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Binding of Vav to Grb2 through dimerization of Src homology 3 domains

(signal transduction/two-hybrid cloning/hematopoietic differentiation)

ZHENG-SHENG YE* AND DAVID BALTIMORE†

Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT **The protooncogenic protein Vav has the structure of an intracellular signal transducer. It is exclusively expressed in cells of hematopoietic lineage and plays a crucial role in hematopoietic cell differentiation. Here we report that both in cell extracts and within intact mammalian cells Vav binds to Grb2 (Sem-5/ASH/Drk), an adaptor molecule which plays a key role in Ras activation. The interaction became evident from a yeast two-hybrid screen and its specificity was demonstrated by *in vitro* binding assays. It is mediated by an unusual protein–protein binding reaction: dimerization of specific intact Src homology 3 domains of each of the partners. Signaling during hematopoietic lineage differentiation may therefore involve the tissue-specific signal transducer Vav linking into the ubiquitous pathway involving Grb2 and ultimately Ras.**

Intracellular signal transduction pathways play crucial roles in all decisions about cell development and function. A key step in the relay of signals from membrane receptors is performed by a group of proteins which exchange guanine nucleotides on Ras and its close relatives (1). Some of these factors are specifically expressed in certain cell lineages, where they may be mediators of differentiation and functional specialization (reviewed in ref. 2). The protooncogenic protein Vav (3–6) is a candidate lineage-specific mediator in that it is expressed only in cells of the hematopoietic lineages (3, 7). It contains multiple structural motifs commonly used by intracellular signaling molecules, including Src homology 2 and 3 (SH2 and SH3) and pleckstrin homology (PH) domains as well as an acidic CDC24 homology region, and a region homologous to the diacylglycerol (DAG) binding site of protein kinase C (Fig. 1*A*). Vav binds to tyrosine-phosphorylated intracellular tails of cell surface receptors through its SH2 domain and is itself phosphorylated on tyrosine after activation of hematopoietic cells through their surface receptors (8–11). Blocking *vav* expression with antisense transcripts completely abolishes hematopoietic lineage development in embryonic stem cell cultures (12), indicating the importance of the Vav signaling pathway during the differentiation of hematopoietic cells. It has been suggested that Vav may activate Ras through its ability to exchange GDP for GTP (13, 14), despite the fact that CDC24, to which Vav bears sequence homology, is an activator of Rho, not Ras (reviewed in ref. 1). Truncating an N-terminal segment of Vav converts it into an oncogenic protein (3, 15, 16).

To study the signaling mechanism controlling hematopoietic development, we have searched for proteins bound to Vav, using the yeast two-hybrid approach (17–20). We identified one Vav-associated protein as the adaptor molecule Grb2 (Sem-5/ASH/Drk) (21–24). Its interaction with Vav involves an SH3/SH3 interaction. This finding suggests that Vav is part of a hematopoietic lineage-specific signaling mechanism that can link to a ubiquitous signal transduction pathway.

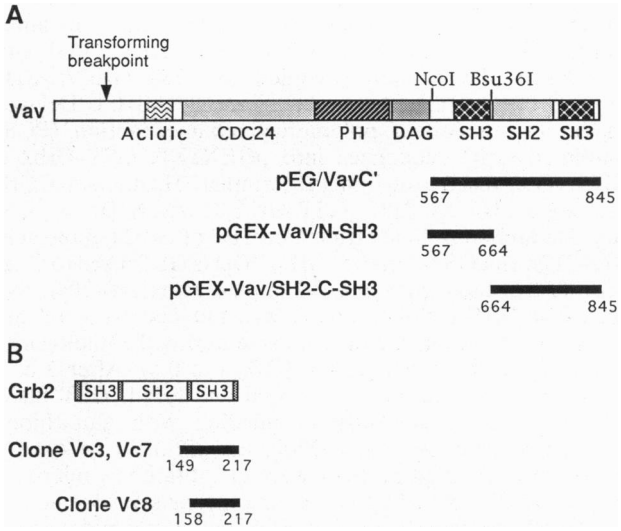

FIG. 1. Vav and Grb2 structures and clones isolated from the yeast two-hybrid system. (*A*) Domain structure of Vav. Arrow indicates a breakpoint which greatly increases its transforming activity (3). pEG/VavC′ is the yeast expression plasmid which encodes the C terminus of Vav and was used for two-hybrid cloning. *E. coli* expression plasmids pGEX-Vav/N-SH3 and pGEX-Vav/SH2-C-SH3 produced glutathione *S*-transferase (GST)–Vav fusion proteins for filter binding assays. (*B*) Partial Grb2 clones isolated from the HeLa cell expression library are compared with full-length Grb2. Two clones (Vc3 and Vc7) started from a common amino acid within the SH2 domain; Vc8 started from a region between the SH2 and C-terminal SH3 domains.

MATERIALS AND METHODS

Yeast Two-Hybrid Cloning. The two-hybrid cloning system developed by R. Brent and colleagues was used in this study (18, 19). The yeast expression plasmid pEG/VavC′ (Fig. 1*A*) was constructed as follows: plasmid pBSK-mVav.10 (6) (generously provided by J. M. Adams) was cleaved with restriction endonuclease *Nco* I (at a murine *vav* internal site) and *Xho* I (at a site present in the pBluescript multiple cloning region 3′ to *vav* sequence); the 0.84-kb DNA fragment encoding the C terminus of Vav protein was purified and ligated into *Eco*RI- and *Xho* I-digested yeast expression

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Abbreviations: GST, glutathione *S*-transferase; SH2 and SH3, Src homology 2 and 3; HA, hemagglutinin.

*Present address: Department of Medicine, New York Veterans Affairs Medical Center, 423 East 23rd Street, New York, NY 10010.
†Present address: Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139.

vector pEG202 (19) together with an *Eco*RI–*Nco* I adaptor to restore the opening reading frame. A yeast expression library derived from HeLa cell cDNA was generously provided by R. Brent (19) and screened as described (20). Sequence analysis was performed with a Sequenase kit (United States Biochemical) according to the manufacturer's instructions.

Generation of GST Fusion Proteins and Biotinylation of Probes for *in Vitro* Binding Assays. Most GST–Vav fusion protein expression vectors were prepared by ligating various restriction fragments of *vav* (illustrated in Fig. 3*A*) into *Escherichia coli* expression plasmid pGEX1ZT (these including GST–Vav/N-SH3, GST–Vav/SH2-(C-SH3), GST–VavB, GST–VavD, and GST–VavE) or pGEX2T (GST–VavF; see Fig. 3*A*). GST–VavPA expression plasmid was made by inserting a synthetic *vav* fragment encoding amino acids 603–622 (YYGIPPPPGAFGPFLRLNPG) into pGEX1ZT. Expression plasmids for GST–Grb2/C-SH3, GST–Grb2/C-SH3N'Del, and GST–Grb2/C-SH3C'Del were made by subcloning polymerase chain reaction (PCR)-amplified Grb2 sequences into pGEX1ZT. GST–Grb2/C-SH3 is GST fused to an intact C-terminal SH3 domain of Grb2 (amino acids 158–217), GST–Grb2/C-SH3N'Del is GST fused to an N-terminally truncated SH3 of Grb2 (amino acids 170–217), and GST–Grb2/C-SH3C'Del is GST fused to C-terminally truncated SH3 of Grb2 (amino acids 158–204). Synthesis of GST fusion proteins was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside during the midlogarithmic phase of bacterial growth (OD_{600} of 0.5). After 2 hr of induction, bacteria were harvested and lysed. GST fusion proteins were either affinity purified with glutathione-conjugated agarose beads (Molecular Probes) or used in crude form. Purified proteins were quantitated by microprotein assay (Pierce) or by visualizing Coomassie blue-stained full-length protein bands on SDS/polyacrylamide gels if proteins were partially degraded. GST fusion proteins were biotinylated with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma).

***In Vitro* Filter Binding Assay (25, 26).** Protein samples were fractionated by electrophoresis through an SDS/10% polyacrylamide gel and transferred to a nitrocellulose membrane filter. One microgram of protein or equivalent full-length form (if proteins were partially degraded) was loaded per lane. After the filter was blocked with 0.2% gelatin in cold (4°C) TBST buffer (10 mM Tris, pH 8.0/150 mM NaCl/0.05% Tween 20) for 30 min, biotinylated probes (1 μg/ml) were added and incubation continued for 2 hr with gentle shaking. The filters were washed with cold TBST four times (5 min per wash). Streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim) was diluted 1:5000 with 0.2% gelatin/TBST and incubated with the filter for 1 hr at 4°C (with gentle shaking) and washed as described above. Blots were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega) at room temperature in 100 mM Tris, pH 9.5/100 mM NaCl/5 mM $MgCl_2$. Reactions were stopped by changing filters into pure water when background color started to appear.

Adsorption of Endogenous Vav Protein to Immobilized GST–Grb2 Proteins. GST fusion proteins were produced in *E. coli* and crude bacterial lysates were mixed with glutathione-conjugated agarose beads for 1 hr. Sufficient bacterial lysate was used to ensure that the binding sites on the glutathione beads were saturated by the GST or GST–Grb2 fusion proteins. After extensive washes with cold (4°C) phosphate-buffered saline, 20 μl (bed volume) of protein-bound beads was added to cytosolic cell extracts containing 3 mg of protein in lysis buffer [20 mM Hepes, pH 7.4/150 mM NaCl/10% (vol/vol) glycerol/1% (vol/vol) Triton X-100/1 mM EGTA/1.5 mM $MgCl_2$/10 mM NaF/1 mM Na_3/VO_4/1 mM phenylmethylsulfonyl fluoride with aprotinin (10 μg/ml) and leupeptin (10 μg/ml)]. Incubation was continued at 4°C for 4 hr with end-to-end rotation. The beads were washed four times with lysis buffer and suspended in 2× SDS gel loading buffer (0.1 mM Tris, pH 6.8/20% glycerol, 2% SDS/0.01% bromophenol blue/5% 2-mercaptoethanol). After boiling for 5 min, the eluted proteins were analyzed by SDS/PAGE and electrotransferred to nitrocellulose filter. The filter was blotted with a monoclonal anti-Vav antibody (Upstate Biotechnology, Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-mouse Fc antibody (Pierce) was used as secondary antibody and blots were developed with ECL reagents (Amersham).

Establishment of a Vav/Grb2-Expressing 3T3 Cell Line. Retroviral expression construct pGD/HAVavF was made by ligating full-length Vav cDNA into vector pGD; an oligonucleotide encoding influenza hemagglutinin (HA) epitope was inserted in-frame at the 5′ end of the *vav* gene. Grb2 expression construct pBABEGrb2myc was a generous gift from R. A. Weinberg (27). Retroviral stocks were produced for both plasmids and used to infect NIH 3T3 cells (28). Clones were selected with G418 and puromycin, and protein expression was detected by Western analysis.

Coimmunoprecipitation of Vav and Grb2 from Cell Lysates. VG5/3T3 cells, which express HA-tagged Vav and Myc-tagged Grb2 proteins, were grown in 150-mm culture plates to near confluency and harvested. For each immunoprecipitation, 20 plates were used. The immunoprecipitation was performed as described earlier (20), using monoclonal anti-HA antibody (12CA5) or anti-Myc antibody (9E10). Immunoprecipitates were analyzed by Western blotting using either anti-Vav antibody or anti-Grb2 antibody. Secondary reagents were either goat anti-rabbit antibody conjugated to alkalian phosphatase (Boehringer Mannheim) or ^{125}I-labeled protein A (Amersham).

RESULTS

The C terminus of Vav was subcloned into the yeast expression vector pEG202, which was designed for yeast two-hybrid interaction cloning (17–19). The full length Vav–LexA fusion protein had a weak transcriptional activity, precluding its use as a suitable "bait," but the LexA–Vav C-terminus fusion gave no basal activity. This portion of Vav includes its SH2 domain flanked by two SH3 domains (Fig. 1*A*), which are structures commonly used for protein–protein interactions by signaling molecules (29, 30). This construct (pEG/VavC′) was used to screen a yeast expression library derived from the cDNA of HeLa cells. Two million clones were screened, and 18 clones were selected. These clones conferred galactose-dependent phenotypes indicating protein interaction on yeast strain EGY48, and the phenotypes segregated with the plasmids from the cDNA library. Thus, these cDNAs represented candidates encoding Vav-binding proteins.

Six cDNA clones were randomly selected from the positive pool from the HeLa library and were subjected to sequence analysis. Nucleotide sequences of three clones showed a 100% match to the cDNA of human Grb2 (Sem-5/ASH/Drk) (21–24), a ubiquitously expressed adaptor molecule involved in the Ras activation pathway (reviewed in ref. 31). Grb2 cDNA thus represented about 50% of the Vav-interacting molecules isolated from HeLa cells. The other three cDNAs contained proline-rich sequences which probably were binding sites for Vav SH3 domains. Of the three Grb2 partial cDNAs, two (Vc3 and Vc7) had identical fusion junctions with LexA and probably represented the same original plasmid in the library, while the other one (Vc8) contained a slightly shorter Grb2 sequence (Fig. 1*B*). All three clones contained an intact C-terminal SH3 domain (C-SH3) of the Grb2 protein.

We used an *in vitro* filter binding assay (25, 26) to confirm the interaction between Grb2 and Vav and to map the structural domains responsible for the interaction. The N-terminal SH3 domain (N-SH3) and the SH2-(C-SH3) region of Vav were separately expressed as GST fusion proteins in *E. coli* (pGEX-Vav/N-SH3 and pGEX-Vav/SH2-C-SH3 in Fig. 1*B*) and affinity purified. Approximately 1 μg of each fusion protein was electrophoresed through an SDS/polyacrylamide gel, transferred to a nitrocellulose filter, and blotted with biotinylated GST–Grb2/C-SH3 fusion protein. The Grb2 C-terminal SH3 domain bound with high affinity to the N-terminal-SH3 domain of Vav (Fig. 2). The specificity of the interaction in this assay was demonstrated by the inability of Grb2/C-SH3 to bind to Vav/SH2-C-SH3 or to either SH3 domain of Grb2 (Fig. 2). Since both Vav and Grb2 seemed to be binding via SH3 domains, we expanded the test for binding specificity by using an additional nine biotinylated GST–SH3 fusion proteins as probes in an attempt to show binding to any of the four SH3 domains present in Vav and Grb2. These probes were Grb2/N-SH3, Abl/SH3, Src/SH3, neural Src/SH3, Lyn/SH3, Fyn/SH3, Lck/SH3, Hck/SH3, and Btk/SH3. Although all the probes were able to bind to their specific cognate proline-rich peptides, none of them bound to any of the four SH3 domains in Vav and Grb2 (data not shown). Of the total of 38 combinations of SH3 domains tested for binding, only the Vav N-terminal SH3 domain and the Grb2 C-terminal SH3 domain specifically interacted with each other. Thus, Vav/Grb2 binding is a highly specific interaction between SH3 domains.

SH3 binding sites are generally short, linear, proline-rich peptide motifs (20, 26, 32). The only such candidate sequence was a continuous run of four prolines in the N-terminal SH3 domain of Vav (Fig. 3*A*). However, when we truncated the Vav N-terminal-SH3 domain and tested its abilities to bind to

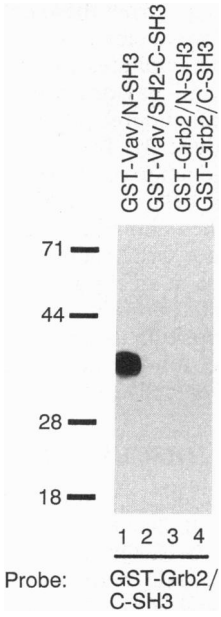

FIG. 2. Mapping of interacting domains of Vav and Grb2 with *in vitro* filter binding assays. Various segments of Vav and Grb2 were cloned into pGEX vectors and produced as GST fusion proteins in *E. coli*. These included Vav's N-terminal SH3 domain (GST–Vav/N-SH3; lane 1), Vav's SH2 and C-terminal SH3 domains (GST–Vav/SH2-C-SH3; lane 2), Grb2's N-terminal SH3 domain (GST–Grb2/N-SH3; lane 3), and Grb2's C-terminal SH3 domain (GST–Grb2/C-SH3; lane 4). The Vav sequences in GST fusion proteins are illustrated in Fig. 1*A*. One microgram of purified protein for each sample was subject to SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. Biotinylated GST–Grb2/C-SH3 (1 μg/ml) was used as probe to detect the binding.

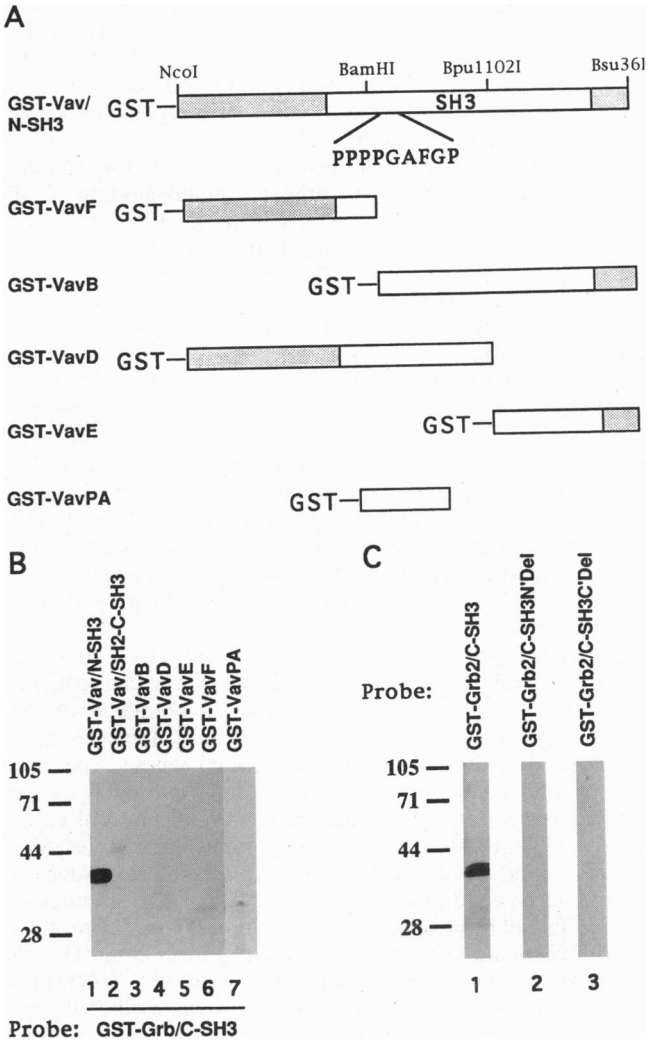

FIG. 3. Defining structural requirements for Vav/Grb2 interaction (*A*) Vav sequences present in various GST–Vav fusion proteins. (*B*) GST–Vav fusion proteins (indicated on top of each lane) were blotted onto filters and tested for their ability to interact with the biotinylated GST–Grb2/C-SH3 domain probe. Each lane contained 1 μg of purified GST fusion protein, except for GST–VavPA (lane 7). In lane 7, a crude bacterial lysate was used that contained >1 μg of fusion protein as judged by Coomassie blue staining. Molecular size markers (kDa) are at left. (*C*) Nitrocellulose filter strips were blotted with 1 μg of GST–Vav/N-SH3 and were probed with biotinylated GST–Grb2/C-SH3 (lane 1), GST–Grb2/C-SH3N'Del (containing the Grb2 C-terminal SH3 domain truncated from its N terminus) (lane 2), or GST–Grb2/C-SH3C'Del (containing the Grb2 C-terminal SH3 domain truncated from its C terminus). Molecular size markers (kDa) are at left.

an intact Grb2 C-terminal SH3 domain, none of the GST–truncated Vav proteins was able to detectably interact with Grb2 (Fig. 3 *A* and *B*). In particular, fragments containing the tetraproline sequence did not bind to Grb2. It appears that an intact N-terminal SH3 domain of Vav is required for Grb2 interaction, although it remains likely that the tetraproline element plays a role.

We then tested whether Grb2's C-terminal SH3 domain needs to be intact for binding. We truncated this domain from both N- and C-terminal ends and made GST fusion proteins. The intact and deleted Grb2/C-SH3 GST fusion proteins were biotinylated and used to bind to an intact Vav N-terminal-SH3 domain blotted onto filter paper. The GST fusion with an intact Grb2 C-terminal SH3 domain bound to Vav, whereas deletion of 12 or 13 amino acids from either end

abolished binding (Fig. 3C), indicating that a complete SH3 domain of Grb2 is required for binding to Vav. Thus, the interaction between the Vav/N-terminal SH3 and Grb2 C-terminal SH3 domain represents another mode of protein interaction among signal transducers—namely, a highly specific dimerization of two intact SH3 domains.

To examine whether endogenous full-length Vav protein binds to full-length Grb2, we prepared cellular lysates from WEHI-231, a murine B-cell line; murine EL4, a T-cell line; and 293, a human kidney cell line. The lysates were incubated with bacterially produced GST–Grb2 proteins immobilized on agarose beads to assay for Vav binding. After incubation of beads with the lysates, proteins bound to the beads were eluted and subjected to Western blot analysis using an anti-Vav antibody. Both full-length Grb2 and Grb2 C-terminal SH3 domain precipitated endogenous Vav from the lymphoid cells (Fig. 4A). Truncated Grb2 C-terminal SH3 domains failed to interact with endogenous Vav protein. No Vav protein was found in the nonhematopoietic cell line 293. The authenticity of the Vav protein was confirmed with another anti-Vav antibody (data not shown). These results showed that the endogenous Vav protein is structurally open for Grb2 binding and that both proteins in their full-length form can interact with each other.

A recent report (14) showed that diacylglycerol and phorbol esters activate Vav's guanine nucleotide-exchanging activity on Ras through a Vav region homologous to the diacylglycerol binding site in protein kinase C (designated as the DAG domain in Fig. 1A). We tested whether phorbol ester treatment of cells would affect the binding of Vav to Grb2. Lysates made from unstimulated and phorbol ester-treated WEHI-231 cells had comparable amounts of Vav protein bound to GST–Grb2 (Fig. 4B). Therefore, under the experimental conditions we used, the Vav/Grb2 interaction is not affected by phorbol ester activation of the Vav protein.

To investigate whether Vav and Grb2 form complexes in mammalian cells, we established a 3T3 cell line (VG5/3T3) expressing both Vav and Grb2 by retrovirus-mediated gene transfer (28). These cells express Vav protein tagged with the influenza virus HA epitope and Grb2 protein tagged with a Myc epitope. Cell lysates from these cells were immunoprecipitated with either monoclonal anti-HA or anti-Myc antibodies. The immunoprecipitates were analyzed by Western blotting using either anti-Vav or anti-Grb2 antibody (Fig. 5). Some Vav was coimmunoprecipitated by antibody against Grb2–Myc protein (lane 3). In a control experiment, the anti-Myc antibody did not precipitate a significant amount of Vav from WEHI-231 cells, where Vav is highly expressed (data not shown). When anti-HA antibody was tested for its ability to coprecipitate Grb2 (lane 2), the experiment was indeterminate because in an exposure long enough to reveal a coimmunoprecipitated protein, a contaminating band was evident at the same position as Grb2 (Fig. 5B Lower). Thus, there is evidence that full-length Vav and Grb2 can form a complex in mammalian cells.

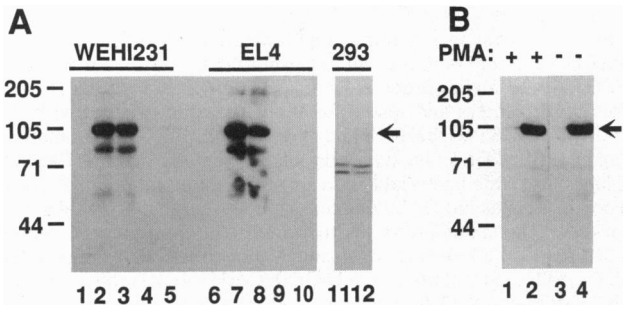

FIG. 4. Binding of endogenous Vav protein to immobilized GST–Grb2. (A) Twenty microliters (bed volume) of glutathione-conjugated agarose beads which were saturated with GST (lanes 1, 6, and 11), GST–full-length Grb2 (lanes 2 and 7), GST–Grb2/C-SH3 (lanes 3, 8, and 12), GST–Grb2/C-SH3N'del (lanes 4 and 9), or GST–Grb2/C-SH3C'del (lanes 5 and 10) was incubated with WEHI-231 cell lysates (lanes 1–5), EL4 cell lysates (lanes 6–10), or 293 cell lysates (lanes 11 and 12). Three micrograms of proteins was used for each sample. Proteins bound to beads were eluted and analyzed by Western blotting using a monoclonal anti-Vav antibody (Upstate Biotechnology). Arrow indicates the position of Vav. Molecular size markers are at left. (B) A culture of 1.6×10^8 WEHI-231 cells grown to a density of 5×10^5 per ml was divided in half. Phorbol 12-myristate 13-acetate (PMA, 10 ng/ml; Sigma) was added to one half and cells were incubated at 37°C for 10 min. Both cell samples were then harvested and lysed. PMA-stimulated cell lysates (lanes 1 and 2) and unstimulated cell lysates (lanes 3 and 4) were incubated either with GST (lane 1 and 3) or GST–Grb2/C-SH3 (lanes 2 and 4) adsorbed to agarose beads. The proteins eluted from the beads were analyzed as in A.

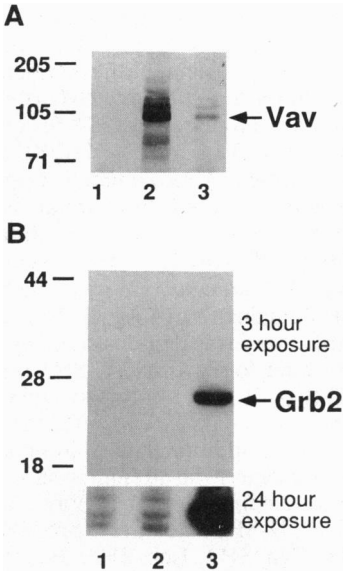

FIG. 5. Vav/Grb2 complex in mammalian cells. Cell lysates prepared either from NIH 3T3 cells (lanes 1) or from HA–Vav/Grb2–Myc-expressing 3T3 clone VG5/3T3 (lanes 2 and 3) were immunoprecipitated with monoclonal anti-HA antibody 12CA5 (lanes 2), anti-Myc antibody 9E10 (lanes 3), or a mixture of the two antibodies (lanes 1). Immunoprecipitates were analyzed by Western blotting using polyclonal anti-Vav antibody (A) or anti-Grb2 antibody (B) (both from Santa Cruz Biotechnology, Santa Cruz, CA). A longer exposure of the same blot in B (Lower) shows a contaminating band in the same position as Grb2, obscuring any possible coimmunoprecipitated Grb2.

DISCUSSION

In this study we have demonstrated that the hematopoietic cell-specific signaling molecule Vav can bind to the ubiquitous adaptor protein Grb2 through a highly specific dimerization of two intact SH3 domains. This interaction first became evident through a two-hybrid screen in yeast cells and was then studied *in vitro* by binding of the Grb2 C-terminal SH3 domain to the Vav SH3 domain immobilized on a filter. When either SH3 domain was truncated, binding was abolished. Binding of the full-length proteins was demonstrated, with GST–Grb2 selecting Vav from cell lysates of lymphoid cells. Using a 3T3 clone overexpressing both proteins, we also showed that a Vav/Grb2 complex can form in mammalian cells.

Vav is a complicated and ambiguous molecule. Its structural features imply that its SH2 domain should be able to link the activated tail of a membrane receptor to downstream

intracellular events through its apparent guanine nucleotide-exchange function (CDC24 homology). However, Vav also has the properties of an "adaptor" protein in that it has an SH3–SH2–SH3 segment as well as a PH domain (Fig. 1*A*). Our results suggest that its N-terminal SH3 domain may link it to Grb2 and thus potentially to an even more extensive network of interactions. A Sos/Grb2 complex is evident in many cells (reviewed in ref. 31), and Grb2's N-terminal SH3 domain is mainly responsible for Sos binding (27, 33). Since Vav binds to Grb2's C-terminal SH3 domain, it may be able to recruit both Grb2 and Sos into one multiprotein complex. This ternary protein complex should activate Ras, because Sos is a strong GDP/GTP exchanger for Ras. This scenario may explain a puzzle surrounding the biochemical function of Vav. The CDC24 homology region of Vav should activate Rho, not Ras, but it has been reported that Vav has GDP/GTP exchange activity on Ras (13, 14). It is possible that in those experiments, the Vav contained the Grb2/Sos complex. In fact, it was shown that a protein of ≈150-kDa, the molecular size of Sos, could be coimmunoprecipitated by anti-Vav antibody (13).

The Vav/Grb2 interaction entails an apparently novel mode of protein/protein interaction through SH3 dimerization. Previous cases of SH3 binding (20, 26, 32) have involved intact SH3 domains associating with a short, linear peptide containing multiple proline residues. In all of those cases, the site of SH3 binding could be deleted down to an ≈10-amino acid peptide. For the Vav/Grb2 interaction, however, we were unable to demonstrate binding to structures any shorter than an intact SH3 domain. The interaction could be similar to the dimer interface interaction between SH3 and SH2 domains in the Lck molecule (34). The interacting Vav and Grb2 SH3 domains contain multiple proline residues, so that the binding might still involve the same specificity of interaction seen in other cases, but if that is true, the proline-containing peptide must be "presented" by the intact SH3 and not by shorter fragments. We have, in fact, identified a proline-rich substrate to which the Vav N-terminal SH3 domain can bind (data not shown), suggesting that this same SH3 domain also functions to bind proline-containing sequences.

It is interesting that Grb2 can serve as a convergence point for Ras activation, where various factors send their inputs. In addition to Vav, signaling molecules such as Shc (35, 36), Abl (20), Bcr–Abl (37, 38), and IRS-1 (39–41) all bind to Grb2. Therefore Grb2 may be a focal point for cross talk between Ras and various signaling pathways. Because Vav is a molecule limited to hematopoietic cells, it may be that Vav provides a cell-specific mode of Ras activation appropriate to the developmental or activation functions of such cells.

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