Identification of Two Tyrosine Phosphoproteins, pp70 and pp68, Which Interact with Phospholipase Cγ, Grb2, and Vav after B Cell Antigen Receptor Activation

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Chong Fu‡§ and Andrew C. Chan§***‡‡

From the ‡Program in Genetics, §Center for Immunology, ‖Howard Hughes Medical Institute, and the **Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Tyrosine phosphorylation of cellular proteins mediates the assembly and localization of effector proteins through interactions facilitated by modular Src homology 2 (SH2) and phosphotyrosine binding domains. We describe here two tyrosine-phosphorylated proteins with Mr values of 70,000 and 68,000 that interact with Grb2, phospholipase C (PLCγ1 and PLCγ2), and Vav after B cell receptor cross-linking. The interaction of pp70 and pp68 with PLC and Vav is mediated by the carboxyl-terminal SH2 domain of PLC and the SH2 domain of Vav. In contrast, the interaction of pp70 and pp68 with Grb2 requires cooperative binding of the SH2 and SH3 domains of Grb2. Western blot analysis demonstrated that neither pp70 nor pp68 represented the recently described linker protein SLP-76, which binds Grb2, PLC, and Vav in T cells after T cell receptor activation. Moreover, SLP-76 protein was not detected in a number of B cell lines or in normal mouse B cells. Hence, we propose that pp70 and pp68 likely represent B cell homologs of SLP-76 which facilitate and coordinate B cell activation.

Antigen binding to surface Ig of the B cell receptor (BCR) complex results in activation of receptor-associated protein-tyrosine kinases (PTKs). Three families of PTKs have been implicated in BCR-mediated signal transduction: the Src family PTKs (Lyn, Fyn, and Blk), Syk, and Bruton’s tyrosine kinase (for review, see Refs. 1–3). These PTKs are activated sequentially after BCR cross-linking and play requisite roles in B cell development and function (4–16). Tyrosine phosphorylation of these PTKs increases their enzymatic activities and results in hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (20–22). Whereas diacylglycerol activates protein kinase C, inositol 1,4,5-trisphosphate triggers calcium release from intracellular stores and results in nuclear translocation of the nuclear factor of activated T cells to regulate gene transcription (22–24).

Activation of the Ras pathway is mediated by protein kinase C-dependent and protein kinase C-independent mechanisms (25–27). The latter is thought to be mediated by two adaptor proteins, Shc and Grb2 (for review, see Ref. 28). Shc undergoes tyrosine phosphorylation after BCR cross-linking and is recruited to the BCR complex (29–33). The phosphorylated tyrosine residues on Shc mediate its interaction with the SH2 domain of Grb2. Grb2 is constitutively associated with Sos, a guanine nucleotide exchange factor that facilitates exchange of GDP to GTP for Ras. Hence, tyrosine phosphorylation of Shc can result in the formation of a Shc Grb2 Sos complex to regulate Ras-mediated cellular proliferation (30). In addition to Sos, recent studies have demonstrated that Vav serves as a guanine nucleotide exchange factor for the Rho family of small GTPases (34, 35). Vav undergoes tyrosine phosphorylation after BCR cross-linking and is required for B cell development and BCR function (36–41). Studies by Altman and colleagues (42) have demonstrated that Vav can bind tyrosine-phosphorylated Syk to mediate activation of the nuclear factor of activated T cells. Moreover, tyrosine phosphorylation of Vav results in c-Jun N-terminal kinase activation (34, 35).

Although proximal PTKs are required for these three signaling pathways, the mechanisms by which the proximal PTKs regulate these distinct signaling pathways remain uncertain. To investigate these mechanisms, we analyzed the spectrum of tyrosine-phosphorylated proteins that interact with PLCγ, Grb2, and Vav. We describe here the identification of two tyrosine-phosphorylated proteins with apparent Mr values of 70,000 and 68,000 which interact with all three signaling proteins. Together, these data suggest that these two proteins may facilitate the integration of multiple signal transduction pathways to coordinate B cell activation.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Ramos, Daudi, and Raji Burkitt lymphoma cells (ATCC) were maintained in RPMI 1640 supplemented with 10% fetal calf serum. A20, WEHI-231, and 70Z/3 mouse B cell lines (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Goat anti-human IgM F(ab)2, anti-mouse IgM F(ab)2, and anti-mouse IgG F(ab)2 fragments were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Additional antibodies used in this study included PY20, an anti-phosphotyrosine (Tyr(P)) monoclonal antibody (mAb, Signal Transduction Laboratories); an anti-Grb2 mAb (Signal Transduction Laboratories); an anti-Vav mAb (Signal Transduction Laboratories); an anti-PLCγ1 mAb (Upstate Biotechnology, Inc., Lake Placid, NY); an anti-PLCγ1 antisera (Santa Cruz Biotechnology, Santa Cruz, CA); and others.
an anti-Vav antiserum (Santa Cruz Biotechnology); an anti-PLCγ2 antiserum (43); H3, an anti-PLC-6 mAb (44); 9E10, an anti-myc epitope mAb (44); an anti-Syk antiserum generated against a peptide encoding amino acids 308–336 of human Syk (45); and an anti-PLC-6 antiserum raised against a peptide encoding amino acids 301–318 of human PLC-6 (44).

Constructs and GST Fusion Proteins—GST fusion protein constructs were generated in the pGEX-KT vector by in-frame ligation of polymerase chain reaction fragments (46). Grb2 mutants were kindly provided by Dr. Bruce Mayer and subcloned into the pGEX-KT vector (47). Specifically, Trp-36, Arg-86, and Trp-193 were mutated to lysine to disrupt the function of the NH2-terminal SH3, the SH2, and the COOH-terminal SH3 domains, respectively. A myc epitope-tagged version of Grb2 was generated by adding the myc epitope sequence (SMEKLIGEDNL) to the NH2 terminus of Grb2. All constructs were verified by standard diodeoxy sequencing. Induction and purification of GST fusion proteins were performed according to the manufacturer’s recommendations (Pharmacia Biotech Inc.).

BCR Stimulation, Immunoprecipitation, and Protein Analysis—Cells were washed and resuspended in phosphate-buffered saline at a concentration of 106 cells/ml. Prior to stimulation, cells were rested at 37 °C for 15 min. Cells were then stimulated with an anti-surface Ig F(ab)'3 fragment (24 μg/ml) for 1.5 min and lysed immediately in 10 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (lysis buffer) containing protease and phosphatase inhibitors for 15 min (44). Cell lysates were cleared by centrifugation (15,000 x g for 10 min) at 4 °C, and the supernatants were analyzed.

For binding studies to fusion proteins, BCR-stimulated cell lysates (5 x 106 cells) were incubated with 2 μg of fusion protein for 1 h at 4 °C and captured with 20 μl of glutathione-Sepharose beads (Pharmacia). For in vitro competition experiments, BCR-activated cell lysates (2 x 106 cells) were mixed with either unphosphorylated or tyrosine-phosphorylated SLP-76 produced from 2 x 106 Sf9 cells and incubated with 0.5 μg of fusion protein as described above (44). Precipitates were washed three times with lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot analysis. Protocols for immunoprecipitation and Western blotting have been described previously (44).

Stable Transfection of Cells—Stable transfection of cells was performed as described previously (44). In brief, 106 Daudi cells were electroporated with 25 μg of linearized cDNA using a BTX 600 Electroporator (Biotechnologies and Experimental Research Inc., San Diego). Conditions used for electroporation were 1,250 microfarads, 250 V, and a resistance setting of R6. Cells at limiting dilution were then selected in 0.5 μg/ml puromycin to generate stable clones.

Ion Exchange Chromatography—Cell lysates were diluted 6-fold with 10 mM Tris-HCl, pH 8.0, resulting in a buffer concentration of 10 mM Tris, pH 8.0, 25 mM NaCl. The diluted sample was loaded onto a Mono Q anion exchange column (Pharmacia) and eluted with a linear NaCl gradient of 0.025–1 M in 0.5 M Tris, pH 8.0, 0.1% Nonidet P-40 at a flow rate of 1 ml/min at 4 °C. 20 fractions (1 ml each) were collected.

Western blot analysis was performed by incubating the cell lysates with the Grb2 fusion protein and then analyzed the GST fusion proteins used in the precipitation. B, Grb2 and PLC-γ1 interact with common phosphotyrosine containing proteins. BCR-stimulated Ramos cell lysates (5 x 106 cells) were sequentially precipitated with GST fusion proteins encoding either Grb2 (lanes 1–3) or the two SH2 domains of PLC-γ1 (lanes 4–6) and subsequently precipitated by PLC-γ1 (lanes 5–7) and subsequently precipitated by PLC-γ1 (lanes 4) or Grb2 (lane 8) fusion proteins reciprocally. Precipitates were resolved by SDS-polyacrylamide gel electrophoresis and Western blotted with an anti-Tyr(P) mAb (PY20).

RESULTS

Distinct Sets of Tyrosine-phosphorylated Proteins Interact with Grb2 and PLC-γ—Given the critical roles that PLC-γ1 and Grb2 play in regulating [Ca2+]i, mobilization and Ras activation, respectively, we analyzed tyrosine-phosphorylated proteins that interact with these molecules after BCR stimulation. GST fusion proteins containing either full-length Grb2 or the two SH2 domains of PLC-γ1 were incubated with either resting or BCR-activated Ramos B cell lysates. Bound proteins were analyzed by immunoblotting with an anti-Tyr(P) mAb (PY20) (Fig. 1A, left panel). Four major tyrosine-phosphorylated proteins with Mr values of 153,000, 120,000, 70,000, and 68,000 were precipitated with a full-length Grb2 fusion protein from BCR-activated cell lysates (Fig. 1A, left panel, lanes 1–4). In contrast, four and two predominant tyrosine-phosphorylated proteins with Mr values of 76,000, 72,000, 70,000, and 68,000 were precipitated with a fusion protein containing the tandem SH2 domains of PLC-γ1 (Fig. 1A, left panel, lane 6). No phosphoproteins bound Grb2 or PLC-γ1 in unstimulated cells (Fig. 1A, left panel, lanes 3 and 5). For reference, we have designated the four PLC-γ1-interacting phosphoproteins of Mr values 76,000, 72,000, 70,000, and 68,000 as proteins 1, 2, 3, and 4, respectively (Fig. 1A, left panel). These tyrosine-phosphorylated proteins did not interact with GST or a fusion protein containing the dual SH2 domains of ZAP-70 (Fig. 1A, left panel, lanes 1, 2, 7, and 8). Similar results were obtained with the Daudi B cell line (data not shown).

To test whether pp70 and pp68, which interact with the Grb2 fusion protein, were identical to proteins 3 and 4, which interact with the PLC-γ1 fusion protein, we depleted activated B cell lysates with the Grb2 fusion protein and then analyzed the ability of PLC-γ1 to bind proteins 3 and 4. Sequential precipitation by the Grb2 fusion protein depleted proteins 3 and 4, which interacted with PLC-γ1 (Fig. 1B, lanes 1–4). In contrast, proteins 1 and 2 still bound PLC-γ1 after depletion by Grb2 (Fig. 1B, lane 4). Conversely, the PLC-γ1 fusion protein depleted the Grb2-binding proteins pp70 and pp68, but not pp150 or pp120 (Fig. 1B, lanes 5–8). Together, these data demonstrate that pp70 and pp68, which bind Grb2, are identical to phosphoproteins 3 and 4, respectively, which interact with PLC-γ1.

Although these in vitro binding experiments demonstrated common and unique phosphoproteins that bind PLC-γ1 and Grb2, we determined whether these interactions also occurred in vivo. PLC-γ1 was immunoprecipitated from either resting or
BCR-stimulated Ramos cells and analyzed by Western blot analysis with an anti-Tyr(P) mAb. Consistent with the in vitro binding experiments, proteins 1–4 were detected in PLCγ1 immunoprecipitates from BCR-activated cell lysates, although the stoichiometry of binding of proteins 1 and 2 was lower in the PLCγ1 immunoprecipitates than the in vitro binding experiments (Fig. 2A, lanes 2 and 5). In addition to tyrosine-phosphorylated PLCγ1, tyrosine phosphoproteins with Mₘ values of 160,000, 150,000, and 120,000 also coimmunoprecipitated with PLCγ1 after BCR cross-linking. pp120 was identified as the c-cbl proto-oncogene product by Western blot analysis (data not shown). No tyrosine phosphoproteins were detected in PLCγ1 immunoprecipitates from unstimulated cells or in control immunoprecipitates with normal rabbit serum (NRS, Fig. 2A, lanes 1, 3, and 4).

Because many B cells utilize the γ2 isof orm of PLC (PLCγ2) (18, 19, 48), we also analyzed the ability of proteins 1–4 to associate with PLCγ2 in vivo. Proteins 1, 3, and 4 were detected in immunoprecipitates of PLCγ2 from BCR-activated Daudi B cells. Protein 2 was below the level of detection and/or resolvable in these immunoprecipitates (Fig. 2B). Together, these results demonstrate that phosphoproteins 1, 3, and 4 interact with both PLCγ1 and PLCγ2 after BCR activation.

To test whether proteins 3 and 4 also interacted with Grb2 in vivo, we generated Daudi B cell lines that stably express a myc epitope-tagged form of Grb2. This strategy was utilized to exclude the possibility that the immunoprecipitating antibody may inhibit the binding of Grb2-interacting proteins. Two representative clones, D28 and D35, which overexpress Grb2, are shown in Fig. 2C, lanes 2 and 3. Similar to the in vitro binding experiments, proteins 3 and 4 coimmunoprecipitated with Grb2 after BCR cross-linking (Fig. 2D, lane 4). Taken together, these in vitro binding and in vivo immunoprecipitation studies demonstrate that phosphoproteins 3 and 4 represent Grb2- and PLCγ-binding proteins, whereas phosphoproteins 1 and 2 interact with PLCγ.

Structural Domains within Grb2 and PLCγ1 Required for Their Interaction with Phosphoproteins 1–4—Grb2 is composed of a single SH2 domain flanked by two SH3 domains. To identify the structural domains within Grb2 which interact with proteins 3 and 4, we generated GST-Grb2 fusion proteins in which each domain was disrupted by a single point mutation. Interestingly, Grb2 mutants with a single domain disrupted retained their ability to interact in vitro with both proteins 3 and 4, although protein 3 appeared to have a slight preference for the COOH-terminal SH3 domain and protein 4 for the NH₂-terminal SH3 domains (Fig. 3A, top panel, lanes 2–4). Mutation of any combination of SH2 or SH3 domains eliminated the binding of proteins 3 and 4 to Grb2.

**Fig. 2. A, in vivo interaction of proteins 1–4 with PLCγ1.** Resting (lanes 1 and 3) or BCR-stimulated (lanes 2 and 4) Ramos cell lysates were immunoprecipitated (2 × 10⁶ cells/lane) with an anti-PLCγ1 antiserum (lanes 1 and 2), normal rabbit serum (NRS; lanes 3 and 4), or precipitated (5 × 10⁶ cells/lane) with the GST-PLCγ1 fusion protein (lane 5). Precipitates were analyzed by Western blotting with an anti-Tyr(P) mAb (PY20, top panel). The same blot was stripped and reblotted with an anti-PLCγ1 mAb (bottom panel). Lane 5 represents a shorter exposure from the same blot for comparison. B, in vivo interaction of proteins 1, 3, and 4 with PLCγ2. Resting (lane 1) or BCR-stimulated (lane 2) Daudi cell lysates (2 × 10⁶ cells/lane) were immunoprecipitated with an anti-PLCγ2 antiserum and blotted with an anti-Tyr(P) mAb (PY20). C, expression of myc-tagged Grb2 in Daudi cells. Daudi cells were transfected with a cDNA encoding a myc epitope-tagged Grb2 subcloned in the pApuro expression vector. Clones expressing myc-Grb2 were analyzed by Western blotting with an anti-Grb2 mAb. Parental (lane 1) and two representative clones (D28 and D35, lanes 2 and 3) are shown in this panel. D, in vivo interactions of proteins 3 and 4 with Grb2. Resting (lanes 1 and 3) or BCR-stimulated (lanes 2 and 4) cell lysates (2 × 10⁶ cells/lane) from either parental Daudi cells (lanes 1 and 2) or the D35 clone (lanes 3 and 4) were immunoprecipitated with an anti-myc epitope mAb (9E10) and analyzed by Western blotting with an anti-Tyr(P) mAb (PY20). Similar data were obtained with two additional clones (D28 and D17).
Hence, cooperation of SH2 and SH3 domains is required for the efficient interaction of Grb2 with proteins 3 and 4.

To identify the structural domains within PLCγ1 which mediate its interaction with proteins 1–4, we analyzed the ability of individual SH2 domains to bind these proteins. The COOH-terminal, but not the NH2-terminal SH2 domain of PLCγ1 was sufficient to bind proteins 1–4 (Fig. 3B, top panel, lanes 2 and 3). In this experiment, proteins 2 and 3 comigrated, although in many other experiments these two proteins could be resolved (see Fig. 5). In contrast, the SH3 domain of PLCγ1 failed to interact with any of these four phosphoproteins (Fig. 3B, lane 4). Rather, the SH3 domain of PLCγ1 interacted with pp120cbl and pp150 (Fig. 3B, lane 4, and data not shown).

Analysis of Phosphoproteins 1–4—The binding characteristics of proteins 3 and 4 are similar to SLP-76, a Mr 76,000 tyrosine phosphoprotein recently described in T cells which binds PLCγ1 and Grb2 in vitro (49). Low levels of SLP-76 mRNA and protein have been described in B cells (49, 50). To determine whether proteins 3 or 4 represented SLP-76, we analyzed B cell lysates by immunoprecipitation and Western blotting with anti-SLP-76 antibodies. SLP-76 protein was not detected in any of the six B cell lines examined (Fig. 4A, lanes 1–6). In contrast, SLP-76 was readily detected in the Jurkat T cell line (Fig. 4A, lane 7). In addition, although proteins 3 and 4 bound both Grb2 and PLCγ1 fusion proteins (Fig. 1A), they failed to be recognized by an anti-SLP-76 mAb (Fig. 4B, lanes 1–4 and 6–9). In contrast, SLP-76 from activated Jurkat T cells readily bound Grb2 and PLCγ1 (Fig. 4B, lanes 5 and 10). Similar data were obtained using normal mouse B cells (data not shown). To resolve better the separation of proteins 1–4 to facilitate their identification, we separated BCR-activated Ramos cell lysates by anion exchange chromatography. Eluted proteins...
were analyzed by binding to the PLCγ1 fusion protein and immunoblotting with an anti-Tyr(P) mAb (PY20, left panel). Protein 2 was eluted in fractions 7 and 8 (~0.3 M NaCl), proteins 3 and 4 in fractions 9 and 10 (~0.4 M NaCl), and protein 1 in fractions 10 and 11 (~0.45 M NaCl). These fractions were then immunoblotted with antibodies against molecules implicated in BCR activation with similar M₆ mobilities. Protein 2 was recognized by an anti-Syk antisera (Fig. 5, right panel, lane 1) and is consistent with previous reports that Syk interacts with PLCγ1 after BCR cross-linking (51, 52). Western blot analysis with antibodies directed against SHP-1, SHP-2, Bruton’s tyrosine kinase, and Sam68 failed to recognize protein 1, 3, or 4 (data not shown). Hence, proteins 3 and 4 do not represent any of these signaling molecules.

**Interaction of Phosphoproteins 3 and 4 with Vav**—Although SLP-76 was not expressed in normal murine B cells or the B cell lines analyzed, the similar abilities of proteins 3 and 4 to interact with Grb2 and PLCγ1 suggested that these two proteins may represent B cell homologs of SLP-76. Because SLP-76 has been recently demonstrated to interact with the vav proto-oncogene product (53–55), we analyzed the ability of proteins 3 and 4 also to interact with Vav. Vav immunoprecipitates from BCR-activated cells demonstrated the association of Vav with two tyrosine-phosphorylated proteins with mobilities identical to the Grb2- and PLC-binding phosphoproteins 3 and 4 (Fig. 6A, lane 4). These two phosphoproteins were not detected in Vav immunoprecipitates from resting B cells or in control immunoprecipitates (Fig. 6A, lanes 1–3). Mapping studies using GST fusion proteins containing the Vav SH2 or SH3 domains demonstrated that the Vav SH2 domain was sufficient for its interaction with these two phosphoproteins (Fig. 6B, lane 3). Although a tyrosine-phosphorylated protein with mobility identical to that of protein 1 was also precipitated by the SH2 domain of Vav (Fig. 6B, lane 3), this interaction was not observed in vivo (Fig. 6A, lane 4).

To confirm that the two Vav-binding phosphoproteins were the same as the Grb2- and PLCγ1-binding proteins 3 and 4, we again performed sequential depletion studies using Grb2, PLCγ, and Vav fusion proteins. Sequential precipitation with the Grb2 fusion protein depleted proteins 3 and 4, which interact with the Vav SH2 domain (Fig. 6C, lanes 1–4). The binding to the Vav SH2 domain depleted the Vav-interacting proteins 3 and 4 (Fig. 6C, lanes 6–9). Together, these experiments demonstrate that phosphoproteins 3 and 4 interact with at least three distinct signaling adapter/effector molecules, Grb2, PLCγ, and Vav.

**SLP-76 Competes with Proteins 3 and 4 in Their Interaction with Vav**—To provide further evidence that proteins 3 and 4 may be functionally homologous to SLP-76, we analyzed whether SLP-76 could compete with proteins 3 and 4 in their interaction with the SH2 domain of Vav. BCR-activated cell lysates were incubated with a Vav SH2 domain fusion protein in the absence (Fig. 7, top panel, lane 1) or in the presence of unphosphorylated (lane 2) or phosphorylated (lane 3) forms of SLP-76. We have demonstrated previously that SLP-76 serves as a downstream substrate of the ZAP-70 and Syk PTKs and can be phosphorylated by coinfection of SLP-76 with activated ZAP-70 or Syk PTKs in insect Sf9 cells (44). Only tyrosine-phosphorylated SLP-76 significantly decreased the ability of proteins 3 and 4 to bind the Vav SH2 domain (Fig. 7, top panel, lane 4), suggesting either full-length Grb2 (lanes 1–3) or the two SH2 domains of PLCγ1 (lanes 6–8) and subsequently precipitated with a fusion protein encoding the SH2 domain of Vav (lanes 4 and 9). GST mock-depleted lysates were also precipitated with the GST-Vav SH2 domain fusion protein (lanes 5 and 10) for comparison. Bound proteins were analyzed by Western blotting with an anti-Tyr(P) mAb (PY20).
SLP-76 Homologs in B Cells

We also provide evidence here of the in vivo interaction of a M, 76,000 tyrosine phosphoprotein and Syk with PLCγ1. The latter is consistent with previous observations implicating the biochemical and functional requirements of Syk in [Ca2+]i mobilization (7, 51, 52). The interaction of the M, 76,000 phosphoprotein with PLCγ1 was not observed with Vav or Grb2 and likely selectively mediates the [Ca2+]i pathway. Genetic studies have demonstrated the functional importance of PLCγ1, Vav, and Grb2 in B cell function and development. B cells deficient in PLCγ demonstrate a lack of inositol 1,4,5-trisphosphate hydrolysis and an absence of [Ca2+]i mobilization after BCR cross-linking (43). Deletion of vav in mice results in an arrest of both B and T cell development (39–41). Finally, overexpression of Grb2 results in an augmentation of antigen receptor-mediated activation of cytokine synthesis.2 Hence, these three signaling and adaptor proteins play requisite roles in normal B cell function.

The structural domains within PLCγ1, Vav, and Grb2 which mediate their binding to pp70 and pp68 have also been demonstrated to be functionally important in lymphocyte activation. Mutation of the SH2 domains of PLCγ2 or the SH2 domain of Vav abrogates BCR- or T cell receptor-mediated signaling functions (43, 53). Hence, the ability of these structural domains within PLCγ and Vav to interact with pp70 and pp68 is likely to be important in mediating BCR function. Although BCR activation in naive B cells or B cell lines results in activation of all three major signaling pathways, anergized B cells demonstrate a lack of coordination of these three signaling pathways. Studies by Goodnow and colleagues (58) have demonstrated that although anergized B cells activate the [Ca2+]i and Ras pathways, no activation of c-Jun N-terminal kinase or nuclear localization of nuclear factor κB was observed in these cells. Hence, the integration of these major signaling pathways, which potentially could be regulated by pp70 and pp68, is required for normal B cell function.

The interaction of pp70 and pp68 with Grb2, PLCγ, and Vav in B cells is similar to the interaction of a linker protein, SLP-76, with Grb2, PLCγ, and Vav in T cells. SLP-76 interacts in vitro with Grb2 fusion protein as well as with Vav and PLCγ1 after T cell receptor cross-linking (49, 53–55, 59). Moreover, SLP-76 is phosphorylated by ZAP-70 and is required for T cell receptor-mediated activation of the nuclear factor of activated T cells and the IL-2 gene (44, 59, 60). Although SLP-76 mRNA is expressed in several B cell lines and SLP-76 protein has been reported in WEHI-231 cells (49, 50), we failed to detect any SLP-76 protein in WEHI-231 B cells, in five additional B cell lines, or in normal mouse B cells using an anti-SLP-76 mAb. A recent report has shown in vivo SLP-76 expression in normal B cells using anti-SLP-76 antiserum (49). We failed to detect any SLP-76 protein in WEHI-231 B cells, in five additional B cell lines, or in normal mouse B cells using an anti-SLP-76 mAb or an anti-SLP-76 antiserum (49). We failed to detect any SLP-76 protein in WEHI-231 B cells, in five additional B cell lines, or in normal mouse B cells using an anti-SLP-76 mAb or an anti-SLP-76 antiserum (49). We failed to detect any SLP-76 protein in WEHI-231 B cells, in five additional B cell lines, or in normal mouse B cells using an anti-SLP-76 mAb or an anti-SLP-76 antiserum (49).

DISCUSSION

Tyrosine phosphorylation of cellular proteins after BCR activation plays critical roles in PLCγ-, Vav-, and Shc/Grb2-mediated [Ca2+]i mobilization, c-Jun N-terminal kinase activation, and Ras activation, respectively. Phosphorylation can directly mediate activation of enzymes, such as PLCγ and Vav (20, 35). In addition, phosphorylation can mediate the assembly and/or localization of effector proteins through interactions facilitated by SH2 and PTB domains (for review, see Refs. 56 and 57). An example of the latter is represented by the tyrosine phosphorylation of Shc to recruit Grb2-SO5 to the membrane and mediate Ras activation (28). Although activation of the proximal Src family, Syk, and Bruton’s tyrosine kinases are required for the efficient function of all three signaling pathways, the integration of these distinct signaling pathways is also critical for efficient BCR-mediated function.

We provide evidence here of two cellular tyrosine phosphoproteins, pp70 (protein 3) and pp68 (protein 4), which interact with PLCγ, Vav, and Grb2. A M, 70,000 tyrosine phosphoprotein, VAP-1, has been described previously to associate with Vav after BCR cross-linking (38). Given the similarities in binding of pp70 and pp68 to these three effector proteins, pp70 and pp68 may represent differentially phosphorylated forms of the same protein. However, the inability to abrogate the M, difference between pp70 and pp68 by treatment with the serine/threonine protein phosphatase 1 and their identical isoelectric points make this possibility less likely (data not shown). Alternatively, pp70 and pp68 may represent differentially spliced forms of the same gene. Finally, pp70 and pp68 may represent a family of homologous proteins. Molecular characterization of these two proteins is under way to elucidate further their mechanistic and functional roles in BCR activation.

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