Co-cross-linking of the Fc receptor (FcR) to surface immunoglobulin (sIg) on B cells inhibits the influx of extracellular calcium and abrogates the proliferative signal. The mechanism by which this occurs is not well understood. In this report we show that co-cross-linking of the FcR to the antigen receptor gives rise to very selective modulation of signal transduction in B cells. Co-cross-linking of sIg and FcR enhanced the phosphorylation of the FcR, the adapter protein, Shc, and the inositol 5′-phosphatase Ship. Furthermore, phosphorylation of the FcR induced its association with Ship. Cross-linking of the FcR and sIg decreased the tyrosine phosphorylation of CD19, which led to a reduction in the association of phosphatidylinositol 3-kinase. In addition, the phosphorylation of several other proteins of 73, 39, and 34 kDa was reduced. Activation of the cells with either F(ab')2 or intact anti-IgG induced very similar changes in levels of tyrosine phosphorylation of most other proteins, and no differences in the activation of several protein kinases were observed. These results indicate that the inhibitory signal that is transmitted through the FcR is not mediated by a global shutdown of tyrosine phosphorylation but is, rather, a selective mechanism involving localized changes in the interactions of adapter proteins and the enzymes Ship and phosphatidylinositol 3-kinase with the antigen receptor complex.

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Cross-linking of the antigen and Fc receptors leads to a dominant negative signal. In contrast, concomitant activation of B cells through both ligation and/or differentiation of the cells (reviewed in Refs. 1 and 2) gives rise to a sequence of intracellular signals that leads to the proliferation of the cells. The inhibitory pathway triggered following co-ligation of the sIg and FcR is not well understood. Studies have recently been undertaken to try to distinguish the molecular events that give rise to the differences in signaling mediated through the antigen receptor alone from that following the cross-linking of sIg to the FcR. Stimulation of the cells with F(ab')2 anti-IgG, which mimics antigen binding to the receptor, gives rise to the mobilization of calcium within the cell from both intracellular and extracellular pools. Uptake of extracellular calcium appears to be critical for the outcome of activation. Co-cross-linking of the FcR to sIg with intact anti-Ig, which mimics the binding of antibody-antigen complexes, inhibits the influx of extracellular calcium (7, 8). Additionally, co-ligation of the sIg and Fc receptors subsequent to the opening of the plasma membrane calcium channel stimulates the closure of the channel (8), and the level of intracellular calcium rapidly declines. In A20 cells, it is possible to restore the influx of extracellular calcium and to overcome the FcR-mediated inhibition of cell activation and IL-2 production by low concentrations of calcium ionophore (8).

Activation of B cells through the antigen receptor also results in the tyrosine phosphorylation of many proteins, including the cell surface antigens CD19 and CD22 that are associated with the antigen receptor (9–12), the components of the antigen receptor complex, Igα and Igβ (13–15), the tyrosine kinases Syk, Lyn, and Btk (16–18), the phospholipases PLCγ1 and PLCγ2 (19–21), and the adapter and signaling proteins Cbl, Vav, Shc, and Gap (3, 22–29). Phosphorylation and activation of PLCγ1 and PLCγ2 results in the generation of inositol 1,4,5-trisphosphate, which in turn stimulates the release of calcium from intracellular stores. However, the signaling pathway that regulates the opening and closing of the plasma membrane calcium channel has not been well established.

Co-cross-linking of the FcR to sIg also gives rise to the phosphorylation of many intracellular proteins, including the FcR itself, on a tyrosine residue in the intracellular domain (30). The region containing this tyrosine residue is essential for the inhibitory activity of the FcR (30). Subsequently, it has been found that phosphorylation of the FcR on this tyrosine residue results in the interaction of the receptor with the Src homology 2-containing tyrosine phosphatase SHP-1 (31, 32). This association, which may give rise to activation of the phosphatase, is then thought to provide the signal that shuts off activation of the receptor complex and thus to abrogate the proliferation of the cells.

SHP-1 has been found to be associated with the antigen receptor in unstimulated cells (32, 33) and with CD22 in cells activated through sIg (34). Cross-linking of CD22 with antibodies can modulate signaling through the antigen receptor (35), and it has been proposed that one mechanism for this is that...
ligation of CD22, by antibodies or counter receptors on adjacent cells, gives rise to the removal of the SHP-1 from the immediate locale of the sIg signaling complex, thus removing the inhibitory enzyme (35, 36). Thus phosphorylation of CD22 and the FcR and their interactions with SHP-1 may provide opposing regulatory control on the strength or duration of the signal transmitted through the antigen receptor (33, 35, 36).

The events that follow the phosphorylation of the FcR and its association with SHP-1 have not been well characterized. The phosphorylated proteins that are the targets of the phosphatase in this regulatory pathway have not been identified, and the influence of the SHP-1 and FcR interaction on the activation of downstream signal transduction pathways has not been determined. Additionally, it is not known how the phosphorylation of the FcR and its subsequent interaction with SHP-1 leads to the rapid closure of the calcium channel.

In this report we have compared the signaling events that occur following triggering of the antigen receptor either alone or with co-ligation with the FcR. We have found that the phosphorylation and activation of many proteins in the signaling pathways were not significantly different between the two modes of stimulation; however, there were marked changes in the phosphorylation states of a few proteins, including CD19, FcR, Ship, and Shc. These changes resulted in the association of the Ship with the FcR complex and a loss of association of PI3-kinase with CD19.

These results indicate that the inhibitory signal mediated by the FcR on B cells is a very selective modulation of intracellular signaling and is not simply a consequence of the global dephosphorylation of the signaling components of the antigen receptor by the association of SHP-1 with the receptor complex. Rather, the co-ligation of sIg and the FcR gives rise to changes in the phosphorylation state of a limited set of proteins, which then alters their ability to interact with components of the antigen receptor. The results also suggest that the local alterations in phosphatidylinositol phosphate metabolism by enzymes associated with the antigen receptor complex, namely PI3-kinase and Ship, may be critical in the regulation of the opening and closure of the plasma membrane calcium channel.

While this manuscript was in preparation, Chacko and co-workers (41) and Ono and co-workers (51) also reported finding the enhanced phosphorylation of Ship and its association with Shc and the FcR following co-ligation of sIg and the FcR.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents—The murine B cell line A20 was obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium containing 10% fetal calf serum and 50 μM β-mercaptoethanol. Rabbit Fab(′)2 and intact anti-mouse IgG were obtained from Cappel (Durham, NC). Horseradish peroxidase-labeled reagents (sheep Fab(′)), anti-mouse IgG, sheep Fab(′), anti-rabbit IgG, and protein A) and ECL reagents were obtained from Amersham Corp. Gamma-Bind Plus was from Pharmacia Biotech Inc. Monoclonal antibodies to phosphotyrosine (RC20H) and to the signaling intermediates were obtained from Transduction Laboratories (Lexington, KY). Monoclonal antibodies to CD19, CD92 (2.4G2), and CD22 were obtained from Pharmingen (San Diego, CA). Polyclonal antibodies to Cbl, Shc, Grb2, Erk2, PLC-γ2, JAK1, JAK2, and c-Jun amino-terminal kinases 1 and 2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to Ship (5340 and 5367) were provided by Dr. Larry Rohrschneider (Fred Hutchinson Cancer Research Center) and have been described previously (40). Polyclonal antibodies to PLC-γ1 and PLC-γ2 were obtained from within Bristol-Myers Squibb; antibodies to Fyn, Btk, Syk, Lyn, Igs, the Igα, and Igδ were obtained from Dr. Joe Bolen (Bristol-Myers Squibb). The biotinylated peptides CAENTITY(p)SLL and CAENTITYSLL were synthesized by SYNPEP (Dublin, CA) with a biotin molecule followed by an aminoethyl spacer group linked through the amino-terminal cysteine. All other reagents were from Sigma.

Immunoprecipitations, Electrophoresis, and Western Analysis—Cells at 2–3 × 10^6/ml were stimulated with either Fab(′)2 or intact anti-mouse IgG for the indicated times. The reaction was stopped by adding 10 ml of ice-cold PBS to each tube. The cells were pelleted by centrifugation at 4000 rpm for 30 s; the supernatants were removed, and the cells were lysed at 1 × 10^9/ml in CHAPS lysis buffer (8). The lysates were either used immediately or stored frozen at −70 °C. Immunoprecipitations were carried out at 4 °C using 5 μg of antibody/1 ml of cell lysate. After 1 h, 80 μl of a 50% suspension of protein G or protein A was added to the samples, and then these were rocked continuously for an additional 3 h at 4 °C. The samples were washed four times with 1 ml of PBS containing 10 mM CHAPS, 500 μM sodium orthovanadate, and 200 μM phenylmethylsulfonyl fluoride. After the last wash the pellets were resuspended in 100 μl 2 × SDS sample buffer. Lysates and immunoprecipitates were analyzed by SDS-polyacrylamide electrophoresis (PAGE) and transferred to ployvinylidine difluoride membranes. The membranes were blocked with PBS containing 5% bovine serum albumin, 1% ovalbumin, and 1 mM sodium orthovanadate, and the blots were then probed with the indicated antibodies and visualized by ECL (Amersham).

Affinity precipitations with peptides were carried out by adsorbing the biotinylated peptides to streptavidin agarose (50 μg of peptide and 50 μl of packed resin/5 × 10^7 cells) in 1 ml of PBS for 2 h at 4 °C. The beads were washed three times with PBS and once with CHAPS lysis buffer prior to addition to cell lysates. The affinity precipitates were then treated in the same way as the immunoprecipitates as outlined above.

IL-2 Production—The stimulation of IL-2 release from A20 cells was measured by enzyme-linked immunosorbent assay as described previously (8). The cells were stimulated with antibodies and reagents as indicated for 24 h. The cell supernatants were collected and assayed immediately or stored frozen. The viability of the cells after treatments was determined by exclusion of trypan blue and found to be greater than 94% at the highest concentration of Wortmannin that was used (100 nM).

Calcium Mobilization—The mobilization of both intracellular and extracellular calcium was measured on an SLM 8000 spectrophotometer using the indicator dye Indo-1 (Molecular Probes, Eugene, OR), as described previously (8). Briefly, the cells (1 × 10^6/ml) in RPMI 1640 medium containing 10% fetal bovine serum were loaded with Indo-1 AM for 45 min at 37 °C. The cells were washed three times in Hank’s balanced salt solution containing 10 mM HEPES and 1% fetal calf serum. The cells were then resuspended at 1 × 10^6/ml in the same buffer for analysis.

RESULTS

Stimulation of Tyrosine Phosphorylation—Co-cross-linking of the FcR to sIg abrogates the influx of extracellular calcium within 1–2 min of activation of the cells (Ref. 8 and Fig. 7A). To address the changes that occur on activation of the cells through sIg alone or when sIg is cross-linked to the FcR, we studied the early cellular responses that occur within this time frame. As previously reported, stimulation of the cells through sIg with Fab(′)2 rabbit anti-mouse IgG gave rise to a rapid increase in the tyrosine phosphorylation of many intracellular proteins. Furthermore, co-cross-linking the FcR with the sIg using intact anti-mouse Ig did not markedly alter the overall increase tyrosine phosphorylation (Fig. 1A) compared with stimulation with Fab(′)2 rabbit anti-mouse IgG. However, on closer examination some differences could be observed. Immunoprecipitation of the FcR confirmed that, in contrast to stimulation of the cells with Fab(′)2 rabbit anti-mouse Ig, activation of the cells with intact anti-mouse Ig induced the phosphorylation of the FcR (Fig. 1B and Ref. 30). In addition, several changes in the levels of tyrosine phosphorylation of other proteins that co-precipitated with the FcR could also be seen (Fig. 1B). These changes included a decrease in the tyrosine-phosphorylated proteins that run with molecular weights of about 34 kDa, 39 kDa, and 73 kDa. On longer exposure of the blots, an increase in the presence of a tyrosine-phosphorylated protein of 145–150 kDa could also be seen (Fig. 1B, lane 3).

We next examined the effect of co-cross-linking the FcR to sIg on the tyrosine phosphorylation of various intracellular proteins that may be involved in the signal transduction pathways. Immunoprecipitations were carried out to isolate specific
with either 40 μg/ml \( F(ab')_2 \) or 60 μg/ml intact anti-IgG for 3 min at 30 °C and lysed, and the whole cell lysate or FcR immunoprecipitates were separated by SDS-PAGE. After Western blotting the membranes were probed with an antiphosphotyrosine monoclonal antibody and then visualized by ECL.

proteins from lysates of cells activated either through the antigen receptor alone or by the co-cross-linking of slg and FcR. As described earlier (Fig. 1B and Ref. 30), concomitant activation of the cells through the slg and Fc receptors induced the tyrosine phosphorylation of the FcR, but there were also increases in other proteins. Immunoprecipitation of either Grb2 or Shc from cell lysates revealed that there was an increase in the tyrosine phosphorylation of a protein doublet centered around 52 kDa, corresponding to Shc, when the cells were activated with intact anti-IgG (Fig. 2A, lanes 3 and 6). Western analysis of immunoprecipitates of either Shc or Grb2 revealed that there was a weak association between Grb2 and Shc in cells activated with F(ab’)_2 anti-IgG (Fig. 2B, lanes 5 and 8), and this was enhanced in cells activated with the intact anti-IgG (Fig. 2B, lanes 6 and 9). In addition, co-cross-linking of the slg and Fc receptors gave rise to a significant increase in the presence of a tyrosine-phosphorylated protein of 150 kDa in the immunoprecipitates of either Grb2 or Shc (Fig. 2A, lanes 3 and 6). Subsequent analysis of these immunoprecipitates revealed that this phosphoprotein was recognized by antibodies to the inositol 5’-phosphatase Ship and that activation of cells by co-cross-linkage of slg and FcR gave rise to an increase in the levels of Ship found associated with Shc and Grb2 (Fig. 2B, lanes 6 and 9).

Immunoprecipitation of Ship from cell lysates using the two different polyclonal antibodies revealed that there was a small increase in tyrosine phosphorylation of the enzyme following stimulation of the cells with \( F(ab')_2 \) anti-IgG, but this was markedly enhanced on co-cross-linking slg to the FcR (Fig. 3A, lanes 3 and 6). In the same immunoprecipitates there was an additional highly phosphorylated band of about 55 kDa that strongly resembled the phosphorylated FcR. Since antibodies that recognize the FcR on Western blots are not available, it was not possible to directly test this. However, Western analysis, using the polyclonal anti-Ship antibodies, after stimulation of the cells with intact anti-IgG and immunoprecipitation of FcR, revealed that Ship was indeed associated with the FcR when the receptor was phosphorylated (Fig. 2B, lane 3). Thus Ship was the 150-kDa tyrosine phosphophoprotein that was originally detected on antiphosphotyrosine blots of FcR immunoprecipitates (Fig. 1B, lane 3).

To confirm the association between Ship and the FcR, affinity precipitations were carried out using immobilized peptides corresponding to the unphosphorylated and phosphorylated forms of the cytoplasmic domain of FeR-Iib. The immobilized phosphorylated peptide was able to bring down Ship from the lysates of unactivated A20 cells (Fig. 3B, lane 3), whereas the strepavidin beads alone or the beads containing the unphosphorylated peptide did not (Fig. 3B, lanes 1 and 2). Activation of the cells with either \( F(ab')_2 \) or intact anti-IgG did not significantly change the interaction of the peptides with Ship (data not shown).

In contrast to the increase in phosphorylation of FcR, Ship, and Shc, a significant reduction in the tyrosine phosphorylation of CD19 were observed at all times following stimulation of the cells with intact anti-IgG. Stimulation of the cells through slg alone gave rise to a marked increase in the tyrosine phosphorylation of CD19 (Fig. 4A, lane 2). This was greatly reduced when the cells were activated by co-cross-linking slg to the FcR. (Fig. 4A, lane 3). It has been shown that phosphorylation of CD19 results in its association with PI3-kinase (28, 43).
agreement with this, analysis of immunoprecipitates of CD19 from cells activated with F(ab’)2 anti-IgG revealed that PI3-kinase was associated with the phosphorylated CD19 (Fig. 4B, lane 5); however, the enzyme was not detected in immunoprecipitates from cells that had been activated by co-cross-linking sIg to the FcR (Fig. 4B, lane 6). It was not possible to reprobe these blots with an anti-CD19 antibody, since those available do not Western blot.

Stimulation of A20 cells with F(ab’)2 anti-IgG gives rise to an initial mobilization of calcium from intracellular stores, which lasts for about 60 s, followed by a second phase, which involves the influx of extracellular calcium (Fig. 5B, top panel, and Ref. 8). Activation of the cells with F(ab’)2 anti-IgG in the presence of wortmannin, which has been shown to inhibit PI3-kinase (37, 38), resulted in the dose-dependent inhibition of the later, extracellular, component of the calcium mobilization within the cell (Fig. 5A). At higher concentrations of wortmannin (>60 nm) slight inhibition of mobilization of calcium from intracellular stores was observed. When wortmannin was added after the influx of extracellular calcium had been initiated, this influx was rapidly curtailed (Fig. 5B, bottom panel). The inhibitor also blocked the F(ab’)2 anti-IgG-induced production of IL-2 in a dose-dependent fashion (Fig. 5C). Wortmannin at concentrations up to 200 nm had no effect on the patterns of tyrosine phosphorylation of whole cell lysates and immunoprecipitates of CD19, FcR, and Shc induced by F(ab’)2 anti-IgG (data not shown).

As shown in Fig. 1, the phosphorylation of many of the proteins in the cell lysates was not altered with stimulation of the cells with intact anti-IgG. When this was analyzed in more detail, no significant differences in the ability to induce the tyrosine phosphorylation of Syk, Cbl, PLCγ1, CD22, Igβ, PI3-kinase, and JAK2 (Fig. 6, A and B) or Vav, Gap, Tyk, and JAK1 (data not shown) were observed between cells activated by the two different modes of triggering. The changes in the other bands visible in the immunoprecipitates in Fig. 6 reflect the change in phosphorylation of p73 (g) and p150 (h). It was not possible to detect changes in the phosphorylation of Lyn or Btk following activation of the cells through sIg or sIg with the FcR (data not shown). Fyn was constitutively phosphorylated in unactivated cells (Fig. 6A, g); on activation of the cells with either F(ab’)2 or intact anti-IgG there was a slight reduction in mobility of the phosphoprotein, suggesting that there was a change in the posttranslational modification of the enzyme. Stimulation of the cells with intact anti-IgG also induced marked increases in the tyrosine phosphorylation of PLCγ2 and Igα; however, this phosphorylation appeared to be slightly less than that induced with stimulation of the cells through sIg alone using F(ab’)2 anti-IgG (Fig. 6). Overall, these results indicate that co-cross-linking the FcR to sIg does not simply cause a global decrease in tyrosine phosphorylation of intracellular proteins.

**Kinetics of Fc Receptor-induced Changes**—Addition of intact anti-IgG to A20 cells that have previously been activated with F(ab’)2 anti-Ig causes a very rapid drop in intracellular calcium by closing the plasma membrane channel, thereby halting the influx of extracellular calcium (Fig. 7A and Ref. 8). We also found that the addition of intact anti-IgG to previously activated cells stimulated the phosphorylation of FcR, Shc, and Ship, the association of p150 with Shc and Grb2, and a decrease in the phosphorylation of CD19. To determine whether the induction of these changes in tyrosine phosphorylation had kinetics similar to the closing of the calcium channel, cells were activated with F(ab’)2 anti-Ig for 1 min, and then sIg was co-cross-linked to the FcR by the addition of intact anti-Ig to the ongoing reaction. Within 15 s following the addition of intact anti-Ig to the cells an increase in the tyrosine phosphorylation of the FcR could be seen; after 45–60 s phosphorylation of the FcR was maximal (Fig. 7B, a). Similarly, the increases in the phosphorylation of Shc and the associated p150 Ship could be detected in immunoprecipitates of Shc or Grb2 within 15–30 s after the addition of anti-Ig and reached a maximal level after about 60 s (Fig. 7B, c and d). Co-cross-linking the FcR to sIg stimulated the dephosphorylation of CD19 with similar kinetics (Fig. 7B, b). The results indicate that the changes in tyrosine phosphorylation and protein associations of the FcR, CD19, and Shc are consistent with the kinetics of closing of the plasma membrane calcium channel, and one or more of these may induce the change in ion mobilization.

**DISCUSSION**

The FcR plays a crucial role in the regulation of activation of B cells (3–6). Co-cross-linking the antigen and Fc receptors gives rise to the phosphorylation of the receptor and the generation of a dominant inhibitory signal that abrogates the proliferation or differentiation of the lymphocytes (30). FcR phosphorylation promotes its association with other intracellular proteins such as SHP-1 (31–33) via Src homology 2 interactions. It has been proposed that the association between SHP-1 and the FcR activates the phosphatase, which then shuts down the signaling through the antigen receptor (31, 32). It has also been shown that co-cross-linking sIg to the FcR prevents the influx of extracellular calcium by closing the plasma membrane calcium channel (8). However, it is not clear how co-cross-linking the FcR to sIg abrogates the influx of calcium and whether the association of activated SHP-1 with the receptor complex curtails all of the activation signals mediated by the antigen receptor or if the effect is more restricted.

In this study we show that co-cross-linking sIg to the FcR gives rise to very selective modulation of the signaling pathways stimulated by the antigen receptor. Co-ligation of sIg and the FcR neither inhibits nor reverses the stimulation of phosphorylation of many of the intermediate proteins thought to be involved in signaling through the antigen receptor. There was no detectable difference in the phosphorylation of Cbl, Vav, Fyn, Gap, Tyk, JAK2, PI3-kinase Igα, JAK1, and CD22 between activation of the cells through either receptor. Phosphorylation of the Igα chain of the antigen receptor complex was also induced by triggering either with F(ab’)2 or intact anti-IgG, although the
levels of phosphorylation stimulated by the latter treatment were slightly diminished. The significance of this is unclear at present.

It has been shown that both PLCγ1 and PLCγ2 become phosphorylated and activated following stimulation of the cells through the antigen receptor (19–21). This then generates the formation of inositol trisphosphate, which then leads to the release of calcium from intracellular stores. In addition, co-cross-linking sIg to the FcR curtails the influx of extracellular calcium but does not significantly inhibit the mobilization of calcium from intracellular stores (7, 8). In agreement with these observations, our experiments showed that co-cross-linking sIg to the FcR had no significant effect on the phosphorylation of PLCγ1. Phosphorylation of PLCγ2 was still significantly enhanced over unstimulated cells, although the levels were slightly decreased. These results are in contrast to a recent report using murine splenic B cells, in which activation of PLCγ2 was significantly inhibited upon co-ligation of the FcR to sIg (48). The reason for this disparity is not clear. It may reflect the differences in cell populations that were used or the conditions of activation. However, it is difficult to directly compare the two studies, since in the other report the influence of the signals on calcium mobilization from the intracellular and extracellular stores was not described.

Stimulation of B cells through sIg gives rise to the rapid ordered activation of a cascade of protein kinases (16–18, 44–47). No differences were observed in the activation of the kinases Syk, Lyn, Btk, Erk1, Erk2, and c-Jun amino-terminal kinase 1, between stimulation of the cells through either sIg alone or co-ligation of the FcR to sIg (data not shown). This suggests that these enzymes are not the target for FcR-associated SHP-1.

Co-cross-linking Fc and antigen receptors did give rise to some very specific changes in the signal transduction pathway. As reported previously (30), stimulation of the lymphocytes with intact anti-Ig gave rise to a marked increase in the phosphorylation of the FcR. Immunoprecipitation of the FcR from activated cells also revealed other changes in the cells. A decrease in the phosphorylation of three proteins that co-precipitated with the immune complexes was consistently observed.
These proteins, of about 34, 39, and 73 kDa, could also be detected in other immunoprecipitates (see Figs. 2A and 4A). The identity of these proteins is not known, but preliminary experiments have indicated that they are not the Igα and Igβ chains, Syk, SHP-1, SHP-2, or Raf-1. In addition to these changes, it was also possible to detect an increase in the level of a phosphoprotein of about 150 kDa in the immunoprecipitates of the FcR from cells activated with intact anti-IgG.

Immunoprecipitation of Shc or Grb2 following activation of the B cells by co-ligation of sIgG and FcR revealed that there was enhanced phosphorylation of a doublet centered around 52 kDa, corresponding to Shc. In addition, immunoprecipitates of both Grb2 and Shc from cells activated by cross-linking sIg and FcR showed an increase in the levels of an associated phosphoprotein of about 150 kDa. Analysis of the immunoprecipitates of Shc, Grb2, and FcR indicated that this protein was Ship, the inositol 5'-phosphatase (39, 40). Subsequently, immunoprecipitation of Ship revealed that the phosphorylation of the enzyme increased following activation of cells through sIg alone; however, the level of phosphorylation was further significantly enhanced when the cells were activated by co-ligation of sIg and FcR. Ship can associate with Shc through the phosphotyrosine binding domain of the latter (39, 40), and there are also several proline-rich sequences within Ship that may allow interaction with the SH3 domains of other proteins such as Grb2 (40). Since Shc interacts with Grb2, Ship could also indirectly associate with Grb2 via a mutual interaction with Shc. Stimulation of an increase in the phosphorylation of Ship could give rise to an enhanced association with Shc and thereby Grb2. These interactions would account for the increased level of Ship seen in immunoprecipitates of Shc and Grb2 in this study. Alternatively the phosphorylation of Ship may promote the interaction of the enzyme with the Src homology 2 domains of Grb2 or Shc. At present the factors regulating these interactions have not been elucidated.

Ship was also found present in the immunoprecipitates of the FcR after stimulation of the cells by co-ligation of antigen and Fc receptors. It could not be detected in immunoprecipitates of FcR from cells activated through sIgG alone. Additionally, immunoprecipitates of Ship from cells stimulated with intact anti-IgG revealed the presence of a phosphoprotein that closely resembled the FcR. These results suggest that the phosphorylated FcR can associate with Ship, probably through interaction with the Src homology 2 domain of the enzyme. A biotinylated peptide corresponding to the sequence surrounding the phosphorylated tyrosine in the cytoplasmic domain precipitated Ship from the lysate of unstimulated cells, whereas the non-phosphorylated form of the peptide did not. This shows that the phosphorylation of this tyrosine in the cytoplasmic domain of the FcR is sufficient to promote the interaction between the receptor and Ship. Affinity precipitates from cells stimulated with F(ab')2 or intact anti-IgG did not significantly change the ability of the phosphorylated peptide to interact with Ship. This indicates that phosphorylation of an intermediary protein such as Shc is not essential for the interaction.

Ship catalyzes the hydrolysis of the 5-phosphate of inositol tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate (39). Our data indicate that interaction of this enzyme with the antigen receptor complex, through its association with the phosphorylated FcR, is likely to alter locally the metabolism of either inositol phosphates or phosphatidylinositol phosphates. This may directly regulate the activity of the plasma membrane calcium channel. It is interesting to note that retroviral expression of Ship in PD-18-fms cells results in strong inhibition of growth (40). This inhibition may be due to the alteration of the ability of cells to mobilize calcium.

In contrast to the effect on FcR, Ship, and Shc, co-ligation of sIg and FcR resulted in the decrease of phosphorylation of several proteins, including CD19 and other, as yet uncharacterized, proteins. Activation of cells through the antigen receptor alone results in an increase in the tyrosine phosphorylation of both CD19 and CD22 (9, 10, 12, 49). In our experiments, co-ligation of the FcR to sIg greatly decreased the phosphorylation state of CD19 but did not significantly alter the level of phosphorylation of CD22. Several signaling molecules are found associated with CD19 following activation of B cells.
through the antigen receptor; these include PI3-kinase, Vav, and Dr. Joe Bolen (DNAX) for providing antibodies.

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REFERENCES
1. Gold, M. R., and DeFranco, A. L. (1994) Adv. Immunol. 55, 221–295
2. Cambier, J. C., Pleiman, C. M., and Clark, M. R. (1994) Annu. Rev. Immunol. 12, 457–486
3. Sidman, C. L., and Unanue, E. R. (1976) J. Exp. Med. 144, 882–896
4. Klaus, G. G., Hawrylowicz, C. M., Holman, M., and Keeler, K. D. (1984) Immunology 53, 693–701
5. Phillips, N. E., and Parker, D. C. (1983) J. Immunol. 130, 602–606
6. Phillips, N. E., and Parker, D. C. (1984) J. Immunol. 132, 627–632
7. Choquet, D., Partisani, M., Amigorena, S., Bonfetor, C., Fridman, W. H., and Korn, H. (1993) J. Cell Biol. 121, 353–363
8. Diegel, M. L., Rankin, B. M., Bolen, J. B., Dubois, P. M., and Kiener, P. A. (1994) J. Biol. Chem. 269, 11409–11416
9. Chalupny, N. J., Kanner, B., Schen, G. L., Gee, S. F., Gilliland, L. K., Aruffo, A., and Lodhetter, J. A. (1993) EMBO J. 12, 2691–2696
10. Roifman, C. M., and Ke, S. (1993) Biochem. Biophys. Res. Commun. 194, 222–235

The analysis of cells that were stimulated initially through sIg and then subsequently restimulated by co-ligation of sIg and the FeR indicated that the changes in tyrosine phosphorylation of the proteins occurred rapidly. The kinetics of these biochemical changes are of the right time frame to be able to account for the changes in influx of extracellular calcium, which diminishes over the first few minutes following co-ligation of the receptors. It should be pointed out that the effect of intact anti-Ig on tyrosine phosphorylation of all of these proteins was not simply due to the abrogation of an influx of extracellular calcium. Removal of extracellular calcium with 2.5 mM EGTA not only altered the tyrosine phosphorylation state of any of the proteins when the cells were activated with Fab(1/2) anti-Ig (data not shown).

In summary, co-cross-linking of the FeR to sIg gives rise to a very discrete set of changes in signal transduction. Many of the signaling pathways that were stimulated following ligation of the antigen receptor alone were unaltered when sIg was co-cross-linked to the FeR. These include the activation of several different protein kinases, the phosphorylation of many intracellular proteins, and the activation of the transcription factor NFκB (42) (data not shown). Thus the role of SHP-1 in the negative regulation of B cell receptor signaling is very specific and is not a global shutdown of tyrosine phosphorylation at the antigen receptor. Our data suggest that dephosphorylation of CD19 (perhaps by SHP-1) and the subsequent loss of association of PI3-kinase with the FeR are crucial steps in altering calcium mobilization. This may be mimicked by the inhibition of PI3-kinase activity with wortmannin. In addition, our studies have shown that the phosphorylation of the FeR promotes its interaction with Shp, an inositol 5’-phosphatase. Thus co-igation of the FeR and sIg markedly changes the overall activity of lipid-modifying enzymes that are associated with the receptor complex. In particular, it is possible that the local levels of phosphatidylinositol 3,4,5-trisphosphate are critical to the regulation of opening and closing of the calcium channel.