Negative Signaling via FcγRIIB1 in B Cells Blocks Phospholipase Cγ2 Tyrosine Phosphorylation but Not Syk or Lyn Activation*

(Received for publication, December 22, 1995, and in revised form, April 24, 1996)

Sujata Sarkar, Klaus Schlottmann‡, Damon Cooney, and K. Mark Coggeshall§

From the Ohio State University, Department of Microbiology, Columbus, Ohio 43210

Crosslinking of the B cell antigen receptor surface immunoglobulin induces tyrosine phosphorylation and activation of the Src family and Syk tyrosine protein kinases, tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2) and increases in intracellular second messengers inositol phosphates and Ca2+. These activation events, in conjunction with other pathways, culminate in the induction of B cell proliferation and differentiation. In contrast, co-crosslinking surface Ig with the B cell IgG Fc receptor prevents many of these activation events, including B cell proliferation and differentiation. The precise nature of the negative signal(s) derived from Fc receptors that prevent B cell activation is not known. Here, early activation events were examined in B cells stimulated via the antigen receptor alone or under co-crosslinking conditions. The data indicated a selective block in the tyrosine phosphorylation and activation of PLCγ2 but not in activation of the upstream kinases, Syk and Lyn, under co-crosslinking conditions. We conclude that the negative signal acts directly on PLCγ2 and is consistent with recent studies describing an activation-induced association of a phosphotyrosine phosphatase with tyrosine-phosphorylated B cell Fc receptor.

Stimulation of the B cell antigen receptor, surface immunoglobulin (slg),1 initiates a cascade of biochemical events that culminate in proliferation (reviewed in Refs. 1 and 2). B cell proliferation is regulated by several means, among which is the suppressive effect of secreted Ig (3). This effect represents a feedback inhibitory mechanism that limits further antibody secretion. To inhibit B cell proliferation, the suppressive antibody must bind specific antigen and bear an intact Fc domain (3), indicating that Fcγ receptor (FcγRIIB1)-slg co-crosslinking generates a dominant negative signal that prevents B cell proliferation (4–6). It has been proposed that blocking of antibody-mediated FcγRIIB1-slg co-crosslinking by FcγRIIB1-binding rheumatoid factors may play an etiological role in autoimmune diseases by disrupting this inhibitory mechanism, thereby facilitating continuous autoantibody production (3).

Studies by Phillips and Parker (4, 5) established a polydonal model to study this mode of inhibition by using whole anti-Ig antibodies which co-cross-link slg and FcγRIIB1 via their Fab and Fc domains, respectively. Such antibodies inhibit B cell proliferation (5, 6), production of phosphatidylinositol-derived second messengers (diacylglycerol and inositol 1,4,5-trisphosphate (IP3); Ref. 7), and increases in intracellular Ca2+ (8). In contrast, F(ab′)2 fragments of anti-Ig antibodies are stimulatory toward B cells in regards to all these events. The molecular basis of the FcγRIIB1-mediated inhibition of PLC activation and the generation of the intracellular mediators diacylglycerol and IP3 are poorly understood.

Protein-tyrosine kinases (PTKs) of the Src family, namely, p56lck, p58yn, and p59fyn (9, 10), and the unrelated p72syk kinase (11, 12) play an obligatory (13, 14) role in B cell activation. These kinases are associated with slg, and their activity is enhanced upon slg triggering. PTK activation leads to, among other events, tyrosine phosphorylation and activation of phospholipase Cγ2 (PLCγ2; Refs. 15 and 16), which is involved in the production of IP3 and diacylglycerol second messengers (17, 18). B cells deficient in p72syk expression demonstrated an inability to undergo receptor-mediated PLCγ2 tyrosine phosphorylation, IP3 formation, and calcium mobilization (19), suggesting that p72syk mediates its activation via tyrosine phosphorylation of PLCγ2.

It is notable that the activation events blocked by FcγRIIB1-slg co-crosslinking, i.e. production of IP3 and increased intracellular Ca2+, are directly or indirectly dependent on the prior activation of PLC and that PLC activation is dependent on prior activation of PTKs. This fact raises the possibility that the FcγRIIB1-derived negative signal may be due to the abrogation of slg-mediated PTK activation and/or of tyrosine phosphorylation and activation of PLCγ2. Here, we examined these possibilities in murine ex vivo B cells by immunoprecipitation of the various PTKs or PLCγ2 and in vitro assays of their enzymatic activity or tyrosine phosphorylation status. The data indicated that, under stimulatory conditions using F(ab′)2 fragments of anti-Ig, Syk and Lyn were activated and PLCγ2 was tyrosine-phosphorylated. Under inhibitory conditions of FcγRIIB1-slg co-crosslinking, the kinase activity of Syk and Lyn was likewise activated; however, and in contrast to activating conditions, PLCγ2 tyrosine phosphorylation was abrogated. These findings are able to account for previous reports (7, 8) of inhibition of PLC activation under FcγRIIB1-slg co-crosslinking conditions and are discussed in the context of current knowledge of lymphocyte antigen receptor-mediated activation events.

MATERIALS AND METHODS

Animals and Reagents—Mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 4–8 weeks of age. F(ab′)2 fragment and whole molecule of rabbit anti-mouse IgG antibody were obtained from Organon Technika, Westchester, PA. Anti-Thy 1.2 monoclonal antibody and guinea pig complement were purchased from Sigma. Immunoprecipitating and immunoblotting antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); protein

*This work was supported by a grant from the National Science Foundation MCB9317027. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Fellow of the German Research Foundation (Schl. 390/1-1).
‡ To whom correspondence should be addressed: Ohio State University, Dept. of Microbiology, 484 W. 12th Ave., Columbus, OH 43210. Tel: 614-292-5394; Fax: 614-292-8120; E-mail: coggleshall.1@osu.edu.

1 The abbreviations used are: slg, surface immunoglobulin; FcγRIIB1, IgG Fc receptor type IIIB; IP3, inositol 1,4,5-trisphosphate; ITAM/ITIM, immunoreceptor tyrosine-based activation/inhibitory motifs; PKC, protein kinase C; PLCγ2, phospholipase Cγ2; PTK, protein tyrosine kinase.
Deficient PLCy2 Tyrosine Phosphorylation upon sIg-FcγRIIB1 Co-crosslinking

20183

RESULTS

Cellular Tyrosine phosphorylation by F(ab')2 and Whole Anti-Ig—B cells triggered by F(ab')2 fragments of anti-Ig antibodies undergo tyrosine phosphorylation of multiple proteins (1, 2). In order to determine whether inhibition of B cell proliferation by intact anti-Ig results from defective PTK activation, profiles of PTK substrates in total extracts of B cells stimulated with F(ab')2 or whole anti-Ig antibodies were compared (Fig. 1). Both stimuli induced an increase in tyrosine phosphorylation of cellular proteins; the overall profile and time course of tyrosine-phosphorylated substrates induced by the two stimuli were similar. Dominant bands at 56–60, 72, and 110 kDa are indicated with arrowheads and may represent activated Src family PTK p56lck, p58m, and/or p59m (9, 10); p72ph (11, 12) and p110 subunit of phosphatidylinositol 3-kinase (21). Slight reduction in the tyrosine phosphorylation of a series of low molecular weight bands were observed in B cell lysates derived from cells stimulated with whole anti-Ig antibodies. Especially apparent is a band migrating at approximately 35 kDa in lysates derived from sIg-stimulated B cells and reduced in lysates of whole anti-Ig antibody-stimulated B cells. Thus, despite the failure of whole anti-Ig antibodies to induce a proliferative response in ex vivo B cells (4, 5), both forms of anti-Ig antibodies trigger the activation of cellular PTK(s) that are responsive to sIg ligation.

FcyRIIB1-sIg Co-crosslinking Reduces Tyrosine Phosphorylation of PLCy2—The apparently intact activation of PTKs in B cells treated with whole anti-Ig antibodies (Fig. 1) does not rule out a selective defect in tyrosine phosphorylation of a critical PTK substrate. Indeed, the reduced tyrosine phosphorylation of the 35-kDa band (Fig. 1) indicates that some PTK substrates may be differentially phosphorylated following stimulation with the two forms of anti-Ig. Our previous studies defined PLCy2 as the major B cell isoform of PLCy, which responded to PTK activation and which acquired phosphorylated tyrosine residues upon sIg triggering (15, 16). PLCy2 tyrosine phosphorylation was examined after stimulation of B cells with intact or (F(ab')2) fragments of anti-Ig by immunoprecipitation of tyrosine-phosphorylated proteins with 4G10 anti-phosphotyrosine followed by anti-PLCy2 immunoblotting. The results, shown in Fig. 2, demonstrated rapid tyrosine phosphorylation of PLCy2 following F(ab')2 anti-Ig treatment and no detectable PLCy2 phosphorylation with intact anti-Ig treatment. This finding indicates PLCy2 tyrosine phosphorylation is reduced when sIg is co-crosslinked with FcγRIIB1.

To confirm this finding, lysates of B cells, unstimulated or stimulated as described above for various times, were subjected to immunoprecipitation with anti-PLCy2 antibodies and analyzed by 4G10 immunoblotting. The results (Fig. 3, upper panel) demonstrated transient PLCy2 tyrosine phosphorylation when B cells were stimulated under inhibitory sIg-FcγRIIB1 co-crosslinking conditions as compared with activating F(ab')2 anti-Ig stimulation conditions. This difference was not due to differing amounts of PLCy2 in the immunoprecipitations as stripping the above immunoblot and re-probing with anti-PLCy2 antibodies revealed similar amounts in PLCy2 immunoprecipitates but not in normal rabbit Ig immunoprecipitates (Fig. 3, lower panel). Tyrosine Phosphorylation and Activation of p72ph and p58m following sIg Crosslinking or sIg and FcγRIIB1 Co-crosslink-
Deficient PLCγ2 Tyrosine Phosphorylation upon sIg-FcRIIB1 Co-crosslinking

**FIG. 2.** Differential tyrosine phosphorylation of PLCγ2 following sIg crosslinking and co-crosslinking with FcγRIIB1. Murine splenic B cells at 10 × 10^6 cells/100 μl were stimulated with 10 μg/ml of F(ab′)_2 fragment or whole molecule (WM) of rabbit anti-mouse IgG for the indicated times in minutes, and lysates were immunoprecipitated with 10 μg of 4G10 anti-phosphotyrosine antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-PLCγ2 antibody, and developed with horseradish peroxidase-conjugated protein G by chemiluminescence. Immunoblotting of PLCγ2 was blocked by inclusion of immunizing PLCγ2, but not PLCγ1 peptide, as reported previously (Ref. 16 and data not shown). The molecular weight markers are shown on the left of each panel. The experiment is representative of three others.

**FIG. 3.** Differential tyrosine phosphorylation of PLCγ2 following sIg crosslinking and co-crosslinking with FcγRIIB1. Murine splenic B cells at 10 × 10^6 cells/100 μl were left unstimulated (NS) or stimulated with 10 μg/ml of F(ab′)_2 fragment or intact rabbit anti-mouse IgG for the indicated times in minutes, and lysates were immunoprecipitated with anti-PLCγ2 antibody or normal rabbit Ig (NRIg) overnight. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with 4G10 antibody, and developed with horseradish peroxidase-conjugated goat anti-mouse by chemiluminescence (upper panel). The membrane was stripped and reprobed with anti-PLCγ2 antibody and developed by chemiluminescence (lower panel). The experiment is representative of four others.

**FIG. 4.** Tyrosine phosphorylation of p72Syk following crosslinking of sIg and co-crosslinking with FcγRIIB1. 10 × 10^6 murine splenic B cells per sample were treated with F(ab′)_2 fragment or whole molecule of rabbit anti-mouse IgG for the indicated times in minutes, and lysates were immunoprecipitated with anti-p72Syk antibody (20). The immunoprecipitates were separated by SDS-PAGE and probed with 4G10 anti-phosphotyrosine antibody (upper panel). The membrane was stripped and reprobed with anti-p72Syk antibody and developed by chemiluminescence (lower panel). The experiment is representative of four others.

---

Differential tyrosine phosphorylation of PLCγ2 was observed under sIg-FcγRIIB1 co-crosslinking conditions may therefore be the result of reduced tyrosine phosphorylation and/or activation of Syk. To examine this hypothesis, tyrosine phosphorylation of p72Syk following co-crosslinking of sIg with or without co-crosslinking with FcγRIIB1 was studied. Tyrosine phosphorylation status of p72Syk was analyzed by immunoprecipitating p72Syk from stimulated or unstimulated murine splenic B cell lysates using rabbit polyclonal anti-Syk antibody and immunoblotting with 4G10 following SDS-PAGE and membrane transfer. Results (Fig. 4, upper panel) show that p72Syk is tyrosine-phosphorylated following stimulation of sIg with F(ab′)_2 anti-Ig (lanes 2 and 3), as described previously (12). Likewise, no detectable difference in Syk tyrosine phosphorylation was observed following co-crosslinking of sIg with FcγRIIB1 using intact anti-Ig (lanes 4 and 5). Lane 1 shows Syk immunoprecipitated from unstimulated cells; lane 6 shows material immunoprecipitated with normal rabbit IgG. The presence of essentially equivalent amounts of p72Syk in the samples was confirmed by stripping and reprobing the immunoblot with anti-Syk antibody (Fig. 4, lower panel).

The activation status of p72Syk following co-crosslinking sIg or co-crosslinking sIg-FcγRIIB1 was studied by measuring Syk autophosphorylation using [γ−32P]ATP in Syk immunoprecipitates derived from lysates of stimulated or unstimulated murine splenic B cells. Results (Fig. 5) showed that stimulation of B cells with F(ab′)_2 anti-Ig antibody activated p72Syk (lanes 2 and 3), which was detectable after 2 min of stimulation. Likewise, treatment of B cells with intact anti-Ig to induce co-crosslinking of sIg and FcγRIIB1 (lanes 4 and 5) induced p72Syk activation to a similar extent and with similar kinetics. Lane 1 represents the unstimulated sample, and lane 6 shows the...
deficient PLCγ2 Tyrosine Phosphorylation upon slg-FcRIIB1 Co-crosslinking

**Fig. 5.** Activation of p72syk following crosslinking of slg and co-crosslinking slg with FcγRIIB1. 10 × 10⁶ murine splenic B cells per sample were treated with Fabγ2 fragment or whole molecule of rabbit anti-mouse IgG for the indicated times at 37 °C and immunoprecipitated as described in the legend to Fig. 5. The immunoprecipitates were washed in kinase buffer and incubated with [γ-32P]ATP at 30 °C for 10 min. The reactions were stopped by addition of excess kinase buffer, resuspended in SDS sample buffer, separated by SDS-PAGE and subjected to autoradiography at −70°C for 2 h. Immunoblotting analysis (not shown) demonstrated essentially equal amounts of Syk in all lanes. The experiment is representative of three others.

Normal rabbit IgG immunoprecipitate control. Thus, p72syk tyrosine phosphorylation and activation of kinase activity is not distinguishable between stimulation of slg alone or upon co-crosslinking of slg-FcγRIIB1. Quantitation of the autophosphorylated Syk band from the samples stimulated for 2 min with Fabγ2 anti-Ig revealed a 6-fold increase over unstimulated controls; the corresponding sample of intact anti-Ig-stimulated B cells revealed a 5-fold increase over unstimulated controls.

Activation of the Src family PTKs has been described previously (9, 10) and, by analogy with the T cell antigen receptor (22), is likely upstream of Syk activation. The activation of Src family PTKs by Fabγ2 and intact anti-Ig antibodies was assessed using Lyn as a representative B cell Src family PTK. Lyn activity was examined by immunoprecipitation of lysates derived from B cells unstimulated, Fabγ2- or intact anti-Ig-stimulated followed by in vitro kinase activity measurements. Results, shown in Fig. 6, demonstrated an equivalent activation of Lyn (as measured by Lyn autophosphorylation or by enolase phosphorylation) by both forms of anti-Ig, consistent with measurements of Syk activation shown above. Quantitation of the two characteristic (9) autophosphorylated Lyn bands (p56 and p58) revealed a 2-fold increase in Fabγ2 anti-Ig-stimulated cells and a 3-fold increase in intact anti-Ig-stimulated cells; both of these were compared with Lyn activity obtained from unstimulated controls. Likewise, liquid scintillation counting of the enolase band revealed a 2-fold increase in both Fabγ2 and intact anti-Ig-stimulated samples, as compared with enolase phosphorylation in unstimulated controls.

**DISCUSSION**

Negative signaling in B cells is important in the maintenance of immune regulation. Co-crosslinking slg and FcγRIIB1 was proposed to represent a negative feedback mechanism by bridging slg and FcγRIIB1 via surface-bound antigen or via anti-idiotypic antibodies and thereby preventing continuous production of antibodies (3). Fc receptor function in B cells may therefore be important for immune regulation via antigen-antibody complexes by inhibiting B cell proliferation and maturation into antibody secreting cells. Furthermore, rheumatoid factor, an IgM anti-IgFc antibody, was been proposed to prevent normal negative signaling by binding the Fc portion of secreted Ig, thereby potentially playing an etiologic role in the development of rheumatoid arthritis (3).

Previous studies regarding the biochemical nature of the aborted activation process demonstrated transient formation of IP₃ when B cells are treated under slg-FcγRIIB1 co-crosslinking conditions (7), indicating a short-lived PLC activation. In contrast, B cells stimulated with Fabγ2 fragments of anti-Ig showed a more sustained IP₃ formation, as well as later changes in cell activation (7). Thus, cell cycle progression induced upon slg crosslinking with Fabγ2 anti-Ig antibodies may be at least in part the result of increased IP₃ and intracellular calcium, two important intracellular second messengers (7, 8). The lack of these intracellular mediators under slg-FcγRIIB1 co-crosslinking conditions likely contribute to the observed inhibitory effect. Recently, two reports (23, 24) revealed decreased calcium flux upon treating A20 cells, a murine B cell lymphoma, with intact Ig antibodies. Additional experiments indicated that negative signaling required an intact immunoreceptor tyrosine-based Inhibitory motif (ITIM) within FcγRIIB1; furthermore, phosphorylation of the tyrosine residues within the ITIM was necessary for negative signaling (23). However, defective activation of A20 under slg-FcγRIIB1 co-crosslinking conditions could not be accounted for by the decreased tyrosine phosphorylation of PLCγ2 and Syk; both these activation events were induced by intact anti-Ig (23, 24).

Our data indicated that the activating signal under co-crosslinking conditions is identical to that induced by slg crosslinking alone, i.e. both Syk and Lyn are activated and the B cells display similar increases in a number of tyrosine-phosphorylated proteins. We observed the induction of a ~35-KDa phosphotyrosine-containing protein in cells stimulated with Fabγ2 fragments of anti-Ig that appeared to be absent from cells stimulated with intact anti-Ig. The precise nature of this band is not clear; however, preliminary studies established that Ig-α is tyrosine-phosphorylated under both conditions of stimulation. We have not examined the status of Ig-β under either stimulation condition. However, despite the essentially equivalent activation of upstream PTKs and increased tyrosine-phosphorylated proteins, PLCγ2 tyrosine phosphorylation appeared only transiently (Fig. 3) in samples stimulated under negative signaling conditions. This finding can account for the previous reports that IP₃ production and Ca²⁺ influx are transient events under co-crosslinking conditions (7, 23, 24) as well as account for the obstruction of cell cycle progression.

Recently it has been shown that tyrosine 309 in the ITIM motif of FcγRIIB1 associates with a phosphotyrosine phosphatase PTP1C (25). PTP1C is an intracellular phosphotyrosine
phosphatase with two tandem N-terminal SH2 domains (26–28) and associates with tyrosine-phosphorylated c-Kit (29) and the β chain of interleukin-3 receptor (30) via interaction of their SH2 domains. In B cells, the association and activation of PTP1C with FcγRIIB1 was detected upon co-crosslinking slg with FcγRIIB1 and was dependent on the phosphorylation of tyrosine 309 on the ITIM (25). The decreased or abolished tyrosine phosphorylation of PLCγ2 observed upon slg-FcγRIIB1 co-crosslinking and reported here can be accounted for by the phosphatase activity of PTP1C. We therefore propose that upon slg-FcγRIIB1 co-crosslinking, tyrosine 309 in the ITIM of FcγRIIB1 is phosphorylated and PTP1C binds to FcγRIIB1 via its SH2 domain to phosphorylated tyrosine 309. This binding induces activation of PTP1C phosphatase activity, as reported (25). Activated PTP1C then selectively dephosphorylates PLCγ2, while tyrosine phosphorylation and activation of Syk and Lyn activation are not affected. This model is currently under investigation in our laboratory.

REFERENCES
1. Gold, M. R., and DeFranco, A. L. (1994) Adv. Immunol. 55, 221
2. Cambier, J. C., Pleiman, C. M., and Clark, M. R. (1994) Annu. Rev. Immunol. 12, 457
3. Sinclair, M. R., and Panokaltsis, A. (1987) Immunol. Today 6, 76
4. Phillips, N. E., and Parker, D. C. (1983) J. Immunol. 130, 602
5. Phillips, N. E., and Parker, D. C. (1984) J. Immunol. 132, 627
6. Klaus, G. G. B., Harylowicz, C. M., Holman, M., and Keeler, K. D. (1984) Immunology 53, 693
7. Bijsterbosch, M. K., and Klaus, G. G. B. (1985) J. Exp. Med. 162, 1825
8. Choquet, D., Partiseti, M., Amigorena, S., Bonnerot, C., Fridman, W. H., and Korn, H. (1993) J. Cell Biol. 121, 355
9. Burkhardt, A. L., Brunswick, M., Bolen, J. B., and Mond, J. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7410
10. Campbell, M.-A., and Sefton, B. M. (1992) Mol. Cell. Biol. 12, 2315
11. D'Ambrosio, D., Hippen, K. L., Minskoff, S. A., Melman, I., Pani, G., Siminovitch, K. A., and Cambier, J. C. (1995) Science 268, 293
12. Shen, S. H., Bastien, L., Posner, B. I., and Chretien, P. (1991) Nature 352, 736
13. Matthews, R. J., Bowne, D. B., Flores, E., and Thomas, M. L. (1992) Mol. Cell. Biol. 12, 2396
14. Plutzky, J., Neel, B. G., and Rosenberg, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1123
15. Yi, T., and Ihle, J. N. (1993) Mol. Cell. Biol. 13, 3350
16. Yi, T., and Ihle, J. N. (1993) Mol. Cell. Biol. 13, 7577