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Human Type II Fcγ Receptors Inhibit B Cell Activation by Interacting with the p21^{ras}-dependent Pathway*

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Co-ligation of antigen receptors and type II Fcγ receptors (FcγRIIb) on B cells interrupts signal transduction and ultimately inhibits antibody production. We have identified p52 Shc in the FcγRIIb1-specific immunoprecipitates isolated from the membrane fraction of BL41 Burkitt lymphoma cells following B cell receptor-FcγRIIb1 co-ligation. The insolubilized synthetic peptide representing the phosphorylated form of the tyrosine-based inhibitory motif of FcγRIIb also binds Shc from the lysates of activated but not from resting BL41 cells. This suggests that the binding does not depend on the interaction of FcγRIIb1-phosphotyrosine with the SH2 domain of Shc. Tyr phosphorylation of FcγRIIb1-associated Shc is low, indicating an impaired function. Shc is implicated in regulating p21^{ras} activation; thus, we have compared p21^{ras} activities in BL41 cells treated in different ways. p21^{ras} activity is reduced when B cell receptor and FcγRIIb1 are co-ligated. p21^{ras} couples protein-tyrosine kinase-dependent events to the Ser/Thr kinase-mediated signaling pathway leading to the activation of mitogen-activated protein kinases (MAPK). Our results show that B cell receptor-FcγRIIb1 co-cross-linking partially inhibits mitogen-activated protein kinase activity. We conclude that FcγRIIb1-dependent inhibition of human B cell activation may be based on interrupting signal transduction between protein-tyrosine kinases and the p21^{ras}/mitogen-activated protein kinase-dependent activation pathway.

The low affinity, IgG Fc-binding, type IIb receptors (FcγRIIb)[1] expressed on B cells may down-regulate cell activation when cross-linked with the antigen receptor, membrane immunoglobulin (mIg) (1). It has been shown earlier that co-engagement of FcγRII and mIg inhibits B cell activation at the level of signal transduction by uncoupling the antigen receptor signal-transducing machinery upstream of G protein-activated events, resulting in the inhibition of Ca^{2+} influx (2, 3). Association of protein-tyrosine-phosphatase 1C with the 13-amino acid-containing phosphorylated immunoreceptor tyrosine-based inhibitory motif (P-ITIM) of FcγRIIb1 in murine B cells has been demonstrated recently. The authors suggest that protein-tyrosine-phosphatase 1C is an effector of BCR-FcγRIIb1 negative signal cooperativity (4). Others proposed that co-ligation of BCR and FcγRIIb1 may result in closing of a Ca^{2+} channel on the cell membrane, inhibiting thereby the Ca^{2+} influx (5). The exact mechanism of FcγRIIb-mediated inhibition of B cell function is not yet completely clarified.

On human B cells, both FcγRIIb1 and FcγRIIb2 isoforms are expressed. Neither FcγRIIb1 nor FcγRIIb2 transduce activation signals for human B cells; however, we have previously demonstrated that protein-tyrosine kinase Fyn and several unidentified Tyr-phosphorylated polypeptides as well as Ser/Thr kinase activities associate with FcγRII isolated from BCR-cross-linked BL41 Burkitt lymphoma cells (6, 7). The vast majority of FcγRII expressed on BL41 cells represents FcγRIIb1 isoform (8). We have suggested that the association of certain signaling molecules with FcγRIIb1 may modify their function when BCR and FcγRb1 are co-ligated.

According to the early experiments of Klaus *et al.* (2), co-ligation of BCR and FcγRII uncouples BCR from the signal-transducing machinery upstream of G proteins. The best documented role for G proteins in B cells is the activation of p21^{ras}, the low molecular mass G protein, since (i) co-localization of p21^{ras} and mIg was observed after mIg cross-linking (9), (ii) the p21^{ras} signaling pathway has been shown to be operative in both human and murine B cells connecting protein-tyrosine kinase activation and the regulation of gene transcription (10), and (iii) it has recently been reported that activation of the p21^{ras} pathway couples antigen receptor stimulation to the induction of the primary response gene *egr*-1 in B cells (11). The activity of p21^{ras} is regulated by guanine nucleotide exchange factors and GTPase-activating protein. m-Sos-1 augments the GDP-GTP exchange and thus activates, while GTPase-activating protein inactivates, p21^{ras} (12). It was recently demonstrated that a Tyr-containing motif of the adapter molecule Shc becomes phosphorylated by the Lyn-activated Syk protein-tyrosine kinases in B cells following mIg cross-linking (13). Via the Tyr(P) residue, Shc binds to the SH2 domain of Grb-2 in the Grb-2/m-Sos-1 complex and in turn the complex translocates to the cell membrane, where p21^{ras} is located then Sos activates *ras* (14). Downstream of p21^{ras}, a cascade of kinases becomes activated. Raf activates MAP kinase kinase, MAPK/ERK kinase, which in turn phosphorylates and activates microtubule-associated kinases, MAPK (or extracellular signal regulated kinases, ERK). Activated MAPK then phosphorylates several intracellular substrates on Ser/Thr residues, such as other Ser/Thr kinases, cytoskeletal proteins, or transcription factors (15, 16).

The effect of BCR-FcγRII co-ligation on p21^{ras}/MAPK activa-

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[1] The abbreviations used are: FcγRII, type II Fcγ receptors; BCR, B cell antigen receptors; mIg, membrane immunoglobulin; ITIM, immunoreceptor tyrosine-based inhibitory motif; P-ITIM, phosphorylated immunoreceptor tyrosine-based inhibitory motif; mIg, membrane immunoglobulin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; HRPO, horseradish peroxidase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein.

tion pathway has not been investigated yet. Co-cross-linking of mIg and FcγRII by intact anti-Ig molecules has been shown to inhibit the primary response genes *egr*-1 and *egr*-2 expression in the murine B cell line, BCL_1 (17). The primary response gene *egr*-1 encodes a sequence-specific transcription factor, the expression of which is necessary for antigen receptor-stimulated activation of B cells, and it has been demonstrated that induction of *egr*-1 after BCR cross-linking is mediated by activation of the p21^{ras}/MAPK-signaling pathway (11).

The aim of this work was to define signaling molecules, the activities of which might be regulated by FcγRII-BCR co-cross-linking and which have a definite role in the *ras*/MAPK activation pathway. We show here that when FcγRIIb1 and BCR are co-ligated on human B cells, a portion of the adapter molecule Shc associates with FcγRIIb1. Furthermore, we demonstrate that the Tyr-phosphorylated inhibitory motif of FcγRIIb1 binds Shc from activated BL41 cell lysate. This association might alter the function of Shc or Shc-associated molecules. A partial inactivation of p21^{ras} and a reduced activity of MAPK are also observed in FcγRII-BCR co-ligated samples. We suggest that FcγRIIb1 on human B cells may diminish antibody production by inhibiting the *ras*/MAPK activation pathway.

EXPERIMENTAL PROCEDURES

Reagents—Polyclonal, affinity-purified human IgG- and IgM-specific antibodies, anti-mouse IgG $F(ab)_2$ fragments, biotinylated anti-mouse IgG $F(ab)_2$, anti-human IgG + IgM antibodies, and horseradish peroxidase (HRPO)-conjugated anti-mouse and anti-rabbit IgG $F(ab)_2$ antibodies (Axell); anti-phosphotyrosine monoclonal antibody (mAb) (clone PT66) and anti-phosphotyrosine-coated agarose beads (Sigma); Shc- and MAPK-specific antibodies (Transduction); anti-Erk-1 polyclonal antibodies (UBI); rat anti-p21^{ras} monoclonal antibody and Lyn-specific rabbit antibodies (Santa-Cruz); CD32, CD79a, and CD79b monoclonal antibodies (PharMingen). The monoclonal antibody specific for FcγRII used for Western blots was a kind gift from Dr. J. Frey, Bielefeld, Germany, and polyclonal antibody recognizing mb1/Igα was a kind gift from Dr. L. Smit, Amsterdam, The Netherlands. [γ-^{32}P]ATP, $^{32}P_i$, enhanced chemiluminescence reagents, and nitrocellulose membranes were purchased from Amersham, Gamma-Bind G beads and Percoll from Pharmacia, and reagents and standards for electrophoresis from Bio-Rad. Synthetic peptides representing the ITIM (AENTITYSLLMHP) or P-ITIM (AENTITY(PO_3H_2)SLLMHP) of human FcγRII were synthesized on a TentaGel-NH_2 resin by Dr. R. Reuschel, SFI, Vienna, Austria (18).

Cells—BL41 Burkitt lymphoma cells were maintained in stationary culture in RPMI 1640 medium containing 10% fetal calf serum; ST486 cells were grown in RPMI 1640 medium containing 20% fetal calf serum.

Stimulation—3×10^7 cells were activated with 20 μg of $F(ab)_2$ fragment or intact IgG of affinity-purified rabbit anti-IgM + IgG for 2 min at 37 °C, the cells were pelleted for 20 s and immediately freezed in liquid nitrogen. To cross-link BCR and FcγRII, the cells were preincubated with different doses of heat-aggregated human IgG or 2 μg of CD32 antibodies for 10 min. In the latter case, co-cross-linking was obtained by biotinylated goat anti-mouse IgG, followed by biotinylated anti-human Ig and the addition of avidin.

Preparation of Membrane and Cytosolic Fractions—Samples of cells were homogenized in a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 20 mg/ml leupeptin, 20 mg/ml soybean trypsin inhibitor, 20 mg/ml aprotinin, 10 mM phenylmethylsulfonyl fluoride, as described (19). The homogenized samples were fractionated into insoluble (membrane) fraction and soluble cytosolic fraction by ultracentrifugation (100,000 × *g* for 40 min, at 4 °C). The membrane fractions were solubilized by sonication in 1 ml of lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH7.4), 100 mM NaF, 10 mM EDTA, 2 mM sodium *o*-vanadate, 10 mM sodium pyrophosphate, 10% glycerol, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride. After 60 min of incubation on ice, cell lysates were centrifuged at 15,000 × *g* for 20 min, and the supernatants were used. The cytosolic fractions were concentrated five times with Amicon concentrators. The solubilized membrane fractions and the concentrated cytosolic fractions were used in the subsequent immunoprecipitating procedures.

Immunoprecipitation and Western Blotting—Immunoprecipitations were carried out using human IgG Fc fragment covalently coupled to Sepharose beads, or CD32 antibody followed by Gamma Bind protein G beads, and with anti-phosphotyrosine-coated agarose beads, respectively. TentaGel resin-bound ITIM and P-ITIM peptides were washed first with HCl-glycine buffer at pH 2.8 followed by several washings in lysis buffer and used to absorb components from resting and activated cell lysates. After overnight mixing at 4 °C, the immunoprecipitated or affinity-purified samples were washed four times with lysis buffer, and the bound components were eluted by incubation in 40 μl of reducing SDS-PAGE sample buffer for 5 min at 95 °C. The eluted samples were electrophoresed through 8.5 or 10% SDS-PAGE gel; then the proteins were blotted onto nitrocellulose membranes, probed with different antibodies, and developed by using HRPO-conjugated second antibodies followed by enhanced chemiluminescence detection. In some experiments, the membranes were striped according to the manufacturers' instruction and reprobed with a different antibody.

Measurement of p21^{ras} Activity—p21^{ras} proteins were immunoprecipitated by rat anti-Ras antibody from the lysate of $^{32}P_i$-labeled cells as described (20). Labeled guanine nucleotides bound to Ras were eluted and separated by thin layer chromatography. The results were quantitated by comparing p21^{ras}-bound GTP with total GTP + GDP activities in the samples on the autoradiogram as measured by laser densitometry.

Immunocomplex Kinase Activity Assay—Immune complexes were obtained by anti-Erk-1 or CD32 antibodies, washed four times with lysis buffer and once with kinase buffer, and then incubated in 30 μl of kinase buffer containing 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 5 μg of myelin basic protein (MBP) as substrate for MAPK. The reactions were carried out for 20 min at 30 °C and then stopped by adding 30 μl of reducing SDS-PAGE sample buffer. The samples were heated for 5 min at 95 °C, electrophoresed, and blotted, and the MAPK activity (MBP phosphorylation) was detected by autoradiography.

RESULTS

Association of p52^{shc} with FcγRII—BL41 cells were subfractionated into membrane and cytosolic fractions, FcγRII were affinity-purified on human IgG-Fc fragment-coated beads, and the samples were compared with the ones precipitated by phosphotyrosine-specific antibody-coated beads (Fig. 1*a*). Several Tyr-phosphorylated proteins were isolated from both the membrane and the cytosolic fraction of anti-(IgM + IgG)-activated BL 41 cells, although the pattern was somewhat different. Aggregated IgG treatment did not induce Tyr phosphorylation and had no detectable effect on Tyr phosphorylation of either membrane or cytosolic proteins when BCR and FcγRII were co-ligated (Fig. 1*a, lane 4* as compared to *lane 2* on first two panels). Some of the Tyr-phosphorylated molecules selectively associated with FcγRII isolated from the membrane of anti-Ig-activated cells (200 kDa, 130–140 kDa, 110 kDa, 75 kDa, and 50–60 kDa), while control, nonactivated, or aggregated IgG-treated samples were negative. When anti-Ig and aggregated IgG were simultaneously applied to co-ligate BCR and FcγRII, a significantly higher Tyr phosphorylation of FcγRII-associated bands, in particular between 50 and 60 kDa (Fig. 1, *third panel, lane 4*), was observed as compared to the BCR-cross-linked samples. No Tyr-phosphorylated molecules of the cytosolic fraction attached to Fc fragment-coated beads except a 75-kDa component which was present in both BCR-cross-linked and BCR-FcγRII co-cross-linked samples.

After the first antibodies were stripped, the same membranes were probed with rabbit antibodies specific for Shc (Fig. 1*b*). The 52-kDa Tyr-phosphorylated band in the anti-phosphotyrosine immunoprecipitates was recognized by the specific antibodies, showing its identity with Shc, the majority of which was detected in the membrane fraction. Shc was also recognized among the FcγRII-co-purified molecules, indicating that a portion of Shc bound to FcγRII isolated from the membrane fraction of BCR-FcγRII co-ligated cells (Fig. 1*b, third panel, lane 4*). A weak band co-migrating with Shc may be detected occasionally in FcγRII precipitates obtained from nonstimu-

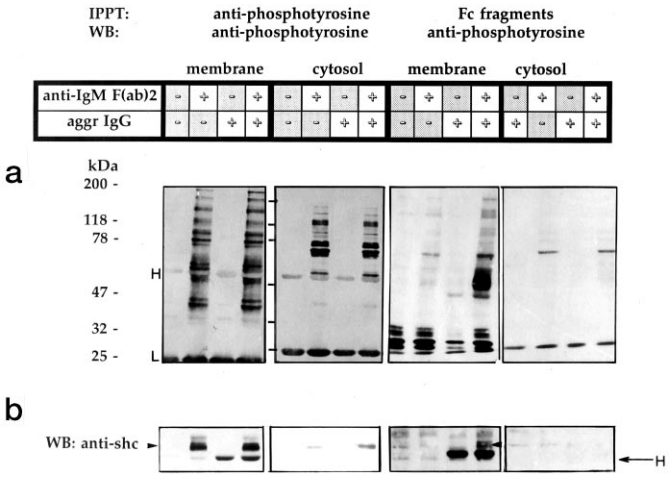

FIG. 1. **Shc associates with FcγRIIb affinity-purified from the membrane fraction of FcγRII-BCR co-ligated BL41 cells.** 5×10^7 cells were stimulated with 30 μg of the reagents indicated for 2 min at 37 °C. The anti-IgM F(ab)$_2$ antibodies recognized the heavy (H) + light (L) chain and thus were cross-reactive with human IgG. The cells were pelleted and immediately frozen in liquid nitrogen. The samples were homogenized and then fractionated into cytosolic and membrane fractions, and the latter were lysed in lysis buffer containing 1% Triton X-100. *a*, Tyr-phosphorylated molecules were isolated by anti-phosphotyrosine-coated agarose beads, and FcγRII were affinity-purified by human IgG Fc fragments covalently coupled to Sepharose beads. The eluted samples were exposed to SDS-PAGE using 10–15% continuous gradient gel, and the proteins were blotted and probed with anti-phosphotyrosine antibody followed by anti-mouse IgG F(ab)$_2$-HRPO. The reaction was detected by enhanced chemiluminescence. *b*, after the first antibodies were stripped, the same membranes were reprobed using Shc-specific rabbit antibodies followed by HRPO-conjugated anti-rabbit IgG F(ab)$_2$. *aggr*, aggregated; *IPPT*, immunoprecipitation; *WB*, Western blot.

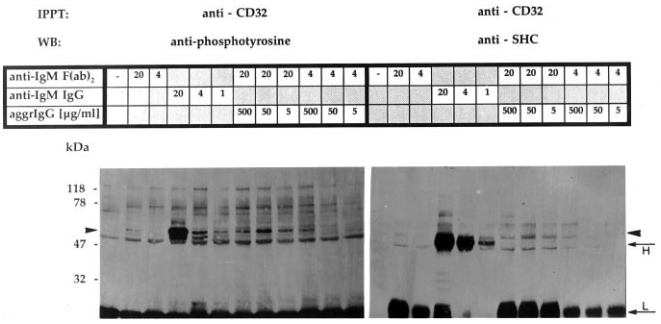

FIG. 2. **Correlation between the association of Tyr-phosphorylated components and the binding of Shc to FcγRIIb isolated by CD32 mAb.** 5×10^7 BL41 cells were stimulated with various doses of reagents as indicated. FcγRIIb were immunoprecipitated (*IPPT*) using CD32 mAb, and the immunocomplexes were collected by Gamma Bind protein G beads. After SDS-PAGE, the proteins were blotted into nitrocellulose membrane and consecutively probed by anti-phosphotyrosine and anti-Shc antibodies. *Arrowheads*, position of Shc. *aggr*, aggregated; *WB*, Western blot; *H*, heavy chain; *L*, light chain.

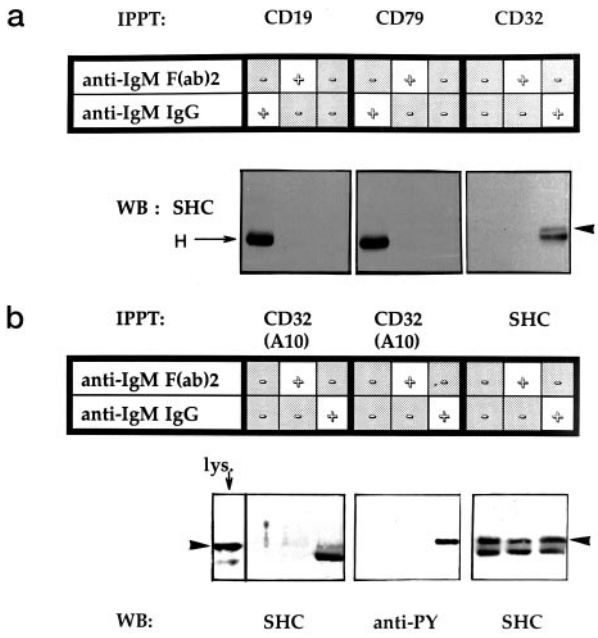

FIG. 3. **Specificity of the association of 52-kDa Shc with FcγRIIb in the membrane of FcγRIIb-BCR-co-ligated BL41 cells.** The cells were treated with 30 μg of anti-IgM F(ab)$_2$ fragment or anti-IgM intact IgG for 2 min at 37 °C. CD19, CD79 (*a*), CD32 (*a* and *b*), and Shc (*b*) were immunoprecipitated (*IPPT*) from the cell membrane fraction by the corresponding antibodies followed by the addition of Gamma Bind protein G beads. The Western blots (*WB*) were probed with the reagents indicated. Cell lysates were used as positive control for Shc (*lys*). *H*, heavy chain.

lated cells, which does not seem to represent a specific interaction.

In the following experiments, FcγRII was isolated using CD32 monoclonal antibody followed by protein G-coated beads, and the precipitated components were analyzed by Western blotting using phosphotyrosine and Shc-specific antibodies, respectively. Fig. 2 shows that co-cross-linking of BCR and FcγRII with intact rabbit IgG anti-IgM antibodies dose-dependently induced the association of a 52-kDa Tyr-phosphorylated molecule with FcγRII, as detected just above the heavy chain of the anti-IgM antibody (*first panel*). Shc-specific antibodies recognized a co-migrating 52-kDa band in the same samples (*second panel*). When BCR and FcγRII were co-ligated using aggregated IgG and F(ab)$_2$ fragment of anti-(IgM + IgG) antibodies, similarly to the affinity-isolated samples, the 52-kDa adapter molecule Shc was co-isolated with FcγRII. The association required an optimal ratio of anti-Ig and aggregated IgG for co-cross-linking. Comparing phosphotyrosine- and Shc-specific Western blots, a correlation between the association of the main Tyr-phosphorylated component and the binding of Shc can be observed.

Similar results were obtained using a different CD32 monoclonal antibody (A10) (Fig. 3*b*), while we could not detect Shc in CD19 and CD79a immunoprecipitates (Fig. 3*a*). Only the 52-kDa Shc, and not the 47-kDa form, was found in CD32 immunoprecipitates (Fig. 3*b*).

Shc was detected in CD32 immunoprecipitates of BCR-FcγRII-co-ligated samples obtained from human tonsil B cells as well (data not shown).

Although under the conditions applied, in the presence of 1% Triton X-100 in the lysis buffer, no association of Shc with CD79a was observed (Fig. 3*a*), to exclude the possibility that the signal-transducing subunit of BCR, mb1/Igα (CD79a) contaminates the isolated FcγRII, immunoprecipitates obtained with CD32 and CD79a mAb were compared by probing the blots with CD79a- and CD32-specific antibodies. No cross-contamination was detected in CD32 and CD79a precipitates isolated from resting, activated, or FcγRII-BCR co-ligated samples (data not shown).

Synthetic Peptide Representing the Phosphorylated Form of ITIM of Human FcγRII Binds Shc and Other Tyr-phosphorylated Molecules—Phosphorylation of ITIM on Tyr induced by BCR-FcγRIIb1 co-ligation in mice was shown to be obligatory to inhibit B cell activation (4, 21). We have compared the molecules bound to insolubilized human ITIM and P-ITIM peptides from the lysates of resting and activated BL41 cells, respectively. P-ITIM bound Shc from activated but not from resting cell lysates, while eluates of the nonphosphorylated ITIM were

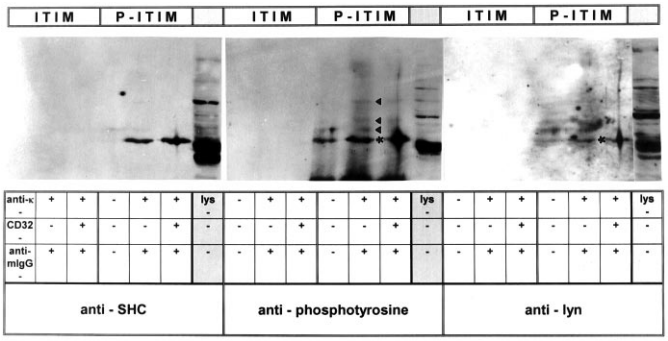

FIG. 4. **Shc binds to the synthetic peptides representing the P-ITIM motif of FcγRII.** 5×10^7 BL41 cells were treated with 5 μg/ml CD32 mAb for 5 min and then activated by 20 μg/ml anti-κ chain mAb followed by 10 μg/ml anti-mouse IgG $F(ab)_2$ to induce co-cross-linking. TentaGel-bound peptides were washed and then mixed for 1 h with the lysates of resting and activated cells, respectively. The associated molecules were eluted and tested by SDS-PAGE and Western blotting. After probing with anti-Shc (*left*), the membranes were stripped and tested with phosphotyrosine-specific mAb (*middle*). This was followed by an additional stripping, and the same membrane was probed with anti-lyn (*right*). The reactions were developed by HRPO-conjugated species-specific antibodies and enhanced chemiluminescence. *Arrowheads*, positions of Tyr-phosphorylated molecules; *asterisk*, position of lyn. Total BL41 cell lysate is shown (*lys*).

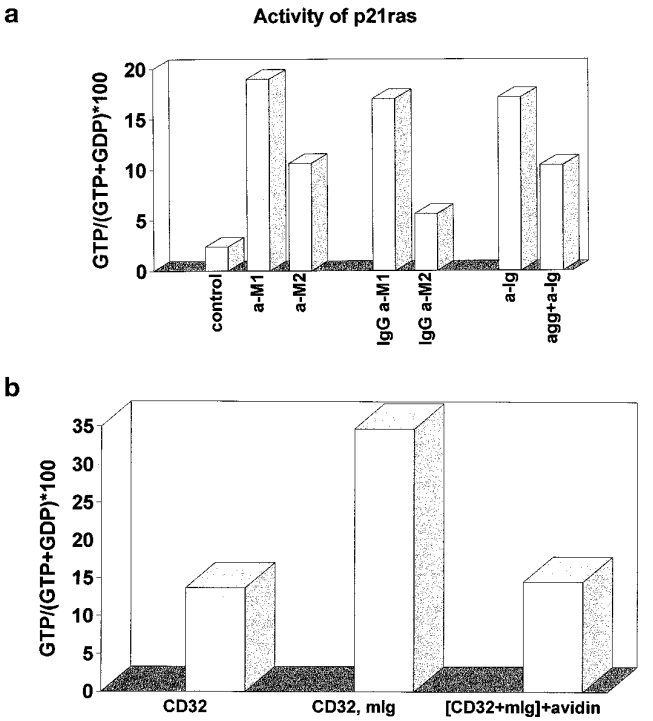

FIG. 5. **Co-ligation of FcγRIIb and BCR reduces p21^{ras} activity compared with the BCR-cross-linked samples.** The cells were labeled by $^{32}P_i$, stimulated by various doses of anti-IgM $F(ab)_2$ fragment (*a-M*), intact IgG anti-IgM (*IgG a-M*), (1:50 μg/ml, 2:10 μg/ml), and aggregated IgG (200 μg) followed by anti-Ig $F(ab)_2$ fragments (40 μg/ml) (*a*) and by 2 μg/ml CD32 mAb followed by 4 μg/ml biotinylated anti-mouse IgG and 30 μg/ml biotinylated anti-human Ig $F(ab)_2$ fragments without (*CD32, mIg*) or with avidin (*[CD32+mIg]+avidin*) (*b*), respectively. p21^{ras} was precipitated; then the bound GDP and GTP were eluted and analyzed after thin layer cellulose chromatography. The nucleotides were detected by autoradiography, and the quantitative comparison was done by laser densitometry.

negative (Fig. 4, *left*). Reprobing the same blots with anti-phosphotyrosine antibodies showed that P-ITIM but not ITIM representing peptide bound a major 53-kDa molecule (just above Shc) and other minor Tyr-phosphorylated components (60, 75, and 110 kDa) from both resting and activated cell samples. The 75- and 110-kDa bands can be seen in nonactivated control cells only after longer exposure. Higher Tyr phosphorylation of the P-ITIM-associated components, in particular the 75-kDa and the 110-kDa ones, was observed in activated samples as compared with resting cells (Fig. 4, *middle*). Further reprobing of the blots with Lyn-specific antibody indicated that the 53-kDa Tyr-phosphorylated component was identical with Lyn protein-tyrosine kinases. P-ITIM bound Lyn from both resting and activated cells (Fig. 4, *right*).

Co-cross-linking of BCR and FcγRII Reduces the Activity of p21^{ras} in BL 41 Cells—Shc/Grb-2/Sos complexes are implicated in controlling p21^{ras} activity and thus in the regulation of the downstream serine/threonine kinase cascade (22). BL41 cells were metabolically labeled with $^{32}P_i$, and the cells were activated by BCR cross-linking or by co-cross-linking FcγRII and BCR as indicated. The p21^{ras}-bound GTP:GDP+GTP ratios were compared in the immunoprecipitated samples. Co-ligation of the receptors in three ways, with intact IgG anti-IgM, by aggregated IgG plus anti-Ig (Fig. 5*a*), or after pretreating the cells with CD32 antibody followed by biotinylated anti-mouse IgG and biotinylated anti-human Ig plus avidin (Fig. 5*b*), all reduced the ratio of GTP-bound p21^{ras}. This result indicates that the activity of p21^{ras} is lower in the BCR-FcγRII co-ligated-samples than in those activated via BCR cross-linking.

Activity of MAPK Is Lower in BCR-FcγRII Co-ligated Samples Compared with the BCR-cross-linked Controls—MAPK were isolated from BL41 cells using ERK1 + 2-specific antibodies. The samples were precleared for CD32 to avoid CD32-associated kinases. The activities of MAPK were compared on the basis of MBP phosphorylation in the immunocomplex kinase assays, and the data were normalized for the quantities of MAPK present in the same samples. As shown in Fig. 6, co-cross-linking of FcγRII and BCR induced a lower MBP phosphorylation as compared to samples activated via BCR, suggesting that MAPK activity decreased in the co-ligated samples.

DISCUSSION

B cell activation by T-independent type 2 antigens is simulated by cross-linking BCR with $F(ab)_2$ fragments of anti-Ig. The immune response to such particular antigens as erythrocytes was suppressed when IgG-antigen complexes were administered for immunization (23). Co-cross-linking of BCR and FcγRII by intact IgG anti-Ig interrupts signal transduction in B cells as detected by the reduction of Ca^{2+} influx (2, 3). It has been described recently that FcγRIIb possesses an inhibitory motif containing 13 amino acid residues in the cytoplasmic tail, including a Tyr residue, which becomes phosphorylated after co-ligation of mIg and FcγRII (21). D'Ambrosio *et al.* (4) have described that in the mouse FcγRIIb1 phosphorylated on Tyr recruit protein-tyrosine-phosphatase 1C after BCR-FcγRIIb1 co-ligation, and more recent results (24) suggested that protein-tyrosine-phosphatase 1C specifically induces dephosphorylation of a 35-kDa BCR-associated protein, probably representing Igα in murine B cells . However, this might not be the only way of the inhibition of B cell signaling since in human B cells, only phosphorylation of Tyr^{292} in FcγRIIb1 isoform is required for the inhibition of Ca^{2+} influx, while Tyr^{273} in FcγRIIb2 is not phosphorylated in the co-cross-linked samples, although Ca^{2+} influx is inhibited (25).

We have shown earlier that several Tyr-phosphorylated molecules associate with FcγRII in activated human B cells and have now tested which molecules associate when BCR and FcγRIIb were co-ligated. Under the conditions applied, in 1% Triton X-100 detergent, 200-, 130-, 110-, and 75-and 50–60-kDa Tyr-phosphorylated proteins were observed in FcγRII immunoprecipitates isolated from the membrane of BCR-FcγRII-

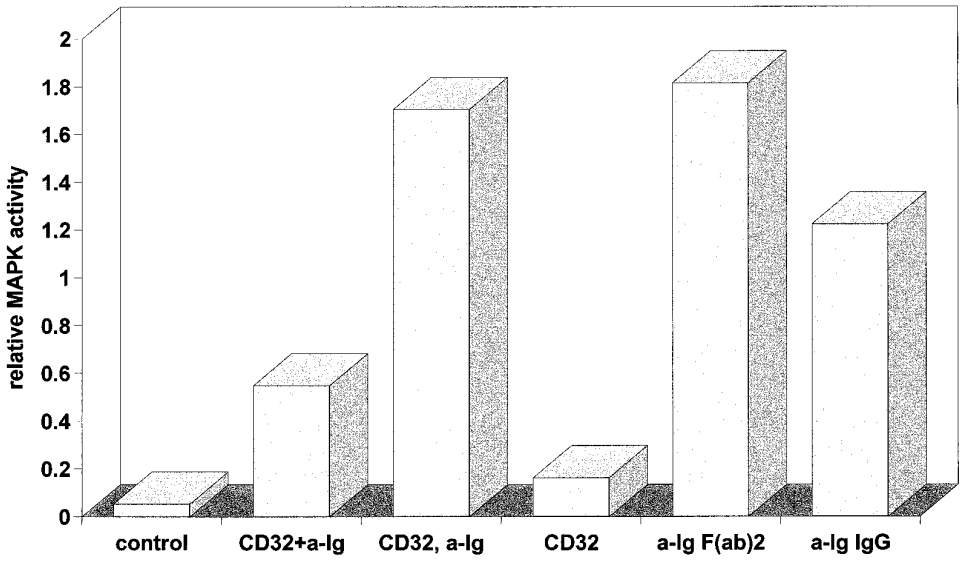

FIG. 6. **Co-ligation of FcγRIIb and BCR reduces MAPK activity.** The samples were treated as follows: unstimulated sample (*control*); CD32 mAb plus goat anti-mouse IgG F(ab)$_2$ and goat anti-human Ig F(ab)$_2$ co-cross-linked by anti-goat IgG (*CD32+a-Ig*); the same treatment as for the previous sample but without co-cross-linking (CD32, *a-Ig*); CD32 mAb plus anti-mouse IgG F(ab)$_2$ (CD32); anti-human Ig F(ab)$_2$; and anti-human Ig intact IgG. After preclearing, the samples were precipitated by ERK1-specific antibodies. MAPK activities were tested in immune complex kinase assays, using MBP as substrate. MBP phosphorylation was detected by autoradiography. The same membrane was probed by antibody specific for ERK1. Values are the result of a typical experiment evaluated by laser densitometry, normalized for the amounts of ERK1 detected in the same samples.

co-cross-linked samples, the 50–60-kDa protein being the most prominent one. This band seems to be composed of several Tyr-phosphorylated proteins, and a fraction of it is identical with Shc. Comparing the total amount of Shc present in the membrane of activated B cells, the FcγRII-associated Shc represent only a portion of it. Shc-FcγRIIa association in murine macrophages was described previously (26). BL41 cells express only FcγRIIb; we could not discern staining with the FcγRIIa-specific antibody, IV.3.[2] More than 95% of FcγRIIb represents the b1 isoform (8); thus, our data indicate that Shc and unidentified Tyr-phosphorylated proteins interact with human FcγRIIb1 in BCR-FcγRIIb1-co-cross-linked samples. Contamination of FcγRIIb1 immunoprecipitates with Igα is excluded, since we could not detect Igα in the isolated CD32 or FcγRIIb in the isolated Igα precipitates. Furthermore, in agreement with Smit *et al.* (27), we did not observe Igα coprecipitated with Shc from the Triton X-100-solubilized samples. Shc has a special adapter function coupling several Tyr-phosphorylated molecules together, since it possesses two Tyr(P)-reactive groups; one is an SH2 domain while the other is a phosphotyrosine-binding domain interacting with a consensus sequence, NPx-pY (28). Since this latter sequence cannot be seen in the intracellular tail of FcγRIIb1 and the SH2 domain of Shc was shown to interact with the motif P-Y-hydrophobic residue *x-X*-I/L (29), which is present in the ITIM motif of FcγRIIb1 (YSLL), we supposed that the SH2 domain of Shc binds to the Tyr-phosphorylated inhibitory motif of FcγRIIb1. To control this hypothesis, molecules absorbed by insolubilized ITIM and P-ITIM peptides were compared. ITIM did not bind any of the tested proteins, while P-ITIM bound Shc from activated but not from resting cells. In the contrary, the 53-kDa Tyr-phosphorylated components were associated with P-ITIM at a similar extent from both resting and activated cells, indicating that these are not identical with Shc. The same bands were recognized on the reprobed blots by Lyn-specific antibodies. These results suggest that the binding of Shc to FcγRIIb1 does not depend on the interaction of Shc SH2 domain with the phosphotyrosine within the ITIM motif of FcγRIIb1 but was probably mediated by the binding of Shc to some of the P-ITIM-associated, inducible Tyr-phosphorylated, unidentified molecules. It has been reported recently that Shc is able to interact with other molecules (adaptins) via its collagen-homologous domain (30); we cannot exclude the possibility of such an interaction.

Association with FcγRIIb1 might alter the function of Shc, a multifunctional signaling molecule. Since the P-ITIM-associated Tyr-phosphorylated 53-kDa molecules co-migrated with Lyn kinase but not with Shc, we assume that FcγRIIb1-bound Shc is not phosphorylated or dephosphorylated due to Shc-FcγRIIb1 interaction. Indeed, when Shc was isolated from the membrane fraction of BL41 cells and its Tyr phosphorylation was compared in BCR-cross-linked and BCR-FcγRII-co-cross-linked samples, a reduced Tyr phosphorylation was observed in the latter (data not shown). These results also suggest that Shc is not a direct substrate of Lyn. In accordance with this finding interaction of Shc with Syk but not with Lyn is described (13). Lyn might be responsible for phosphorylating FcγRIIb1 in the co-ligated samples. This latter suggestion is in agreement with recent finding of Bewarder *et al.* suggesting that Lyn is the most likely candidate for FcγRIIb1 phosphorylation *in vivo* (31).

Shc is implicated in the regulation of p21^{ras} by forming Shc/Grb-2/Sos/p145 complexes after being Tyr phosphorylated and translocating these complexes to the cell membrane where p21^{ras} is located (14). Monitoring p21^{ras} activity we have found that various types of co-ligation of BCR and FcγRIIb1 reduce p21^{ras} activity and this depends on the degree of co-cross-linking. The number of mIg molecules per cell is approximately 10 times higher than that of FcγRIIb1 molecules. Thus, a high concentration of intact IgG anti-IgM could not sufficiently induce inactivation of *ras*, probably since more BCR were cross-linked inducing cell activation than co-cross-linked with the inhibitory FcγRIIb1. The highest degree of inhibition was observed in samples when FcγRII and BCR were co-cross-linked with biotinylated antibodies followed by avidin. Thus, we suggest that FcγRIIb1 interrupts B cell signaling when co-ligated

[2] G. Sármay, Z. Rozsnyay, and J. Gergely, unpublished observations.

with BCR by inhibiting p21^{ras} activation.

p21^{ras} induced phosphorylation of the Ser/Thr kinase, raf, which phosphorylates MAPK kinase; in turn, this dual specificity kinase phosphorylates MAPK on Ser and Tyr residues. When MAPK activities were compared by detecting MBP phosphorylation in the MAPK immunocomplex kinase assays and the values were normalized for the amount of MAPK present, a partially reduced MAPK activity in FcγRIIb1-BCR-co-cross-linked samples was observed when compared with that in BCR-cross-linked ones. These data suggest that BCR-FcγRIIb1 co-ligation may lower p21^{ras}-dependent MAPK activity.

Taking these data together, we suggest the following model for the FcγRIIb1-mediated inhibition of B cell activation. Co-ligation of FcγRIIb1 and BCR induces the Tyr phosphorylation of the ITIM motif allowing the association of SH2 domain-containing molecules with FcγRIIb1. Among these, Shc and lyn were identified. Some of the FcγRIIb1-associated inducible Tyr-phosphorylated molecules bind Shc. Although we could not detect protein-tyrosine-phosphatase 1C in FcγRIIb1 precipitates obtained from the human Burkitt lymphoma line, BL41, we cannot exclude that FcγRIIb1-bound Shc might be the target of phosphatases under certain conditions. Alternatively, Shc might just be sterically hindered to become a target of protein-tyrosine kinases. As a result, Tyr phosphorylation and thus the function of the FcγRIIb1-associated Shc are impaired. Since FcγRII and BCR are co-ligated, FcγRIIb1-bound Shc is transferred to the cell membrane where Shc cannot exert its proper function, the translocation of the Grb-2/Sos complexes to the vicinity of *ras*. Thus, p21^{ras} is less active and has a reduced ability to activate the Ser/Thr kinase cascade leading to MAPK activation and *egr*-1 induction. This mechanism might be responsible for the inhibition of antibody synthesis by the FcγRIIb1-BCR co-ligated cells and *in vivo* may help to avoid production of autoantibodies.

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