-Csk association.

Similar results were obtained with PBMC; in resting cells all detectable CD5 is in the soluble fraction, and upon CD5 activation a large fraction of CD5 translocates to lipid rafts (Fig. 7*A*). To evaluate whether translocation of CD5 to lipid rafts would result in the association with raft-resident molecules other than Lck and Fyn, we further analyzed the raft and soluble fractions before and after CD5 ligation. We immunoprecipitated CD5 from four sets of sucrose gradient fractions; one corresponding to the CD5-containing soluble fractions of non-activated PBMC (*NS-Sol*), a second to the rafts fraction of non-activated cells (*NS-Raft*, that contains no detectable CD5), a third set consisted of sucrose gradient fractions from CD5-ligated

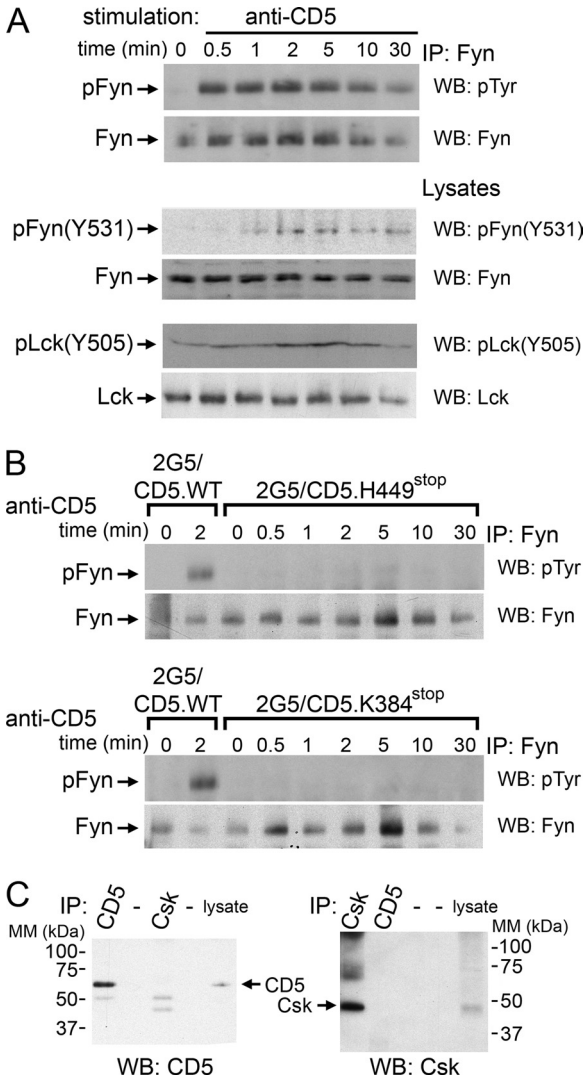

FIGURE 5. **The tyrosine kinases responsible for CD5-induced Fyn phosphorylation and for CD5 phosphorylation interact with distinct sequences of the cytoplasmic tail of CD5.** *A*, 2G5/CD5.WT cells were activated with the anti-CD5 mAb Y-2/178 for the indicated time points at 37 °C or incubated for 2 min with isotype-control antibody (*time 0*). Subsequently, cells were rapidly collected and resuspended in lysis buffer. Lysates were subjected to anti-Fyn immunoprecipitation followed by 7.5% SDS-PAGE and immunoblotting with the HRP-conjugated anti-phosphotyrosine mAb 4G10 or with polyclonal Fyn. Additionally, lysates were run and blotted with polyclonal antibodies reacting with phosphorylated Tyr^{531} of Fyn or $Tyr(P)^{505}$ of Lck. Lysates were also blotted with anti-Fyn and anti-Lck polyclonal antibodies, as controls. *B*, 2G5/CD5.H449^{stop} and 2G5/CD5.K384^{stop} cells were activated with CD5 mAb Y-2/178 for the times indicated. After cell lysis, Fyn was immunoprecipitated, and IPs were loaded into SDS-PAGE followed by immunoblotting using 4G10. In parallel with the activation of (mutant CD5-expressing cells shown in stimulations from 0 to 30 min), 2G5/CD5.WT cells were activated under the same conditions (shown for the 2-min time point) as the positive control. 2G5/CD5.WT cells displayed a clear increase in Fyn tyrosine phosphorylation, indicating that cells were properly stimulated. *C*, JKHM cells were lysed, immunoprecipitated with Csk polyclonal antibodies or CD5 mAb Y-2/178, subjected to SDS-PAGE, and immunoblotted with the indicated antibodies. *Arrows* show the detection of CD5 and Csk. Empty lanes are indicated by *dashes*.

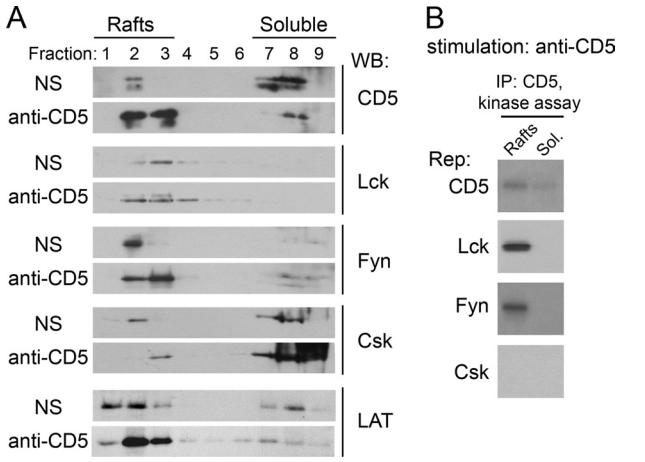

FIGURE 6. **CD5 associates with signaling effectors within lipid rafts upon stimulation.** *A* and *B*, JKHM cells were stimulated with Y-2/178 (anti-CD5) or incubated with isotype-control antibody (*NS*) for 10 min at 37 °C. Subsequently, cells were collected, washed, and lysed in 1% Triton X-100 lysis buffer. Cell lysates were prepared and subjected to sucrose density centrifugation as described under "Experimental Procedures." In *A*, equal volumes of the collected fractions 1–9 were separated by SDS-PAGE and immunoblotted with CD5, Lck, Fyn, or Csk antibodies. Immunodetection of LAT was used as indicative of samples enriched in rafts fractions. Proteins were visualized by enhanced chemiluminescence. In *B*, raft fractions 1–3 and soluble fractions 7–9 of CD5-activated cells were combined, immunoprecipitated with anti-CD5, and subjected to *in vitro* kinase assays. Labeled CD5 immune complexes were denatured in 2% SDS and reprecipitated with polyclonal anti-CD5, anti-Lck, anti-Fyn, or anti-Csk antibodies. Products were subjected to SDS-PAGE and visualized by exposure of the dried gels to Kodak BioMax MR films. *Rep*, reprecipitation.

PBMC, corresponding to the portion of CD5 that did not translocate to lipid rafts (*Act-Sol*), and a final set included CD5 molecules that shifted to lipid rafts upon CD5 ligation (*Act-Raft*).

By immunoblotting and detecting CD5 from immunoprecipitates, we confirmed that in resting cells virtually all CD5 is contained in the soluble fractions and that upon activation CD5 is split at similar amounts between the soluble and rafts fractions (Fig. 7*B*, *top panel*). The CD5 immunoprecipitates were then subjected to kinase assays to label the signaling molecules, after which the immune complexes were denatured, run on SDS-PAGE in non-reducing conditions, and exposed to autoradiography. In the lanes corresponding to CD5 immune complexes isolated from the soluble phase of non-activated (*NS-Sol*) as well as of activated cells (*Act-Sol*), CD5 itself seems to be the dominant phosphorylated species, whereas no phosphoproteins are evident from the NS-Raft set (Fig. 7*B*, *bottom panel*). In the lane corresponding to the Act-Raft fraction, several phosphoproteins were visible, suggesting that upon translocation to lipid rafts, CD5 is able to interact with rafts-associated phosphoproteins. We then attempted to identify by reprecipitation using specific antibodies some of the proteins associating with CD5 in the different fractions. Of the set of proteins we targeted, ZAP-70, Lck, Fyn, Csk, PAG, and LAT, none was detected in association with CD5 in the soluble fractions of either resting (*NS-Sol*) or activated cells (*Act-Sol*) (Fig. 7*C*). CD5 was in fact the only phosphoprotein detected in these fractions. In contrast, CD5 recovered from lipid rafts (*Act-Raft*) associates with Lck, Fyn, PAG, and LAT but not with ZAP-70 or Csk (Fig. 7*C*, *left panel*).

CD5 Forms Homodimers—We have shown that CD5 mutants fail to establish some of the interactions displayed by wild-type CD5. The underlying assumption is that the interactions of CD5 depend on given regions of the cytoplasmic tail interacting with a corresponding ligand. Alternatively, it could be possible that the integrity of the cytoplasmic tail is essential for

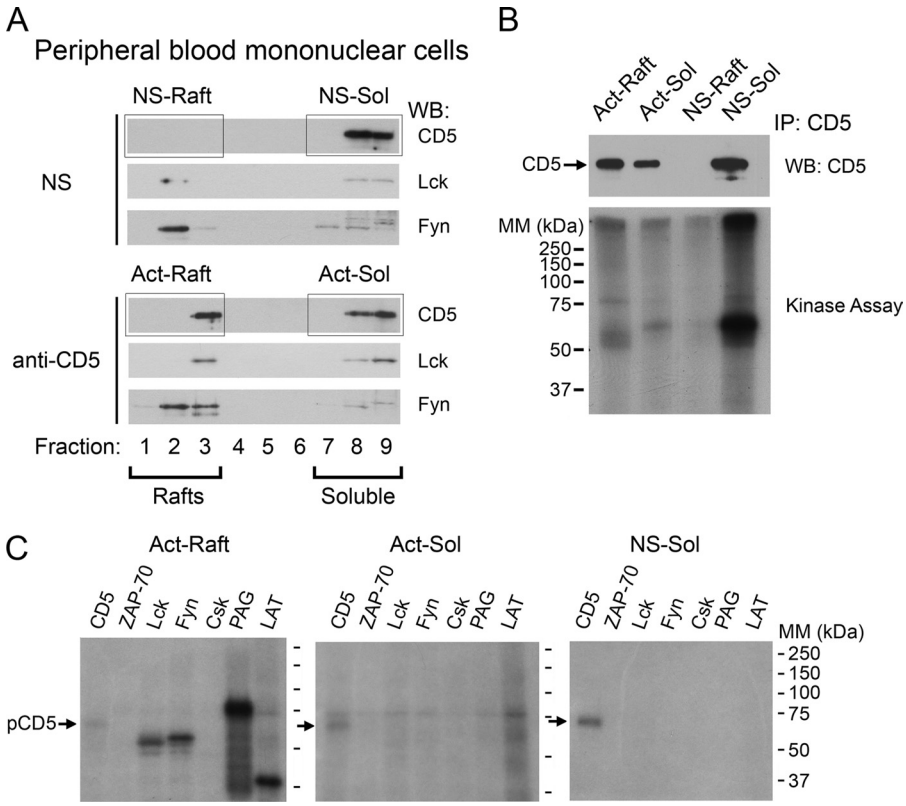

FIGURE 7. **Upon CD5 ligation CD5 translocates to lipid rafts and associates with signaling effectors.** *A*, PBMC were stimulated with anti-CD5 mAb Y-2/178 or incubated with isotype-control antibody (*NS*) for 10 min at 37 °C. Total cell lysates were subjected to sucrose density centrifugation as described under "Experimental Procedures." Fractions recovered were numbered 1–9 from the top of the gradient. An equal volume of each fraction was separated by SDS-PAGE and immunoblotted with either CD5, Lck or Fyn antibodies. The preferential distribution of LAT into the rafts allowed for the identification of raft fractions (not shown). Indicated (*framed*) fractions were pooled, corresponding to CD5 immune complexes in the "soluble" fraction in non-stimulated PBMC (*NS-Sol*), CD5 immune complexes in lipid rafts in non-activated PBMC (*NS-Raft*), CD5 immune complexes in the soluble fraction in CD5-stimulated cells (*Act-Sol*), and CD5 immune complexes in lipid rafts in CD5-stimulated PBMC (*Act-Raft*). *B*, *top panel*, CD5 was immunoprecipitated from the different pools, run on SDS-PAGE, and immunoblotted using anti-CD5 antibodies. *Bottom panel*, CD5 immune complexes from the different sets were then subjected to *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. After denaturation, samples were run on SDS-PAGE in non-reducing conditions and exposed to autoradiography. *C*, CD5 complexes were disrupted by heat and reprecipitated with polyclonal CD5, ZAP-70, Lck, Fyn, Csk, PAG, and LAT antibodies. After SDS-PAGE using reducing conditions, radioactive products were visualized by exposure of dried gels to Kodak BioMax MR films. Phosphorylated CD5 in each gel is indicated by an *arrow*. *Numbers on the right* represent the molecular mass (*MM*) of proteins in kDa.

the folding/expression of the protein or its correct localization in the membrane microdomains. For example, it is possible that the lack of association between CD5.H384^{stop} and Lck or between CD5.H449^{stop} and the elusive Fyn-phosphorylating kinase is not due to the lack of any interaction domains. Instead, it could be possible that the CD5 mutants simply did not fold correctly or that they created a defect that disrupted the migration to lipid rafts, where CD5 would bind to its signaling effectors.

During a study addressing the oligomerization state of transmembrane proteins (34), we had gathered preliminary indications that CD5 was expressed as a dimer at the cell surface. Thus, we enquired whether the oligomerization state of mutant CD5 could be different from the native protein, as this might explain the differences in associations of the CD5 mutants. For this purpose we used the BRET assay, which measures the efficiency of resonance energy transfer between luciferase (donor) and GFP (acceptor) and is useful for determining whether proteins oligomerize *in situ* because the transfer of energy is critically dependent on the separation of the two fluorophores, falling to 0 beyond a distance of 10 nm. In BRET assays, randomly interacting proteins give much weaker signals than oligomers due to their larger average separation distance. Moreover, the signal for randomly interacting proteins, in contrast to that for oligomers, exhibits independence from the acceptor/donor ratio under conditions in which the acceptor level is kept essentially constant (34) as predicted by theory (37).

CD5 fused to either luciferase or GFP[2] retained native CD5-like reactivity with a series of antibodies (data not shown). When expressed as a "BRET pair" in 293T cells, CD5 gave values for the efficiency of energy transfer (up to 0.5) that were substantially higher than those for the control monomeric protein, CD2 (up to 0.3 (34)). Also, in contrast to CD2, the BRET efficiency measured for CD5 exhibited the dependence on acceptor/donor ratio characteristic of oligomeric proteins. The extent of energy transfer and its dependence on the acceptor/donor ratio is comparable with that observed for CD80 in BRET experiments (34), which in solution self-associates with an affinity of ~35 μM (38). This implies that, as likely for CD80, monomeric and dimeric forms of CD5 exist in a dynamic equilibrium. To test whether the cytoplasmic domain of CD5 is involved in dimerization, we generated a chimeric protein, CD5Ext/CD2Int, composed of the extracellular and transmembrane regions of CD5 fused to the cytoplasmic tail of the CD2

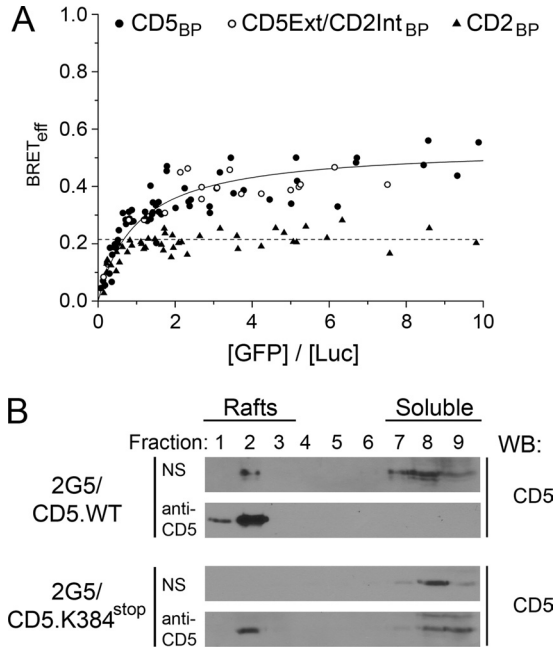

FIGURE 8. **CD5 forms dimers at the cell surface; the cytoplasmic tail of CD5 is not involved in CD5 dimerization or in its translocation to lipid rafts.** *A*, CD2 or CD5 or CD5Ext/CD2Int (a chimera consisting of the extracellular and transmembrane domains of CD5 and the intracellular tail of CD2) was expressed at the surface of 293T cells as BRET pairs (for example by cotransfecting genes encoding $CD5_{Luc}$ together with $CD5_{GFP}$ in the same cell or $CD2_{Luc}$ and $CD2_{GFP}$). BRET analysis showed that the CD5 BRET pair (*$CD5_{BP}$*) shows $BRET_{eff}$ values that can readily be fitted by a dimerization model (34). The CD5Ext/CD2Int chimera follows the same profile as seen for the CD5 wild-type BRET pair, whereas $CD2_{BP}$ shows low $BRET_{eff}$ values and independence of the acceptor:donor ratio, characteristics of monomers. *B*, 2G5/CD5.WT and 2G5/CD5.K384^{stop} cells were stimulated with anti-CD5 mAbY-2/178 or incubated with isotype-control antibody (*NS*) for 10 min at 37 °C. Cells were collected, washed, and lysed, and total cell lysates were subjected to sucrose density centrifugation. Equal volumes of each fraction were resolved by SDS-PAGE and blotted onto PVDF filters. Membranes were incubated with CD5 mAb followed by HRP-conjugated rabbit anti-mouse antibody, and proteins were visualized by enhanced chemiluminescence.

monomer (34). The $BRET_{eff}$ levels obtained for the CD5Ext/CD2Int chimera were indistinguishable from those obtained for the wild-type molecule (Fig. 8*A*), indicating that it is the extracellular region of CD5 that mediates dimerization, as is also the case for CD80.

To test whether the lack of the cytoplasmic tail would impair CD5 translocation to lipid rafts, we analyzed the translocation to rafts of wild-type CD5 as well as of a cytoplasmic deletion mutant. After stimulation of cells with the CD5 mAb, the tailless mutant CD5.K384^{stop} could also be found at significant amounts in lipid raft fractions (Fig. 8*B*), although this translocation was not as complete as in the control-reconstituted cells 2G5/CD5.WT.

We can thus conclude that the lack of the cytoplasmic domain of CD5 does not severely impair its raft-localization or have a role in dimerization. Taken together, our results show that the associations of CD5 with Lck and with the Fyn-phosphorylating kinase are determined by well defined stretches of the cytoplasmic tail of CD5, as we had previously hypothesized.

CD5 Stimulation Prevents Raft-associated Signaling Module Disassembly—Summarizing, we have shown that antibody-mediated stimulation of CD5 induced a significant decrease in the activity of Fyn, caused at least partially by the phosphorylation of the Tyr^{531} inhibitory tyrosine residue. However, we were not able to show a direct interaction of CD5 with Csk. This may point to the existence of a second kinase that, associating with the cytoplasmic tail of CD5, phosphorylates the C-terminal tyrosine of Fyn. It is, however, possible that Csk is still the major enzyme involved and that the interaction with CD5 is not direct or has a complex mechanism of regulation.

In resting T cells, Csk is bound via its SH2 domain to the constitutively phosphorylated tyrosine 317 residue of the membrane-bound adaptor PAG (39). This phosphorylation is kept under equilibrium, controlled by the kinase activity of Fyn, and the activation loop is switched-off as follows; Fyn phosphorylates PAG-Tyr^{317}, and this phosphorylation allows the docking of Csk, which in turn phosphorylates Fyn at its C-terminal inhibitory tyrosine, leading to a decrease in Fyn activity (40, 41). In the onset of T cell activation, TCR engagement and signaling leads to the rapid dephosphorylation of PAG, Csk is displaced from this membrane bound adaptor, and Fyn (as well as Lck) is no longer phosphorylated at the C terminus and thus becomes active, all concurring to the release from tonic inhibition of T cell activation (42). However, 3–5 min later the PAG-Csk complex reassembles, and activation is terminated.

Given that CD5 associates with both Fyn and PAG in lipid rafts and that CD5 stimulation results in the C-terminal phosphorylation of Fyn and the down-regulation of its activity, we questioned the effect of triggering of CD5 on the level of PAG phosphorylation and on the PAG-Csk interaction. After stimulation of JKHM cells with CD5 antibodies, the level of phosphorylation of PAG-Tyr^{317} decreased significantly, as detected cytometrically (Fig. 9*A*) as well as by immunoblotting (Fig. 9*B*) using anti-phospho-PAG antibodies specific for Tyr^{317}. We, therefore, tested whether or not CD5 stimulation and PAG dephosphorylation resulted in the release of Csk from the PAG adaptor. JKHM cells were stimulated with Y-2/178 antibody or with OKT3 to invoke full activation. Cell lysates were subjected to immunoprecipitation with PAG-C1 antibody, and the immune complexes were analyzed by immunoblotting with a Csk polyclonal antibody. As shown in Fig. 9*C*, stimulation with OKT3 resulted in the rapid release of Csk from PAG as reported (39, 43), but there was a fast reassembly of the complex 2 min after activation. On the other hand, CD5 stimulation resulted in the gradual, and only ever partial, dissociation of Csk from PAG.

In toto these observations suggest that after direct ligation, CD5 is targeted to lipid rafts where it associates with both Fyn and PAG (illustrated in Fig. 9*D*). As there is no abrupt displacement of Csk from PAG, CD5-associated Fyn is positioned close to the PAG-Csk complex and is phosphorylated by Csk, and its activity is progressively down-modulated. However, the residual activity of Fyn that we detect is enough to keep PAG-Tyr^{317} sufficiently phosphorylated to retain Csk and keep the Csk-PAG-Fyn complex in an inactive mode. Thus, through this regulatory mechanism, CD5 further contributes to signaling inhibition.

DISCUSSION

Tyrosine kinases are key mediators of signaling by cell surface receptors. The regulation of their activities is, therefore,

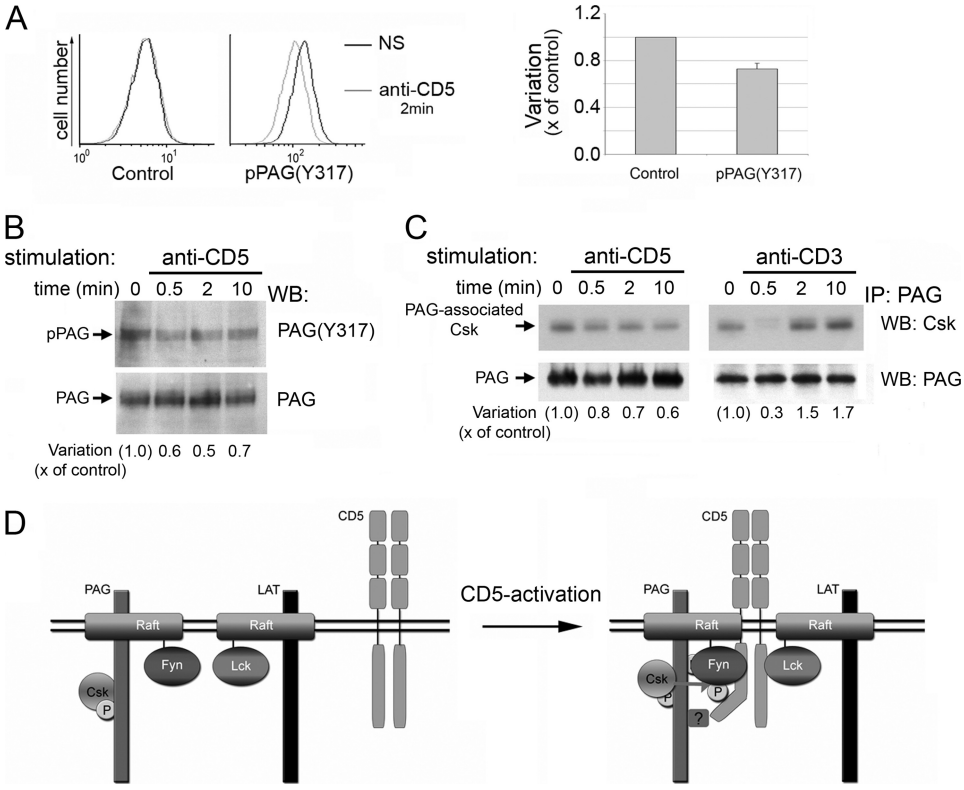

FIGURE 9. **CD5 stimulation induces PAG dephosphorylation and PAG-Csk dissociation.** *A*, JKHM cells were incubated with the CD5 mAb Y-2/178 for 2 min or with isotype-control antibody for 2 min (*NS*). Subsequently, cells were collected, fixed, and permeabilized for 30 min at 4 °C. For flow cytometric analysis, cells were stained with anti-pPAG(Tyr^{317}) antibody followed by FITC-labeled anti-rabbit. Histograms are from one of three independent experiments, and graphs shown are the mean values and S.D. of the variation of PAG-Tyr^{317} phosphorylation relative to time 0 from the three experiments. *B*, detection of pPAG(Tyr^{317}) was also performed by immunoblotting. After CD5 activation, cells were lysed, and lysates were run on SDS-PAGE and immunoblotted with antibodies recognizing the Tyr^{317} residue of PAG, and total PAG. The *x*-fold decrease of PAG(Tyr^{317}) phosphorylation was evaluated by densitometric analysis, corrected for the densitometric values of the PAG Western blotting, and is indicated as values *under the panels*. *C*, JKHM cells were stimulated with anti-CD5 (Y-2/178) or anti-CD3 (OKT3) for the indicated time periods at 37 °C and lysed in lysis buffer. PAG immunoprecipitates were resolved by 7.5% SDS-PAGE and immunoblotted with anti-Csk polyclonal antibody or with anti-PAG as loading control. The *x*-fold modulation of PAG-associated Csk was evaluated by densitometric analysis, corrected for the densitometric values of the PAG Western blotting, and is indicated as values *under the panels*. *D*, a model for CD5-mediated inhibition of T lymphocyte signaling is shown. CD5 molecules are readily recruited to lipid rafts upon CD5 ligation. CD5 may function as a scaffold bringing together signaling effectors such as Lck, Fyn, and PAG, which can be expressed in different subsets of lipid rafts. Within the complex of pooled rafts, CD5 interactions with PAG and Fyn result in a continuous phosphorylation of the tyrosine residue 317 of PAG catalyzed by Fyn, blocking or slowing down the release of Csk that binds to phosphorylated Tyr^{317} of PAG. Captured or unreleased Csk targets Fyn at the C-terminal inhibitory tyrosine residue, but as Fyn is docked through the SH2 domain and thus kept in an open conformation, is resistant to total inhibition (67). The activity of Fyn is nevertheless decreased and consequently impairs downstream signaling, including ZAP-70 activity. This CD5-controlled modulation of the PAG/Csk/Fyn loop ultimately down-regulates long term T cell responses, such as T cell proliferation.

critical for generating the appropriate responses to external stimuli. In T cells, Fyn and Lck, which associate with the T cell receptor and the CD4 or CD8 co-receptors, respectively, are the main Src kinases involved in the early stages of T cell activation (44, 45). Src-family kinases adopt a closed and inactive conformation when the C-terminal inhibitory tyrosine residue is phosphorylated and binds to its own SH2 domain (46). Conversely, dephosphorylation of this residue by tyrosine phosphatases favors the "open," kinase-active state. These effects are mediated predominantly by the tyrosine phosphatase CD45 and the tyrosine kinase Csk (36, 47). Increases in the activity of Src-type kinases can be further promoted via their autophosphorylation of activation loop tyrosines (48).

CD5 belongs to the group B of the scavenger receptor cysteine-rich family of extracellular receptors and secreted glycoproteins that also include CD6, CD163, M160, DMBT1, Spα, scavenger receptor cysteine-rich-4D (SRCR-4D), and SSc5D (49, 50). Although some of these proteins have been associated with specific leukocyte functions, a unifying role for the ensemble of this family of proteins has not been uncovered. CD5 inhibits T cell activation and differentiation without decreasing antigen-presenting cells/T cell adhesion or interfering with immunological synapse formation (51). Instead, CD5 associates with signaling inhibitors, of which the most relevant appears to be the SHP-1 phosphatase (21, 23–25). Binding of SHP-1 to CD5 was mapped at the Tyr^{378} residue of CD5 (21), but the fact that CD5 mutants that do not contain this residue still retain the capacity of inhibit signaling (52, 53) implies that SHP-1 is not the only inhibitory effector of CD5. Other molecules such as rasGAP and Cbl are likely to contribute to the role of CD5 as a signaling attenuator as well (21, 23, 26, 27). In the present report we show that CD5 may also inhibit cell activation through a parallel and previously undescribed mechanism involving the phosphorylation of Fyn at the C-terminal inhibitory tyrosine, thus reducing the activity of this kinase.

An interaction between CD5 and Fyn may be facilitated by the net movement of CD5 to lipid rafts. These membrane

microdomains are heterogeneous (54) and highly mobile (55), and receptors such as CD5 may serve as scaffolds that allow distinct types of rafts and their components to interact (56). CD5 associates with CD2 and with the TCR (23, 57), and these two surface receptors signal via Src kinases in the context of lipid rafts (33, 58). As we show here that CD5 is present at the cell surface as a homodimer, this may promote further the establishment of interaction arrays with a variety of lipid raft and signaling receptors.

Activation of CD5 results in Fyn phosphorylation at Tyr^{531}. In T cells the phosphorylation of the C-terminal inhibitory tyrosine residues of the Src family kinases Lck and Fyn is controlled by the opposing activities of the transmembrane phosphatase CD45 and Csk (59). TCR phosphorylation and ensuing T cell activation may in fact be set off simply by changing the phosphorylation equilibrium through the inhibition of cellular phosphatases (60). Nevertheless, phosphorylation of the C-terminal inhibitory tyrosine residue per se does not necessarily imply an obligatory repression of the kinase. In resting T cells, a significant proportion of Lck is kept permanently active despite being phosphorylated on the C-terminal tyrosine, and changes in phosphorylation status do not always correlate with changes in activity (61, 62).

Notwithstanding the complexity of Src family kinase regulation, our own observation that upon CD5 activation the activity of Fyn decreases nearly by half strongly supports that Fyn phosphorylation at the C-terminal tyrosine contributes to CD5-mediated inhibition. On the other hand, although we did not find major changes in the activity of Lck upon CD5 triggering, a role for Lck in the CD5-mediated inhibition should not be ruled out. Lck still appears to be the main kinase phosphorylating CD5 and it is possibly via phosphorylation of the last tyrosine residue of CD5, Tyr^{463}, that the phosphorylation of Fyn is regulated. CD5 can interact with Lck, and the cytoplasmic domain of CD5 appears to be responsible for the association. Using a series of CD5 truncation mutants, we show that the region between amino acids Lys^{383} and Ala^{417} is sufficient for the association with Lck. Interestingly, the relevant mutant, $CD5.E418^{stop}$, does not contain the tyrosine residue (Tyr^{429}) assumed to be the docking site for the SH2 domain of Lck. Thus, without ruling out a possible role of Tyr^{429} in the Lck-CD5 association, we conclude that Lck can bind to CD5 through a tyrosine-independent mechanism.

The distal region of the cytoplasmic domain of CD5 (amino acids 449–471) is required for the phosphorylation of Fyn. As Csk is the only kinase known to perform this task, we anticipated that CD5 could associate with Csk and, when brought to the vicinity of Fyn, would promote Fyn inhibitory phosphorylation. However, we have not been able to detect such association between CD5 and Csk. It is possible that an indirect interaction of CD5 with Csk occurs, perhaps mediated by an adaptor that binds the distal part of the CD5 tail. However, a further possibility, which we favor, is that CD5 couples Fyn to the regulation of the Csk-PAG interaction, which then feeds back to the inhibition of Fyn (Fig. 9*D*).

In resting T cells, through the interaction with PAG, Csk is maintained at high levels of activity, necessary for inactivating the Src-family kinases (43, 63, 64). According to the current models (41), upon TCR-mediated activation, cellular phosphatases dephosphorylate the Csk-docking tyrosine of PAG (Tyr^{317}), resulting in the release of Csk to the cytoplasm. Interruption of the Csk-PAG association results in the abrupt decline of phosphorylation of the C-terminal inhibitory residue of Lck and Fyn, which becomes active to sustain a burst of phosphorylation reactions, leading to full-scale cellular activation. Active Fyn is then able to induce the phosphorylation of PAG-Tyr^{317}, concurring to the down-modulation phase of the response.

Because raft-associated CD5 interacts with Fyn and PAG, its role may be to sustain the interaction between these two proteins and avoid the disengagement of Csk. We propose the following stages of T cell inhibition by CD5. Coincident with a TCR trigger, the extracellular ligation of CD5 induces its translocation to the sites of signaling initiation, where it closely interacts with Lck and Fyn as well as with the raft-resident adaptors PAG and LAT. CD5-associated Fyn is positioned to continuously phosphorylate PAG at the Csk-docking site, contributing to the retention of Csk at the membrane level, whereas at the same time Fyn is phosphorylated in turn by PAG-bound Csk. Hence, Fyn's own kinase capacity, although partially inhibited, is likely to keep the Csk docking site of PAG phosphorylated. Consistent with this interpretation, our results show that, in contrast to TCR mediated activation, there is no abrupt dissociation of Csk from PAG after CD5 ligation. It is not fully clear what may be the mechanistic role of CD5 in the assembly/disassembly of the Csk-PAG-Fyn complex, whether it functionally blocks the release of Csk from PAG or it simply accelerates the coupling of Fyn with PAG. Given that CD5 associates with many of these signaling components within lipid rafts, it may be instrumental in the architecture of this regulatory platform.

It seems that CD5 may inhibit early TCR signaling via several routes involving ZAP-70. For example, CD5 may associate with SHP-1, which might directly dephosphorylate ZAP-70, inhibiting its activity (65, 66). In parallel, CD5 may inhibit Fyn, which is also important for the activation of ZAP-70 (67). The convergence of these pathways may ensure effective control of this pivotal signaling molecule.

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