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Human Type II Fcγ Receptors Inhibit B Cell Activation by Interacting with the p21ras-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 FcγRIIb-specific immunoprecipitates isolated from the membrane fraction of BL41 Burkitt lymphoma cells following B cell receptor-FcγRIIb 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 FcγRIIb-phosphotyrosine with the SH2 domain of Shc. Tyr phosphorylation of FcγRIIb-associated Shc is low, indicating an impaired function. She is implicated in regulating p21ras activity, thus, we have compared p21ras activities in BL41 cells treated in different ways. p21ras activity is reduced when B cell receptor and FcγRIIb are co-ligated. p21ras 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-FcγRIIb co-cross-linking partially inhibits mitogen-activated protein kinase activity. We conclude that FcγRIIb-dependent inhibition of human B cell activation may be based on interrupting signal transduction between protein-tyrosine kinases and the p21ras/mitogen-activated protein kinase-dependent activation pathway.

The low affinity, IgG Fc-binding, type IIb receptors (FcγRIIb) 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 Ca2+ influx (2, 3). Association of protein-tyrosine-phosphatase 1C with the 13-amino acid-containing phosphorylated immunoreceptor tyrosine-based inhibitory motif (P-ITIM) of FcγRIIb in murine B cells has been demonstrated recently. The authors suggest that protein-tyrosine-phosphatase 1C is an effector of BCR-FcγRIIb negative signal cooperativity (4). Others proposed that co-ligation of BCR and FcγRIIb may result in closing of a Ca2+ channel on the cell membrane, inhibiting thereby the Ca2+ influx (5). The exact mechanism of FcγRIIb-mediated inhibition of B cell function is not yet completely clarified.

On human B cells, both FcγRIIb and FcγRIIb2 isofoms 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 unidentifed Tyr-phosphorylated polypeptides as well as Ser/Thr kinase activities associated with FcγRII isolated from BCR-cross-linked BL41 Burkitt lymphoma cells (6, 7). The vast majority of FcγRII expressed on BL41 cells represents FcγRIIb1 isorm (8). We have suggested that the association of certain signaling molecules with FcγRIIb1 may modify their function when BCR and FcγRIIb 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 p21ras, the low molecular mass G protein, since (i) co-localization of p21ras and mlg was observed after mlg cross-linking (9), (ii) the p21ras 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 p21ras pathway couples antigen receptor stimulation to the induction of the primary response gene egr-1 in B cells (11). The activity of p21ras 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 inactives, p21ras (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 mlg cross-linking (13). Via the Tyr(P) residue, Shc binds to the SH2 domain of Grb-2 in the Grb-2/Sos-1 complex and in turn the complex translocates to the cell membrane, where p21ras is located then Sos activates ras (14). Downstream of p21ras, 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 p21ras/MAPK activa-
tion pathway has not been investigated yet. Co-cross-linking of mlg and FcyRII by intact anti-lg molecules has been shown to inhibit the primary response genes egr-1 and egr-2 expression in the murine B cell line, BCL1 (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 p21ras/MAPK-signaling pathway (11).

The aim of this work was to define signaling molecules, the activities of which might be regulated by FcyRIII-BCR co-cross-linking and which have a definite role in the ras/MAPK activation pathway. We show here that when FcyRIIB1 and BCR are co-ligated on human B cells, a portion of the adapter molecule Shc associates with FcγRII. Furthermore, we demonstrate that the Tyr-phosphorylated inhibitory motif of FcyRIIB1 binds Shc from activated BL41 cell lysate. This association might alter the function of Shc or Shc-associated molecules. A partial inactivation of p21ras and a reduced activity of MAPK are also observed in FcyRII-BCR co-ligated samples. We suggest that FcyRIIB1 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, 10% glycerol, 10 mM EGTA, 2 mM EDTA, 0.25M sucrose, 20 mg/ml leupeptin, 20 mg/ml aprotinin, 5 µg/ml aprotinin, 10 mM sodium pyrophosphate, 10% glycerol, 10 µg/ml aprotinin, 10 mM vanadate, 10 mM sodium pyrophosphate, 5 µg/ml leupeptin, and 0.2 mM phenylmethylsulfonylfluoride. After 60 min of incubation on ice, the cell lysates were centrifuged at 100,000 × g for 40 min, at 4 °C. 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 secondary antibodies followed by enhanced chemiluminescence detection. In some experiments, the membranes were stripped according to the manufacturers’ instruction and reprobed with a different antibody.

Measurement of p21ras Activity—p21ras proteins were immunoprecipitated by rat anti-Ras antibody from the lysate of 32P-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 p21ras-bound GTP with total GTP + GDP activities in the samples on the autoradiogram as measured by laser densitometry.

Immunocomplex Kinase Activity Assay—Immunocomplexes 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 µCi of [γ-32P]ATP, 5 µCi of [γ-32P]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 p52shc with FcγRII—BL41 cells were subfractionated into membrane and cytosolic fractions, FcyRII 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. 1a). 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 FcyRII were co-ligated (Fig. 1a, lane 4 as compared to lane 2 on first two panels). Some of the Tyr-phosphorylated molecules selectively associated with FcyRII isolated from the membrane of anti-Ig-activated cells (200 kDa, 130–140 kDa, 110 kDa, and 50–60 kDa), while control, nonactivated, or aggregated IgG-treated samples were negative. When anti-lg and aggregated IgG were simultaneously applied to co-ligate BCR and FcyRII, a significantly higher Tyr phosphorylation of FcyRII-associated bands, in particular between 50 and 60 kDa (Fig. 1b, 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-FcyRII co-cross-linked samples.

After the first antibodies were stripped, the same membranes were probed with rabbit antibodies specific for Shc (Fig. 1b). 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 FcyRII-co-purified molecules, indicating that a portion of Shc bound to FcyRII isolated from the membrane fraction of BCR-FcyRII co-ligated cells (Fig. 1b, third panel, lane 4). A weak band co-migrating with Shc may be detected occasionally in FcyRII precipitates obtained from nonstimu-
labeled cells, which does not seem to represent a specific interaction.

In the following experiments, FcyRII 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 FcyRII with intact rabbit IgG anti-IgM antibodies dose-dependently induced the association of a 52-kDa Tyr-phosphorylated molecule with FcyRII, 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 FcyRII were co-cross-linked 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 FcyRII. 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 Tyr-phosphorylated components and the binding of Shc to FcyRIIb isolated by CD32 mAb was detected in CD32 and CD79a precipitates isolated from resting, activated, or FcyRII-BCR co-ligated samples (data not shown).

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

Shc was detected in CD32 immunoprecipitates of BCR-FcyRIIb-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. 3a), to exclude the possibility that the signal-transducing subunit of BCR, mb1/Lig (CD79a) contaminates the isolated FcyRII, 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 FcyRII-BCR co-ligated samples (data not shown).

**Fig. 1.** Shc associates with FcyRIIb affinity-purified from the membrane fraction of FcyRII-BCR co-ligated BL41 cells. 5 x 10^6 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 FcyRII were affinity-purified by human IgG for co-cross-linking. 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 FcyRIIb isolated by CD32 mAb. 5 x 10^6 BL41 cells were stimulated with various doses of reagents as indicated. FcyRII 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 FcyRIIb in the membrane of FcyRIIb-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, CD79a (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.
MAPK present in the same samples. As shown in Fig. 6, co-

bodies. The samples were precleared for CD32 to avoid CD32-

on 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.

Fig. 4. Shc binds to the synthetic peptides representing the

P-ITIM motif of FcγRII. 5 x 10^4 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 mol-

ecules 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 (right). This was followed

by an additional stripping, and the same membrane was probed with

anti-lyn (right). The reactions were developed by HRP-conjugated

species-specific antibodies and enhanced chemiluminescence. Arrow-

heads, positions of Tyr-phosphorylated molecules; asterisk, position of

lyn. Total BL41 cell lysate is shown (lys).}

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 component (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 nonacti-

vated control cells only after longer exposure. Higher Tyr phos-

phorylation 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). Fur-

ther 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

p21ras in BL 41 Cells—Shc/Grb-2/Sos complexes are implicated in

controlling p21ras activity and thus in the regulation of the

downstream serine/threonine kinase cascade (22). BL41 cells

were metabolically labeled with 32P, and the cells were acti-

vated by BCR cross-linking or by co-cross-linking FcγRII and

BCR as indicated. The p21ras-bound GTP/GDP + GTP ratios were

compared in the immunoprecipitated samples. Co-liga-

tion of the receptors in three ways, with intact IgG anti-IgM, by

aggregated IgG plus anti-Ig (Fig. 5a), or after pretreating the

cells with CD32 antibody followed by biotinylated anti-mouse

IgG and biotinylated anti-human Ig F(ab)2 fragments (Fig. 5b),

all reduced the ratio of GTP-bound p21ras. This result indicates

that the activity of p21ras 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 antibi-

todies. 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 ki-

nase 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 phos-

phorylation as compared to samples activated via BCR, sug-

gesting that MAPK activity decreased in the co-ligated

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 represents only a portion of it. Shc-FcγRIIα association in murine macrophages was described previously (26). BL41 cells express only FcγRIIb; we could not discern staining with the FcγRII-specific antibody, IV.3. 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-coupling adapter function coupling several Tyr-phosphorylated 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-FcγRIIb1 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, the interaction of Shc with Syk but not with Lyn is described (13). Lyn might be responsible for phosphorylating FcγRIIb1 in the co-precipitated 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 vitro.

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 precipitated 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.

REFERENCES