Sos, Vav, and C3G Participate in B Cell Receptor-induced Signaling Pathways and Differentially Associate with Shc-Grb2, Crk, and Crk-L Adaptors*

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B cell antigen receptor (BCR)-mediated signal transduction controls B cell proliferation and differentiation. The BCR activates Ras, presumably by the formation of a Shc-Grb2 adaptor complex, which recruits the Grb2-associated guanine nucleotide exchange factor Sos to the plasma membrane. In order to reveal additional BCR-induced signaling events involving the Grb2 adaptor, we undertook the isolation of Grb2-binding proteins. Using the yeast two-hybrid system and bacterial fusion proteins, Vav and C3G were identified as Grb2 binders. Vav is a putative nucleotide exchange factor and a target for BCR-induced tyrosine phosphorylation. C3G exerts nucleotide exchange activity on the Ras-related Rap1 protein. While Sos binds to both Grb2 Src homology-3 (SH3) domains, Vav was found to associate selectively with the carboxyl-terminal SH3 domain, while C3G bound selectively to the amino-terminal SH3 domain of bacterially expressed Grb2. Despite the association of Vav with Grb2 in vitro, we could not demonstrate an interaction between endogenous Vav and Grb2 molecules in primary B cells. Instead, Vav was found to inducibly associate with the Grb2-related adaptor protein Crk upon BCR stimulation. C3G did not bind to either Grb2, Shc, or Crk in vivo. Instead, C3G was found in association with the Crk-L adaptor, both before and after BCR stimulation. We show that Crk-L also participates in BCR signaling, since it inducibly interacts with tyrosine-phosphorylated Cbl. We conclude that, in addition to Sos, Vav and C3G play a role in BCR-mediated signal transduction. These guanine nucleotide exchange factors selectively associate with Grb2, Crk, and Crk-L, respectively, which may serve to direct them to different target molecules. Since Cbl binds to Grb2, Crk, as well as Crk-L, we hypothesize that Cbl may affect the function of all three exchangers.

Src homology-3 (SH3)1 domains are found in a variety of signaling molecules, involved in the response of cells to external stimuli (1). SH3 domains specifically interact with proline rich sequences (2) and thereby control cellular localization (3), substrate specificity (4), or catalytic activity (5) of associating proteins. One example illustrating the importance of SH3 domain-mediated intermolecular interactions is the complex formation between the adaptor protein Grb2 and the guanine nucleotide exchange factor (GEF) son of sevenless (Sos) (6). Grb2 consists of an SH2 domain, flanked by two SH3 domains. Sos is constitutively associated with the SH3 domains of Grb2 and regulates Ras activity by converting GDP-Ras to the GTP-bound state (7, 8). Translocation of Sos from the cytosol to the plasma membrane is thought to play a key role in Ras activation (9). Recruitment of the Grb2-Sos complex to the plasma membrane may be mediated by direct association of the Grb2 SH2 domain with a tyrosine-phosphorylated membrane molecule or by indirect association via the tyrosine-phosphorylated adaptor protein Shc. Receptors with intrinsic tyrosine kinase activity (7, 8), as well as receptors that activate associated cytoplasmic protein-tyrosine kinases, such as cytokine receptors (10), the T cell antigen receptor (11), and the BCR (12–14) induce association of Grb2-Sos with tyrosine-phosphorylated Shc. In lymphocytes, stimulation of T cell antigen receptor and BCR induces Ras activation, which most likely involves Sos.

Vav is exclusively found in hematopoietic cells. It contains multiple structural motifs, including one SH2 and two SH3 domains, a pleckstrin homology domain, as well as a region found in the human Dbl oncogene product (15, 16). Dbl is a GDP-GTP exchange factor for the Ras-like polypeptide Cdc42 (18), but Cdc42 has not been defined as a target for Vav. Vav was reported to exert nucleotide exchange activity on Ras (19). However, other authors found that Vav cooperates with Ras to transform fibroblasts, but is not an exchange factor for Ras (20, 21). Therefore, the target of the Dbl domain in Vav remains unidentified. Vav is phosphorylated on tyrosine upon T cell antigen receptor, Fc receptor (22, 23), BCR (24), and cytokine receptor (25) stimulation. Mice with a disrupted Vav gene die early during development, indicating that Vav has an essential function prior to the onset of hematopoiesis (26). Studies in chimeric mice have indicated that Vav also plays a role in the differentiation and function of T and B cells (27–29).

C3G was identified as a protein binding to a bacterially expressed SH3 domain of the adaptor protein Crk (30) and it associates with endogenous Crk in PC12 cells (31). In vitro, C3G can also interact with SH3 domains of Grb2 (30). The carboxyl terminus of C3G has homology with GEFs for Ras and C3G can complement Cdc25 function in yeast (30). Recently, however, Rap1 rather than Ras, was identified as a target for C3G in mammalian cells (32). The function of C3G in lymphocytes has not yet been addressed.

In this study, we have identified Vav and C3G as Grb2...
binders, using the yeast two-hybrid system and bacterial fusion proteins. However, in vivo in B lymphocytes, only Sos participates in a Shc-Grb2 complex. Vav does not bind detectably to Shc or Grb2, but inducibly associates with Crk upon BCR stimulation. C3G was not detected in Shc, Grb2, or Crk complexes, but constitutively interacts with Crk-L. We demonstrate that Grb2, Crk, as well as Crk-L interact with the Cbl proto-oncogene product (33), which is a major target for BCR-induced protein-tyrosine kinase activity (34). This indicates that all three adaptors participate in BCR-induced signaling. Given their differential interaction with Sos, Vav, and C3G, we postulate that Grb2, Crk, and Crk-L selectively target the functions of these nucleotide exchangers in response to BCR stimulation.

MATERIALS AND METHODS

Cells—The human Burkitt lymphoma lines Ramos and Daudi, which are mature B cell lines expressing transmembrane immunoglobulin (Ig)M complexes, were cultured in Iscove’s modified Dulbecco’s medium (IMDM), supplemented with 5% fetal calf serum. Primary human B cells were isolated from tonsils by depletion of T cells. The total cell suspension was incubated with a mixture of anti-CD3 and anti-CD19 mAb, followed by goat anti-mouse Ig-conjugated magnetic beads (Advanced Magnetics, Cambridge, MA). The remaining cell suspension, containing 80% B cells, was cultured for 1 h in IMDM supplemented with 10% fetal calf serum. Prior to stimulation with anti-Ig reagent, the cells were incubated for 30 min at 37 °C in IMDM without serum.

A synthetic rat anti-Grb2 and anti-Shc sense and anti-Sos anti-Grb2, and anti-Sos sense were kindly provided by Dr. J. L. Bos (Utrecht University, Utrecht, The Netherlands). They were raised against a fusion protein of glutathione S-transferase (GST) and the complete Grb2 protein and against a fusion protein of GST and the SH2 domain of Shc, respectively. Anti-Crk mAb, which recognizes Crk-I and Crk-II forms, but not Crk-L, was purchased from Transduction Laboratories (Lexington, KY). Anti-Crk-L serum, which recognizes Crk-I and Crk-II forms, but not Crk-L, was purchased kindly provided by Dr. J. L. Bos (Utrecht University, Utrecht, The Netherlands). Anti-Crk-L serum, which recognizes Crk-I and Crk-II forms, but not Crk-L, was purchased from Transduction Laboratories (Lexington, KY). Anti-Crk-L serum, which recognizes Crk-I and Crk-II forms, but not Crk-L, was purchased from Transduction Laboratories (Lexington, KY). Anti-Crk-L serum, which recognizes Crk-I and Crk-II forms, but not Crk-L, was purchased from Transduction Laboratories (Lexington, KY).

Immunoblotting—Polyclonal goat anti-human Ig reagent (Tago Immunologicals, Burlingame, CA) was used for B cell stimulation. The anti-phosphotyrosine, anti-Vav, and anti-C3G reagents, nitrocellulose membrane-bound proteins, were detected using an enhanced chemiluminescence (Amersham, Little Chalfont, United Kingdom).

RESULTS

Isolation of the Vav Protein as a Grb2 Binder by the Use of the Yeast Two-Hybrid System—To identify B cell-derived molecules that can interact with Grb2, the yeast two hybrid system was used (36). Full-length Grb2 was fused to the BD of the Gal4 transcription factor. This Gal4 DBD-Grb2 fusion protein bait was expressed in a yeast strain containing lacZ and his3 reporter genes, together with an Epstein-Barr virus-transformed human B lymphocyte cDNA library fused with the Gal4 TAD. Colonies expressing a B cell-derived protein that effectively binds to Grb2 were revealed by blue staining and the capacity to grow on medium without histidine. Out of 38 colonies selected in this way, one contained cDNA sequences of the Vav oncoprotein. The Gal4 TAD-Vav fusion protein only activated the
Endogenous Vav, Sos, and C3G Differentially Associate with SH3 Domains of Grb2, Vav, and C3G from resting and activated B cells selectively interact with SH3 domains of bacterial GST-Grb2 fusion proteins. A, primary B cells were isolated by immunodepletion of T cells from tonsil cell suspensions. Cells were incubated in medium (−) or with anti-human Ig polyclonal antibody to trigger the BCR (+). Nonidet P-40 lysates were prepared, precleared with bacterial GST protein and incubated with GST fusion proteins of full-length Grb2, its SH2 domain, its carboxyl-terminal (SH3-C) or amino-terminal (SH3-N) SH3 domains. Part of the lysates was used for immunoprecipitation with anti-Vav mAb. Isolated proteins were separated by SDS-PAGE transferred to nitrocellulose sheets, incubated with anti-Vav mAb, and second antibody-conjugated to horseradish peroxidase and developed by enhanced chemiluminescence. The arrow indicates the 95-kDa Vav protein. The asterisks indicate the positions of the fusion proteins. B, Ramos B cells were treated as described for A, lysates were incubated with GST-Grb2 fusion proteins, and samples were unblotted with anti-Sos polyclonal antibody. C, Ramos B cells were treated as described for A, lysates were incubated with GST-Grb2 fusion proteins or with anti-C3G polyclonal serum, and samples were unblotted with anti-C3G mAb. The asterisks indicate the positions of the fusion proteins.

70 kDa is detected. Both Vav species associate with equal efficiency with full-length Grb2. Vav is known to be inducibly phosphorylated on tyrosine upon BCR stimulation, but triggering of the BCR and ensuing Vav phosphorylation (results not shown) did not affect the potential of endogenous Vav to associate with Grb2 (Fig. 2A). As in the yeast two-hybrid system, B cell-derived Vav selectively interacted with the SH3-C domain of Grb2 and not with the SH3-N or SH2 domains.

Proline-rich motifs in the guanine nucleotide exchange factor Sos are known to interact with Grb2. In contrast to Vav, Sos derived from human tonsillar B cells, did not discriminate between the SH3-N and SH3-C domains of Grb2. It bound to both SH3 domains, and not to the SH2 domain (Fig. 2B), as expected (41).

The C3G protein is known to bind to Grb2 in vitro (30). Its expression and function in lymphocytes has thus far not been examined. Fig. 2C shows that the 145-kDa C3G molecule can effectively be isolated from Ramos B cells with specific anti-
body. Endogenous C3G from both resting and activated B cells bound with equal efficiency to GST-Grb2 fusion proteins (Fig. 2C). Upon stimulation of the BCR, no tyrosine phosphorylation of the C3G molecule could be detected (data not shown). Interestingly, C3G showed yet another Grb2 binding specificity than Vav and Sos. It preferentially interacted with the amino-terminal SH3 domain and to a lesser extent with the carboxyl-terminal SH3 domain. It did not bind at all to the Grb2 SH2 domain (Fig. 2C). We conclude that in vitro, Vav, Sos, and C3G proteins from human B cells all have the ability to bind to Grb2, which is not significantly affected by BCR stimulation. The three proteins selectively interact with the two SH3 domains of Grb2. While Sos binds with similar efficiency to both domains, Vav preferentially associates with SH3-C and C3G with SH3-N.

Sos, but Not Vav or C3G, Participates in BCR-induced Shc-Grb2 Complexes—We have reported previously that activation of the BCR leads to tyrosine phosphorylation of Shc and the formation of a Shc-Grb2 complex (14). These complexes were shown to contain the Sos molecule, which is associated with Grb2 in vivo (12, 13). In Ramos and Daudi cells, we could indeed demonstrate the BCR-inducible association of Sos with the Shc adaptor (Fig. 3A). Anti-Shc immunoprecipitates from resting and activated B cells were immunoblotted with anti-Sos serum, revealing the presence of Sos exclusively in the Shc-Grb2 complexes from activated B cells.

In contrast to Sos, Vav could not be detected by immunoblotting in anti-Shc or -Grb2 immunoprecipitates from Burkitt lymphoma cell lines (results not shown), or from primary tonsillar B cells either prior to, or after BCR stimulation (Fig. 3B). Similarly, C3G was not found in either anti-Grb2 or -Shc immunoprecipitates from resting or BCR-activated B cells (Fig. 3C). We conclude that, despite the shared in vitro reactivity of Sos, Vav, and C3G with Grb2-SH3 domains, only Sos has detectable affinity for Grb2 in human B lymphocytes and is found in a BCR-inducible Shc-Grb2 complex.

Vav, but Not C3G, Interacts with the Crk Adaptor in a BCR-inducible Fashion—Although not found in anti-Shc or -Grb2 precipitates, the 95-kDa Vav species was detected in an anti-Crk immunoprecipitate from tonsillar B cells (Fig. 3B). The Crk adaptor, like Grb2, exclusively contains SH2 and SH3 domains and occurs in three different forms: a 28-kDa Crk-I species with one SH2 and one SH3 domain and 40- and 42-kDa Crk-II species with the structure SH2-SH3-SH3 (17). The anti-Crk mAb employed here detects both Crk-I and Crk-II. The association of Vav with Crk was induced by BCR stimulation. In independent experiments using tonsillar B cells, a Crk-Vav complex was occasionally found prior to BCR stimulation, but the amount of Vav associated with Crk was always significantly less than in activated B cells (data not shown). This variability might be caused by differences in the amount of activated B cells present in the tonsil preparations.

In PC12 pheochromocytoma cells, C3G was shown to constitutively interact with endogenous Crk (31). However, we could not identify such an association in B cells, either before or after BCR stimulation (Fig. 3C). We conclude that Sos, Vav, and C3G differentially interact with SH3 domain containing adaptor proteins in intact human B lymphocytes. Whereas Sos associates in a BCR-induced Shc-Grb2 complex, Vav and C3G do not. Vav instead associates with Crk, but C3G does not.

C3G Is Associated with Crk-L, Which Participates in a BCR-induced Signaling Pathway—Clearly, Sos and Vav lie in BCR-induced signaling cascades: Sos is recruited into a Shc complex upon BCR stimulation, while Vav is tyrosine-phosphorylated upon BCR stimulation and recruited to the Crk adaptor. Given its expression in B cells, we wondered whether C3G is functionally connected to the BCR. To this end, we first examined whether C3G binds to another adaptor protein that might be subject to BCR regulation. Crk-L was recently cloned from a hematopoietic cell line (42). It is a 36-kDa Crk-related molecule, containing one SH2 domain and two SH3 domains. The Crk-L protein is expressed in human B cells as shown by immunoprecipitation of Crk-L and blotting with the same antibody (Fig. 4). Interestingly, blotting of the Crk-L immunoprecipitates with anti-C3G antibody indicated that Crk-L is associated with C3G in these B cells, both before and after BCR stimulation (Fig. 4).

To determine whether Crk-L is regulated by the BCR, we first investigated whether it is phosphorylated in response to receptor stimulation. However, no inducible tyrosine phosphorylation of either Crk or Crk-L was observed (results not shown). We were able to link Crk and Crk-L to BCR-induced
Grb2 a BCR-induced signaling pathway. The Cbl proto-oncogene product inducibly associates with both adaptors upon B cell signaling by the following observation. The Cbl proto-oncogene participates in the BCR signaling by its inducible interaction with the Cbl protein, indicating the position of the Ig heavy chains of the immunoprecipitating antibodies.

signaling by the following observation. The Cbl proto-oncogene product inducibly associates with both adaptors upon B cell activation (Fig. 5). We conclude that Crk-L, and possibly C3G, which constitutively interacts with this adaptor, participate in a BCR-induced signaling pathway.

DISCUSSION

In this paper, we show that Sos, Vav, and C3G all bind to Grb2 in vitro, with different specificities for the amino-terminal or carboxyl-terminal SH3 domains. However, the endogenous GEFs in B lymphocytes bind to different adaptor proteins. Only Sos binds to Grb2, whereas Vav binds to Crk and C3G to Crk-L. The BCR communicates with all three adaptor proteins. BCR stimulation induces recruitment of Grb2 to Shc and the association of Crk with Vav. Crk-L was shown to be connected to BCR signaling by its inducible interaction with the Cbl molecule.

It is of interest that Sos, Vav, and C3G associate with different adaptor proteins in B lymphocytes. The Grb2, Crk, and Crk-L adaptors may serve to differentially regulate intracellular targeting, substrate specificity, and/or catalytic activity of Sos, Vav, and C3G. Sos is known to activate Ras and therewith the MAP kinase pathway leading to cell proliferation. Sos is constitutively associated with Grb2 SH3 domains, leaving the SH2 domain available for interaction with other proteins. The BCR-inducible ShcGrb2 association is mediated by the SH2 domain of Grb2 and presumably serves to recruit the Grb2-Sos complex to the plasma membrane where Ras is located. It is much less clear which are the in vivo targets of the GEF activity of Vav and C3G and which cellular responses are regulated by these two proteins.

The homology of Vav with Dbl and Cdc24 suggests specificity for the Cdc25, Rho, and/or Rac small GTP-binding proteins, which play a role in cytoskeletal organization (16). However, no activity of Vav on these molecules has been demonstrated. Its specificity for Ras is debated, but Vav has been implicated in cellular activation and mitogenesis. Its overexpression can enhance MAP kinase activity in fibroblasts, in a Ras-independent manner (21). In T lymphocytes, Vav overexpression can activate the NFAT nuclear factor, involved in interleukin-2 expression (43). In this case, Vav function is dependent on Ras. It is not known whether the effects of Vav on mitogenic pathways can be ascribed to its GEF domain or to functions exerted by other domains.

Originally, C3G was thought to regulate Ras, based on the sequence homology of its carboxyl-terminal domain with Cdc25 and Sos and on the suppression of a Cdc25 loss of function mutation in yeast by this domain (30). Recently, however, C3G was shown to have GEF activity for Rap1, rather than Ras (32). Rap1 has been implicated in negative regulation of Ras (44). It will be of interest to determine whether C3G may have targets other than Rap1 and whether it is involved in the regulation of mitogenesis and/or other cellular responses.

Vav associated with Grb2 in yeast and with bacterial Grb2-GST fusion proteins, but we could not detect a Vav-Grb2 interaction in vivo. Vav was found earlier to bind to Grb2 SH3 domains in yeast and in transfected mammalian cells (45). More importantly, coimmunoprecipitation experiments showed that Vav inducibly associates with Grb2 and Shc upon pervanadate treatment of T lymphocytes (46). This suggests that in vivo, tyrosine phosphorylation of Vav is involved in its binding to Grb2 an raises the question whether the interaction involves a Grb2 SH2 domain rather than an SH3 domain. We do not exclude the possibility that Vav can associate with Shc-Grb2 in B cells, but failed to detect it under conditions that allowed binding of Vav to Crk. Therefore, the Vav-Crk interaction might be prevalent in BCR-mediated signaling.

Vav was shown to bind in vivo to Crk-I and/or Crk-II proteins. The v-crk avian retroviral gene has transforming properties and induces tyrosine phosphorylation of intracellular substrates. Since Crk-I and -II do not contain enzymatic activity, these properties must be ascribed to associating proteins (17). Crk can interact via one of its SH3 domains with the Abl tyrosine kinase, which might be involved in Crk-induced cellular transformation. In addition, Abl can phosphorylate Crk (4), indicating that Crk function might be regulated by phosphorylation. In B cells, we have not observed BCR-induced tyrosine phosphorylation of Crk, but the Crk-Vav association was induced upon BCR stimulation. It is possible that BCR-induced tyrosine phosphorylation of Vav allows the Crk-Vav interaction to occur. We do not know whether this interaction involves Crk SH3 or SH2 domains.

The Crk SH3 domain can bind Sos as well as C3G and Crk overexpression enhanced nerve growth factor-induced Ras activity, indicating that Crk-associated proteins can regulate Ras (31). We found that in B cells, C3G is not associated with Crk, but with the product of a related gene, Crk-L. Crk-L was identified at the genomic level, by cross-reactivity with a Crk cDNA (42) and contains one SH2 and two SH3 domains. Like Crk, Crk-L can associate with the Abl protein-tyrosine kinase and can be phosphorylated by it (47). Crk-L is not phosphorylated in response to BCR stimulation (results not shown) and its association with C3G appears to be constitutive. Therefore, it was not immediately clear whether the Crk-L-C3G complex is linked to BCR signaling. However, we found that BCR stimulation induces association of Crk-L with the Cbl protein, indi-
cating that Crk-L is regulated by the BCR. We don’t know whether the pool of Crk-L that is associated with Cbl can simultaneously interact with C3G, but it is an interestingpossibility. Thus far, we could not detect phosphorylation or plasma membrane translocation of C3G in response to BCR stimulation. Obviously, the most important aim is to define the in vivo activity of C3G and assess whether this can be modulated by BCR stimulation.

The Cbl protein has emerged as a major target for antigen receptor-induced tyrosine phosphorylation both in T cells (48) and in B cells (34). Recently, epidermal growth factor was also shown to induce tyrosine phosphorylation of Cbl and its association with the epidermal growth factor receptor (49). Interestingly, Cbl associates with all three adaptors described here: it constitutively interacts with Grb2 and inductively associates with Crk-I and -II and Crk-L upon BCR triggering. These three adaptors could bring Cbl in proximity of Sos, Vav, and C3G, respectively. The function of Cbl is as yet unclear, but it was identified as the transforming gene of a murine retrovirus, which induces pre-B cell lymphomas and myeloid leukemias (50). In Caenorhabditis elegans, the Cbl homologue sl-1 was recently identified as a negative regulator of the Let-23 receptor tyrosine kinase pathway (51), which involves Grb2 and Ras homologues. We hypothesize, therefore, that by participating in the three BCR-regulated adaptor complexes, Cbl might affect the function of associated Sos, Vav, and C3G and therewith regulate mitogenesis and possibly other responses to BCR stimulation.

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REFERENCES

1. Kuriyan, J., and Cowburn, D. (1993) Curr. Opin. Struct. Biol. 3, 828–837
2. Cicchetti, P., Mayer, B. J., Thiel, G., and Baltimore, D. (1993) Science 257, 803–806
3. Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V., and Schlessinger, J. (1993) Cell 74, 83–91
4. Feller, S. M., Knudsen, B., and Hanafusa, H. (1994) EMBO J. 13, 2341–2351
5. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) J. Biol. Chem. 269, 21463–21465
6. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Levinsky, R. J., and Kinnon, C. (1995) J. Biol. Chem. 270, 1168–1172
7. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Levinsky, R. J., and Kinnon, C. (1995) J. Biol. Chem. 270, 1168–1172
8. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Levinsky, R. J., and Kinnon, C. (1995) J. Biol. Chem. 270, 1168–1172
9. Feller, S. M., Knudsen, B., and Hanafusa, H. (1994) EMBO J. 13, 2341–2351
10. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) J. Biol. Chem. 269, 21463–21465