

## Association of Bcr-Abl with the Proto-oncogene Vav Is Implicated in Activation of the Rac-1 Pathway\*

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Florian Bassermann‡, Thomas Jahn‡§, Cornelius Miething§, Petra Seipel, Ren-Yuan Bai, Sunita Coutinho, Victor L. Tybulewicz¶, Christian Peschel, and Justus Duyster||

From the Department of Internal Medicine III, Laboratory of Leukemogenesis, Technical University of Munich, 81675, Germany and the ¶National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

Vav is a guanine nucleotide exchange factor for the Rho/Rac family predominantly expressed in hematopoietic cells and implicated in cell proliferation and cytoskeletal organization. The oncogenic tyrosine kinase Bcr-Abl has been shown to activate Rac-1, which is important for Bcr-Abl induced leukemogenesis. Previous studies by Matsuguchi *et al.* (Matsuguchi, T., Inhorn, R. C., Carlesso, N., Xu, G., Druker, B., and Griffin, J. D. (1995) *EMBO J.* 14, 257–265) describe enhanced phosphorylation of Vav in Bcr-Abl-expressing Mo7e cells yet fail to demonstrate association of the two proteins. Here, we report the identification of a direct complex between Vav and Bcr-Abl in yeast, *in vitro* and *in vivo*. Furthermore, we show tyrosine phosphorylation of Vav by Bcr-Abl. Mutational analysis revealed that the SH2 domain and the C-terminal SH3 domain as well as a tetraproline motif directly adjacent to the N-terminal SH3 domain of Vav are important for establishing this phosphotyrosine dependent interaction. Activation of Rac-1 by Bcr-Abl was abrogated by co-expression of the Vav C terminus encoding the SH3-SH2-SH3 domains as a dominant-negative construct. Bcr-Abl transduced primary bone marrow from Vav knock-out mice showed reduced proliferation in a culture cell transformation assay compared with wild-type bone marrow. These results suggest, that Bcr-Abl utilizes Vav as a guanine nucleotide exchange factor to activate Rac-1 in a process that involves a folding mechanism of the Vav C terminus. Given the importance of Rac-1 activation for Bcr-Abl-mediated leukemogenesis, this mechanism may be crucial for the molecular pathogenesis of chronic myeloid leukemia and of importance for other signal transduction pathways leading to the activation of Rac-1.

The proto-oncogene p95 Vav-1 is expressed predominantly in hematopoietic cells (1). The protein consists of several conserved domains, among them a calponin homology domain, a Dbl homology domain, a pleckstrin homology domain, and a cysteine-rich region, as well as an Src homology 2 (SH2)<sup>1</sup> do-

main framed by two SH3 domains, suggesting a role for Vav in multiple signal transduction pathways (2, 3). Deletion of the N-terminal region encoding the calponin homology domain results in oncogenic activation of Vav (1). Vav has been shown to be important for the transduction of signals from the T-cell receptor; Vav knock-out animals have defects in T cell development, and their T-cells proliferate poorly and produce little IL-2 in response to T-cell receptor stimulation (4–7). In addition, Vav-deficient T-cells have specific defects in T-cell receptor-induced calcium fluxes and in the activation of the extracellular signal-related kinase (ERK), mitogen-activated protein kinase (MAPK), and NF- $\kappa$ B pathways (8). Vav is phosphorylated in response to T-cell receptor stimulation and to cytokines and growth factors like IL-3, SCF, platelet-derived growth factor, or granulocyte/macrophage colony-stimulating factor, suggesting that it is also involved in the signal transduction pathways activated by these receptors (9–12). Through this mechanism, the SH2 domain of Vav mediates association with the tyrosine kinase receptor itself or with cytoplasmic tyrosine kinases like Lyn, JAK, or ZAP-70 (3, 9, 11, 13). Vav has been shown to be a GDP/GTP exchange factor (GEF) for members of the Rho family of GTPases (14). It has been demonstrated that tyrosine phosphorylation of Vav *in vitro* and *in vivo* by the tyrosine kinase Lck can activate its Rac GEF activity (14). The GEF activity of Vav is encoded by the Dbl homology domain. In addition to tyrosine phosphorylation, products of phosphatidylinositol 3'-kinase activation have been proposed to contribute to the GEF activation of Vav via the PH domain (15). Rac activation leads to a wide array of cellular responses including membrane ruffling, JNK (c-Jun N-terminal kinase) activation, and transcriptional activation from Rho/Rac-responsive elements such as NF-AT and NF- $\kappa$ B.

Both the SH2 and SH3 domains of Vav have been shown to bind to a whole array of signaling molecules. Among them, as mentioned above, are tyrosine kinases such as Lck, JAK-2, and ZAP-70, which bind to the SH2 domain, and adapter molecules like Cbl, Grb2, and SLB70, binding to the SH3 domain. In addition, heterogeneous ribonucleoprotein (hnRNP), the focal adhesion protein zyxin, Ku-70, hnRNP-c, and hShah2 have been identified as binding partners of Vav (reviewed in Ref. 3).

The chimeric oncogene Bcr-Abl is crucial for the pathogenesis of chronic myeloid leukemia (CML) and a subset of acute lymphoblastic leukemia (16). The dysregulated tyrosine kinase activity of Bcr-Abl has been shown responsible for the transformation of fibroblasts and hematopoietic cells and for the

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‡ These authors contributed equally to this work.

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¶ To whom correspondence should be addressed: Dept. of Internal Medicine III, Technical University of Munich, Trogerstr. 32, D-81675 Munich, Germany. Tel.: 49-89-41404104; Fax: 49-89-41407432; E-mail: justus.duyster@lrz.tum.de.

<sup>1</sup> The abbreviations used are: SH2, Src homology 2; SH3, Src homol-

ogy 3; GEF, GDP/GTP exchange factor; CML, chronic myeloid leukemia; WT, wild type; FCS, fetal calf serum; SCF, stem cell factor; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; IMDM, Iscove's modified Dulbecco's medium; IL, interleukin; TS, temperature-sensitive.

induction of a CML-like disease in mice (17–20). Bcr-Abl contains several autophosphorylation sites, which can serve as docking sites for SH2 domain-containing signaling molecules, some of which may be critical for the transformation ability of Bcr-Abl. One of the best studied and defined interactions is the binding of Grb2 to tyrosine 177 in the Bcr part of Bcr-Abl, which links Bcr-Abl to Sos/Ras pathway (21, 22). In addition, a multitude of other adapter proteins and substrates of Bcr-Abl have been identified such as Grb10, Grb4, SHC, Crk, Crkl, phosphatidylinositol 3-kinase, and STAT (signal transducers and activators of transcription), which are thought to be involved in the molecular pathogenesis of CML (23–33). Thus, screening for Bcr-Abl-interacting proteins seems an efficient method of identifying molecules important for Bcr-Abl-induced leukemia.

Recently it has been shown that Bcr-Abl expression in Mo7e cells results in constitutive phosphorylation of Vav (11). However, the authors could not detect coprecipitation of Vav and Bcr-Abl. Instead it was reported that Vav associates and is phosphorylated by JAK-2 in these cells (11). In this paper, in contrast to the previous report, we demonstrate that Vav interacts directly with Bcr-Abl. This interaction is dependent on the tyrosine kinase activity of Bcr-Abl and an unusual binding mechanism involving a proline-rich region and the two SH3 domains framing the SH2 domain of Vav. We present evidence that complex formation of Bcr-Abl and Vav is necessary for activation of the Rac pathway by Bcr-Abl, which has been shown to be crucial for Bcr-Abl-mediated leukemogenesis (34).

#### MATERIALS AND METHODS

**Yeast Two-hybrid System**—The DNA for Bcr-Abl $\Delta$ Sal was cloned into the yeast expression vector BTM116 (35). A cDNA library derived from K562 cells was used (CLONTECH, Heidelberg, Germany) as the prey. Various Bcr-Abl mutants were used in the screen as described previously (23, 24).

**Plasmid Construction**—For *in vitro* translation and expression in 293 cells, the cDNAs of Bcr-Abl WT, Bcr-Abl mutants, F46Wt, and the F46 mutants were cloned in pCDNA 3.1, a mammalian expression vector containing the cytomegalovirus promoter and the SV40 origin of replication (Invitrogen). Cloning of the corresponding human F46/Vav mutant into the full-length murine Vav c-DNA generated VavR696L and VavAA $\Delta$ CSH3. Expression of these chimerical constructs was performed using the pCMV Tag II vector (Invitrogen). Tyrosine to phenylalanine mutants Bcr-Abl/115F, Bcr/177F-Abl, Bcr/246F-Abl, Bcr/246F-Abl/449F, Bcr/246F-Abl/342F, 449F, Bcr/246F-Abl/353F, 449F, and Bcr/177F-Abl/393 were generated by site-directed mutagenesis using overlapping oligonucleotides and *Pfu* DNA polymerase (Stratagene, Heidelberg, Germany). The numbers correspond to the human Bcr and murine c-Abl sequence. The Bcr-Abl temperature-sensitive mutant (TS-Bcr-Abl) was generated by subcloning a temperature-sensitive v-Abl mutant (DP) into Bcr-Abl (23). The Bcr/1–509-Abl, Bcr/1–242-Abl, and Bcr/1–63-Abl constructs have been described previously (4). Point mutations of the F46/Vav cDNA were conducted using the QuikChange mutagenesis kit following the instructions of the supplier (Stratagene), and deletion mutants were made by PCR using oligonucleotides containing *EcoRI* and *XhoI* restriction sites and standard cloning procedures. All constructs derived from site-directed mutagenesis and PCR were verified by automatic sequence analysis.

**Cell Culture and Transfections**—293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% FCS and penicillin/streptomycin (200 units of penicillin/ml and 200  $\mu$ g of streptomycin/ml). K562, Jurkat, Mo7e, and Mo7e/p210 cells were maintained in RPMI 1640 medium (Invitrogen) with 10% fetal calf serum (Seromed, Berlin, Germany) and penicillin/streptomycin. The medium for Mo7e was supplemented with granulocyte/macrophage colony-stimulating factor (R&D Systems DPC Biernan GmbH, Wiesbaden, Germany). SCF stimulation of Mo7e cells was performed at a final concentration of 200 ng/ml SCF at 37 °C for 5 min and subsequent resuspension in ice-cold phosphate-buffered saline. Transfections were performed with the DOTAP transfection reagent (Roche Molecular Biochemicals).

**GST Fusion Proteins and Pull-down Assay**—The cDNAs of F46/Vav and F46/Vav mutants were cloned in-frame into the vector pGEX KG to

make GST (glutathione *S*-transferase) fusion proteins. WT and mutant Bcr-Abl proteins were *in vitro* translated and  $^{35}$ S-radiolabeled using the TNT system (Promega, Heidelberg, Germany). The translation mix was diluted to a final concentration of 25 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 100  $\mu$ M cold ATP and incubated for 30 min at 4 °C to allow for autophosphorylation of the translated proteins. Reactions were stopped by dilution to a final concentration of 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml each phenantroline, aprotinin, leupeptin, and pepstatin (lysis buffer). GST fusion proteins were added and incubated for 1 h at 4 °C. Protein complexes were collected on glutathione-agarose beads (Amersham Biosciences, Inc.), washed thoroughly with NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine), and subjected to SDS-PAGE. *In vitro* translated proteins were visualized by autoradiography. For binding experiments with cell extracts,  $1 \times 10^7$  cells were solubilized in lysis buffer, precleared with glutathione-beads, and incubated with the GST fusion protein for 3 h at 4 °C. Protein complexes were collected on glutathione-beads, washed thoroughly with lysis buffer or NETN buffer, and subjected to SDS-PAGE. Immunoblotting was performed with the antibodies indicated.

**Antibodies**—Bcr-Abl and Abl was detected by immunoblotting using an Abl-specific antibody 8E9 (BD Pharmingen, Hamburg, Germany). Tyrosine phosphorylation was detected with the monoclonal anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology, Lake Placid, NY), and PY20 (Transduction Laboratories, Lexington, KY). Polyclonal anti-Vav and anti-Rac-1 antibodies came from Santa Cruz Biotechnology, Heidelberg, Germany. Monoclonal anti-Xpress<sup>®</sup> and anti-Flag antibodies were from Invitrogen.

**Immunoprecipitation and Immunoblotting**—Immunoprecipitation was done as described previously (36). Briefly,  $1 \times 10^7$  cells were solubilized in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10  $\mu$ g/ml each phenantroline, aprotinin, leupeptin, and pepstatin. After clarification by centrifugation, antibody-protein complexes were brought down with 30  $\mu$ l of protein A-Sepharose (Amersham Biosciences, Inc.). Immunoprecipitations were analyzed by SDS-PAGE followed by immunoblotting with anti-Abl (8E9), monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology), PY20 (Transduction Laboratories), anti-Flag (Invitrogen), and polyclonal anti-Vav antibodies (Santa Cruz) as described previously (36). Bands were visualized using the ECL system (Amersham Biosciences, Inc.).

**In Vitro Kinase Assay**—Bcr-Abl and kinase-defective Bcr-Abl were *in vitro* translated using the TNT system (Promega). The translation mix was diluted to a final concentration of 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10  $\mu$ g/ml each phenantroline, aprotinin, leupeptin, and pepstatin. Immunoprecipitation was performed with 2  $\mu$ g of anti-Abl (Ab-3). The immune complexes were collected on protein A-Sepharose beads, washed twice each with lysis buffer containing 500 mM NaCl, lysis buffer plus 100 mM NaCl, and lysis buffer alone and twice with kinase buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol. The pellet was resuspended in 20  $\mu$ l of kinase buffer with 1  $\mu$ g of GST-F46/Vav as a substrate. Reactions were started by the addition of 15  $\mu$ M cold ATP and 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP and incubated for 30 min at 30 °C. Reactions were stopped by the addition of Laemmli buffer, heated at 95 °C for 10 min, and resolved by SDS-PAGE.

**Rac-1 Activity**—293 cells ( $3 \times 10^5$ ) were cotransfected in 60-mm dishes using the DOTAP transfection reagent (Roche Molecular Biochemicals) with 3  $\mu$ g of plasmid encoding Rac-1, 2.5  $\mu$ g of the plasmid expressing WT-Bcr-Abl, KD-Bcr-Abl, or the control vector, and 5  $\mu$ g of the dominant-negative construct Vav/F46 or Vav/F46AA $\Delta$ CSH3 or the control vector. 65 h post-transfection, cells were starved in FCS-free media and harvested 12 h later. Cell lysates were precleared with glutathione-beads and incubated with equal amounts of GST-PAK for 1 h at 4 °C. Samples were separated by SDS-PAGE, and proteins were detected with a Rac-1 specific antibody (Santa Cruz). To normalize transfection efficiencies, lysates were analyzed by immunoblotting with anti-Rac-1 (Santa Cruz), anti-Abl 8E9 (BD Pharmingen), and anti-Xpress<sup>®</sup> (Invitrogen).

**Generation of Retrovirus Stocks**—Retroviral supernatant was produced by transiently transfecting the Phoenix ecotropic producer line (Gary Nolan, Stanford, CA) with the Mig-p210 or Mig vectors (W. Pear, Philadelphia, PA) as described previously (37). Medium was changed at 36 h after transfection and collected after 48 h. Titers of  $\sim 1 \times 10^5$ – $1 \times 10^6$  helper virus-free viral particles were obtained as measured by

TABLE I  
Binding of Vav/F46 mutants to Bcr-Abl

Equal amounts of GST fusion proteins of the represented Vav/F46 mutants were tested for their binding affinity to Bcr-Abl from K562 lysates of  $5 \times 10^6$  cells in a GST pull-down assay as described in Fig. 4. The amino acid numbers of the F46/Vav fragments are indicated. +++, strong binding; (+), very weak binding; -, no binding. R696L is an SH2 mutant defective in phosphotyrosine binding. AASH3-SH2-SH3 is a proline-to-alanine mutant of four prolines (amino acids 607–610). Dbl, Dbl homology; PH, pleckstrin homology; SH, Src homology; p-rich, proline-rich.

		Bcr-Abl binding
SH2	655–775	-
SH2-SH3	655–845	(+)
SH3-SH2	614–775	-
SH3-SH2-SH3	614–845	(+)
PPSH3-SH2	591–775	-
PPSH3-R696L-SH3	591–845	-
PPSH3-SH2-SH3	591–845	+++
AASH3-SH2-SH3	591–845	(+)
AAF46	560–845	(+)
F46	560–845	+++

	SH2	SH3	SH3
	Vav/F46		
	655	775	845
	p-rich		
	Dbl		
	PH		
	PPPP		
	SH3		
	SH2		
	SH3		
	845		

fluorescence-activated cell sorter titer of EGFP-positive infected Rat-1 cells.

**Soft Agar Assays**— $2 \times 10^4$  cells were seeded onto each 60-mm culture dish in IMDM containing 10% FCS and 0.1% agarose on a base of bottom agar (IMDM, 10% FCS, 0.6% agar). The growth layer was allowed to harden and was subsequently overlaid with a top agar layer (IMDM, 10% FCS, 0.1% agar). Colonies were analyzed ~2 weeks after plating.

**Bone Marrow Transformation Assay**—Wild-type and Vav<sup>-/-</sup> mice (4) were treated with 150 mg/kg 5-fluorouracil 4 days prior to bone marrow harvest. BM cells flushed from tibia and femur were preincubated for 1 day in bone marrow medium (IMDM, Invitrogen) supplemented with 30% FCS (Seromed), 1% bovine serum albumin, 1% glutamine, 0.5% penicillin-streptomycin, 100  $\mu$ M 2-mercaptoethanol, and 1  $\mu$ M hydrocortisone) 50 ng/ml mouse SCF, 8 ng/ml mouse IL-3, and 12 ng/ml human IL-6 (all from R&D Systems). After 1 day of preincubation,  $1 \times 10^6$  bone marrow cells were coincubated with 3 ml of viral supernatant and 1 ml of new medium in the presence of the above mentioned growth factors and 4  $\mu$ g/ml polybrene. New supernatant was added after 24 h. 3 days after infection, cells were washed in phosphate-buffered saline. Transformation of bone marrow cells in liquid culture was determined as described previously (39). Briefly,  $1 \times 10^5$  infected cells were plated in bone marrow medium in 12-well plates in a volume of 2 ml. Nonadherent cells were counted after 9 days.

## RESULTS

**A Vav Fragment Encoding the C-terminal SH3-SH2-SH3 Domain Interacts Specifically with Bcr-Abl in a Phosphotyrosine-dependent Manner**—To isolate signaling intermediates important for the oncogenic signal transduction of Bcr-Abl, a yeast two-hybrid screen was performed using Bcr-(1–509)-Abl/Lex A as a bait and a cDNA library obtained from a cell line established from a CML patient in blast crisis of the disease (K562). An identical 800-bp clone was isolated four times, which encoded the C-terminal SH3-SH2-SH3 region of p95 Vav (F46/Vav, amino acids 560–845) (Table I). Co-expression of kinase-active Bcr-Abl and F46/Vav resulted in histidine autotrophy and growth of the yeast cells, indicating interaction of Bcr-Abl with F46/Vav (Fig. 1A). The interaction of Vav/F46 and Bcr-Abl was specific, as the control protein lamin showed no binding (Fig. 1A). Yeast cells co-expressing F46/Vav and a tyrosine kinase-defective mutant of Bcr-Abl (KDBcr-Abl) failed to grow, indicating that Bcr-Abl autophosphorylation is required for this interaction (Fig. 1A). Binding of F46/Vav to Bcr-Abl in

A

Constructs	-TL*	-TLH†
F46/Vav + WT Bcr-Abl	+	+
F46/Vav + KD Bcr-Abl	+	-
F46/Vav + lamin (control)	+	-

B

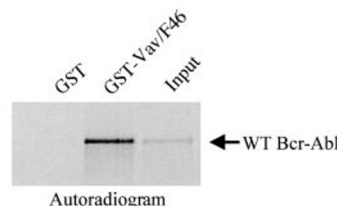
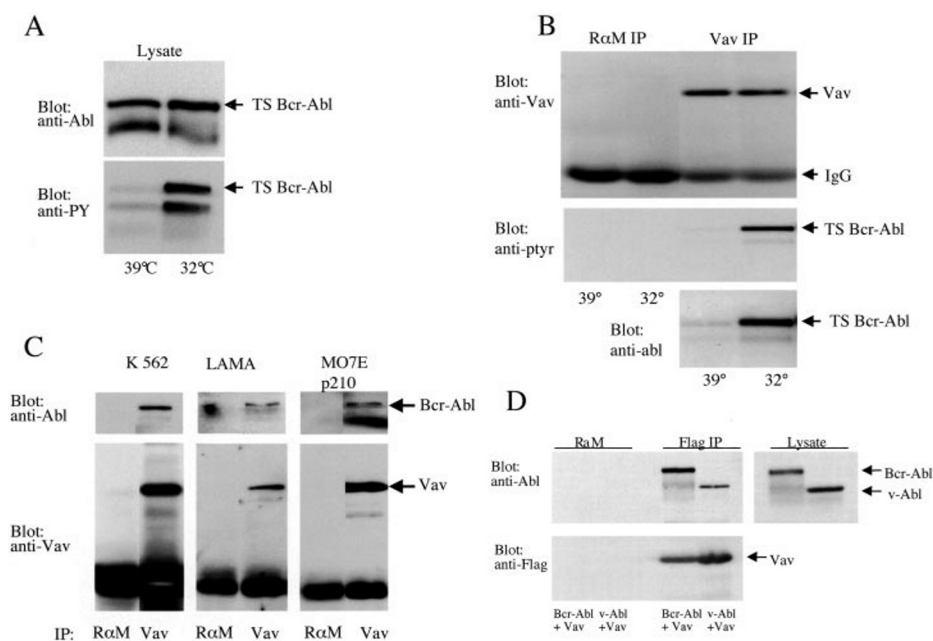


FIG. 1. A, A C-terminal Vav fragment binds to kinase-active Bcr-Abl in yeast. \*, growth under tryptophan and leucine selection reflecting the transfection efficiency; †, growth under additional histidine selection reflecting the specificity of interaction. B, the Vav/F46 fragment binds directly to Bcr-Abl *in vitro*. Bcr-Abl was *in vitro* translated and <sup>35</sup>S-labeled. 50  $\mu$ l of the translation mix was allowed to autophosphorylate and was then incubated with 5  $\mu$ g of GST-Vav/F46 or GST alone as a control. Input (right lane) and bound fractions on GST-Vav/F46 (middle lane) and GST as control (left lane) were resolved by SDS-PAGE and visualized by autoradiography.

yeast suggested a direct interaction between Vav and Bcr-Abl. To confirm a direct association of Bcr-Abl and Vav/F46, we tested the ability of GST-Vav/F46 to bind to *in vitro* translated Bcr-Abl. Fig. 1B shows that Vav/F46 is able to directly bind to Bcr-Abl *in vitro*. Moreover, we found two constructs carrying internal deletions in the Bcr part of Bcr-Abl (BCR/1–242-Abl and BCR/1–63-Abl) to bind to GST-Vav/F46 (data not shown). BCR/1–63-Abl is missing the autophosphorylation site Tyr<sup>177</sup> responsible for the binding of Bcr-Abl to Grb2, indicating that an Abl phosphorylation site other than Tyr<sup>177</sup> is responsible for this phosphotyrosine-dependent interaction. These results together with the data obtained in yeast suggested that the Vav SH2 domain binds to an autophosphorylation site located in the Abl part of Bcr-Abl, which is different from Tyr<sup>177</sup>. Concordant with this hypothesis, complex formation of Vav could also be demonstrated with v-Abl (Fig. 2D). One tyrosine residue within the Abl portion of Bcr-Abl resembles, at least in part, the predicted consensus site for the Vav SH2 domain, YMEP, which is tyrosine 115 with the sequence -YITP- (40). In an attempt to identify the tyrosine residue in Abl that is responsible for complex formation with Vav, a series of tyrosine to phenylalanine mutations and combinations thereof were introduced into Bcr-Abl. These mutants, including Abl Tyr<sup>115</sup>, Abl Tyr<sup>393</sup>, the major Abl autophosphorylation site, and the Grb2 binding site, Bcr Tyr<sup>177</sup>, were tested for their ability to bind to the Vav/F46 fragment in a GST pull-down assay (Abl numbers refer to the Abl Ia isoform). All of the tested tyrosine-to-phenylalanine mutants retained the ability to bind Vav/F46 (data not shown). Thus, the complex between Vav and Bcr-Abl may involve multiple tyrosine residues or involve other binding motifs and intermediate proteins (for example, c-Cbl). Alternatively the binding may be dependent on a so-called “open” conformation that can only be adopted by kinase-active Bcr-Abl, as shown by the paper of Schindler *et al.* (41).

**Only Kinase-active Bcr-Abl Forms a Complex with Vav in Vivo**—To demonstrate the complex of Vav and Bcr-Abl *in vivo* and to determine the role of tyrosine kinase activity, a temperature-sensitive (TS) mutant of Bcr-Abl was transiently expressed in 293 cells. These cells express endogenous p95 Vav. Bcr-Abl was expressed to comparable levels at the restrictive and permissive temperature in these cells (Fig. 2A, upper panel). Shifting to the permissive temperature (32 °C) for 3 h



**FIG. 2. Vav interacts with Bcr-Abl and v-Abl *in vivo*.** *A*, a temperature-sensitive mutant of Bcr-Abl (TS Bcr-Abl) was transiently transfected into 293 cells. TSBcr-Abl-transfected 293 cells were kept at 39 °C (restrictive temperature) or shifted to 32 °C (permissive temperature) for 4 h. Cell lysates were run on SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody (PY20) (upper panel) or an anti-Abl antibody (8E9) (lower panel). *B*, cell lysates from  $1 \times 10^7$  TSBcr-Abl-transfected 293 cells kept at the restrictive (39 °C) or permissive (32 °C) temperature were immunoprecipitated with an anti-Vav antibody or a rabbit anti-mouse (RaM) antibody as control. Protein complexes were washed thoroughly and resolved by SDS-PAGE followed by immunoblotting with anti-Vav antibody (upper panel), anti-phosphotyrosine antibody (anti-ptyr, middle panel), or anti-Abl antibody (lower panel). *C*, lysates of  $5 \times 10^6$  K562 cells (left panel), LAMA cells (middle panel), and MO7E overexpressing Bcr-Abl (right panel) were immunoprecipitated with anti-Vav antibody or rabbit anti-mouse antibody as control. Bound fractions were resolved by SDS-PAGE and immunoblotted with anti-Vav antibody. *D*, 293 cells were transiently cotransfected with v-Abl or Bcr-Abl together with Flag-tagged full-length Vav (Vav). Cell lysates were prepared and immunoprecipitated with rabbit anti-mouse antibody as control or anti-Flag antibody to precipitate Flag-tagged Vav. Precipitated proteins (upper left and lower left panels) and lysates (right panel) were run on SDS-PAGE and visualized with anti-Abl and anti-Flag antibodies as indicated.

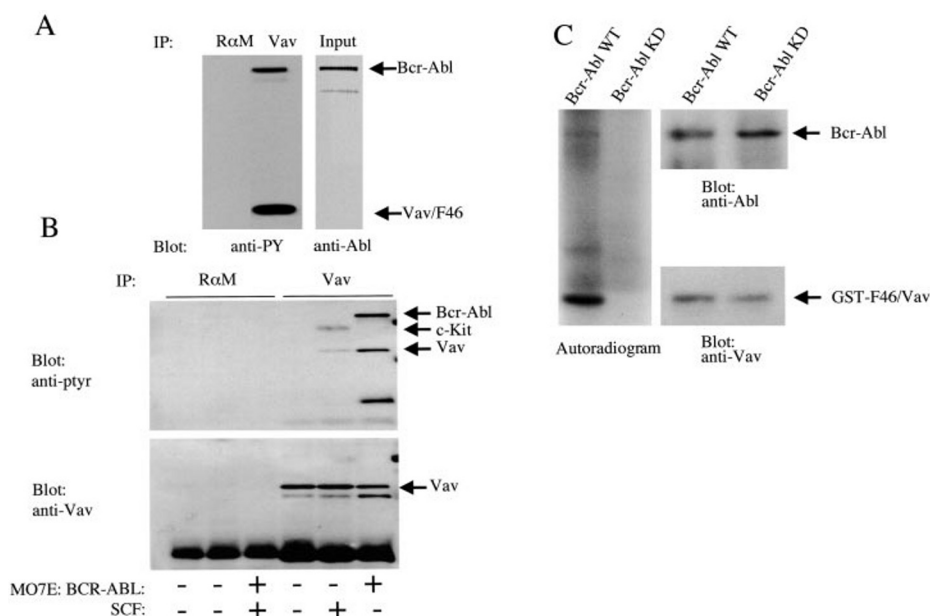
induced tyrosine kinase activity of TSBcr-Abl as anticipated (Fig. 2*A*, lower panel). Cells kept at the restrictive (39 °C) or shifted to the permissive temperature (32 °C) were analyzed by an anti-Vav immunoprecipitation (Fig. 2*B*). Equal amounts of Vav were precipitated at both temperatures (Fig. 2*B*, upper panel). The phosphotyrosine blot shows a highly phosphorylated protein coprecipitating with Vav only at the permissive temperature (Fig. 2*B*, middle panel). This protein was proven to be TSBcr-Abl by probing the blot with an anti-Abl antibody (Fig. 2*B*, lower panel). Only trace amounts of TSBcr-Abl were precipitated at the restrictive temperature, probably because of some leaky kinase activity at 39 °C. The same results were obtained in a GST pull-down assay with GST-F46/Vav and TS Bcr-Abl (data not shown). Thus, Bcr-Abl and Vav are complexed *in vivo* in a phosphotyrosine-dependent manner, confirming the data obtained in yeast and *in vitro*.

**Vav Forms a Complex with Bcr-Abl in the CML Cell Lines K562 and Lama**—To demonstrate the Vav-Bcr-Abl complex in CML cells, co-immunoprecipitation experiments were performed using two cell lines established from patients with Bcr-Abl-positive CML and a hematopoietic cell line engineered to overexpress Bcr-Abl. The anti-Vav antibody was able to precipitate a complex of Vav and Bcr-Abl in all three cell lines (Fig. 2*C*). This complex formation is specific because no Bcr-Abl protein was detected using an unrelated rabbit antiserum (Fig. 2*C*). Similar complex formation of endogenous Vav and Bcr-Abl was observed in other cell lines expressing Bcr-Abl (data not shown).

**Vav Forms a Complex with v-Abl**—If complex formation of Bcr-Abl and Vav is mediated by an autophosphorylation site located in the Abl part of Bcr-Abl, a kinase-active Abl construct should also show complex formation with Vav. To test this

theory we performed co-immunoprecipitation experiments with cells overexpressing Bcr-Abl, v-Abl (v-Abl is activated by deletion of the Abl SH3 domain), and Flag-tagged Vav. Both Bcr-Abl and v-Abl were co-immunoprecipitated using an antibody recognizing tagged Vav (Fig. 2*D*), supporting the notion that the Bcr-Abl-Vav complex is mediated by the Abl part of Bcr-Abl.

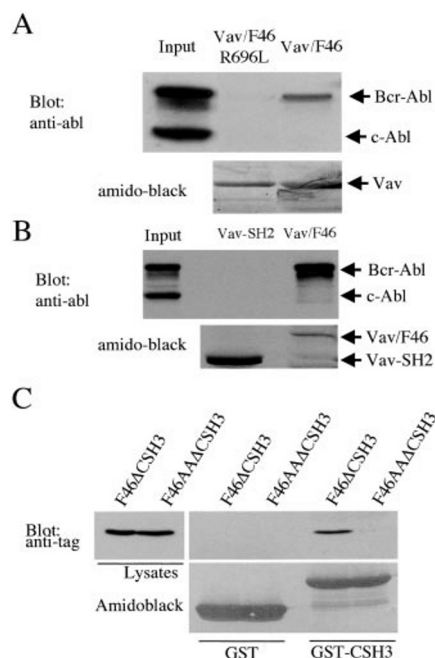
**Vav Serves As a Direct Substrate for the Bcr-Abl Tyrosine Kinase**—The isolated C-terminal tail of Vav (F46) contains several potential phosphorylation sites, among which are sites matching the consensus Abl substrate phosphorylation site (42). To determine whether complex formation between Bcr-Abl and Vav leads to phosphorylation of Vav by Bcr-Abl, we expressed the tagged C-terminal tail of Vav (F46/Vav) together with Bcr-Abl in 293 cells. An immunoprecipitation using an anti-tag antibody was performed and analyzed by anti-phosphotyrosine immunoblotting (Fig. 3*A*). Only two phosphorylated bands were detectable representing the immunoprecipitated Vav tail itself and complexed Bcr-Abl. To determine the phosphorylation status of endogenous Vav in response to Bcr-Abl in hematopoietic cells, we immunoprecipitated equal amounts of Vav from parental Mo7e cells and Mo7e cells overexpressing Bcr-Abl (Fig. 3*B*). SCF stimulation in parental Mo7e cells leads to tyrosine phosphorylation of Vav (Fig. 3*B*, upper panel, second lane from right). Mo7e cells expressing Bcr-Abl show significantly enhanced levels of Vav phosphorylation compared with parental Mo7e cells maximally stimulated with SCF (Fig. 3*B*, compare the two rightmost lanes). Again, Bcr-Abl coprecipitated with Vav together with a phosphorylated ~50-kDa protein of unknown identity. The resulting complexes were specific because no phosphorylated protein could be detected using a nonspecific rabbit antiserum (Fig. 3*B*, left



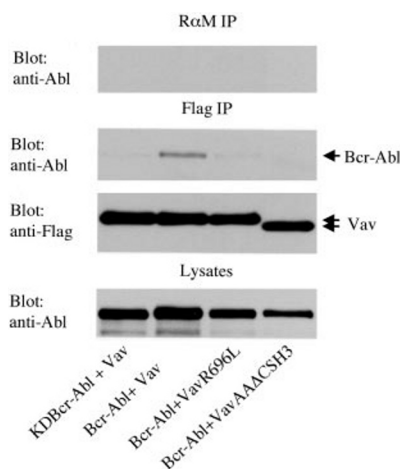
**FIG. 3. The Vav/F46 fragment serves as a substrate for Bcr-Abl.** A, Bcr-Abl and Vav/F46 were transiently cotransfected into 293 cells. Cell lysates were immunoprecipitated with monoclonal anti-tag antibody to detect F46/Vav or rabbit anti-mouse (RαM) antibody as control. Immunoprecipitates (IP) were analyzed by Western blotting using monoclonal anti-phosphotyrosine antibodies (a mixture of 4G10 and PY20) (left panel). The input was analyzed using anti-Abl antibody (right panel). B, Mo7E stably transfected with Bcr-Abl or empty vector were stimulated with 200 ng/ml SCF for 5 min as indicated. Cell lysates were prepared and immunoprecipitated with a polyclonal anti-Vav antibody or rabbit anti-mouse (RαM) antibody as control. Immunoprecipitates were analyzed by Western blotting using monoclonal anti-phosphotyrosine antibody (upper panel) or anti-Vav antibody (lower panel). C, wild-type (WT) and kinase-defective KD-Bcr-Abl were *in vitro* translated, immunoprecipitated, and incubated with GST-F46/Vav in the presence of [ $\gamma$ - $^{32}$ P]ATP in kinase buffer. The reaction products were separated by SDS-PAGE and autoradiographed (left panel). Equal levels of Bcr-Abl or KDBcr-Abl (upper right panel) and GST-F46/Vav (lower right panel) were ascertained by Western blotting as described.

half). To reconstitute this kinase reaction, *in vitro* purified Vav and purified kinase-active and kinase-defective Bcr-Abl were used in an *in vitro* kinase reaction (Fig. 3C). Kinase-active but not kinase-defective Bcr-Abl incorporated  $^{32}$ P into F46/Vav (Fig. 3C, left panel). Thus, the C-terminal tail of Vav serves as a direct substrate of Bcr-Abl *in vitro*.

**SH2-dependent Binding of Bcr-Abl Requires Both the Proline-rich Region Upstream of the N-terminal SH3 Domain and the C-terminal SH3 Domain**—To define the domains of Vav which are required for the phosphotyrosine-dependent binding to Bcr-Abl different mutants of F46/Vav were tested for their ability to form a complex with Bcr-Abl from lysates of K562 cells. Vav/F46R696L carries a point mutation in the SH2 domain replacing the arginine of the central FLVR motif with leucine, resulting in a nonfunctional SH2 domain. As anticipated, this mutant is no longer able to interact with Bcr-Abl (Fig. 4A, upper panel). However, surprisingly, the isolated Vav SH2 domain failed to bind Bcr-Abl (Fig. 4B, upper panel). Further binding studies utilizing different truncated mutants of F46/Vav as shown in Table I could delineate the region minimally required for binding to the SH3-SH2-SH3 formation including a short 4-amino acid proline repeat upstream of the N-terminal SH3 domain of Vav (amino acids 607–610). This proline repeat was absolutely essential for the complex formation of Bcr-Abl and Vav because a mutant in which the tetraproline motif was replaced by alanines no longer bound Bcr-Abl (AASH3-SH2-SH3, Table I). In addition, deletion of the C-terminal SH3 domains rendered F46/Vav equally unable to bind Bcr-Abl (PPSH3-SH2, Table I). These results indicated that in addition to the Vav SH2 domain, the C-terminal SH3 domain and the proline repeat play an equally important role. The Abl SH3 domain is not able to bind to the Vav proline-rich region, thus excluding an Abl SH3-mediated binding to Vav (data not shown). However, the isolated C-terminal SH3 domain of Vav is capable to bind the F46/Vav fragment



**FIG. 4. The Vav SH2 domain is required but not sufficient to mediate the complex with Bcr-Abl in K562 cells.** Lysates of  $5 \times 10^6$  K562 cells were incubated with GST-Vav/F46 and GST-Vav/F46R696L (SH2-mutant) (A) or GST-Vav-SH2 (B). Input and bound fractions were resolved by SDS-PAGE. Proteins were visualized by immunoblotting with anti-Abl antibody and by Amido Black staining as indicated. C, 293 cells were transiently transfected with Xpress<sup>®</sup>-tagged F46 lacking the C-terminal SH3 domain (F46ΔCSH3) or F46 with a proline-to-alanine substitution of 4 proline residues and a deletion of the C-terminal SH3 domain (F46ΔAΔCSH3). Cell lysates were prepared and incubated with GST-F46CSH3. Input and bound fractions were resolved by SDS-PAGE. Proteins were visualized by immunoblotting with anti-Xpress<sup>®</sup> antibody and by Amido Black staining as indicated.



**FIG. 5. The Vav SH2 and SH3 domain and a proline-rich region are required for the Vav/Bcr-Abl complex *in vivo*.** 293 cells were transiently transfected with wild-type Bcr-Abl or kinase-defective Bcr-Abl (*KDBcr-Abl*) together with Flag-tagged full-length Vav (*Vav*), Flag-tagged full-length Vav with a mutation in the SH2 domain (*VavR696L*), or Flag-tagged full-length Vav with a proline-to-alanine substitution of 4 proline residues and a deletion of the C-terminal SH3 domain (*VavAAΔCSH3*). Cell lysates were prepared and immunoprecipitated (*IP*) with rabbit anti-mouse (*RαM*) antibody as control (*upper panel*) or anti-Flag antibody to precipitate Flag-tagged Vav (*middle two panels*). Precipitated proteins and lysates (*lower panel*) were run on SDS-PAGE and visualized with anti-Abl and anti-Flag antibodies as indicated.

(F46ΔSH3) in a pull-down assay (Fig. 4C). This binding is mediated by the proline repeat, because a F46/Vav mutant in which the prolines have been replaced by alanines (F46AAΔSH3) no longer was complexed by the Vav SH3 domain (Fig. 4C). This finding supports the hypothesis of an intramolecular folding mechanism involving the C-terminal SH3 domain and the short proline-rich domain, which enables the SH2 domain to develop its full binding potential. Similarly stronger binding of Vav to ZAP-70 and to other tyrosine phosphorylated proteins required the proline-rich region upstream of the N-terminal SH3 domain (data not shown). Thus, the general binding capacity of the Vav SH2 domain is influenced by the adjacent SH3 domains and the proline repeat.

**Mutational Analysis of the *in Vivo* Complex Formation between Vav and Bcr-Abl**—To determine whether the SH3 domains and the proline repeat are also required for the *in vivo* complex formation between Vav and Bcr-Abl, we introduced the mutations into full-length Vav. Co-expression of WT Vav with kinase-active and -inactive Bcr-Abl confirmed the phosphorylation dependence of the interaction (Fig. 5, *first two lanes*). Mutation of the Vav-SH2 domain (*VavR696L*) or deletion of the C-terminal SH3 domain and mutation of the prolines (*VavAAΔSH3*) resulted in abrogation of the complex formation between Bcr-Abl and Vav *in vivo* (Fig. 5, *last two lanes*).

**The C-terminal Vav Tail Has a Dominant-negative Effect on Bcr-Abl-induced Rac Activation**—Bcr-Abl has been reported to activate Rac (34). Vav is a GEF for Rac. Therefore, it is possible that Bcr-Abl-induced Rac activation is mediated by binding and phosphorylation of Vav through Bcr-Abl. To test this hypothesis we employed a pull-down assay for GTP-bound Rac using the p21-binding domain of p21-activated kinase 1, which has been shown to specifically bind Rac-GTP (43). With this assay the amount of precipitated GTP-Rac in a cell can easily be quantified by anti-Rac immunoblotting.

Bcr-Abl expression in 293 cells leads to Rac activation (Fig. 6). Co-expression of the C-terminal Vav fragment resulted in inhibition of Bcr-Abl induced Rac activation, suggesting a dominant-negative function of the Vav tail. Coexpression of a C-terminal fragment incapable to bind Bcr-Abl had no dominant-

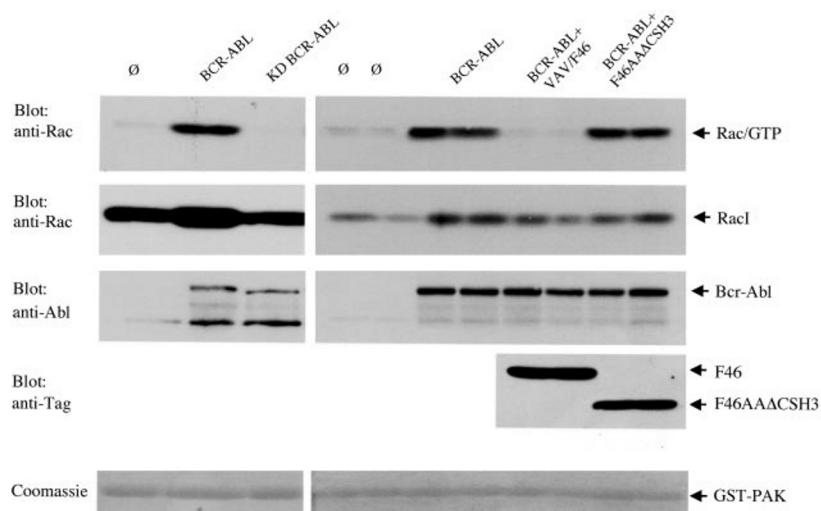
negative effect. These data indicate that Bcr-Abl-induced Rac activation is mediated by the Bcr-Abl-Vav complex and probably by Vav phosphorylation because a kinase-defective Bcr-Abl could not activate Rac (Fig. 6) (34).

**Bcr-Abl Utilizes Vav to Transform Primary Bone Marrow Cells**—Because the isolated C-terminal tail of Vav showed dominant-negative activity on Bcr-Abl-induced Rac activation and Rac activation has been shown to be important for the oncogenic potential of Bcr-Abl, we wished to determine the importance of Vav for Bcr-Abl-induced mitogenicity. We first investigated whether Vav1 overexpression in fibroblasts could contribute to the transformation potential of Bcr-Abl in NIH3T3 fibroblasts. The transformation property was scored by anchorage-independent growth in soft agar. In the absence of Bcr-Abl, neither the control vector nor Vav gave rise to morphologically transformed foci. Retroviral infection of Bcr-Abl induced outgrowth of soft agar colonies in both the control vector and two different Vav-expressing stable NIH3T3 cell lines. No significant difference in colony number and size was observed between these entities (Fig. 7). Next we used bone marrow from wild-type and Vav knock-out mice, which was retrovirally transduced with Bcr-Abl, to perform a bone marrow transformation assay as previously described (39). Primary cells were retrovirally transformed with Bcr-Abl, and the outgrowth of cells was measured. When analyzed for EGFP fluorescence, the liquid cultures of Bcr-Abl-transformed bone marrow cells of both the Vav-deficient mice as well as the wild-type cells than the mock-EGFP-infected bone marrow cultures (data not shown). Bcr-Abl induces the outgrowth of transformed cells in wild-type bone marrow (Fig. 8, *left panel*). However, in Vav knock-out bone marrow, Bcr-Abl-induced bone marrow growth is severely impaired (Fig. 8). Both control wild-type and Vav knock-out bone marrow showed comparable growth after the addition of IL-3 (Fig. 8). Thus, although Bcr-Abl also mediates a proliferative advantage in Vav-deficient bone marrow cells, efficient transformation of primary bone marrow cells by Bcr-Abl requires Vav.

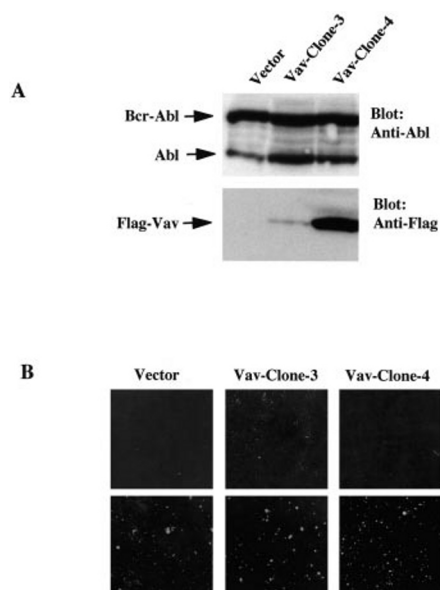
## DISCUSSION

We report here that p95 Vav interacts directly with Bcr-Abl in yeast, both *in vitro* and *in vivo*. This interaction was identified in a yeast two-hybrid screen using Bcr-Abl as bait and a cDNA library from the CML cell line K562 as prey. An 800-bp clone was isolated that interacted with Bcr-Abl and encoded for the C-terminal SH3-SH2-SH3 region of p95 Vav. Deletion mutants show that the interaction occurs at an Abl autophosphorylation site within Bcr-Abl. Although the Abl sequence within Bcr-Abl contained a consensus binding site for the Vav SH2 domain, mutation of that site and combinations thereof did not abolish binding suggesting that additional domains may be involved. An Abl-SH2-pTyr interaction contributing to the complex is possible because the Vav C terminus contains several Abl-SH2 consensus binding sites and is heavily phosphorylated (44). A Vav GST fusion protein encoding this region was shown to bind strongly to Bcr-Abl but not c-Abl using whole cell lysates of Bcr-Abl-expressing K562 cells. This probably reflects the poor autophosphorylation status of c-Abl in normally cycling cells. A recent study solved the crystal structure of the Abl kinase domain binding to the inhibitor STI571 (41). Whereas kinase-active Abl displays an open conformation of the kinase domain, a pseudo-substrate loop is observed in kinase-inactive Abl. Thus, binding of Vav to Bcr-Abl may depend on a conformation that can only be adopted by the active Bcr-Abl.

Co-immunoprecipitation studies clearly showed that Vav forms a complex with Bcr-Abl in the CML cell line K562 and LAMA cells, as well as in Bcr-Abl-expressing Mo7e/p210 cells,

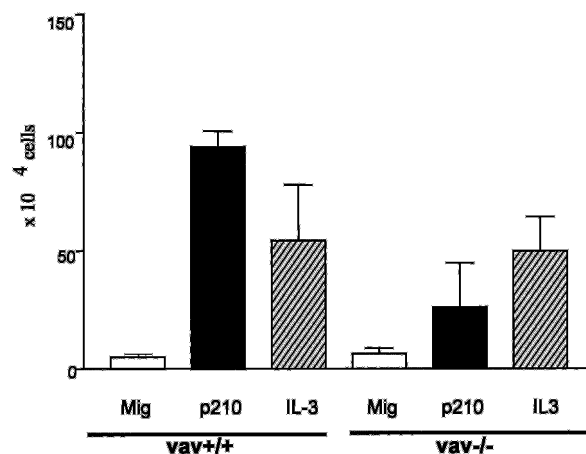


**FIG. 6. Bcr-Abl utilizes Vav to activate Rac-1.** 293 cells were transiently transfected with Rac-1 and empty vectors ( $\emptyset$ ); Rac-1, WT-Bcr-Abl, or KD-Bcr-Abl and empty vector (*Bcr-Abl*, *KD-Bcr-Abl*); Rac-1, Bcr-Abl, and Vav/F46 as a dominant-negative; or Rac-1, Bcr-Abl, and Vav/F46AA $\Delta$ CSH3. The amount of DNA was equal in all transfections. 72 h after transfection, lysates were prepared from  $1 \times 10^7$  starved cells. A GST pull-down assay with the GST-p21-binding domain of PAK, which specifically precipitates Rac and Cdc42 in their GTP-bound form (43), was done in duplicates. Bound fractions were resolved by SDS-PAGE and analyzed by immunoblotting with a monoclonal anti-Rac antibody, a monoclonal anti-Abl antibody, and an anti-tag antibody to detect Flag-tagged Vav mutants as indicated. The amount of GST-PAK was analyzed by Coomassie staining of the gel. A representative experiment is shown that was done independently three times with similar results.



**FIG. 7. Overexpression of Vav does not enhance the transformation potential of Bcr-Abl in NIH3T3 fibroblasts.** *A*, immunoblot analysis showing the expression of Vav and Bcr-Abl proteins in stable NIH3T3 cell lines used in *B*. NIH3T3 cells were transfected with the pcDNA 3.1-Flag-Vav construct containing a Zeocin<sup>®</sup> selection marker or the empty vector, respectively. Zeocin<sup>®</sup>-resistant clones were picked, expanded, and retrovirally transduced with a Mig-Bcr-Abl construct. *B*, the indicated NIH3T3 cell lines were plated into soft agar, and colonies were photographed after 2 weeks.

and is constitutively phosphorylated. A previous paper also reported Vav phosphorylation in Mo7E cells transfected with Bcr-Abl (11); in this paper, however, the authors did not detect complex formation of Bcr-Abl and Vav. Therefore, the authors (11) speculated that Vav may not be a direct substrate of Bcr-Abl but is phosphorylated by other tyrosine kinases in these cells. Here, we clearly show that Bcr-Abl and Vav form a complex in different experimental systems including *in vitro* purified proteins, yeast cells, and mammalian cells. We also show complex formation of Vav and Bcr-Abl by coprecipitating Bcr-Abl with a Vav specific antibody. However, we were not



**FIG. 8. Reduced transformation of Vav<sup>-/-</sup> compared with Vav<sup>+/+</sup> bone marrow by Bcr-Abl.**  $1 \times 10^5$  bone marrow cells from wild-type (Vav<sup>+/+</sup>) or Vav knock-out (Vav<sup>-/-</sup>) mice retrovirally transduced with empty vector (Mig) or Bcr-Abl (p210) were plated in bone marrow medium as described under "Materials and Methods." As a further control, growth of cells in the presence of 1 ng/ml IL-3 was measured. Bars represent the numbers of cells after 10 days of culture. Data represent means  $\pm$  standard deviation of an experiment done in triplicate. Similar results were obtained in three independent experiments.

able to coprecipitate Vav with a Bcr-Abl-specific antibody. This may be due to the fact that the Abl antibody interferes with the binding site for Vav on Bcr-Abl. In addition, the Vav antibody used in the present study detects the native protein very well but the denatured protein only poorly. Thus, the differences between their study (11) and ours may be because of the Vav antibody used to coprecipitate Bcr-Abl.

We show that this interaction is strictly dependent upon the tyrosine kinase activity of Bcr-Abl. Concurring with the yeast data showing a tyrosine-dependent interaction of the Vav fragment with Bcr-Abl, a TS mutant of Bcr-Abl showed detectable complex formation with and phosphorylation of Vav only at the permissive temperature for the TSBcr-Abl tyrosine kinase. We identified the region that mediates this interaction as the C-terminal SH3-SH2-SH3 domain of Vav. Even though mutational studies showed that the interaction is entirely phospho-

tyrosine-dependent and requires the intact SH2 domain of Vav, the C-terminal SH3 domain as well as a proline repeat next to the N-terminal SH3 domain of Vav are required for complex formation between Vav and Bcr-Abl; the Vav SH2 domain alone was not able to bind to Bcr-Abl. The deletion of the C-terminal SH3 domain as well as the replacement of the four adjacent prolines with alanine completely abolished the phosphotyrosine-dependent binding of the C-terminal Vav fragment. The C-terminal SH3 domain or the N-terminal SH3 domain alone does not bind to Bcr-Abl.

Additionally, we found that the C-terminal SH3 domain binds with high affinity to the proline-rich stretch adjacent to the N-terminal SH3 domain. Therefore a model is possible in which an intramolecular folding mechanism involves the C-terminal SH3 domain and the short spanning proline-rich domain next to the N-terminal SH3 domain within Vav. This intramolecular folding then enables the SH2 domain to exert its full binding potential, leading to complex formation between the C terminus of Vav and Bcr-Abl. Alternatively, our data would also support a model in which a head-to-tail dimerization process of two Vav molecules results in better accessibility of the Vav SH2 domain. However, by running protein gels under native conditions no experimental evidence of Vav dimerization could be obtained.<sup>2</sup> It is very likely that many direct phosphotyrosine-mediated interactions that involve Vav have been undetected because the binding ability of the single SH2 domain, often used for binding studies, is greatly reduced. In a pull-down experiment we found a panel of tyrosine-phosphorylated proteins precipitating only with the pp-SH2-SH3-SH3 fragment but not with the isolated SH2 domain (data not shown). In addition, one of the known Vav-SH2-binding proteins was precipitated with much higher affinity by the C-terminal Vav fragment than by the isolated SH2 domain. A 3–4-fold increase in binding to ZAP-70 from CD3-stimulated Jurkat cells was observed when both the proline-rich region adjacent to the SH3N and the SH3C were present compared with the binding of the SH2 domain alone (data not shown). In fact, other proteins have been reported recently to bind to Vav in a phosphotyrosine-dependent manner requiring the SH3-SH2-SH3 configuration (45, 46). However, these authors did not explore the contribution of the proline-rich stretch in Vav to this complex.

We show that Vav itself and the Vav fragment are phosphorylated in cells expressing Bcr-Abl. Phosphorylation of Vav strictly depends on the activity of the Bcr-Abl tyrosine kinase as demonstrated by use of a temperature-sensitive mutant of Bcr-Abl. *In vitro* kinase reactions with purified Bcr-Abl and Vav proteins indicate that Vav is a direct substrate of the Bcr-Abl kinase. The N-terminal Vav fragment contains several potential tyrosine phosphorylation sites, among them a tyrosine residue matching the predicted Abl substrate consensus site (42). Alternatively, Vav could also be phosphorylated by a different kinase in which activation strictly depends on kinase-active Bcr-Abl. For example the Src family kinases were shown to be able to phosphorylate Vav and to be activated by Bcr-Abl (47).

Vav phosphorylation by LCK has been shown to activate its GEF activity. Much effort has been made to identify the tyrosine residues in Vav that are responsible for the activation of its GEF activity. Most phosphorylation sites characterized so far are located in the N-terminal part of Vav. Tyr<sup>174</sup> is phosphorylated by Lck *in vitro* and *in vivo* and is suggested to be important for the activation of Vav GEF activity. However, it

was recently demonstrated that mutation of this site and mutation of additional tyrosine residues in the N terminus leads to oncogenic activation rather than inactivation of Vav (38). Thus, the mechanism by which Vav is activated by phosphorylation is still enigmatic and may involve additional phosphorylation sites including sites present in the C terminus. It will be interesting to determine the Vav tyrosine residues phosphorylated in Bcr-Abl-expressing cells and mutational studies thereof.

Rac activation by Bcr-Abl was completely abrogated by co-expression of the Vav C terminus encoding the SH3-SH2-SH3 domains as a dominant-negative construct. However, expression of a construct lacking the C-terminal SH3 domain and bearing the proline to alanine mutation had no effect on Rac activation. Thus, Bcr-Abl utilizes Vav to activate Rac-1. Rac activation has been shown to be crucial for Bcr-Abl mediated leukemogenesis (34). Bcr-Abl transforms cell lines of hematopoietic origin more effectively than for example fibroblasts. Vav is a GEF for Rac and is predominantly expressed in hematopoietic cells. Hence it is attractive to speculate that Bcr-Abl utilizes Vav to activate Rac-1 and therefore transforms hematopoietic cells more efficiently. This notion is supported by our data employing Vav knock-out bone marrow. To elucidate the contribution of Vav to Bcr-Abl induced leukemia it will be interesting to determine the leukemic potential of mice reconstituted with Bcr-Abl transduced WT compared with  $-/-$  Vav bone marrow.

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