Vav Binding to Heterogeneous Nuclear Ribonucleoprotein (hnRNP) C
EVIDENCE FOR Vav-hnRNPs INTERACTIONS IN AN RNA-DEPENDENT MANNER

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The vav proto-oncogene is exclusively expressed in hematopoietic cells and encodes a 95-kDa protein that contains multiple structural domains. Vav is involved in the expansion of T and B cells, in antigen-mediated proliferative responses, and in the induction of intrathymic T cell maturation. It becomes rapidly and transiently tyrosine-phosphorylated upon triggering of a large number of surface receptors and catalyzes GDP/GTP exchange on Rac-1. We now provide evidence for the specific interaction of Vav with heterogeneous nuclear ribonucleoprotein (hnRNP) C. Vav and hnRNP C interact both \textit{in vivo} and \textit{in vitro} mediated through the carboxyl Src homology 3 domain of Vav and the proline-rich motif located in the nuclear retention sequence of hnRNP C. More importantly, Vav-hnRNP C complexes are present in living hematopoietic cells and both proteins localize in the nuclei, mainly on perichromatin fibrils but also on clusters of interchromatin granules. The Vav-hnRNP C interaction is regulated by poly(U) RNA, although a basal association is still detected in the absence of RNA. Furthermore, RNA homopolymers differentially alter the binding affinity of Vav to hnRNP C and hnRNP K. We propose that Vav-hnRNP interactions may be established in an RNA-dependent manner.

Oncogenic \textit{vav} was originally isolated on the basis of its transforming properties in NIH3T3 fibroblasts (1). The corresponding cellular gene, the proto-oncogene \textit{vav}, is only expressed in cells of hematopoietic lineage (2). However, recently, a homologue of \textit{vav}, \textit{vav}2, has been identified and is more widely expressed (3, 4). Vav and Vav2 have over 50% identical residues, and both harbor a number of conserved domains found in molecules involved in protein-protein interactions and signaling events (Fig. 1A) (see Ref. 5 for review).

Vav becomes rapidly and transiently tyrosine-phosphorylated upon triggering of a variety of surface receptors in almost every hematopoietic cell. These include T cell receptor/CD3, CD2, and CD28 on T cells, IgM receptor and CD19 on B cells, the IgE high affinity FceRI receptor on mast cells, the lipopolysaccharide receptor on monocytes/macrophages, and several cytokine and tyrosine kinase receptors (5). The rapid tyrosine phosphorylation of Vav in all these cell types suggests an important role for this protein in signal transduction of hematopoietic cells. The Syk-Zap protein-tyrosine kinases have recently been shown to participate in tyrosine phosphorylation of Vav (6). Vav also binds to a hematopoietic specific protein, SLP-76, and after antigen receptor activation, the two proteins synergistically activate \textit{NF-AT} and \textit{IL-2} gene expression (7). Furthermore, chimeric \textit{vav}+/−/rag-2−/− mice have shown that Vav participates in the expansion of both T and B cells and is essential for antigen-mediated proliferative responses and for induction of intrathymic T cell maturation (8–10).

Despite the importance of Vav, little is known about its function. Recently, it has been shown that tyrosine-phosphorylated Vav catalyzes GDP/GTP exchange on Rac-1. Rac-1 is implicated in cell proliferation and cytoskeletal organization, and stimulates c-Jun kinase, a downstream element in the signaling pathway of this GTPase (11, 12). As Vav exhibits a striking combination of structural motifs and is localized both in the cytoplasm and in the nucleus (13, 14), it is likely that Vav is involved in several distinct cellular functions. It has been reported that the carboxyl SH3 (C-SH3) of Vav interacts with the proline-rich sequences of hnRNP K, which has a role in the regulation of the late steps of RNA biogenesis and transport of mRNA (15, 16). This interaction has been detected mainly in the cytoplasmic fraction (16). On the other hand, we have shown that the same C-SH3 domain of Vav also interacts with Ku-70 independently of the presence of a proline motif (14). Ku-70 is the DNA-binding element of the DNA-dependent protein kinase, which has been implicated in DNA repair, replication, recombination, and transcription. In addition, it has recently been shown that the leucine zipper motif of Vav associates with ENX-1 (17). \textit{enx-1} is a novel human gene homolog of the \textit{Drosophila Enhancer of Zeste} gene, a member of the Polycomb group of genes, which code for transcriptional regulators of homeobox gene expression.

To further identify proteins capable of physically associating with Vav, we studied a positive clone isolated from a Jurkat cDNA library by the yeast two-hybrid system using as bait the boxyl-SH; hnRNP, heterogeneous nuclear ribonucleoprotein; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; RBD, RNA-binding domain; NLS, nuclear localization signal; NRS, nuclear retention sequence; IG, interchromatin granule(s); PF, perichromatin fibril(s).
to the intron polypyrimidine tract of pre-mRNA (19), and this binding is negatively regulated by serine/threonine hyperphosphorylation (20). In vitro, hnRNPs C proteins have been involved in pre-mRNA splicing (21). Using a variety of approaches, we characterized the Vav-hnRNPs C association and analyzed the influence of RNA homopolymers on this interaction as well as the potential role of Vav as a hnRNP-interacting molecule.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cloning, and Mutagenesis—** Yeast pVJL10 derivatives included those containing SHHAV (residues 623–837 of human Vav), point mutant SHHAV P833L, SHS-C Vav (residues 787–837), SHS-C’ Vav (residues 813–837), and SHS-N’SH2 Vav (residues 623–812) (14). Plasmids encoding the Rasα3 and Rap proteins were used as controls (14). A bacterial pGEX2T derivative containing Vav SHS2/SHS-C (residues 666–840) (15) was also used. v90 from pGAD-v90 (residues 120–290 of hnRNP C1) was cloned in pGEX-4T-2 (Pharmacia Biotech Inc.) digested with EcoRI and NotI endonucleases. The mutant SHHAV Y836F and v90PA were constructed in the yeast two-hybrid vectors mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM dithiothreitol performed on ice. The pellet was resuspended in ice-cold buffer A (100 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM sodium vanadate, 10% glycerol, 0.03 mM MgCl2) and centrifuged at 100,000×g for 20 min. The extract was centrifuged at 4°C for 60 min at 4°C. Supernatants were incubated overnight with polyclonal anti-Vav or preimmune serum, followed by protein A-Sepharose beads. The beads were washed six times in lysis buffer and dissolved into sodium dodecyl sulfate (SDS)-sample buffer, incubated at 95°C for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

**Electrophoresis and Western Blot Analysis—** The gels were electrophoresed and blotted with PVDF membranes and probed with the different antibodies. Peroxidase-coupled anti-rabbit IgG from donkey, anti-mouse IgG from sheep, and anti-rat IgG from sheep were from American (Les Ulis, France). Immune-reactive bands were visualized using an enhanced chemiluminescence Western blotting system (ECL, Amersham) according to the manufacturer’s protocol.

**Antibodies—** Anti-Vav polyclonal antibodies were from Dr. A. Altman2 and Drs. M. Barbacid and X. R. Bustelo (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). Anti-Vav polyclonal antibody for electron microscopy was from Transduction Laboratories (Lexington, KY). Anti-Vn monoclonal antibody was from UBI (Lake Placid, NY). Anti-Raf1 polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-hnRNPs C (4F4 antibody, provided by Dr. Gene Dreyfuss, University of Pennsylvania) (28), N3H10 (anti-Ku-70) monoclonal antibody by Dr. W. H. Reeves (University of North Carolina) (29), and anti-hnRNPs K (B4B6) monoclonal antibody by Drs. J. Celis and K. Dejgaard (30).

**Electron Microscopy—** After centrifugation of UT7-S Epo cells, the cell pellets were fixed for 1 h at 4°C in 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer, pH 7.5. After another centrifugation at the end of fixation, the resulting pellets were washed with phosphate buffer for 1 h at 4°C, dehydrated in increasing concentrations of methanol (30, 50, 70, and 90%), and embedded in Lowicryl K4M (31). Polymerization was carried out under long wavelength UV light (Philips fluorescence tubes T6, 6 watts) for 5 days at −20°C and for 1 day at room temperature. Ultrathin sections were mounted on carbon-Formvar-coated gold grids (200-mesh). For immunolabeling, thin sections were incubated for 30 min at room temperature either on a drop of anti-Vav polyclonal antibody (diluted 1/50 in PBS) or anti-hnRNPs C monoclonal antibody (diluted 1/5000). After 15 min in PBS, the grids were floated for 10 min on a drop of either 1/50 dilution of anti-rabbit IgG or 1/50 of anti-mouse IgG. Both secondary antibodies being conjugated to gold particles 10 nm in diameter (Biocell Research Laboratories, Cardiff, United Kingdom). After 20 min of incubation at room temperature, the grids were washed with distilled water and air-dried. Before observation, grids were processed according to the RNP preferential staining method of Bernhard (32). For controls, a 1/10 dilution of normal rabbit serum or 1/10 dilution of normal mouse ascites fluid was used instead of anti-Vav and anti-hnRNPs C, respectively.

**Competition Experiments—** The peptide blocking experiments were done in GST fusion proteins preincubated for 2 h at 4°C with 50–100 μM amounts of the polyprotein peptide YPARYPPPPPAPAR derived from hnRNP C proteins (residues 126/139–140/153 of hnRNP C1/C2) and subjected to SDS-PAGE, respectively. For RNA experiments, nuclear extracts were preincubated for 30 min at 4°C with poly(U), poly(A), poly(G), or poly(C) RNA (PharMacia) or nuclear RNA (preheated 10 min at 65°C) at several concentrations before adding antibodies for immunoprecipitation experiments.

**Immuno precipitation Experiments—** Nuclear extracts from Jurkat and UT7-S Epo cells (1.5–3 × 109) were diluted 2.5 times with buffer C (see “Subcellular Fractionation”) lacking NaCl and supplemented with 0.05% Brij 96. After centrifugation, supernatants were incubated with preimmune serum for 30 min and protein A-Sepharose beads (Pharmacia) for 1 h at 4°C. Supernatants were incubated overnight with polyclonal anti-Vav or preimmune serum, followed by protein A-Sepharose beads for 1 h. After centrifugation, the beads were washed six times in PBS, 10% glycerol, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Eluted proteins were dissolved into SDS-sample buffer, incubated at 95°C for 10 min, and subjected to SDS-PAGE. Equivalent amounts of 100 fractions were directly used for immunoprecipitation experiments.

**Isolation of Nuclear RNA and RNase Digestion—** Nuclear RNA was prepared by the acid/guanidinium isothiocyanate/phenol/chloroform extraction method (33). Digestions with RNase (400 μg/ml) were carried out with preboiled bovine RNase I (PharMacia) for 45 min at 4°C.

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2 Altman, unpublished data.
Domains are at scale. The length of SHVAV is also indicated.

The interaction between the two-hybrid proteins is indicated by the induction of 

lysates from 10^7 Jurkat and UT7-S Epo cells. After being washed, proteins eluted in sample buffer were resolved on SDS-PAGE, blotted on nitrocellulose filters, and developed with monoclonal anti-Vav antibody. A total of 1 μg of each GST protein was used. Lysate, total lysate from 2 x 10^9 cells.

After centrifugation, the supernatants were used for coimmunoprecipitation experiments.

RESULTS

Interaction of Vav and hnRNP C—To study the signaling mechanism involved in hematopoietic cell development, we searched for proteins bound to Vav using the yeast two-hybrid approach. A Jurkat T cell cDNA library was screened using as bait SHVAV (residues 623–837 of human Vav) fused to the carboxyl-terminal of the LexA DNA-binding domain. This portion of Vav includes the SH2 domain flanked by two SH3 domains (Fig. 1A), structures commonly involved in protein-protein interactions of signaling molecules. The v90 polypeptide was identified as interacting specifically with SHVAV both by the induction of lacZ expression and by the growth of yeast in absence of histidine, the two reporter genes used in the two-hybrid system. The specificity of this interaction was confirmed because neither the RasV12/v90 polypeptide nor the LexA DNA-binding domain/v90 polypeptide transactivated the reporter genes (Fig. 1B). Furthermore, full-length Csk, another protein with SH2 and SH3 domains, did not interact with the v90 polypeptide, whereas the full-length Vav was clearly positive (data not shown).

Sequence analysis of the yeast plasmid revealed that v90 encoded a 170-amino acid-long polypeptide identical to residues 120–290 of hnRNP C1/C2 (34) (Fig. 1C). hnRNP C1 and C2 proteins are two isoforms generated from a single gene by alternative splicing of a common pre-mRNA (35) and are restricted to the nucleus in interphase cells (36). They have an amino-terminal domain containing an RNA-binding domain (RBD) with two highly conserved sequences called RNPI and RNPII, and a carboxyl-terminal essentially composed of an acidic domain, a nuclear localization signal (NLS), and a nuclear retention sequence (NRS) (18, 35, 36). These carboxyl-terminal domains are included in the v90 polypeptide. Interestingly, the NRS contains a proline-rich motif classically involved in protein-protein interaction with SH3-containing proteins.

To validate the results obtained with the yeast two-hybrid system, the interaction of hnRNP C and Vav was studied in another expression system. The cDNA fragment of v90 was subcloned into a pGEX derivative to generate a chimeric GST-v90. The expressed fusion protein was purified from bacterial lysates by affinity chromatography with glutathione-agarose beads and incubated with lysates from 10^7 Jurkat and UT7-S Epo cells. After being washed, proteins eluted in sample buffer were resolved on SDS-PAGE, blotted on nitrocellulose filters, and developed with monoclonal anti-Vav antibody. A total of 1 μg of each GST protein was used. Lysate, total lysate from 2 x 10^9 cells.

The C-SH3 Domain of Vav Is Involved in the Interaction with hnRNP C—To further delineate the Vav interacting domain, three fragments of SHVAV cDNA subcloned in frame with the LexA DNA-binding domain were used (14): the intact C-SH3 of Vav (SH3-C Vav), the COOH-terminal half of Vav C-SH3 (SH3-C’ Vav), and the SHVAV lacking the COOH-terminal half of C-SH3 (SH3-N/SH2). The study of these subclones by the two-hybrid system showed that the C-SH3 of Vav is responsible

Fig. 1. Interaction of Vav and hnRNP C in the yeast system and in vitro. A, structural domains of Vav. Leu-rich, leucine-rich domain; LZ, leucine zipper; CH, calponin homology domain; Acidic, acidic domain; DH, Dbl homology domain; PH, pleckstrin homology domain; NLS, nuclear localization signals; Cys-rich, cysteine-rich domain; SH3,Src homology domain 3; Pro, proline-rich motif; SH2,Src homology domain 2. The domains are at scale. The length of SHVAV is also indicated. B, interaction of v90 polypeptide with SHVAV in the two-hybrid system. v90 was obtained after screening a lymphoid cell cDNA library using SHVAV as bait. The L40 reporter strain was cotransformed with the indicated plasmids. The interaction between the two-hybrid proteins is indicated by the induction of lacZ expression (dark gray patches). L40 carrying pLex-RasV12 and pGAD-Raf was used as a positive control. Each patch represents an independent transformant. There are two patches for every strain. DB, fusion with the DNA-binding domain of LexA; AD, fusion with the activation domain of Gal4. C, structure of hnRNP C proteins. The amino acid sequence of C2 is identical to that of C1 except for an extra 13 residues near the middle of the protein. RBD, RNA-binding domain; RNPI, ribonucleoprotein motif 1; RNPII, ribonucleoprotein motif 2; NRS, nuclear retention sequence; Pro, proline-rich motif; NLS, nuclear localization signal; Acidic, acidic domain. The domains are at scale. The length of v90 polypeptide in the carboxyl-terminal portion of hnRNP C1/C2 is also indicated. D, Vav interacts with hnRNP C in vitro. Expression of the GST fusion proteins was induced by addition of isopropyl-1-thio-β-D-galactopyranoside, and proteins were isolated from bacterial lysates by affinity chromatography with glutathione-agarose beads and incubated with lysates from 10^7 Jurkat and UT7-S Epo cells. After being washed, proteins eluted in sample buffer were resolved on SDS-PAGE, blotted on nitrocellulose filters, and developed with monoclonal anti-Vav antibody. A total of 1 μg of each GST protein was used. Lysate, total lysate from 2 x 10^9 cells.
for the interaction with v90 polypeptide (Fig. 2A). The SH3-C' Vav was still able to bind the COOH-terminal region of hnRNP C, but SH3-N'SH2 Vav could not interact with the v90 polypeptide.

Further confirmation of the association of hnRNP C with the C-SH3 domain of Vav was obtained by analysis of the P833L mutation of SHVAV. This mutation is analogous to an SH3 mutation found previously to abrogate the function of the Grb2 homolog Sem5 in the vulva differentiation pathway of Caenorhabditis elegans (37, 38). As shown in Fig. 2B (third lane), the P833L mutation completely abolished the interaction of SH-VAV with the v90 polypeptide in the yeast two-hybrid system. As a negative control, a SHVAV Y836F mutant, which maintains the ability to bind polyproline motifs, interacted with v90 at the same level as the wild-type polypeptide (Fig. 2B, fourth lane). As all Vav subclones and point mutants were produced in similar amount in L40 strain, these experiments define the C-SH3 domain of Vav as the binding site for hnRNP C.

The Proline-rich Motif of hnRNP C Is Implicated in the Binding to Vav—We next sought to identify the hnRNP C sequence involved in the interaction with Vav. hnRNP C proteins have a proline-rich sequence stretch, YPARVPPPPPIARAV (35) (see Fig. 1C), which resembles previously identified SH3 binding motifs (39) and is contained in the v90 subclone. We examined whether this proline-rich sequence was responsible for the Vav-hnRNP C interaction.

First, the ability of GST-Vav SH2/SH3-C (15) to bind hnRNP C was tested. As hnRNP C proteins are completely restricted to the nucleus (36), subcellular fractionations were performed (see “Experimental Procedures”). As a control, GST alone was used. After washing, proteins were resolved by SDS-PAGE and blots incubated with anti-hnRNP C antibody. No interaction was detected with GST alone. Similar results were obtained with extracts from UT7-S Epo cells (data not shown). Therefore, the chimeric GST-Vav SH2/SH3-C recognized full-length human hnRNP C proteins in vitro.

Next, a proline-rich peptide (YPARVPPPPPIARAV) was used to block the Vav-hnRNP C interaction. To this end, GST-Vav SH2/SH3-C was preincubated with various concentrations of polyproline peptide YPARVPPPPPIARAV before incubation with Jurkat nuclear extracts (37, 38). As a control, GST alone was used. After washing, blots were incubated with anti-hnRNP C antibody. 0.5 μg of each GST proteins were used. Extracts, nuclear extracts from 4 × 10^6 cells. E, the same filters were developed with monoclonal antibody anti-Ku-70 (N3H10). F, binding site of v90 to SHVAV using a proline-rich mutant of v90 (v90PA) in the yeast two-hybrid system. See the legend to Fig. 1B for details.
100 μm almost completely abolished this association (Fig. 3D). The same peptide was tested in the Vav-Ku-70 interaction, which implicates the same C-SH3 domain of Vav but is independent of the presence of a proline-rich motif (14). Therefore, these experiments were used as a control of specificity of the blocking of Vav-hnRNP C association. Reblotting these filters with anti-Ku-70 (N3H10) monoclonal antibody showed that the proline-rich peptide was unable to block the Vav-Ku-70 interaction, indicating that the Vav-hnRNP C association is specifically blocked by the peptide (Fig. 3E). Furthermore, a proline-rich mutant of v90 polypeptide did not interact with SHVAV in the two-hybrid system (Fig. 3F). These results demonstrate that the proline-rich motif of hnRNP C is involved in the binding to Vav.

In Vivo Binding of Vav and hnRNP C—To examine whether Vav and hnRNP C formed complexes in intact hematopoietic cells, we performed a series of coimmunoprecipitation experiments. For this purpose, nuclear extracts from UT7-S Epo cells were incubated with an anti-Vav polyclonal antibody (raised against a specific peptide of Vav corresponding to residues 456–469) or with the preimmune serum. The resulting immunocomplexes were blotted with anti-hnRNP C and, as illustrated in Fig. 4 (right panel), Vav coimmunoprecipitated with the endogenous hnRNP C proteins. Coimmunoprecipitations using the S100 fractions were performed as controls and no hnRNP C was detected. In fact, hnRNP C proteins were not present in lysates from S100 fractions of UT7-S Epo cells (Fig. 4, left panel), whereas Vav was detected in both fractions (data not shown). Similar experiments were carried out with a second anti-Vav polyclonal antibody, which recognizes a different epitope (27), and with extracts from Jurkat cells, and the results were equivalent to those described above (data not shown). In similar experiments, we also detected Vav proteins in the anti-hnRNP C immunoprecipitates. Thus, our findings show that full-length Vav and hnRNP C can form a complex in the nucleus of intact hematopoietic cells.

Nuclear Localization of Vav and hnRNP C—By confocal microscopy, we have shown previously that Vav is partially localized in the nucleus of UT7-S Epo cells (14). To evaluate the in vivo association of Vav and hnRNP C, the localization of both proteins in the nucleus of UT7-S Epo cells was observed by electron microscopy. After EDTA staining, chromatin was bleached while nuclear RNP components, including nucleolar and extranucleolar RNP components, remained contrasted (see “Experimental Procedures”). The localization of proteins recognized by anti-Vav and anti-hnRNP C antibodies were similar except that the former was always less intense (Fig. 5, A and B). In both cases, gold particles were clearly localized on perichromatin fibrils extending in the nucleoplasm between clumps of bleached condensed chromatin. The clusters of interchromatin granules (IG) were only slightly labeled. When visible, nuclear bodies, including beaded bodies, coiled bodies, and IG-associated zones, were never labeled, and no labeling occurred on the nucleoli. The cytoplasm was not labeled with anti-hnRNP C, whereas Vav was detected on a few non-structured spots, mainly distributed within polyribosomes containing areas. The distribution of Vav in the nucleus and in the cytoplasm was estimated by counting the number of gold particles in both structures. From five different electron micrographs, we evaluated that 27% of cellular Vav was localized in the nucleus of UT7-S Epo cells. We did not detect gold particles by incubation of grids with normal rabbit serum or with normal mouse ascites fluid (both 1/10 in PBS) followed by immunogold labeling.

Therefore, the subcellular fractionation studies and the immunolabeling using confocal microscopy (14), electron microscopy, and light microscopy show that Vav is partially localized in the nucleus of UT7-S Epo cells.

Poly(U) RNA Increases the Vav-hnRNP C Interaction—Previous studies have shown that hnRNP C binds to RNA molecules containing oligouridie stretches (18). Thus, to examine the physiological relevance of the Vav-hnRNP C interaction, we
investigated the role of poly(U) RNA in this association. Coimmunoprecipitation experiments were performed with nuclear extracts from Jurkat or UT7-S Epo cells preincubated with increasing concentrations of poly(U) RNA (10–200 μg/ml) followed by the addition of anti-Vav antibodies. Blots were probed with anti-hnRNP C antibody and show that increasing amounts of poly(U) RNA enhanced the Vav-hnRNP C interaction (Fig. 6A) although similar amounts of Vav protein were immunoprecipitated (Fig. 6B). The opposite question, that of whether the total absence of RNA might impede the Vav-hnRNP C interaction, was also addressed. Coimmunoprecipitation experiments were performed as described above with nuclear extracts from UT7-S Epo cells either treated with or without RNase. Western blot analyses with anti-hnRNP C antibody revealed that a basal interaction was still detected in RNase treated extracts (Fig. 7, A and B). Similar results were obtained with nuclear extracts from Jurkat cells (data not shown). These findings indicate that, although Vav can bind to hnRNP C proteins that are either associated or free of poly(U) RNA, the higher affinity of Vav for hnRNP C-poly(U) RNA suggests that the folding of hnRNP C induced by poly(U) RNA is more favorable for an interaction with Vav.

**Role of RNA Homopolymers in Vav-hnRNP Interactions**—We next studied the Vav-hnRNP C association in the presence of nuclear RNA (hnRNA). hnRNA from UT7-S Epo cells was isolated and added to nuclear extracts from these cells. The amount of hnRNA used was similar to the amount found in the cells from which the nuclear extracts were isolated. After preincubation, extracts were immunoprecipitated with anti-Vav antibodies. Under these conditions, coimmunoprecipitated hnRNP C was detected, but at much lower levels than from nuclear extracts lacking hnRNA or with poly(U) RNA (Fig. 8, A and B). These data suggest that the presence of RNA molecules other than poly(U) RNA can decrease the Vav-hnRNP C interaction. This hypothesis was tested by analyzing the role of poly(U), poly(rC), poly(rG), and poly(rA) RNA as well as combinations of these RNA homopolymers with poly(U) RNA on Vav-hnRNP C binding. Coimmunoprecipitation experiments showed that both poly(rC) and poly(rG) RNA decreased the interaction of Vav with hnRNP C (6- and 2-fold, respectively), whereas poly(rA) RNA did not affect binding and, as expected, poly(U) RNA increased the association in a concentration-dependent manner (Fig. 9, A and B). The addition of poly(U) RNA to poly(rC) or poly(rG) RNA resulted in higher levels of associated hnRNP C as compared with poly(rC)- or poly(rG) RNA alone, but less than that observed with poly(U) RNA. In contrast, association in the presence of poly(rA)-RNA and poly(U) RNA alone were similar. These results suggest that other RNA-binding proteins may play a role in the association of Vav with hnRNP C. To examine this possibility, we analyzed hnRNP K as a model of an RNA-binding protein with a preference for poly(rC) RNA. Anti-Vav immunoprecipitations were performed in the presence of RNA homopolymers or nuclear RNA and immunoblotted with an anti-hnRNP K monoclonal antibody (B4B6). As shown in Fig. 10 (A and B), the basal Vav-hnRNP K interaction was increased by poly(rC) RNA (5-fold), unchanged by poly(rA) RNA and decreased by the addition of poly(U) or poly(rG) RNA (5-fold each). Furthermore, the addition of nuclear RNA maintained the interaction, although at a low level. Overall, these findings suggest that the binding affinity of Vav for hnRNP proteins depends on the identity of associated RNA molecules. This opens new perspectives for the study of Vav interactions and functions.

**DISCUSSION**

Vav is a complicated and interesting molecule because its structural features suggest that it may participate in several signaling pathways (5). Since it was first observed that Vav is rapidly tyrosine-phosphorylated on hematopoietic cells upon triggering of a large array of surface receptors (27, 40–42), several potential functions for Vav have been described (6, 7, 12, 14–17). Furthermore, in this report, we have demonstrated that Vav associates with hnRNP C proteins, suggesting a role...
for Vav as a hnRNP-interacting molecule. As hnRNPs are ubiquitous whereas vav is expressed solely in hematopoietic cells, Vav2 might replace Vav as an hnRNP partner in non-hematopoietic cells (3, 4).

In human cells, hnRNPs comprise a group of about 20 proteins that associate with pre-mRNA as RNA synthesis proceeds. Their functions are not fully understood, but they are thought to participate in posttranscriptional events such as regulated splicing and mRNA export (43). Although hnRNPs have a general affinity for RNA, the majority have distinct sequence preferences. For example, hnRNP C prefers poly(U) sequence preferences. For example, hnRNP C prefers poly(U) whereas hnRNP K binds poly(rC) RNA (18). The initial model in which pre-mRNAs are packaged into a globular ribonucleoprotein structure, reminiscent of the way DNA is packaged in nucleosomes, contrasts with the current dynamic model. At present, there is evidence for the existence of transcript-specific complexes in which the protein composition varies during processing (43). The most abundant hnRNPs belong to groups A, B, and C, which are referred to as the core proteins. hnRNP C1 and C2 are identical, except for an insertion of 13 residues in the latter (35), and are restricted to the nucleus (36). The hnRNP C proteins have a RBD, acidic domain, NLS, NRS, putative NTP-binding site, and possible phosphorylation sites (18).

Through a two-hybrid screen in yeast, we detected a specific interaction of Vav with hnRNP C. This binding was confirmed in vitro using GST fusion proteins of both Vav and hnRNP C. The proline-rich motif present in the NRS of hnRNP C is involved in this interaction. In fact, a proline-rich peptide (YPARVPPPPPIARAV) contained in hnRNP C proteins or a proline to alanine mutant in this region were indeed capable of blocking the Vav-hnRNP C association. Using various subclones of Vav, we showed that the association with hnRNP C is mediated by the C-SH3 domain of Vav and that a P833L mutation in this region, which abolishes interactions with proline-rich motifs, completely eliminated the binding of Vav to hnRNP C. These data show that the interaction between Vav and hnRNP C is an SH3 domain interaction with a proline-rich sequence (39). Moreover, these findings raise the possibility that Vav participates in the mechanism of nuclear retention of hnRNP C, a hypothesis that remains to be tested.

Coimmunoprecipitation experiments and the nuclear localization of Vav and hnRNP C by electron microscopy suggest a functional role for Vav-hnRNP C association in hematopoietic cells. It was important to use non-transfected cells because ectopic expression of these genes, which would magnify the protein levels, might result in non-physiologically relevant results. In two hematopoietic cell lines (Jurkat and UT7-S Epo) using two different anti-Vav antibodies to immunoprecipitate the endogenous Vav, we were able to recover hnRNP C proteins and in the immunoprecipitate with anti-hnRNP C we detected Vav. These findings confirm the results found in vitro and in the yeast system. As described previously (36), we detected hnRNP C proteins only in the nuclear fraction of cells. Interestingly, electron microscopy of UT7-S Epo cells showed that both proteins localized on perichromatin fibrils (PF) and slightly on clusters of interchromatin granules (IG). The PF are formed at sites of transcription of pre-mRNA at the border of condensed chromatin and are the first extranucleolar structures to be labeled after short pulses with radioactive uridine (44, 45). After their formation, these fibrils migrate toward the interchromatin space while their RNA is being processed (46–48). A series of results using a variety of methodological approaches including parallel biochemical and morphological studies of purified subnuclear fractions (49, 50) led to the model that the PF correspond to structures containing newly synthesized pre-mRNA and to the main substrates of splicing. This conclusion is in agreement with the detection of small nuclear RNP antigens (51), small nuclear RNAs (52), and poly(A)+ RNAs (53) in association with PF. Cluster of IG have also been shown to contain high levels of splicesomal components (51, 53). However, in contrast to PF, the IG were only slightly labeled with radioactive uridine, indicating that they were probably not active sites of transcription and splicing of pre-mRNA. Consequently, IG are thought to be involved in pre- or post-splicing events such as the assembly of splicosomes, intron degradation, etc. (47, 52, 54). However, recently, small
quantities of poly(A)^+ RNA have been detected in some IG clusters (53), suggesting that IG might also be involved in some steps of intranuclear transport or sorting of mRNA molecules.

We and others have detected Vav in the nuclei (13–15, 17, 41). From our subcellular fractionations, we estimated that clusters (53), suggesting that IG might also be involved in some with hnRNP proteins from pre-mRNA synthesis to mRNA ex-

splicing factors to selected sites as well as for mRNA export. We may be involved in the configuration of perichromatin fibrils, implying a biological relevance of these associations.

The role of hnRNPs in splicing derives from the preferential activity of several hnRNPs in splice-site selection. It seems that affinity of these proteins for splicing signals and from the imple-ration of RNA molecules and the identity and phosphorylation state of associated hnRNPs.

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