The Pseudo-immunoreceptor Tyrosine-based Activation Motif of CD5 Mediates Its Inhibitory Action on B-cell Receptor Signaling*

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Genetic studies revealed that CD5 could be a negative regulator of the B-cell antigen receptor (BCR). We explore here the effect of human CD5 on BCR-triggered responses. B cells were obtained expressing a chimera composed of extracellular and transmembrane domains of Fcγ type IIB receptor fused to CD5 cytoplasmic domain (CD5cyt). Coligation of the chimera with the BCR induces CD5cyt tyrosine phosphorylation. A rapid inhibition of BCR-induced calcium response is observed, as well as a partial but delayed inhibition of phospholipase Cγ1 phosphorylation. Activation of extracellular regulated kinase-2 is also severely impaired. Moreover, at the functional level, interleukin-2 production is abolished. Src homology 2 domain-bearing tyrosine phosphatase SHP-1 and Src homology 2 domain-bearing inositol 5'-phosphatase SHIP usually participate in negative regulation of the BCR. We show that they do not associate with the phosphorylated CD5 chimera. We finally demonstrate that the pseudo-immunoreceptor tyrosine based activation motif present in CD5cyt is involved because its deletion eliminates the inhibitory effect of the chimera, both at biochemical and functional levels. These results demonstrate the inhibitory role of CD5 pseudo-immunoreceptor tyrosine based activation motif tyrosine phosphorylation on BCR signaling. They further support the idea that CD5 uses mechanisms different from those already described to negatively regulate the BCR pathway.

CD5, a 67-kDa monomeric type I membrane antigen, belongs to a family of proteins widely expressed by cells of the immune system and whose extracellular domains are characterized by the presence of the highly conserved scavenger receptor cysteine-rich domain (1). CD5 has several potential ligands (2–4). The best known is CD72, an homodimeric membrane glycoprotein commonly present on B cells (2). CD5 is expressed on T and B cells. Most immature T cells express the molecule, including early tyrosine phosphorylation events elicited by TCR activation (18, 19), and it was reported to associate with CD5 in immature thymocytes (20). Moreover, an association of SHP-1 with CD5 has been reported in human thymocytes (15). Thus, it was proposed that CD5 may negatively influence the activity of critical signaling elements between the TCR and the Ca2+ response, particularly in immature T cells. The cytoplasmic domain of CD5 is tyrosine phosphorylated after TCR engagement (12, 16, 17). It was therefore assumed that this phenomenon may help to recruit Src homology 2 (SH2) domain-containing signaling molecules with inhibitory properties. Notably, an implication of the SH2 domain-bearing tyrosine phosphatase SHP-1 was predicted. SHP-1 is known to exert a negative influence on the early tyrosine phosphorylation events elicited by TCR activation (18, 19), and it was reported to associate with CD5 in murine thymocytes (20). Moreover, an association of SHP-1 that requires a tyrosine residue (at position 378) in an immunoreceptor tyrosine-based inhibition motif (ITIM)-like sequence straddling the transmembrane and the cytoplasmic domain of CD5 was also described in Jurkat T cells (21).

CD5 is also associated with the B-cell receptor (BCR) (22), and studies in CD5-deficient mice also suggested a negative role of the molecule on BCR signaling. Normal CD5+ perito-
innocent B-1 cells proliferate poorly in response to an anti-μ stimulation. Proliferation was restored in CD5-negative mice (23). Ca2+ response also increased in parallel. Thus, CD5 molecules may also down-regulate some early signaling events of the BCR biochemical pathway. In agreement with this assumption was the finding that a previous cross-linking of CD5 on normal B-1 cells could restore anti-IgM-induced proliferation, likely by moving these molecules away from the BCR (23). However, no direct evidence has yet been provided showing that CD5 could inhibit early signaling events triggered through the BCR. The present study was undertaken to investigate this possibility.

To analyze the regulatory properties of CD5 on BCR signaling, we expressed in a murine B cell line a chimeric membrane receptor made of the cytoplasmic domain of CD5 (CD5cyt), beginning at residue lysine 379, with the extracellular and transmembrane domains of the low affinity IgG receptor FcyRIIB. Our results demonstrate that when cotigated with the BCR, the CD5 chimera strongly antagonized both biochemical and functional BCR stimulatory events. They also show the key role played by the cytoplasmic sequence of CD5, which comprises two tyrosine residues in a pseudo-immunoreceptor tyrosine based activation motif (ITAM). They further suggest that the inhibitory action of CD5 is mediated by different molecules in B cells and T cells because our results show no clear implication of SHP-1.

**Experimental Procedures**

**Cell Culture**—The murine B lymphoma cell line IIA1.6 (a Fcy receptor-negative variant of the murine B lymphoma cell line A20 (24) was grown in RPMI 1640 medium (Seromed, Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum, antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin), 2 mM l-glutamine, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate. IIA1.6 transfectants expressing wild-type FcyRIIB (IIA B1) or a truncated form of the molecule without the cytoplasmic domain (IIA IC1) were previously described (25). They were cultured in the same medium supplemented with 1 mg/ml G-418 (Geneticin, Life Technologies, Inc.). This medium was also used for selection and culture of stable transfectants (see below).

**Antibodies**—The anti-FcyRIIB 2.4G2 monoclonal antibody (mAb) (26) was produced and purified by affinity chromatography on protein G-Sepharose (Pharmacia). A mouse IgM isotype control (CTC)-labeled mouse anti-rat Fab′ fragments and FITC-labeled donkey anti-rabbit F(ab′)2 fragments were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Rabbit IgG fraction to mouse IgG (RAM) (whole molecule) and F(ab′)2 fragments were from Cappel (ICN Pharmaceuticals, Inc., Aurora, OH). Anti-phosphotyrosine mAb 4G10 and anti-PLCγ1 mAb were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phosphotyrosine mAb PY-20 directly coupled to horseradish peroxidase was from Chemicon (Temecula, CA). mAbs against SHP-1 and SH2 domain-bearing inositol 5′-phosphatase (SHIP) were purchased from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology Inc., respectively. Anti-extracellular regulated kinase (ERK) 2 mouse mAb and anti-active ERK1/ERK2 rabbit polyclonal antibody (Ab) were purchased from Upstate Biotechnology Inc. and Promega Corporation (Madison, WI), respectively. Rabbit polyclonal Ab against recombinant extracellular domain of FcyRIIB were a kind gift of Dr. Catherine Saute`s (INSERM U255, Institut Curie, Paris, France).

**Constructs**—The primers used to amplify CD5cyt were chosen according to the Swiss-Prot analysis of human CD5 protein sequence (8). CD5 was first amplified by polymerase chain reaction (PCR) using the following primers 5′-GGGTAACCCAAAGACGTAGAAGAGAGGCGGTTCCTGCATGGGCAGG-3′ and 5′-GGAGCGATGCAGAACCCTCCTTTCCGTTATACCCACCTGGTGGAAGGC-3′, according to the manufacturer’s protocol of the Quick Change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) and using the FcyRIIB-CD5cyt construct in pNT-neo vector as a template. cDNA sequence encoding the transmembrane and cytoplasmic domains of a human killer cell inhibitory receptor (KIR) were the new insert (27) amplified by PCR with the following primers: 5′-CCCAGACAGTACCCCGTCTCCT-3′ and 5′-CTGAACTCTGGAGCTCATGGCGACGGAG-3′. The PCR-introduced restriction sites at the 5′ (KpnI) and the 3′ (SacI) ends were then used for subcloning the purified PCR product into the pNT-neo vector in frame with the extracellular domain of FcyRIIB. All constructs were verified by DNA sequencing.

**Intracellular Ca2+ Measurements**—Constructs were linearized by SstI restriction enzyme digestion and purified by SS-phenol extraction and ethanol precipitation. 5 × 104 IIA1.6 cells were mixed with 20 μg of plasmid DNA in 0.5 ml of a buffer containing 120 mM KCl, 150 μM CaCl2, 10 mM KHPO4/KH2PO4, 2 mM EGTA, 5 mM MgCl2, 1 mM ATP, 5 mM glutathione, and 25 mM HEPES, and electroporated at 260 V, 960 microfarads, in a Gene Pulse cuvette (Bio-Rad, Ivry sur Seine, France). Transfectants were selected by selection with G-418, 24 h after electroporation. FcyRIIB expression on the expanding cells after 10–15 days of culture was detected by indirect immunofluorescence staining with mAb 2.4G2 and FITC-labeled mouse anti-rat Fab′, and the positive cells were sorted with a FACSCalibur before cloning by limiting dilution in 96-well culture plates. For FcyRIIB-CD5cyt construct with deletion of the pseudo-ITAM motif, a cell line was also established after twofold fittings of 2-week intervals of FcyRIIB expression. The cell suspension was excited alternatively at 340 and 380 nm, and the fluorescence was measured at 510 nm. Graphic representations of intracellular free Ca2+ concentration ([Ca2+]]i) were computed by using the fura-2 ratio ([Ca2+]]i = 225 [F(R)]/[F(R)0] = RMS(BS)/RMS(SB), respectively, published by Grynkiewicz et al. (29).

**Cell Stimulation, Immunoprecipitation, and Western Blot Analysis**—Cells were washed once and resuspended in RPMI medium (1 × 105/ml) containing 10 mM HEPES, pH 7.2, and then were equilibrated for 20 min at 37 °C in a humidified atmosphere containing 5% CO2. Cell stimulation was achieved by incubation at 37 °C in lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 50 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate) containing 1% of Nonidet P-40 detergent. Nuclei and cellular debris were removed by centrifugation at 10,000 × g for 10 min. The amount of proteins present in each sample was determined using Bradford test (Bio-Rad).

For FcyRIIB immunoprecipitations, lysates were incubated for 2 h at 4 °C with 2.4G2-coated protein G-Sepharose (Sigma, Saint-Quentin Fallavier, France; 5 μg of purified Ab/50-μl beads diluted 1:2). For PLCγ1 immunoprecipitation, lysates were incubated for 2 h at 4 °C with specific mAb, and immune complexes were precipitated with 10 μl of protein A-Sepharose (Sigma). After four washes in lysis buffer, immune complexes were boiled for 3 min in reducing or nonreducing (for FcyRIIB blotting) sample buffer. Eluted proteins were separated by SDS-PAGE, transferred for 75 min to PVDF membranes, treated with polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) or Immobilion-P membranes (Millipore, Bedford, MA). Membranes were saturated with 3% gelatin (Bio-Rad) or 5% skimmed milk (Régilait, Saint-Martin-Belleruche, France). Following incubation with specific antibodies as indicated, blots were revealed using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Bio-Rad) as secondary antibodies and an enhanced chemiluminescence detection sys-
tem (Amersham Pharmacia Biotech) on Cronex x-ray film (Du Pont de Nemours, Les Ulis, France).

For ERK2 analysis, 20 mg of 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 anti-ERK2 or anti-active ERK1/ERK2 Abs. Scanning densitometry of the films was performed with the Bio-Rad densitometer GS-670. Results were analyzed with the Molecular Analyst/PC image analysis software (Bio-Rad).

RESULTS

Coligation of FcγRIIB-CD5 Chimera with BCR Inhibits IL-2 Production—To investigate the regulatory properties of CD5 on BCR signaling, IIA1.6 cells were stably transfected with a chimera molecule made of the extracellular and the transmembrane (T.M.) domains of the low affinity IgG receptor FcγRIIB (open box) fused to the cytoplasmic domain of human CD5 molecule (CD5cyt, solid black box). The amino acid sequence of the junction is shown. The first CD5 residue in the chimera was a lysine, corresponding to Lys379 of wild-type CD5. The sequence of the pseudo-ITAM motif of CD5 is also shown as well as the position of the three tyrosine residues expressed by the cytoplasmic domain of the chimera and corresponding to Tyr429, Tyr441, and Tyr463 of the human CD5 sequence. B, BCR and FcγRIIB expression. Open histograms show the expression levels of the BCR, measured by indirect immunofluorescence, using rabbit anti-mouse IgG F(ab’)2 fragments and FITC donkey anti-rabbit F(ab’)2 and of the FcγRIIB-CD5 chimera using 2.4G2 mAb and FITC mouse anti-rat F(ab’)2, respectively. Closed histograms show the fluorescence of cells incubated with secondary Abs only. C, inhibition of IL-2 production. FcγRIIB transfectants were stimulated with indicated concentrations of RAM F(ab’)2 (closed symbols) or RAM IgG (open symbols). The IL-2 release in 18-h culture supernatant was assayed for [3H]thymidine incorporation in CTLL-2 cells. Shown is the radioactivity incorporated in CTLL-2 cells as a function of the concentration of RAM Abs or fragments.

To evaluate the functional consequence of CD5 expression on BCR stimulation, the CD5 chimera was coaggregated with the BCR by different concentrations of RAM IgG and IL-2 production obtained under these conditions compared with IL-2 production induced by RAM F(ab’)2 fragments (Fig. 1C). IIA B1 and IIA IC1 were stimulated in parallel using the same procedure. We can see that F(ab’)2 fragments specific for murine IgG triggered marked IL-2 productions in the different cell types, dose-dependently. As with wild-type FcγRIIB1, this production was inhibited when the chimera containing CD5cyt was coligated with the BCR by the stimulating Abs. No inhibition was observed with IIA IC1 control cells. Similar results were consistently obtained with several other clones expressing the FcγRIIB-CD5 molecule (not shown).

CD5 Antagonizes Both Ca2+ and ERK2 Activation Induced after BCR Stimulation—Signal transduction from the BCR triggers various biochemical events (reviewed in Ref. 31). This includes an increase in cytosolic Ca2+ concentration and an activation of Ras, which are essential to activate transcription. We therefore analyzed Ca2+ responses and activation of ERK2, a downstream effector of the Ras pathway, induced by RAM IgG or RAM F(ab’)2 fragments in the different transfectants.
We first measured with the Ca\(^{2+}\) indicator fura-2 the Ca\(^{2+}\) response to BCR stimulation. As shown in Fig. 2A (left panel), the different cells markedly respond to F(ab')\(_2\) fragments specific for murine IgG. As already reported, responses were strongly reduced with whole IgG in IIA B1 cells used here as a positive control of BCR inhibition. No inhibition was observed with IIA IC1 cells used as a negative control. The Ca\(^{2+}\) response was strongly antagonized upon aggregation of the BCR with the Fc\(_{gRIIB}\) molecule in two representative clones (IIA CD5.17 and IIA CD5.21) expressing the chimera. Experiments were also performed in Ca\(^{2+}\)-free medium to analyze the sensitivity of the two phases of the Ca\(^{2+}\) response to CD5 inhibition (Fig. 2A, right panel). We can see that Ca\(^{2+}\) mobilization was moderately inhibited as compared with the influx phase, which was strongly impaired. Note that the initial slope of the Ca\(^{2+}\) curve was identical for RAM IgG and RAM F(ab')\(_2\) fragments.

This inhibitory effect of CD5 on BCR-induced Ca\(^{2+}\) responses led us to analyze PLC\(_{\gamma}\) tyrosine phosphorylation. Shown in Fig. 2B (left panel), is an experiment where we compared PLC\(_{\gamma}\) phosphorylation induced by a 2-min stimulation with RAM IgG or RAM F(ab')\(_2\) fragments. A partial inhibition was observed with RAM IgG in cells expressing the CD5 chimera. Additional kinetic studies were also performed. As shown in Fig. 2B (right panel), the partial inhibition of PLC\(_{\gamma}\) phosphorylation upon coligation of BCR with Fc\(_{gRIIB}\)-CD5 was rather delayed and became evident after only 2 min of stimulation. Note the increase of PLC\(_{\gamma}\) phosphorylation for a short period of stimulation with RAM-IgG, as compared with RAM F(ab')\(_2\) fragments.

Ras activation results in the subsequent phosphorylation and activation of proteins of the ERK family, ERK1 and ERK2. We therefore assessed ERK phosphorylation in transfectants stimulated for 2 min with RAM IgG or RAM F(ab')\(_2\) fragments. As shown in Fig. 3A (upper panel), lysates of activated cells were probed with an anti-ERK2 Ab, which recognizes both the unphosphorylated form and the dually phosphorylated, active form of the molecule. Partial reduction of the upper band corresponding to phosphorylated ERK2 was found upon coligation of the BCR and the chimera with RAM IgG, suggesting an inhibition of the coupling of the BCR to the Ras pathway by CD5. Note the marked inhibition of ERK2 phosphorylation with the wild-type Fc\(_{gRIIB1}\). Lysates were also probed with an Ab specific for the active form of ERK1 and ERK2 (Fig. 3A, lower panel). Only phosphorylated ERK2 was detected in the different cell clones, confirming the previous result, reduced ERK2 phosphory-
lutions were observed with RAM-IgG in clones expressing FcγRIIB1 wild-type or the FcγRIIB-CD5 chimera. Kinetic experiments were also conducted by measuring ERK2 phosphorylation after coligation of the chimera with the BCR for different periods of time. As shown in Fig. 3B, inhibition of ERK2 phosphorylation was almost complete after 10 min of stimulation with RAM IgG. Interestingly, as observed with PLCγ1 phosphorylation, RAM IgG triggered a higher activation of ERK2 than RAM F(ab’)2 for a very short period of stimulation.

The Phosphorylated FcγRIIB-CD5 Chimera Does Not Associate with SHP-1 or SHIP Phosphatases—BCR stimulation has already been reported to induce the tyrosine phosphorylation of CD5 (22). We therefore assessed phosphorylation of the FcγRIIB-CD5 chimera in cells stimulated with RAM IgG or with RAM F(ab’)2 fragments. In Fig. 4A, the chimera was immunoprecipitated with the FcγRIIB-specific mAb 2.4G2 before probing with anti-phosphotyrosine mAb 4G10. IIA B1 and IIA IC1 cells were used as positive and negative controls, respectively. No phosphorylation was observed in the unstimulated cells. A slight phosphorylation of the chimera, migrating just below IgH chains revealed by peroxidase-conjugated goat anti-mouse secondary Abs, was usually induced by F(ab’)2 fragments. A strong labeling occurred with RAM IgG as found for wild-type FcγRIIB1. We conclude that coligation of the FcγRIIB- CD5 molecule with the BCR strongly induces the phosphorylation of the chimera on tyrosine residues.

Negative regulatory receptors for the BCR usually involved interactions of phosphorylated tyrosine residues in the cytoplasmic domain of the inhibitory receptor with SH2 domain(s) of phosphatases like SHP-1 (i.e. for CD22; Refs. 32 and 33) or SHIP (as reported for FcγRIIB; Refs. 34–37). We checked whether similar interactions might take place with the FcγRIIB-CD5 chimera whose phosphorylation was induced upon coligation of the BCR with the chimera. FcγRIIB-CD5 immunoprecipitates obtained from cells stimulated or not with RAM-IgG were probed with SHP-1- and SHIP-specific Abs. IIA B1 cells expressing FcγRIIB1 wild-type molecules were used in parallel as a positive control of SHIP interaction. We also used a IIA1.6 transfectant expressing a chimeric molecule made of the FcγRIIB extracellular domain and of the transmembrane and the cytoplasmic domain of human p58.183 KIR, IIA KIR, as a positive control of SHP-1 interaction in B cells (38, 39). As shown in Fig. 4B, neither SHP-1 nor SHIP was detected in FcγRIIB-CD5 immunoprecipitates, contrasting with the results obtained with wild-type FcγRIIB1 and KIR receptors. Similarly, we did not detect any association of SHP-2 with the FcγRIIB-CD5 chimera (data not shown).

A CD5 Chimera Deleted of Its Pseudo-ITAM Motif Does Not Inhibit BCR Signaling—A BCR-activated Src kinase, Lyn, is likely involved in FcγRIIB1 phosphorylation after coligation of the two receptors (40). Moreover, CD5 is a substrate of Src PTKs (17). CD5cyt in our chimera expresses three tyrosine residues. Among them, the first one, Tyr429, is in a canonical Src autophosphorylation site (DNEY); moreover, Tyr429 and Tyr441 are in a pseudo-ITAM motif YSQP3YPA (Fig. 1). We therefore constructed a FcγRIIB-CD5 molecule deleted within CD5cyt of this short sequence to stably transfect IIA1.6 cells. We first established a cell line by sorting FcγRIIB positive cells from the bulk culture of IIA1.6 cells transfected with the deleted chimera. Clones were also selected. Fluorescence analysis of the cell line (IIA CD5ΔITAM.L) and of one representative clone (IIA CD5ΔITAM.3) is shown in Fig. 5A. Phosphorylation studies were then conducted after coligation of the mutated receptor with BCR by RAM-IgG. We found that CD5 phosphorylation was reduced by comparison with the undeleted FcγRIIB-CD5 chimera. A typical experiment performed with the IIA CD5ΔITAM.L cell line is shown in Fig. 5B.

We next checked the Ca2+ response and the IL-2 production induced after coligation of the deleted chimera with BCR. Results are shown in Fig. 5 (C and D). Ca2+ responses elicited by RAM IgG in cells expressing the chimera deleted of the pseudo-ITAM motif of CD5 were now high and sustained. By comparison with the response induced by RAM F(ab’)2 fragments, we even usually observed a higher Ca2+ peak. Moreover, the CD5 chimera was no more able to inhibit IL-2 production upon its coligation with the BCR. Taken together, these findings demonstrate that the deleted region is involved in the inhibitory effect of CD5 on BCR signaling.

DISCUSSION

Only few studies investigated the signaling properties of CD5 in B cells. As with its T-cell counterpart, a privileged physical link between CD5 and the B-cell antigen-specific receptor in CD5+ B cells was demonstrated (22). Several reports also suggested a possible cooperation between the two receptors at the functional level leading to the hypothesis that CD5 positively contributes to B cell activation (41, 42). Other arguments arising from studies in CD5 knockout mice promote instead a negative role of CD5 on BCR-induced responses. To further elucidate this problem, we studied in a murine B cell model the consequences of coligation of BCR with CD5 on BCR-mediated responses.

The IIA1.6 murine B cell model, a FcγRIIB-negative variant of the A20 lymphoma B cell line, is ideally suited to analyze the effects of receptors that may interfere with BCR signaling. Indeed, by constructing a chimeric molecule containing the extracellular domain of FcγRIIB and the cytoplasmic domain of the receptor, one can use a straightforward procedure to bring...
Fig. 4. The FcγRIIB-CD5 chimera is phosphorylated after its coligation with the BCR but does not associate with SHP-1 or SHIP phosphatases. A, phosphorylation of the FcγRIIB-CD5 chimera. FcγRIIB transfectants (1 × 10⁵ cells) were stimulated for 2 min with RAM IgG (45 μg/ml) or RAM F(ab')₂ (30 μg/ml) at 37 °C or left unstimulated. Cell lysates were immunoprecipitated with FcγRIIB-specific mAb 2.4G2 immobilized on protein G-Sepharose. Immune complexes were fractionated by SDS-PAGE, transferred onto membranes, and blotted with anti-pTyr mAb 4G10 and peroxidase-conjugated goat anti-mouse secondary Ab (upper panel). Position of IgH chains revealed by the secondary Ab is shown. B, the phosphorylated FcγRIIB-CD5 chimera does not recruit SHP-1 or SHIP. FcγRIIB transfectants (1 × 10⁵ cells) were stimulated for 2 min with RAM IgG (45 μg/ml) at 37 °C or left unstimulated. Cell lysates were immunoprecipitated with FcγRIIB-specific mAb 2.4G2 immobilized on protein G-Sepharose. Immune complexes were fractionated by SDS-PAGE, transferred onto membranes, and blotted with specific Abs as indicated. Anti-phosphotyrosine mAb PY-20 directly coupled to horseradish peroxidase was used in this experiment. A FcγRIIB blot is also shown. A whole cell lysate (WCL) of IIA B1 cells was migrated and blotted in parallel with Abs against SHP-1 and SHIP to indicate the position of the two proteins.

into close contact the chimera with the BCR using a single Ab. We therefore used this system to investigate the effects of CD5 on BCR stimulatory events. Our results unambiguously demonstrate an inhibition of B cell activation by the cytoplasmic domain of CD5. Striking effects were especially observed at the functional level because IL-2 gene (43). This ultimately suggests that the outcome of CD5-mediated inhibition may be more severe in B cells than in T cells. Accordingly, CD5 seems to have a stronger effect on BCR-induced proliferation, as illustrated in mice by the results obtained with B1a cells whose proliferation is restored in CD5 knockout animals or, more spectacularly, after keeping CD5 well away from the BCR (23).

Coligation of the FcγRIIB-CD5 chimera with BCR induced a strong tyrosine phosphorylation of CD5cyt. The Src PTK Lyn is presumably involved because it has already been shown to be responsible for FcγRIIB1 phosphorylation in similar stimulatory conditions in mast cells (40). The B-cell coreceptor CD22 is also phosphorylated by Lyn (44). This phosphorylation of CD5 is likely required to observe the inhibitory effect. This is in agreement with our observations using the YSQQPXYPAL CD5 deletant together with the general scheme explaining how cell surface antigens, using particular motifs of their cytoplasmic domain, the so-called ITIM sequences, inhibit neighboring activation receptors. Tyrosine phosphorylated ITIMs recruit SH2-containing phosphatases switching off the activity of PTKs and/or dephosphorylating key downstream elements of the response (reviewed in Refs. 45 and 46). Whether phosphorylated tyrosine residues inside CD5cyt may also represent docking sites for SH2-containing phosphatases was therefore an important question.

SHP-1 is a cytosolic tyrosine phosphatase, widely expressed in hematopoietic cells. SHP-1 exerts a negative regulatory influence on the early tyrosine phosphorylation events elicited after activation of different tyrosine kinase-associated receptors, like the TCR (18, 19) or the BCR (47, 48). By means of its two SH2 domains, SHP-1 binds to the phosphorylated tyrosine of the ITIM motifs present in the cytoplasmic domain of various inhibitory receptors, for example molecules of the KIR family in NK cells (49, 50) or CD22 (32, 33, 51, 52) and PIR-B (53) in B cells. Its participation in the inhibitory effects of these receptors is now well accepted, raising the possibility that SHP-1 is also involved in the effects of CD5. This question has been addressed for the first time by Pani et al. (20) in TCR-activated murine thymocytes. They showed that CD5 became heavily phosphorylated after TCR stimulation, and they detected SHP-1 in CD5 immunoprecipitates. However, SHP-1 was also associated with CD5 in resting thymocytes where CD5 was not significantly phosphorylated, suggesting a mechanism not involving SH2-ITIM interaction. In a recent work performed in Jurkat human T cells, an interaction of SHP-1 with CD5, increased after TCR stimulation, was also reported (21). Strikingly, it was shown that a particular tyrosine residue of CD5, Tyr⁷⁷⁸ in an ITIM-like motif, was crucial for the binding of SHP-1 and the inhibition of T cell activation mediated by CD5.

However, our results show that the effect of CD5 on BCR signaling is independent of SHP-1 for several reasons. First, and importantly, this tyrosine residue, which is in a very charged region of the molecule just at the junction of trans-
FIG. 5. A CD5 chimera deleted of its pseudo-ITAM motif no more inhibits BCR signaling. A, BCR and FcγRIIB expression. Open histograms show the expression levels of the BCR and of the FcγRIIB-CD5 chimera as assessed by indirect immunofluorescence using rabbit anti-mouse IgG F(ab')2 and FITC donkey anti-rabbit F(ab')2 and 2.4G2 mAb and FITC mouse anti-rat F(ab')2, respectively. Closed histograms show the fluorescence of cells incubated with secondary Abs only. B, phosphorylation of the FcγRIIB-CD5 chimera. FcγRIIB transfectants (1 × 10⁶ cells) were stimulated for 2 min with RAM IgG (45 μg/ml) or RAM F(ab')2 (30 μg/ml) at 37 °C or left unstimulated. Cell lysates were immunoprecipitated with FcγRIIB-specific mAb 2.4G2 immobilized on protein G-Sepharose. Immune complexes were fractionated by SDS-PAGE, transferred onto membranes, and blotted with anti-pTyr mAb PY-20 (upper panel) and anti-FcγRIIB Abs (lower panel). Positions of the FcγRIIB-CD5 chimera and of the chimera deleted of the pseudo-ITAM motif (FcγRIIB-CD5ΔITAM) are indicated. C, Ca²⁺ responses to BCR stimulation. FcγRIIB transfectants were loaded with the fluorescent Ca²⁺ indicator fura-2 AM and fluorescence of the cell suspension monitored with a spectrofluorimeter in a 1 mM Ca²⁺ containing medium at 37 °C after addition of RAM IgG (45 μg/ml) (dotted lines) or RAM F(ab')2 (30 μg/ml) (continuous lines). D, IL-2 production. FcγRIIB transfectants were stimulated with indicated concentrations of RAM F(ab')2 (closed symbols) or RAM IgG (open symbols). The IL-2 release in 18-h culture supernatant was assayed for [³²P]thymidine incorporation in CTLL-2 cells. Shown is the radioactivity incorporated in CTLL-2 cells as a function of the concentration of RAM Abs or fragments.
membrane and cytoplasmic domains, was missing in our CD5 chimera. Second, we did not observe any coprecipitation of SHP-1 with the phosphorylated FcγRIIB-CD5 chimera in IAA 1.6 transfected cells. We cannot exclude that a faint amount of SHP-1, not detectable in our assay conditions, is present. A supplementary argument, however, does not support this possibility. Indeed, experiments made with phosphopeptides of CD5cyt showed that the SH2 domains of SHP-1 did not bind to any CD5cyt phosphopeptides (54). Interestingly, we can notice from the sequence of CD5 (Fig. 1) that a particular motif, with a serine in position –2 (SAYPAL), is also present inside the pseudo-ITAM. It is different from the prototypal inhibitory sequence (IVVXXXYLVV) found in most inhibitory receptors. Nevertheless, this motif is present in the mast cell function-associated antigen, a murine inhibitory co-receptor member of the C-type lectin family where the IV residue at position –2 is replaced by a serine residue (55). Thus, we also controlled with a phosphorylated peptide of the pseudo-ITAM that this sequence did not precipitate SHP-1 or SHP-2 from lysates of the lymphoma B cells (data not shown). As a supplementary argument, it should be emphasized that in IIA 1.6 B cells, inhibitory kinases (66, 67), was reported. CD5 also activates in the cytoplasmic domain of a KIR, abolished the Ca2+-dependent kinases (64). More recently, association of CD5 with different signaling molecules for the Ca2+ response containing a PI-3,4,5-P3-binding PH domain, like the subunit of phosphatidylinositol 3-kinase (73–75) as well as the cold-regulated Syk PTK (71, 72) and interacts in vivo with numerous other signaling molecules like Fyn, Grb2, and Shc, the p85 subunit of phosphatidylinositol 3-kinase (73–75) as well as the SH3 domain of Bruton’s tyrosine kinase (76). By altering BCR targeting of such key signaling molecules, CD5 may therefore profoundly influence signaling downstream of the BCR.

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REFERENCES

1. Resnick, D., Pearson, A., and Krieger, M. (1994) Trends Biochem. Sci. 19, 5–8
2. Van de Velde, H. I. von Hoeven, L., Luo, W., Farnes, J. R., and Thielemans, K. (1991) Nature 351, 662–671
3. Biancone, L., Bowen, M. A., Lim, A., Aruffo, A., Andres, G., and Stamenkovic, I. (1996) J. Exp. Med. 184, 811–819
4. Posnett, R. H., Potts, M. G., and Mage, R. G. (1996) J. Exp. Med. 184, 1279–1284
5. Weiss, A., Daxin, P. F., Shields, R., Fu, S. M., and Lanier, L. L. (1987) J. Immunol. 139, 3245–3250
6. Hayakawa, K., Hardy, R. R., Park, D. R., and Herzenberg, L. A. (1983) J. Exp. Med. 157, 262–281
7. Herzenberg, L. A., Stall, A. M., Lalter, P. A., Sidman, C., Moore, W. A., and Parke, D. R. (1986) Immunol. Rev. 93, 81–102
8. Hayakawa, K., and Hardy, R. R. (1989) Annu. Rev. Immunol. 6, 197–218
9. Kips, T. J. (1989) Adv. Immunol. 47, 117–185
10. Kantor, A. B. (1991) Immunol. Today 12, 389–392
11. Tarakhovsky, A. (1997) J. Exp. Med. 185, 981–984
12. Burgess, K. E., Yamamoto, M., Prasad, K. V., and Ruddle, C. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9311–9315
13. Osman, N., Ley, S. C., and Crompton, M. J. (1992) Eur. J. Immunol. 22, 2995–3000
14. Tarakhovsky, A., Kanner, S. B., Hombach, J., Ledbetter, J. A., Muller, W., Killeen, N., and Rajewsky, K. (1996) Science 270, 535–537
15. Grygorczyk, H., Lang, V., Sarven, S., Beumsl, M., and Bismuth, G. (1997) J. Immunol. 159, 3739–3747
16. Davies, A. A., Ley, S. C., and Crompton, M. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6036–6037
17. Raab, M., Yamamoto, M., and Ruddle, C. E. (1994) Mol. Cell. Biol. 14, 2862–2870
18. Plas, D. R., Johnson, R., Pingel, J. T., Matthews, R. J., Dalton, M., Roy, G., Chan, A. C., and Thomas, M. L. (1996) Science 272, 1173–1178
19. Raai, M., and Rudder, C. E. (1996) Biochem. Biophys. Res. Commun. 229, 55–57
20. Pani, G., Fischer, K. D., Miniaric-Rascan, I., and Siminovich, K. A. (1996) J. Exp. Med. 184, 839–852
21. Perpezat-Jill, J. T., Whitney, G. G., Bowen, M. A., Hewitt, D. H., Aruffo, A. A., and Kanner, S. B. (1999) Mol. Cell. Biol. 19, 2903–2912
22. Lankester, A. C., van Schijndel, G. M. W., Cordell, J. L., van Noesel, C. J. M., and van Lier, R. A. W. (1994) Eur. J. Immunol. 24, 812–816
23. Bikah, G., Careey, J., Cailleon, I., Tarakhovsky, A., and Bonada, S. (1996) Science 274, 1906–1909
24. Jones, B., Titie, J. P., and Janeway, C. A., Jr. (1986) J. Immunol. 136, 348–356
25. Amigorena, S., Bonnerot, C., Drake, J. R., Choquet, D., Hunziker, W., Guillot, J. G., Webster, P., Sautes, C., Mellman, I., and Fridman, W. H. (1992) Science 256, 1808–1812
26. Unkeless, J. C. (1979) J. Exp. Med. 150, 580–596
27. Takebe, Y., Seki, M., Fujimura, J., Hay, P., Yokota, K., Ariai, K., Yoshida, M., and Ariai, N. (1988) Mol. Cell. Biol. 8, 466–472
28. Waggman, N., Biaison, R., Canton, C., Verduini, S., Malnati, M. S., Vitale, M., Bottino, C., Moretta, L., Moretta, A., and Long, E. O. (1995) Immunol. Rev. 149, 439–449
29. Grnkiewicz, G., Pienie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
30. Gillis, S., and Smith, K. A. (1977) Nature 268, 154–156
31. Campbell, K. S. (1999) Curr. Opin. Immunol. 11, 256–264
32. Doddy, G. M., Justement, L. B., Delibrac, C. A., Matthews, R. J., Lin, J., Thomas, M. L., and Pearen, D. T. (1995) Science 269, 242–244
33. Law, C. L., Sidorenko, S. P., Chandran, K. A., Zhao, Z. H., Shen, S. H., Fischer, E. H., and Clark, E. A. (1996) J. Exp. Med. 183, 547–560
34. Ono, M., Bolland, S., Tempst, P., and Ravetch, J. V. (1996) Nature 383, 459–462
