Shc Contains Two Grb2 Binding Sites Needed for Efficient Formation of Complexes with SOS in B Lymphocytes

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Cross-linking of the B-cell antigen receptor (BCR) induces tyrosine phosphorylation of Shc, which is believed to lead to the activation of Ras. Previous work has shown that tyrosine-phosphorylated Shc forms complexes with another adapter protein, Grb2, and the Ras guanine nucleotide exchange factor SOS. Here, we demonstrate that phosphorylation of Shc by the hematopoietic cell-specific tyrosine kinase Syk induces binding of Grb2 to Shc, suggesting that Syk phosphorylates Shc in stimulated B cells. Surprisingly, Syk-phosphorylated Shc possesses two Grb2 binding sites rather than the one site that has been previously reported. Both of these sites are required for efficient formation of Shc-Grb2-SOS complexes in vitro and in vivo. We suggest that two Grb2 proteins anchored by a single Shc protein bind simultaneously to one SOS molecule, resulting in a complex that is more stable than a complex containing only a single Grb2 protein bound to one SOS molecule. This model is consistent with our observation that BCR stimulation greatly increases the amount of SOS associated with Grb2.

Signal transduction through the B-cell antigen receptor (BCR) is essential for the proper development and functioning of B lymphocytes. Depending on the developmental state of the cell, engagement of the BCR may cause differentiation, proliferation, growth arrest, or apoptosis (17). BCR signaling induces many of the well-described biochemical events that are observed upon growth factor stimulation of cells, such as the rapid activation of tyrosine kinases and the subsequent activation of Ras (12, 19, 29, 54). The importance of these events is underscored by the ability of tyrosine kinase inhibitors or a dominant negative Ras mutant to interfere with B-cell activation following BCR cross-linking (35, 39). We are interested in understanding the mechanisms by which these signaling cascades are initiated after BCR stimulation of B lymphocytes. Unlike growth factor receptors, the BCR does not have intrinsic tyrosine kinase activity. Instead, cross-linking of the BCR promotes the phosphorylation of the receptor complex by Src family tyrosine kinases, allowing the cytoplasmic tyrosine kinase Syk to bind to the receptor and undergo enzymatic activation (12). Genetic studies have demonstrated that Syk is necessary both for the proper development of B cells and for the cellular responses of B cells to BCR stimulation (8, 52, 55).

Among the many proteins that are tyrosine phosphorylated upon BCR cross-linking is the adapter protein Shc (24, 27, 48, 49). B lymphocytes express two Shc isoforms, of 46 and 52 kDa, which are the products of a single mRNA translationally initiated at two different sites (41). Shc has been implicated in Ras activation in many systems. For example, overexpression of Shc in PC12 cells leads to their neuronal differentiation; significantly, this differentiation can be blocked by overexpression of a dominant negative form of Ras (46). Similarly, overexpression of Shc in myeloid cells enhances the granulocyte-macrophage colony-stimulating factor-induced activation of mitogen-activated protein (MAP) kinases and cellular proliferation, two responses which are downstream of Ras (26).

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Phosphopeptide mapping and mutational analysis of murine p526c revealed that tyrosine 313, the previously identified Grb2 binding site (47), is a target of Syk phosphorylation in vitro, as are tyrosines 239 and 240. Upon phosphorylation, both tyrosine 239 and tyrosine 313 act as Grb2 binding sites in BCR-stimulated B cells, suggesting that they are the primary sites of tyrosine phosphorylation in vivo. Both of these Grb2 binding sites are required for the efficient formation of Shc-Grb2-SOS complexes in vitro and in vivo, suggesting that the stable binding of SOS to Shc requires two Grb2 molecules rather than one as previously envisioned.

**MATERIALS AND METHODS**

**Cell lines and culture conditions.** The murine B-cell line WEHI-231 was obtained from Lewis Lanier (DNA Research Institute, Palo Alto, Calif.), and the human B-cell line Ramos and murine B-cell line Bal-17 were obtained from the American Type Culture Collection (Rockville, Md.). These cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM sodium pyruvate, 1 mM glutamine, and 50 μM 2-mercaptoethanol. To induce maximal expression of transfected hemagglutinin (HA)-Shc gene products, cells were incubated for 48 h in medium plus 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Research Products International Corp., Mount Prospect, Ill.). The *Spodoptera frugiperda* cell line SE21 (Clontech, Palo Alto, Calif.) was grown in Grace’s medium supplemented with 10% fetal bovine serum, 10% F-68 (Sigma Chemical Co., St. Louis, Mo.), and penicillin-streptomycin.

**Cell stimulation and lysate preparation.** Murine or human B lymphocytes (5 × 10⁶ cells/ml in culture medium) were stimulated at 37°C for 5 min (unless otherwise indicated) with medium alone, with goat anti-mouse immunoglobulin M (IgM) antibodies, or with goat anti-human IgG plus IgM (both antibodies from Jackson Immunological Research Laboratories, West Grove, Pa.) at 20 μg/ml. Cell suspensions were then diluted with cold phosphate-buffered saline containing 1 mM Na₂VO₃, pelleted by centrifugation, and resuspended in ice-cold lysis buffering consisting of 20 mM Tris (pH 8.0), 90 mM NaCl, 10% glycerol, 1 mM PMSF, and 1 mM Na₃VO₄. Recombinant His₆-Syk was purified from the *Escherichia coli* Biochemical Corp., Cleveland, Ohio). The fusion proteins were expressed in *Escherichia coli* by standard methods (50). Expression of the fusion proteins in bacteria was induced with 0.1 mM IPTG treatment for 3 h, and the fusion proteins were affinity purified by using glutathione–agarose beads (Sigma).

**Baculoviral expression and purification of Syk.** The baculoviral transfer vector was made as follows. Murine Syk (44) was subcloned into the pQE-11 (Qiagen, Chatsworth, Calif.) bacterial expression vector. Syk was then excised as a HindIII/EcoRI fragment, retaining the His₆ tag from pQE-11, and subcloned into the Smal/EcoRI sites of plV1392. Recombinant baculovirus was created by cotransfecting SE21 cells with pVL1392-Syk and BaculoGold baculovirus DNA (Pharmingen, San Diego, Calif.), according to the manufacturer’s instructions. SE21 cells were harvested 48 h after infection with high-titer baculovirus-Syk stocks at a multiplicity of infection of 5. Cells were washed in phosphate-buffered saline and then resuspended and sonicated in ice-cold buffer consisting of 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 0.1% Triton X-100, 1 mM PMSF, and 1 mM Na₃VO₄. Recombinant-Syk-Syk was purified from the cell lysate via immobilized metal chelate affinity chromatography. Immonidocid acid coupled to Sepharose 6B (Sigma) was loaded with 200 mM CoCl₂, washed well with water, and equilibrated with buffer A (50 mM HEPES [pH 6.0], 300 mM NaCl, 10% glycerol, 1 mM PMSF, 1 mM Na₂VO₄, 1 mM DTT), and finally with buffer B (buffer A plus 5 mM imidazole). The column was then washed with buffer D (50 mM HEPES [pH 8.0], 300 mM NaCl, 10% glycerol, 1 mM imidazole). Syk-positive fractions were pooled and stored at −80°C until used.

**GST-Shc affinity purification.** Purified GST-Shc proteins (approximately 0.5 μg of kinase-active GST fusion protein and 0.5 μg of baculoviral protein) were added to 106 cell equivalents; or with anti-Shc antibody (affinity-purified rabbit antibody). Next, the immunoblots were incubated with anti-Grb2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) for 2 h at 4°C. The beads were then washed three times with lysis buffer and the associated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

In vitro kinase assay. Both purified recombinant Syk (r-Syk) and immunoprecipitated Syk were used in kinase assays. Immunoprecipitated Syk was prepared as described above and then washed with kinase buffer. Kinase buffer containing 10 μCi of [γ-32P]ATP (duPont NEN) was added, and in some cases approximately 1 μg of purified GST fusion protein (on beads) was added as well. Reaction mixtures were incubated for 15 min and were stopped by the addition of SDS-sample buffer. Kinase assays with r-Syk were performed in the same way except that 100 ng of purified r-Syk rather than immunoprecipitated Syk was used. These kinase reactions were separated on SDS-polyacrylamide gels and visualized using a PhosphoImager (Molecular Dynamics, Sunnyvale, Calif.) according to the manufacturer’s instructions.

**Expression of HA- Shc in B cells.** The Strategene LacSwitch system was used to express HA-Shc in WEHI-231 cells. p3/5S was linearized with XmnI and then electroporated into WEHI-231 cells in a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) at 320 V and 500 μF. After 24 to 48 h of unselected growth, cells were selected with 0.35 μg of hygromycin B per ml, and resistant clones were screened for Lac expression by immunoblot analysis (anti-Lac antiserum from Stratagene). One positive clone was selected for further transfections with HA-Shc vectors. HA-Shc expression vectors were prepared as follows. Wild-type and mutant Shc cDNAs were released from pGSTag-Shc vectors as EcoRI fragments and subcloned into the EcoRI site of pBS (Stratagene). 5′ Phosphorylation and mutational analysis of murine p526c: revealed that tyrosine 313, the previously identified Grb2 binding site (47), is a target of Syk phosphorylation in vitro, as are tyrosines 239 and 240. Upon phosphorylation, both tyrosine 239 and tyrosine 313 act as Grb2 binding sites in BCR-stimulated B cells, suggesting that they are the primary sites of tyrosine phosphorylation in vivo. Both of these Grb2 binding sites are required for the efficient formation of Shc-Grb2-SOS complexes in vitro and in vivo, suggesting that the stable binding of SOS to Shc requires two Grb2 molecules rather than one as previously envisioned.

**Phosphopeptide mapping.** Approximately 1 μg of purified GST-Shc (wild type or mutated at tyrosine 239, tyrosine 313, or both) was phosphorylated with r-Syk, using 50 μCi of [γ-32P]ATP (duPont NEN) as described above. The phosphorylated protein was purified by SDS-PAGE, and the phosphopeptide mapping was carried out using 4 (Peptides were electrophoresed in pH 1.9 buffer for 25 min at 1 kV and subjected to chromatography in isobutyric acid buffer. A synthetic peptide with the sequence MAGFD GSAWD EEEEE (corresponding to residues 268–277) which contains tyrosines 239 and 240) was obtained from Quality Controlled Biochemicals (Hopkinton, Mass.), phosphorylated with r-Syk, purified by SDS-PAGE, and used as a control.
immunoprecipitations were incubated with lysates were immunoprecipitated (IP) with anti-Syk (a)
ylation of Shc (reference 11 and data not shown) and is thus association correlated with the induction of tyrosine phosphor-
cross-linking induced the association of Shc with Grb2. This increase in Grb2-SOS association correlated with the in-
duction of association between Grb2 and Shc, suggesting that these events might be mechanistically linked. We also noted a time-dependent retardation in the electrophoretic mobility of Grb2-associated SOS1 after BCR stimulation (Fig. 1 and data not shown). This reduction of SOS1 mobility is likely due to its phosphorylation by MAP kinases, a process that plays a role in SOS regulation (9, 10).

**RESULTS**

Cross-linking of the BCR induces formation of a Shc-Grb2 complex and enhances the association between Grb2 and SOS. BCR cross-linking results in Ras activation, a process shown in other systems to be dependent on the adapter proteins Shc and Grb2 and the guanine nucleotide exchange factor SOS. We therefore examined the interactions of these proteins following BCR cross-linking. We stimulated Ramos B cells by using antibodies to cross-link cell surface BCR for various amounts of time. As shown in Fig. 1, Grb2 was then immunoprecipitated and the levels of associated Shc were determined. BCR cross-linking induced the association of Shc with Grb2. This association correlated with the induction of tyrosine phosphorylation of Shc (reference 11 and data not shown) and is thus consistent with the model that the SH2 domain of Grb2 binds to tyrosine-phosphorylated Shc. Similar data have been previously reported by others (24, 27, 48, 49).

We also examined the associations between Grb2 and SOS1 or SOS2. We observed a low basal level of SOS1 and SOS2 bound to Grb2 in unstimulated B cells. However, the amount of SOS1 and SOS2 coimmunoprecipitating with Grb2 markedly increased upon BCR cross-linking (2). Genetic studies have indicated that all three types of kinases are required for the induction of signaling events downstream of the BCR (52, 53). However, previous work has demonstrated that Syk is likely to be the kinase responsible for the BCR-induced (37, 44) and the FcεRI-induced (21) tyrosine phosphorylation of Shc. We therefore examined whether Syk could directly phosphorylate Shc in vitro. First, we immunoprecipitated Syk or Shc alone, or we mixed antibodies recognizing both Syk and Shc and immunoprecipitated the two proteins together. The immunoprecipitated proteins were incubated with [γ-32P]ATP, phosphorylation reactions were allowed to proceed at room temperature, and the products were then resolved by SDS-PAGE. In this assay, Shc was phosphorylated only when it was coimmunoprecipitated with Syk and was most efficiently labeled when both proteins were isolated from lysates made from stimulated cells (Fig. 2A). To further characterize this reaction, we isolated a recombinant GST-Shc fusion protein produced in *E. coli* and a His-tagged Syk protein produced in insect cells (r-Syk). We found that GST-Shc, like Shc immunoprecipitated from B cells, was phosphorylated efficiently by Syk immunoprecipi-
tated from stimulated B cells and by r-Syk (Fig. 2C). GST itself was not a substrate for Syk in these experiments.

In vitro kinase assays performed with truncated versions of Shc demonstrated that its central domain was efficiently phosphorylated by Syk while the PTB and SH2 domains were not (data not shown). Interestingly, two of the three tyrosines (Y239 and Y240) in this central region are conserved in all Shc homologs, while the remaining tyrosine (Y313) is conserved in all mammalian members of the Shc family (Fig. 3 and reference 38). Tryptic phosphopeptide mapping of in vitro-labeled GST-Shc revealed three prominent spots (Fig. 4A). GST-Shc mutants and a synthetic peptide were used to identify these three phosphopeptides as two tryptic fragments from the middle region of Shc. Mutation of Y313 to phenylalanine caused the disappearance of spot 1 (Fig. 4C and D), indicating that this spot corresponds to the phosphopeptide containing Y313. Mutation of Y239 to phenylalanine caused the disappearance of spot 2 (Fig. 4B and D). In this case, analysis is more complicated, as the tryptic peptide containing Y239 also contains Y240. To determine where phosphorylated forms of this peptide migrate, a peptide corresponding to amino acid residues 219 to 246 of murine p52Shc was synthesized, purified to 96% homogeneity, and phosphorylated in vitro with r-Syk. This tyrosine-phosphorylated peptide migrated as two separate spots (Fig. 4E), which probably represent differentially phosphorylated peptides. Analysis of a mixture of in vitro-phosphorylated GST-Shc and synthetic peptide revealed that these phosphopeptides comigrate with spots 2 and 3 (Fig. 4F). As spot 2 exhibits decreased electrophoretic mobility compared to spot 3 and is lost upon mutation of Y239 to phenylalanine (Fig. 4B and D), it is likely that spot 2 represents the peptide that has been phosphorylated upon both Y239 and Y240 whereas spot 3 represents monophosphorylated forms of the peptide. Thus, it appears that both Y239 and Y240 are phosphorylated by r-Syk in vitro. Intriguingly, both Y239 and Y313 are present in a YxNx motif (where x indicates any amino acid), the consensus binding sequence for the SH2 domain of Grb2 (51). Indeed, phosphorylation of Y317 in human p52Shc (equivalent to Y313 in murine p52Shc) has been shown to create a binding site for the SH2 domain of Grb2 (47). The identification of both Y239 and Y313 as phosphorylation sites suggested that Syk-phosphorylated Shc might possess two Grb2 binding sites.

Phosphorylation of Shc by Syk creates two Grb2 binding sites in vitro. We used an affinity purification technique to determine if Grb2 bound to Syk-phosphorylated Shc. GST-Shc was bound to glutathione-agarose beads and phosphorylated in vitro with purified r-Syk, which was then removed by sequential washing of the beads. The phosphorylated GST-Shc was next incubated with lysates made from unstimulated B cells. Specifically associated proteins were eluted from the glutathione beads, resolved on SDS-polyacrylamide gels, and analyzed by anti-Grb2 immunoblotting. We found that Grb2 bound to GST-Shc that had been tyrosine phosphorylated by Syk but did not bind to GST-Shc that was not phosphorylated (Fig. 5A).

We next wanted to determine whether Grb2 was binding to Shc at Y239, at Y313, or at both sites. Site-directed mutagenesis of the peptides containing these tyrosines indicated that spot 2 is likely to be the peptide containing both Y239 and Y313, while spot 3 is likely to be the monophosphorylated form of the peptide.
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FIG. 5. Shc has two Grb2 binding sites. (A) Approximately 1 μg of GST-Shc was purified on glutathione-agarose beads and then split into halves. One half was phosphorylated in vitro with r-Syk, while the other half was left unphosphorylated. Both sets of beads were washed extensively and then added to lysates from unstimulated Ramos cells (30 × 10^6 cell equivalents per sample) and incubated at 4°C. Specifically associated Grb2 was detected by SDS-PAGE and immunoblotting with anti-Grb2 (αGrb2) antiserum. An anti-Shc immunoprecipitation (IP) from BCR-stimulated Ramos cells was included as a positive control. Sizes are indicated in kilodaltons. (B) Wild-type GST-Shc and GST-Shc in which either tyrosine 239, tyrosine 313, or both tyrosines were mutated to phenylalanine were purified on glutathione-agarose beads and then split into halves. One half was probed first with antiphosphotyrosine (α-P-Y) antibody (4G10; top panel) and then stripped and reprobed with anti-glycoproteins (αG) to verify equal loading of protein. Proteinolytic fragments of GST-Shc were isolated along with full-length GST-Shc and are visible in these blots.

A

GST-Shc

r-Syk: - +

αGrb2

Immunoblot

φG

SOS1

SOS2

GST-Shc

Grb2

B

Blots:

αY

αG

αGrb2

αSOS1

αSOS2

αP-Y

αG

αGrb2

αG

FIG. 6. Tyrosines 239 and 313 of Shc are the primary sites of tyrosine phosphorylation in BCR-stimulated B cells. WEHI-231 B cells were stably transfected with cDNAs encoding wild-type or mutant forms of HA-tagged p52Shc. The indicated transfectants were incubated with or without anti-IgM (αIg) for 3 min and then lysed. The transfected HA-Shc proteins were immunoprecipitated from 12 × 10^6 cell equivalents of lysate by using anti-HA antibody, separated on an 8% gel by SDS-PAGE, and transferred to nitrocellulose. The blot was probed with antiphosphotyrosine (α-P-Y) antibody (4G10; top panel) and then stripped and reprobed with anti-Shc antibody (bottom panel) to verify equal loading of protein. Sizes are indicated in kilodaltons.

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Grb2 sites are required for efficient coupling of Shc to SOS in vitro. As described above, BCR cross-linking induced the formation of a Shc-Grb2-SOS complex (Fig. 1). We therefore tested whether endogenous SOS1 and SOS2 present in B-cell lysates would copurify with Grb2 that was bound to tyrosine-phosphorylated, recombinant GST-Shc. Both SOS1 and SOS2 associated with wild-type GST-Shc in a phosphorylation-dependent manner (Fig. 5B). However, the amount of SOS1 or SOS2 associated with either GST-Shc Y239F or GST-Shc Y313F was greatly diminished and was usually undetectable (Fig. 5B and data not shown). This result indicates that in vitro, both tyrosine 239 and tyrosine 313 are required for efficient binding of SOS1 and SOS2 to the Shc-Grb2 complex. As was true for Grb2, SOS1 and SOS2 were not detected in association with GST-Shc Y239F/Y313F (Fig. 5B).

Tyrosine 239 and tyrosine 313 are phosphorylated and are Grb2 binding sites in vivo. To evaluate the relative importance of Y239 and Y313 as Grb2 binding sites in vivo, we stably transfected expression vectors containing wild-type and mutated Shc cDNAs into the B-cell line WEHI-231. These Shc genes were tagged with an HA epitope so that their protein products could be distinguished from endogenous Shc. While wild-type HA-Shc was efficiently tyrosine phosphorylated following BCR stimulation, we found that phosphorylation of HA-Shc Y313F was much reduced in comparison (Fig. 6). The induced level of tyrosine phosphorylation of HA-Shc Y239F was even more reduced. When we examined HA-Shc in which both Y239 and Y313 were mutated to phenylalanine, we found that this protein exhibited very low but detectable levels of tyrosine phosphorylation in response to BCR cross-linking (Fig. 6). These results suggest that Y239 and Y313 are the major but not the only sites of tyrosine phosphorylation in BCR-stimulated B cells.

We next examined Grb2 association with wild-type and mutant HA-Shc proteins following BCR cross-linking. First, anti-HA immunoprecipitates were immunoblotted with anti-Grb2 antibodies. Grb2 coimmunoprecipitated with wild-type HA-Shc isolated from stimulated cells (Fig. 7A), consistent with the efficient in vivo tyrosine phosphorylation of this protein upon stimulation of the BCR. Much less Grb2 could be detected in association with HA-Shc Y313F, and even less associated with HA-Shc Y239F. In most experiments, no Grb2 was observed in association with HA-Shc Y239F/Y313F (Fig. 7A), although in a few experiments very low but detectable levels of Grb2 were present.

We next performed the reciprocal experiment, immunoprecipitating Grb2 from stimulated cells and immunoblotting with anti-Shc antibody to detect associated HA-Shc, which migrates more slowly on SDS-polyacrylamide gels than does endogenous p52Shc (Fig. 7B). Wild-type HA-Shc was efficiently coimmunoprecipitated with Grb2. However, lower levels of HA-Shc Y313F associated with Grb2, and there was an even further...
Tyrosines 239 and 313 of Shc are both Grb2 binding sites in BCR-stimulated B cells. (A) The indicated transfectants were stimulated with anti-IgM for 3 min and then lysed. 20 × 10⁶ cell equivalents of lysate were immunoprecipitated with anti-HA antibody and analyzed by immunoblotting with anti-Grb2 (αGrb2) antisemur (bottom panel) and with anti-Shc antibody (top panel) to ensure comparable loading of the lanes. (B) BCR-stimulated cell lysates were immunoprecipitated with anti-Grb2 antisemur. Associated proteins were analyzed by immunoblotting with anti-Shc antibody (top panel); note that HA-Shc has a slower mobility on SDS-PAGE than does endogenous p52cbl. The tyrosine-phosphorylated forms of wild-type HA-Shc and HA-Shc Y313F migrated slightly more slowly than did tyrosine-phosphorylated HA-Shc Y239F. The bottom portion of the immunoblot was probed with anti-Grb2 antiserum to verify similar loading of lanes (bottom panel). Sizes are indicated in kilodaltons.

Both Grb2 binding sites are required for the efficient formation of Shc-Grb2-SOS complexes in vivo. We next examined the ability of the transfected HA-Shc proteins to form complexes with SOS. SOS1 was immunoprecipitated from lysates of unstimulated or stimulated B cells expressing wild-type or mutant forms of HA-Shc. These immunoprecipitations were then analyzed for the presence of HA-Shc by immunoblotting. As shown in Fig. 8A, a small amount of all forms of HA-Shc associated with SOS1 isolated from unstimulated cells. This is a specific interaction, as no HA-Shc is present in control immunoprecipitations (Fig. 8A, lanes 1 and 2). BCR stimulation caused a dramatic increase in the amount of wild-type HA-Shc communoprecipitated with SOS1 (lanes 5 and 6). Expression of wild-type HA-Shc reproducibly enhanced the amount of endogenous Shc associated with SOS1 in stimulated cells, perhaps mediated by HA-Shc–Shc dimerization (data not shown). In contrast, cell stimulation did not cause an increase in the association between SOS1 and any of the mutant forms of HA-Shc. This is despite the efficient stimulation of the cells expressing these mutated versions of HA-Shc, as is demonstrated by the shift in electrophoretic mobility of SOS1 and the increased amounts of both endogenous Shc and Grb2 copurifying with SOS1. Similar results were observed when SOS2 complexes were immunoprecipitated and analyzed for association of HA-Shc (Fig. 8B). The lack of stimulation-induced association between either SOS1 or SOS2 and HA-Shc in which either Y239 or Y313 had been changed to phenylalanine cannot be fully explained by a lack of Grb2 binding, since significant amounts of Grb2 did associate with both single mutants (Fig. 7). These results demonstrate that in vivo as well as in vitro, both of the two Grb2 binding sites on Shc are required for efficient formation of Shc-Grb2-SOS complexes.

**DISCUSSION**

We have examined the BCR stimulation-dependent interactions between three proteins, Shc, Grb2, and SOS, which have been implicated in Ras activation. Since the ability of the BCR to induce both the tyrosine phosphorylation of Shc and the downstream activation of MAP kinase is dependent on the expression of the cytoplasmic tyrosine kinase Syk (37, 44), we investigated the ability of recombinant Syk to phosphorylate Shc in vitro. Tryptic phosphopeptide mapping of wild-type and mutant forms of Shc indicated that Syk phosphorylates Shc on Y239, Y240, and Y313. Surprisingly, Syk phosphorylation of Shc created two distinct Grb2 binding sites, rather than the one site that has been previously reported (47). This second Grb2 binding site was identified as Y329. Mutation of either Y239 or Y313 to phenylalanine led to a decrease in the in vitro association of Grb2 with Syk-phosphorylated GST-Shc, while mutation of both tyrosines led to a complete loss of Grb2 binding. Thus, both tyrosines act as Grb2 binding sites upon phosphorylation by Syk. As this report was in preparation, van der Geer et al. (56) and Gotoh et al. (18) reported that EGF stimulation of COS cells and interleukin-3 stimulation of Ba/F3 cells also induce tyrosine phosphorylation of Y239, Y240, and Y317 of Shc. In addition, van der Geer et al. (56) provided evidence that Y239 is a second site of Grb2 binding.

The importance of Y239 and Y313 for Grb2 binding in vivo was examined by introducing epitope-tagged wild-type and mutant Shc proteins into the B-cell line WEHI-231. Upon BCR cross-linking, wild-type HA-Shc was tyrosine phosphorylated and formed complexes with Grb2. Mutation of either tyrosine considerably decreased both the tyrosine phosphorylation of HA-Shc and the amount of Grb2 associated with it. HA-Shc in which both tyrosines 239 and 313 were changed to phenylalanine exhibited a very slight but detectable increase in tyrosine phosphorylation upon BCR stimulation. Although mutation of these sites may indirectly affect phosphorylation at other sites, the most likely interpretation of these results is that Y239 and Y313 are the predominant sites of tyrosine phosphorylation and that other tyrosines on Shc such as Y240 are phosphorylated to a lesser extent. Interestingly, although Grb2 binding to HA-Shc Y239F/Y313F was greatly decreased compared to binding to the single-point mutants, it was not completely abrogated. This residual binding may be due to the formation of HA-Shc–She dimers (data not shown) or the result of an indirect association between HA-Shc and Grb2 mediated by some other signaling molecule. For example, it has been demonstrated that p150SHIP (22) and p120SHIP (15) can bind to both Shc and Grb2 in antigen receptor-stimulated B cells.

Our finding that Shc has two Grb2 binding sites suggests the
We and others have noted that this stimulation-dependent increase in Grb2 binding to Shc (5, 43, 57). Ravichandran and colleagues (43) have demonstrated that wild-type, phosphorylated GST-Shc can induce an increased association between Grb2 and SOS in vitro, suggesting that the binding of Grb2 to Shc might enhance Grb2’s affinity for SOS or otherwise promote this interaction.

With this in mind, we assessed the importance of Shc’s two Grb2 binding sites for the formation of complexes containing both Shc and SOS1 or SOS2. Both in vivo and in vitro, we found that mutation of either Y239 or Y313 to phenylalanine greatly reduced the association of SOS1 and SOS2 with Shc despite the presence of significant amounts of associated Grb2. Thus, both tyrosines are required for efficient formation of Shc-Grb2-SOS complexes and are presumably necessary for the efficient activation of Ras and induction of downstream events such as MAP kinase activation in BCR-stimulated cells. We therefore examined the BCR-mediated activation of the MAP kinases ERK-1 and ERK-2 in cells transfected with wild-type and mutant forms of HA-Shc. Stimulation of all transfec-
tants produced similar levels of hyperphosphorylated ERK-1 and ERK-2 (data not shown). We hypothesize that low levels of Grb2 and/or SOS1 and SOS2 limit the amount of Shc-Grb2-SOS complexes formed in stimulated WEHI-231 cells, so that overexpression of wild-type Shc does not promote formation of this complex (Fig. 8) and does not enhance MAP kinase activation. We attribute the recessive nature of all three mutated versions of Shc to their reduced ability to form complexes with Grb2 (Fig. 7) and their inability to block tyrosine phosphorylation of endogenous Shc (data not shown). However, it remains formally possible that Shc-Grb2-SOS complexes do not mediate Ras activation in BCR-stimulated cells or that there are multiple pathways leading to Ras activation in these cells.

Why are the two Grb2 binding sites both required for efficient formation of Shc-Grb2-SOS complexes? It is possible that they are redundant and allow Shc that has been phosphorylated to remain Grb2 molecule to Shc, exposing any tyrosine-phosphorylated residues to cellular phosphatases. This could explain the larger than twofold decrease in both Grb2 binding and Shc tyrosine phosphorylation observed upon mutation of either Y239 or Y313 to phenylalanine.

Most previously published studies have indicated that Grb2-SOS association is constitutive and unaffected by cell stimulation (13, 16, 30, 31, 46). Although it has been reported that SOS2 has a higher affinity for Grb2 than does SOS1 (58), we observed that SOS1 and SOS2 behaved in qualitatively similar manners in all experiments. We found that BCR stimulation of B lymphocytes led to an increase in association between Grb2 and both SOS1 and SOS2. It is possible that simple Grb2-SOS complexes, formed when the SH3 domains of Grb2 bind to proline-rich regions in the C-terminal tails of SOS1 and SOS2 (58), are for some reason not very stable in unstimulated B cells. But in accord with our finding that cell stimulation can enhance the association between Grb2 and SOS, there are reports that EGF treatment of fibroblasts (5, 57) and T-cell receptor stimulation of T cells (43) can also have this effect. We and others have noted that this stimulation-dependent increase in Grb2 and SOS association correlates with the induction of Grb2 binding to Shc (5, 43, 57).

The possibility that two Grb2 proteins bound to Shc interact with one another, further stabilizing this complex. Soluble Grb2 has been reported to dimerize at high concentrations (7). Additionally, crystallized Grb2 dimerizes in a manner that buries a large protein-protein interface but that leaves all of the SH2 and SH3 domains fully accessible (32). The large protein surface area that interacts in the Grb2 dimer suggests that Grb2 proteins may physically interact under certain physiological conditions. Such an interaction might be possible when two Grb2 proteins are simultaneously bound to Shc. Loss of either Grb2 molecule would then destabilize the binding of the remaining Grb2 molecule to Shc, exposing any tyrosine-phosphorylated residues to cellular phosphatases. This could explain the larger than twofold decrease in both Grb2 binding and Shc tyrosine phosphorylation observed upon mutation of either Y239 or Y313 to phenylalanine.

We therefore examined the BCR-mediated activation of the MAP kinases ERK-1 and ERK-2 in cells transfected with wild-type and mutant forms of HA-Shc. Stimulation of all transfec-
tants produced similar levels of hyperphosphorylated ERK-1 and ERK-2 (data not shown). We hypothesize that low levels of Grb2 and/or SOS1 and SOS2 limit the amount of Shc-Grb2-SOS complexes formed in stimulated WEHI-231 cells, so that overexpression of wild-type Shc does not promote formation of this complex (Fig. 8) and does not enhance MAP kinase activation. We attribute the recessive nature of all three mutated versions of Shc to their reduced ability to form complexes with Grb2 (Fig. 7) and their inability to block tyrosine phosphorylation of endogenous Shc (data not shown). However, it remains formally possible that Shc-Grb2-SOS complexes do not mediate Ras activation in BCR-stimulated cells or that there are multiple pathways leading to Ras activation in these cells.

Why are the two Grb2 binding sites both required for efficient formation of Shc-Grb2-SOS complexes? It is possible that they are redundant and allow Shc that has been phosphor-
ylated at Y239 and Y313 to bind to two separate Grb2-SOS complexes (Fig. 9A). According to this model, one would expect mutation of either tyrosine would cause a reduction in SOS association that is commensurate with the reduction in the amount of Grb2 that is bound to Shc. However, we found that mutation of either tyrosine 239 or 313 to phenylalanine led to a more than twofold decrease in Grb2 binding and an almost complete loss of SOS binding. These observations suggest an alternate possibility, that a single SOS protein binds simultaneously to two Grb2 proteins that are anchored by a single Shc molecule (Fig. 9B). Intriguingly, SOS1 and SOS2 contain multiple proline-rich regions that can act as Grb2 binding sites (58), suggesting that either of them might be able to simultaneously bind to the four SH3 domains contained in two Grb2 proteins. Since the affinity of SH3 domains for proline-rich sequences is relatively low (14), it is possible that these multiple interactions are required for the stable formation of Shc-Grb2-SOS complexes. The multiple protein-protein interactions afforded by a four-way complex between one Shc protein, two Grb2 proteins, and one SOS protein could be considerably more stable than a three-way complex between one Shc protein, one Grb2 protein, and one SOS protein. Such a model could account for our observation that BCR stimulation enhances the association between Grb2 and SOS (Fig. 1) and the ability of phosphorylated GST-Shc to induce a similar enhancement (43). This model may also explain why Grb2 which has been artificially translocated to the plasma membrane does not activate Ras whereas translocated SOS does (20).

The recruitment of SOS to the plasma membrane has been implicated in Ras activation in many systems (34), and it has been shown that BCR cross-linking leads to the translocation of Shc to the plasma membrane (27, 48). We have demonstrated that Shc possesses two Grb2 binding sites and that both of these sites are necessary for efficient binding of Shc to SOS in BCR-stimulated cells. It therefore seems likely that when phosphorylated, these two sites act together to promote translocation of SOS to the membrane, where SOS activates Ras and thus initiates downstream cellular responses.

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