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CD5-Negative Regulation of B Cell Receptor Signaling Pathways Originates from Tyrosine Residue Y429 Outside an Immunoreceptor Tyrosine-Based Inhibitory Motif¹

Hélène Gary-Gouy,²* Julie Harriague,²† Ali Dalloul,* Emmanuel Donnadieu,† and Georges Bismuth³†

CD5 is a cell surface receptor that negatively regulates B cell function, but whose relationship to the immunoreceptor tyrosine-based inhibitory motif (ITIM) family of B cell inhibitory receptors is unclear. Using Fcγ type IIB receptor-CD5 chimeras encompassing the cytoplasmic domain of CD5, we previously showed that a particular region of the molecule containing two tyrosine residues, Y429 and Y441, in an amino acid stretch similar to the Src autophosphorylation motif and a putative ITIM, respectively, antagonized early signaling events triggered through the B cell receptor (BCR). In this study, we provide evidences that only Y429 is mandatory for the inhibition by CD5 of the calcium response activated via the BCR. This residue also efficiently controls coaggregation of the two receptors. Thus, CD5 uses an ITIM-independent strategy, centered on Y429, the major tyrosine-phosphorylation residue in its cytoplasmic domain, to inhibit BCR activation. The Journal of Immunology, 2002, 168: 232–239.

Inhibitory receptors of signals delivered through the B cell Ag receptor (BCR)² upon Ag binding require the presence of one or two copies of a consensus cytoplasmic amino acid sequence, the so-called immunoreceptor tyrosine-based inhibitory motif (ITIM) characterized by the prototypic following sequence: (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val) (X = any amino acid) (1). To obtain the inhibitory signal, the tyrosine residue of the ITIM(s) must be phosphorylated, a function essentially assumed by Lyn (2–5), to recruit phosphatases such as the Src homology 2 (SH2) domain-bearing tyrosine phosphatase SHP-1 (6–10) or the SH2 domain-bearing inositol 5'-phosphatase SHIP-1 (11, 12). SHP-1 dephosphorylates various transduction proteins required for B cell activation, such as the protein tyrosine kinase Syk, the B-linker protein BLNK, or phospholipase Cγ enzymes (13–15). As for SHIP-1, it inhibits the relocalization to the cell membrane of critical signaling proteins with a pleckstrin homology (PH) domain, such as the Tec family Bruton’s tyrosine kinase (BTK) (16, 17) or the serine/threonine kinase AKT (18, 19), by hydrolyzing phosphatidylinositol (PtdIns) 3,4,5-trisphosphate (3,4,5-P₃) produced after PtdIns 3-kinase (PI3-K) activation (20). This current ITIM model explains quite satisfactorily the functioning of most inhibitory receptors in B cells, including the FcyRIIB1, the CD22, the CD72, and the paired Ig-like B receptors that share more or less these repressing mechanisms to impair BCR-triggered responses.

Another membrane receptor, the CD5 molecule, constitutively expressed by a subset of B lymphocytes termed B1-a (21–23) or after surface IgM cross-linking of peripheral B cells (24), is also considered now as an authentic B cell inhibitory receptor. Its function in B cells remained unclear until the establishment of genetically engineered mice lacking the CD5 molecule (25). Contrary to other B cells, B1-a cells are poorly responsive to BCR stimulation. It was discovered that in these mice the absence of CD5 restored their capacity to fully proliferate in response to an anti-μ stimulation (26). More recently, it was shown using a BCR transgenic model that CD5 was necessary to maintain tolerance in anergic B cells in vivo (27). Collectively, these data therefore suggested that CD5 was exerting at the functional level a negative signal on the BCR, probably contributing to set threshold levels for activation signals. Using a reconstitution approach in a murine B cell line, we thus recently challenged the possibility of an alteration by the CD5 cytoplasmic domain (CD5cyt) of the early biochemical signals downstream the BCR. We demonstrated that both calcium (Ca²⁺) response and extracellular signal-related kinase-2 (ERK-2) activation following BCR stimulation were antagonized by CD5 upon their clustering, resulting in complete loss in lymphokine release (28). We showed, however, that neither SHIP-1 nor SHIP-1 could be precipitated with phosphorylated CD5 molecules.

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4 Abbreviations used in this paper: BCR, B cell Ag receptor; BTK, Bruton’s tyrosine kinase; CD5cyt, CD5 cytoplasmic domain; ERK-2, extracellular signal-related kinase-2; GFP, green fluorescent protein; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; 3,4,5-P₃, 3,4,5-trisphosphate; PH, pleckstrin homology; PtdIns, phosphatidylinositol; PI3-K, PtdIns 3-kinase; RAM, rabbit IgG to mouse IgG; SH2, Src homology 2; SHIP, SH2 domain-bearing inositol 5'-phosphatase; SH2D, SH2 domain-bearing tyrosine phosphatase.

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CD5cyt contains two potential ITIMs according to the prototypic sequence for these motifs. One, LAY378KKL, straddles the transmembrane and CD5cyt. In T cells, in which an inhibitory effect of CD5 is also disputed (29), its contribution to CD5 phosphorylation and inhibitory effects by recruiting the tyrosine phosphatase SHP-1 (30) has been, however, a great matter of debate. Several studies reported in fact that this putative ITIM did not participate in the phosphorylation of human CD5cyt in Jurkat T cells (31, 32). It was also superfluous for CD5-mediated inhibition in murine T cell models (33, 34). The reason for this discrepancy is not clear, but we demonstrated in our previous work that CD5 constructs without this residue were still inhibitory in B cells. In contrast, we showed the key role played by a sequence of CD5cyt, close to an immunoreceptor tyrosine-based activation motif (ITAM), with two tyrosine residues in a sequence (DNEY429) identical with the autophosphorylation motif of Src protein tyrosine kinases and in the second additional putative ITIM of CD5cyt (SAY441PAL), respectively (28).

The present study was undertaken to make clear the structural basis of CD5-mediated inhibitory effects in B lymphocytes. It demonstrates the essential role played by tyrosine Y429 expressed in the motif of CD5cyt similar to the Src autophosphorylation site. This residue controls all the inhibitory effects of the molecule on critical BCR-induced signaling events, and, consequently, is also required for the phosphorylation of CD5 and inhibition of B cell functional responses. These results definitively demonstrate that CD5 escapes from the classical ITIM model to inhibit B cell activation.

Materials and Methods

Cell culture and IL-2 production

The murine B lymphoma cell line IIA1.6 (a Fc\γRIIB receptor-negative variant of the murine B lymphoma cell line A20) was grown in RPMI 1640 medium (Seromed, Biochrom KG, Berlin, Germany) supplemented with 10% FCS, antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin), 2 mM L-glutamine, 50 μM 2-ME, and 1 mM sodium pyruvate (complete medium). CD5.21, CD5GAITAM.3, and ICI IIA1.6 transfectants were previously described (28). CD5.21 express a chimeric receptor made of the FcγRII1 extracellular and transmembrane domains fused to human CD5cyt, CD5GAITAM.3 express the same chimeric deleted of the pseudo-ITAM motif of CD5cyt, ICI express a truncated form of the molecule without the cytoplasmic domain. They were cultured in the same medium supplemented with 10% FCS, antibiotics and F(ab\(^\prime\))\(_2\) mouse IgG (RAM) (whole molecule) and F(ab\(^\prime\))\(_2\) chimeras expressed in IIA1.6 cells of the mutated Fc\γRIIB-CD5cyt chimeras

Stable expression in IIA1.6 cells of the mutated Fc\γRIIB-CD5cyt chimeras

Constructs were linearized by Scal restriction enzyme digestion and purified by salt-saturated phenol extraction and ethanol precipitation. A total of 5 × 10\(^3\) IIA1.6 cells was mixed with 20 μg plasmid DNA in 0.5 ml of a buffer containing 120 mM KCl, 150 μM CaCl\(_2\), 10 mM K\(_2\)PO\(_4\)/KH\(_2\)PO\(_4\), 2 mM EGTA, 5 mM MgCl\(_2\), 1 mM ATP, 5 mM glutathione, and 25 mM HEPES, and electrophorated at 260 V, 960 μF, in a Gene Pulser (Bio-Rad, Evry, Yonne, France). Transfectants were selected by addition of 1 mg/ml G-418, 24 h after electroporation. Fc\γRIIB expression on the expanding cultures after 10–15 days of culture was detected by indirect immunofluorescence staining with mAb 2.4G2 and FITC-labeled goat anti-rat F(ab\(^\prime\))\(_2\), and the positive cells were sorted with a FACS before cloning by limiting dilution in 96-well culture plates. For each tyrosine mutagenesis, a cell line was also established after two sortings at a 2-wk interval of Fc\γRIIB-positive cells.

Ca\(^{2+}\) response analysis

Intracellular Ca\(^{2+}\) measurements on cell suspensions were performed with fura 2-AM (Molecular Probes, Eugene, OR), as described previously (28). For single cell Ca\(^{2+}\) video imaging, cells loaded with fura 2-AM were seeded in a 10 mM HEPES buffer, pH 7.2, supplemented with 120 mM NaCl, 0.5 mM MgCl\(_2\), 5 mM KCl, 1 mM Na\(_2\)PO\(_4\), and 1 μM flavopiridol on a glass coverslip mounted on a 30-mm petri dish in a final volume of 100 μl. After 5 min to allow the cells to deposit, the medium was carefully removed and replaced with fresh medium at 37°C supplemented with RAM IgG or RAM F(ab\(^\prime\))\(_2\). Measurements were performed at 37°C with a Diaphot 2 microscope (Nikon, Melvlie, NY) and an IMSTAR imaging system, as described previously (35).

Cell stimulation, immunoprecipitation, and Western blot analysis

Cells were washed and resuspended in RPMI medium (1 × 10\(^5\)/ml) containing 10 mM HEPES, pH 7.2, and were then equilibrated for 10 min at 37°C. Cell stimulation was achieved by incubation at 37°C in medium alone or in the presence of the indicated Ab. Activation was stopped by brief centrifugation and lysis at 4°C for 30 min in lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 50 U/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate) containing 1% Nonidet P-40 detergent. Nuclei and cellular debris were removed by centrifugation at 10,000 × g for 10 min, and the amount of proteins in lysates was determined using Bradford test (Bio-Rad). For Fc\γRIIB immunoprecipitations, lysates were incubated for 2 h at 4°C with 2.4G2-coated protein G-Sepharose (Sigma, Saint-Quentin Fallavier, France; 5 μg purified Ab per 50 μl beads diluted 1:2), followed by four washes in lysis buffer. Proteins were then separated by SDS-PAGE (10%) and blotted onto polyvinylidene difluoride membranes (Amersham, Paris, France). For ERK-2 analysis, 20 μg whole cellular lysates were separated on 10% acrylamide SDS gels containing 0.1% bisacrylamide (instead of 0.3%) for accurate separation of the unphosphorylated and phosphorylated forms of the protein and blotted with the anti-ERK-2 Ab. Blots were revealed with an ECL detection system (Amersham). Scanning densitometry was performed with the Bio-Rad’s densitometer GS-670.

Fluorescence analysis

For fluorescence analysis with GFP, cells were centrifuged and resuspended in complete RPMI medium (2 × 10\(^5\)/ml). Cells were then incubated at room temperature for 15 min with 5 μg plasmid DNA and electrophorated at 280 V, 950 μF. After a 16-h culture in complete culture medium, cells were seeded in their culture medium on a glass coverslip mounted on a 30-mm petri dish in a final volume of 100 μl. Cells were left unstimulated or stimulated with RAM IgG or RAM F(ab\(^\prime\))\(_2\) for 5 min at 37°C in a CO\(_2\) incubator. Fluorescence microscopy was performed with a Nikon Eclipse TE300 inverted microscope equipped with fluorescein filters using a ×60 objective. Fluorescence images were collected with a cooled CCD camera (CoolSNAPfx; Roper Scientific, Evry, France) and the MetaView Imaging software (Universal Imaging, West Chester, PA). Digital images (8-bit scale) were printed directly.

Results

Y429, but not Y441, contributes to the inhibitory effect of CD5cyt on BCR-induced Ca\(^{2+}\) response

We previously used Fc\γRIIB1-CD5cyt chimeras expressed in IIA1.6, a FcγR-negative B cell derived from the A20 murine lymphoma, to show that a short sequence alike an ITAM motif in...
human CD5cyt was required to inhibit BCR-induced $\text{Ca}^{2+}$ responses (28). However, the contribution of the two tyrosine residues expressed in this sequence, Y429 and Y441, was not evaluated. To this end, we constructed FcγRIIB1-CD5cyt chimeras in which either residue was mutated to phenylalanine. After transfection in IIA1.6 cells, clones were selected for stable and similar expression of FcγRIIB and BCR (not shown).

$\text{Ca}^{2+}$ responses triggered by F(ab')$_2$ or whole Abs against murine IgG were first analyzed using the different cell clones. CD5.21 cells, expressing the wild-type FcγRIIB1-CD5cyt chimera, and CD5ΔITAM.3 cells, a cell clone in which the deletion of the entire pseudo-ITAM motif of CD5cyt has been shown to restore the $\text{Ca}^{2+}$ response to RAM IgG (28), were used as controls. Upon coligation of the BCR with the chimera, the $\text{Ca}^{2+}$ increase was antagonized in cells expressing the Y441F FcγRIIB1-CD5cyt mutant (Fig. 1A). As with CD5.21 cells, the initial $\text{Ca}^{2+}$ peak (especially in clone Y441F.8), but mainly the sustained $\text{Ca}^{2+}$ influx, was reduced, giving a transient response to BCR stimulation. On the opposite, the response to RAM F(ab')$_2$ or RAM IgG was roughly identical in cells expressing the Y429F mutant. Note that we did not find any significant influence in our experimental cell system of anti-FcγRIIB 2.4G2 mAb addition on the $\text{Ca}^{2+}$ response induced by RAM F(ab')$_2$ (not shown).

To strengthen this finding, $\text{Ca}^{2+}$ experiments at the single cell level were next performed with the Y429F.L and the Y441F.L cell lines. Measurements were done with an imaging system allowing us to analyze the response of a large panel of individual cells. Fig. 1B, four upper panels, shows the $\text{Ca}^{2+}$ response of 40 individual cells stimulated with RAM F(ab')$_2$ or RAM IgG. As compared with RAM F(ab')$_2$ stimulation, the initial $\text{Ca}^{2+}$ increase induced by RAM IgG was regularly reduced in Y441F.L cells. Moreover, the prolonged influx phase was hardly ever observed. In contrast, Y429F.L cells responded strongly to both stimuli. Mean responses are shown in the two lower panels, confirming the results found at the individual cell level. Taken together, these findings demonstrate the prominent role of Y429 in the inhibitory effect of CD5cyt on BCR-induced $\text{Ca}^{2+}$ responses.

ERK-2 inhibition after BCR aggregation with CD5 is ITIM independent

As recently shown for the FcγRIIB1 (36), different inhibitory pathways could originate from distinct regions of the CD5 molecule to alter B cell activation. In parallel to $\text{Ca}^{2+}$ inhibition, ERK-2 activation was also impaired upon BCR coligation with CD5 (28).

**FIGURE 1.** The inhibitory properties of CD5 on BCR-induced $\text{Ca}^{2+}$ response in IIA1.6 murine lymphoma B cells are mediated by a unique tyrosine residue, Y429, in the cytoplasmic domain of the molecule. A, FcγRIIB transfectants were loaded with the fluorescent $\text{Ca}^{2+}$ indicator fura 2-AM, and fluorescence of the cell suspension was monitored with a spectrometer in a $\text{Ca}^{2+}$-containing medium at 37°C after addition of RAM IgG (45 µg/ml) (dotted lines) or RAM F(ab')$_2$ (30 µg/ml) (continuous line). B, Cells were loaded as in A with fura 2-AM, and the $\text{Ca}^{2+}$ response of individual cells to RAM F(ab')$_2$ and RAM IgG stimulation was measured with an imaging system. Traces of 40 individual cells are shown in the upper panels. Mean responses were calculated in the two lower panels.
However, the region of CD5cyt involved in this phenomenon was not defined yet, and a contribution of the putative SAY441PAL ITIM motif could not be excluded. To investigate this, lysates from clones (upper panel) or cell lines (lower panel) expressing the Y429F or Y441F mutants were probed in Western blot experiments with an anti-ERK-2 Ab after stimulation with RAM F(ab')₂ or RAM IgG (Fig. 2). CD5.21 cells and CD5ΔITAM.3 cells were used in parallel as positive and negative controls of inhibition, respectively. As previously reported, a strong reduction of the upper band corresponding to phosphorylated ERK-2 was found in CD5.21 cells upon coligation of the BCR with the wild-type chimera by RAM IgG. This inhibition was maintained in cells expressing the Y441F mutant. In contrast, ERK-2 activation was fully restored in CD5ΔITAM.3 and largely in cells expressing the Y429F-mutated chimera. This result demonstrates that inhibition of BCR coupling to ERK-2 by CD5 is also governed by the pseudo-ITAM of CD5cyt and requires Y429, but not the putative SAY441PAL ITIM motif.

The Y429 residue controls the inhibition by CD5 of the membrane translocation of the 3'-PtdIns-specific PH domain of AKT

Collectively, the previous results showed that CD5 inhibition of Ca²⁺ and ERK-2 pathways may originate from a unique site of CD5cyt outside an ITIM. This led us to examine whether this region of the molecule could also impair additional important metabolic events such as production of 3'-PtdIns. This pathway is critical in signaling processes downstream the BCR by recruiting at the cell membrane proteins bearing specific PH domains (37). To investigate this, we used a fluorescent selective probe made of GFP fused to the PH domain of the protein kinase AKT, one well-known cellular effector of PI3-K after BCR engagement (38, 39). This probe has been shown to be a very sensitive tool to monitor localized changes in plasma membrane PtdIns 3,4-bisphosphate and PtdIns 3,4,5-P₃ levels in living B cells (40).

We first analyzed whether wild-type CD5 could inhibit AKT membrane relocalization using CD5.21 cells and cells expressing a Fc-RIIB-truncated receptor without the cytoplasmic domain (IC1), as a control. Fig. 3A shows that AKT PH-GFP translocation did not occur significantly upon RAM IgG-induced aggregation of the BCR with the FcγRIIB-CD5cyt chimera in CD5.21 cells. No inhibition was observed in IC1 control cells. Quantitative analysis is shown in Fig. 3B, in which we measured the percentage of cells having either a diffuse fluorescent pattern or a membrane localization of AKT PH-GFP. Only fluorescent cells exhibiting an unambiguous pattern (usually >70%) were taken into account. The data clearly showed that the relocalization induced by RAM F(ab')₂ in most CD5.21 cells was totally suppressed upon coligation of the BCR with CD5cyt. Again, no difference was observed in IC1 control cells with RAM F(ab')₂ and RAM IgG, both stimuli triggering the membrane translocation of the probe with the same efficacy. Similar results were obtained with a BTK PH-GFP probe, although in this case the translocation was much less apparent than with AKT (not shown).

The different cell mutants were then checked to see whether the Y429 residue responsible for the Ca²⁺ and the ERK-2 inhibition also controls the inhibitory effect of CD5 on AKT translocation. This was examined after transfection of the different CD5cyt mutants with AKT PH-GFP and analysis of the fluorescence after RAM F(ab')₂ and RAM IgG addition. The percentage of cells in which the probe was translocated to the cell membrane is plotted in Fig. 3C. All transfected responded equally well to BCR stimulation by RAM F(ab')₂. Upon aggregation of the BCR with the chimera by RAM IgG, the results demonstrate unambiguously that the inhibition of AKT translocation was fully maintained only in the two cell clones and the cell line expressing the Y441F chimera. This inhibition was essentially lost in the different Y429F cell mutants.

Y429 mediates inhibition of IL-2 production

All the biochemical pathways inhibited by CD5 are critical in mediating B cell functional responses. Thus, our finding that Y429 played a prominent role in these inhibitory processes prompted us to analyze the functional consequence of its mutation on inhibition of BCR-induced IL-2 production by CD5. For this aim, the different FcγRIIB-CD5cyt-mutated chimeras were coaggregated with the BCR by RAM IgG, and IL-2 production obtained under these conditions was compared (Fig. 4A). We can see that IL-2 synthesis was totally inhibited both in CD5.21 cells containing the wild-type chimera and in the cell line expressing the Y441F mutant. In contrast, mutation of residue Y429 restored IL-2 production. Similar results were consistently obtained with the cell clones expressing the mutated chimeras (not shown).

A unique tyrosine residue mediates BCR-induced CD5cyt tyrosine phosphorylation

CD5cyt is strongly tyrosine phosphorylated upon its coligation with the BCR, and we assume in our previous report that this phenomenon was required to inhibit B cell responses (28). Because Y429 was essential to block various signaling pathways downstream the BCR, we therefore assessed its phosphorylation status by measuring FcγRIIB-CD5cyt phosphorylation after RAM IgG stimulation of the different cell mutants (Fig. 4B). The chimera was immunoprecipitated with the FcγRIIB-specific mAb 2.4G2 before probing with anti-phosphotyrosine mAb 4G10. A strong labeling, not significantly different from CD5.21 control cells, was observed with RAM IgG in the two Y441F mutant cell clones, Y441F.1 and Y441F.8, and in the Y441F.L cell line (upper panel). In contrast, phosphorylation was almost undetectable in clone Y429F.5 and in the Y429F.L cell line expressing the Y429F mutant. It was slightly higher in clone Y429F.6. However, we could calculate from the

FIGURE 2. Y429 is also involved in ERK-2 inhibition after BCR aggregation with CD5. FcγRIIB transfectants were stimulated for 10 min with RAM IgG (45 μg/ml) or RAM F(ab')₂ (30 μg/ml) at 37°C, or left unstimulated. Cells were lysed, and 20 μg cellular proteins were fractionated by SDS-PAGE, transferred onto membranes, and blotted with the anti-ERK-2 mAb. The two blots were from separate experiments.
The Y429 residue of CD5 controls the inhibition of the membrane translocation of the 3'-PtdIns-specific PH domain of AKT. 

A. CD5.21 cells expressing the wild-type FcγRIIB-CD5cyt chimera or IC1 cells expressing a FcγRIIB1-truncated receptor without the cytoplasmic domain were electroporated with a construct coding for the PH domain of AKT fused to GFP. Cell fluorescence was analyzed 16 h later on living cells, after 5 min of stimulation with RAM IgG (45 μg/ml) or RAM F(ab')₂ (30 μg/ml), or left unstimulated. Four typical individual cells are shown for each stimulation condition of the two cell types. 

B. CD5.21 cells were electroporated with the AKT PH-GFP construct and stimulated as in A. Fluorescence was then analyzed, and the number of cells with a diffuse fluorescent pattern or a membrane localization of the probe was plotted as the percentage of the total cell number (n > 70).

C. FcγRIIB transfectants, electroporated with the AKT PH-GFP construct, were stimulated for 5 min with RAM IgG (45 μg/ml) or RAM F(ab')₂ (30 μg/ml) at 37°C, or left unstimulated. Fluorescence was then analyzed, and the number of cells with a membrane localization of the probe was plotted as the percentage of the total cell number (n > 70).

experiment shown in the lower panel by scanning densitometry that mutating residue Y429 removed >90% of CD5cyt tyrosine phosphorylation in this clone after 2 min of stimulation with RAM IgG. One has to remark in this work that the pseudo-ITAM-deleted form of FcγRIIB-CD5cyt, which was run in parallel, gave a more important residual phosphorylation than the Y429F mutant, a finding that will be discussed below. No background phosphorylation was observed with the tailless IC1 mutant. Finally, this experiment
also showed no further increase of the Y429F mutant phosphorylation for a longer period of stimulation, excluding a delay in the phosphorylation. Similar results, establishing the key role played by Y429 in CD5cyt phosphorylation in B cells, were found in Daudi human B cells upon coaggregation of their membrane IgM with the Y429-mutated chimera (not shown). Hence, phosphorylation of CD5cyt induced by BCR stimulation is essentially controlled by residue Y429.

Discussion

CD5 is categorized now, at least in B cells, in the enlarging family of inhibitory receptors. This has been shown at the functional level in genetically modified murine cell systems as well as at the biochemical level after its coligation with the BCR (28). Following the common scheme established for other B cell inhibitory receptors with ITIM(s), it was thus suggested that the tyrosine phosphorylation of ITIM sequences expressed by CD5cyt might be used to alter BCR signaling. The present study clearly demonstrates that this assumption is not true, showing that the CD5-inhibitory process is not subordinated to any ITIM, but requires the phosphorylation of the tyrosine residue in the DNEY motif shared by CD5 and the autophosphorylation site of Src protein tyrosine kinases.

The relationship between CD5 tyrosine phosphorylation and BCR inhibition has not been firmly established yet. Thus, our finding that mutation of Y429 to phenylalanine strongly antagonized CD5 tyrosine phosphorylation as well as its inhibitory action on BCR stimulation demonstrates that the phosphorylation of this residue is at the onset of the CD5-inhibitory pathway. A more significant residual phosphorylation of CD5cyt was found in chimera lacking the entire pseudo-ITAM motif (Ref. 28 and Fig. 4). One hypothesis is that in this case conformational changes are induced by the ITAM deletion, exposing Y463, the remaining tyrosine at the C terminus of CD5cyt, in a more accessible position. However, it should be stressed that our finding with the Y429F mutant does not necessarily mean that in normal conditions with the wild-type CD5 molecule Y463 is not phosphorylated. Indeed, as shown for CD19 (41), Y429 might be necessary as a starting point to induce sequential phosphorylation of CD5, presumably by Lyn, which appears to be required for CD5 phosphorylation in B cells (42). Mutational analysis of Y463 in our system would be necessary to clarify this point. Interestingly, the recent studies of Dennehy et al. (31) and Vila et al. (32), analyzing by point mutations the tyrosine phosphorylation sites of CD5cyt in Jurkat T cells, showed that the situation was most likely different in this cellular model. They found that Y463 seemed to be phosphorylated at greater stoichiometry than Y429, a result that might reflect the parallel observation that both could bind Lck in vitro (31). Whether Lyn only adapts to Y429 in the DNEY motif of CD5 is thus a workable possibility. Whatever it may be, there is no doubt that both in B cells (this study) and in T cells (31, 32), Y441 is not phosphorylated. Thus, our parallel observations that Y441 is dispensable for inhibition at biochemical and functional levels demonstrate that CD5 is an ITIM-independent inhibitory receptor in B cells.

Our findings raise the attractive possibility that CD5-inhibitory effects in B cells originate from the Src autophosphorylation site-like sequence of CD5 only. This must be compared with the situation found with the FcγRIIB1 receptor, in which multiple distinct sites participate in inhibitory signaling. Thus, it was recently shown that two tyrosine residues within the cytoplasmic domain, one in the ITIM, the other at the C terminus, could function uniquely by recruiting SHIP and supposedly Grb2, respectively (36). Moreover, an inhibitory function was also attributed to the transmembrane domain of the FcγRIIB1 molecule that could mediate CD19 dephosphorylation (43). CD19 has been shown to participate in PI3-K recruitment and activation of related pathways, such as the AKT pathway (44), and also to up-regulate the Ca^{2+} response (45) and the mitogen-activated protein kinase cascade (46) after BCR stimulation. Mechanistically, the inhibitory effect of FcγRIIB1 on CD19 phosphorylation is not yet understood, but our chimeras encompass the transmembrane FcγRIIB1 receptor domain. An inhibition of BCR signaling by this domain independently of CD5cyt was thus questionable. We checked CD19 phosphorylation after immunoprecipitation and found that all our CD5 constructs reduced CD19 phosphorylation similarly when coligated with the BCR (not shown). This was true in particular for Y429F cells, in which the inhibitory effects of CD5 were strongly reduced, demonstrating that this residue controls BCR signaling independently of CD19 phosphorylation. One can just notice that BCR signaling (see, for instance, AKT translocation in Fig. 3C)
was never totally restored by the Y429F mutation, suggesting conceivably a small participation of the FcγRIIB transmembrane region through CD19 dephosphorylation. Constructions with the CD5 transmembrane domain are now being established to stamp out this parameter.

A primary mechanism by which the FcγRIIB receptor inhibits ERK-2 activation involves the recruitment and the tyrosine phosphorylation of the Ras regulatory adapter DOK-1 onto SHIP molecules bound to the phosphorylated ITIM (47). We previously reported that SHIP-1 could not be precipitated with phosphorylated CD5 molecules, in agreement with the present results showing no phosphorylation of the putative ITIM in our chimera. Accordingly, we found that DOK-1 was not phosphorylated and did not associate with SHIP-1 after FcγRIIB-CD5cyt coligation with the BCR (J. Harriague, unpublished results). Thus, another mechanism(s) must be responsible. Inhibition of PI3-K metabolism by CD5, as suggested by our results with AKT, might be very important, as we know in particular that a molecule like the adapter Gab1 has a PtdIns 3,4,5-P3-specific PH domain and links the BCR to the PI3-K/AKT and the ERK-2 signaling pathways (48–50). Moreover, BTK also links the BCR to the Ca2+-response in a PtdIns 3,4,5-P3-dependent manner (17). So, any alteration of membrane PtdIns 3,4,5-P3 would affect both pathways similarly and could explain all the inhibitory effects seen with CD5. From this point of view, CD5 is not very different from the FcγRIIB receptor, in which degrada-

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