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p95^{vav} Associates with the Nuclear Protein Ku-70

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The proto-oncogene *vav* is expressed solely in hematopoietic cells and plays an important role in cell signaling, although little is known about the proteins involved in these pathways. To gain further information, the Src homology 2 (SH2) and 3 (SH3) domains of Vav were used to screen a lymphoid cell cDNA library by the yeast two-hybrid system. Among the positive clones, we detected a nuclear protein, Ku-70, which is the DNA-binding element of the DNA-dependent protein kinase. In Jurkat and UT7 cells, Vav is partially localized in the nuclei, as judged from immunofluorescence and confocal microscopy studies. By using glutathione *S*-transferase fusion proteins derived from Ku-70 and coimmunoprecipitation experiments with lysates prepared from human thymocytes and Jurkat and UT7 cells, we show that Vav associates with Ku-70. The interaction of Vav with Ku-70 requires only the 150-residue carboxy-terminal portion of Ku-70, which binds to the 25 carboxy-terminal residues of the carboxy SH3 domain of Vav. A proline-to-leucine mutation in the carboxy SH3 of Vav that blocks interaction with proline-rich sequences does not modify the binding of Ku-70, which lacks this motif. Therefore, the interaction of Vav with Ku-70 may be a novel form of protein-protein interaction. The potential role of Vav/Ku-70 complexes is discussed.

p95^{vav} is a complicated and interesting molecule because of a number of structural features, several of which have suggested a role for Vav in cell signaling (51). Vav is expressed solely in hematopoietic cells, and its temporal pattern of expression during development precedes and coincides with the onset of hematopoiesis (67). Multiple types of evidence show that Vav is involved in signal transduction. We and others have shown that in T cells, Vav is tyrosine phosphorylated upon activation through the CD2 or the CD3 receptor (8, 44, 52), and it has been suggested that Zap-70 could be responsible for Vav tyrosine phosphorylation (38). Vav is also tyrosine phosphorylated upon activation of B and mast cells through the immunoglobulin M (IgM) antigen receptor (7) and the IgE high-affinity FceRI receptor (44), respectively. Furthermore, Vav is tyrosine phosphorylated upon activation of c-Kit by the steel factor (1) and Flk-2 (16) tyrosine protein kinase receptors, by erythropoietin (Epo) stimulation of the Epo receptor (46), and by interleukin-2, interleukin-3, and alpha interferon treatment of hematopoietic cells (16, 20, 50). Recently, the generation of mice deficient in *vav* expression in their lymphoid cells has pointed to the essential role of Vav in antigen receptor-induced proliferation of T and B cells. However, these studies also showed that there are Vav-independent signaling pathways involved in the proliferation of both kinds of cells (24, 61, 66).

Vav has two Src homology 3 (SH3) domains flanking an SH2 domain and a proline-rich region in the amino-terminal SH3 (N-SH3) domain (8, 44). It also contains a cysteine-rich region that displays strong similarity to the zinc butterfly domains of protein kinase C isoforms, a pleckstrin homology region, and a Dbl-like domain with homology to a guanine nucleotide release factor. Therefore, it was postulated that Vav may participate in the activation of small GTP-binding proteins. Vav also has an acidic domain, a leucine zipper motif, a helix-loop-helix domain, and nuclear localization signals, suggesting that Vav could play a role as a nuclear factor (5, 14, 36, 47).

The function of Vav in cell signaling is not clear, and little is known about the proteins implicated in these pathways. The proline-rich region of Vav, located in the N-SH3 domain, is involved in binding to the carboxy-terminal SH3 (C-SH3) domain of Grb2 (53, 65). Moreover, the C-SH3 domain of Vav interacts with the proline-rich region of poly(rC)-specific RNA-binding proteins (10, 34). These data suggest a potential role of Vav in Grb2-dependent signaling and in RNA biogenesis and/or transport. On the other hand, Vav has been reported to be a GDP/GTP exchange factor for Ras (28–31), but later work did not confirm this activity (9, 39).

To identify proteins involved in the Vav signaling pathway, we used the yeast two-hybrid system (23). Our work provides evidence for an interaction between Vav and Ku-70 both in vitro and in vivo.

Ku-70, initially identified as an antigen recognized by sera from some autoimmune patients (45), is a cell cycle-dependent nuclear protein which associates with Ku-80 to form a heterodimeric complex (25, 54, 64). Ku-70 is the DNA-binding component of the DNA-dependent protein kinase, whose catalytic component is a 350-kDa polypeptide (18). In vitro, this trimeric complex phosphorylates several chromatin-bound proteins and has been implicated in DNA repair, replication, recombination, and transcriptional events (22, 26, 41, 42). Ku-70 was originally described as binding to the ends of double-stranded DNA (27), but more recent reports have shown that it binds to a variety of DNA structures bearing single- to double-strand transitions (21).

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The results presented here were obtained by three different approaches: (i) by a genetic approach using the yeast double-hybrid system, (ii) in vitro by the binding of Vav to Ku-70-derived fusion proteins, and (iii) by coimmunoprecipitation of Ku-70 with anti-Vav from lysates of different hematopoietic cells.

MATERIALS AND METHODS

Cloning and site-directed mutagenesis. SHVAV (residues 623 to 837 of Vav) was PCR amplified from pKLS1 (provided by M. Barbacid and X. R. Bustelo) and fused to the DNA-binding domain of LexA (63) in pVJL10. This plasmid is derived from plasmid pBTM116 (2), in which the frame of the cloning site has been modified (11). SH3-C Vav (residues 787 to 837), SH3-C′ Vav (residues 813 to 837), and SH3-N′/SH2 Vav (residues 623 to 812) were PCR amplified from pLexA-SHVAV and fused to pLexA-DB. v41Nter (residues 291 to 483) and v41Cter (residues 484 to 609) were PCR amplified from pGAD-v41 (residues 291 to 609 of Ku-70) and fused to pGal4-AD (activation domain). v41 and v94, from pGAD-v41 and pGAD-v94, respectively, were cloned in pGEX-4T-2 (Pharmacia) digested with *Eco*RI and *Not*I endonucleases. DNAs encoding Nck-$(SH3)_3$/SH2, Csk-SH2/SH3, and Lck-SH2/SH3 were cloned in pGEX-2T as described before (52).

The mutant SHVAV P833L was made in the yeast two-hybrid vector pVJL10 by using the transformer site-directed mutagenesis kit from Clontech. The mutagenic primer was CGG GTT GGC TGG TTC CTT GCC AAC TAC GTG TGA GTC GAC CTG CAG CC. The mutated base that changes a proline to a leucine is underlined.

Two-hybrid screen. A cDNA library from Jurkat cell polyadenylated RNA constructed in fusion with Gal4AD in pGAD1318 (32) was used (3). *Saccharomyces cerevisiae* L40 (*MAT***a** *trp1 leu2 his3 LYS2*::*lexA-HIS3 URA3*::*lexA-lacZ*) was grown at 30°C in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% glucose, and it was sequentially transformed with pLexA-SHVAV and the Jurkat cell cDNA library by the lithium acetate method (58). Double transformants were plated on yeast drop-out medium lacking Trp, Leu, His, Lys, and uracil (58). They were grown for 5 days at 30°C, and then colonies were patched on the same medium and replica-plated on Whatman 40 filters to test for β-galactosidase activity (6). Plasmids pLexA-Ras^{V12} (63) and pGAD-Raf (62) were used as controls. Positive clones were rescued and tested for specificity by retransformation into L40 either with pLexA-SHVAV or with extraneous targets (pLexA-Ras^{V12} or pLexA-Lamin) (63).

Sequence analysis. Sequencing of PCR fragments, point mutations, and cDNA inserts from positive clones of the two-hybrid screening was performed on both strands with an automatic sequencer (Applied Biosystems model 373A) by the dideoxy termination method (57). Sequence comparisons were done with the program FASTA.

Cell culture and lysis. Jurkat cells (clone J77.6.8) were grown in RPMI 1640 medium (Gibco) supplemented with 10% heat-activated fetal calf serum (FCS) (Boehringer Mannheim), 2 mM L-glutamine, penicillin, and streptomycin in a 5% CO_2 humidified atmosphere at 37°C.

UT7-S cells (49) were maintained in α-medium with 10% FCS and Epo at 1 to 2 U/ml (UT7-S Epo). Nonstimulated cells were incubated for 20 h in medium without serum (Iscove's modified Dulbecco's minimum essential medium [DMEM] containing 0.4% bovine serum albumin and 20 μg of iron-saturated transferrin per ml).

Cell lysis was performed at 5×10^7 cells per ml at 4°C in 150 mM NaCl–1 mM EDTA–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4)–1 mM sodium vanadate–10% glycerol–0.875% Brij 96 (polyoxylethylene-10-oleylether)–0.125% Nonidet P-40–1% aprotinin–1 mM phenylmethylsulfonyl fluoride (PMSF)–pepstatin (1 mg/ml)–leupeptin (1 mg/ml) for 20 min. The extract was centrifuged at $20,000 \times g$ for 20 min, and the supernatant was placed in N_2 and stored at −80°C.

Human thymocytes were obtained from normal thymus fragments that had been removed from children (5 days to 27 months of age) during corrective cardiac surgery. Cells were isolated by teasing the fragments in cold RPMI 1640 usually supplemented with 1% FCS. Freshly isolated thymocytes were lysed in a buffer containing 20 mM Tris (pH 7.5), 1 mM EDTA, 140 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, and 50 U of aprotinin per ml plus 1% Brij 96 for 45 min at 4°C. Cell debris was removed by centrifugation at $12,000 \times g$.

Coimmunoprecipitation experiments. Lysates from 5×10^7 UT7-S Epo cells or 10^8 thymocytes were incubated with protein A-Sepharose beads (Pharmacia) for 1 h at 4°C. The supernatants were incubated with polyclonal anti-Vav or preimmune serum for 4 h and with protein A-Sepharose beads for 1 h and then centrifuged, and the beads were washed five times in phosphate-buffered saline (PBS) with 10% glycerol and 0.02% gelatin. Eluted proteins were dissolved into sodium dodecyl sulfate (SDS) sample buffer, incubated at 95°C for 10 min, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), electroblotted, and probed with anti-Ku-70 monoclonal antibodies (N3H10). Immunoreactive bands were visualized with an epichemiluminescence Western immunoblotting system (ECL; Amersham) according to the manufacturer's protocol.

GST fusion proteins, electrophoresis, and Western blot analysis. Expression of the glutathione *S*-transferase (GST) fusion proteins was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the fusion proteins were isolated from bacterial lysates by affinity chromatography with glutathione-agarose beads (Sigma).

Lysates from 5×10^5 Jurkat cells were incubated for 2 h with fusion protein bound to glutathione-coupled agarose beads. The precipitates were washed as described for the coimmunoprecipitation experiments. The gel was blotted onto a nitrocellulose membrane and probed with the different antibodies.

Antibodies. Anti-Vav polyclonal antibody was from M. Barbacid and X. R. Bustelo (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J.) (8). Anti-Vav monoclonal antibody was from Upstate Biotechnology Inc., Lake Placid, N.Y. N3H10 (anti-Ku-70) and 111 (anti-Ku-80) monoclonal antibodies were used as described before (55). Peroxidase-coupled donkey anti-rabbit IgG was from Amersham (Les Ulis, France).

Immunostaining of Vav. After being washed in DMEM, cells were attached to poly-L-lysine-coated coverslips, fixed for 10 min in 3% paraformaldehyde, and permeabilized with 0.1% Triton X-100 for 15 min. Affinity-purified rabbit anti-Vav (Signal Transduction) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson) diluted 1:200 were applied for 30 min each. Rhodamine-labeled wheat germ agglutinin was added together with the anti-rabbit IgG antibody. Coverglasses were mounted in Moviol (Hoechst, Frankfurt, Germany). Confocal laser scanning microscopy and double immunofluorescence analysis were performed with a TCS4D confocal microscope based on a DM microscope interfaced with a mixed-gas argon-krypton laser (Leica Laser Technik). Simultaneous double fluorescence acquisitions were performed with the 488-nm and 568-nm laser lines to excite FITC and tetramethylrhodamine isothiocyanate (TRITC) dyes with a 100× oil immersion Neofluor objective. The fluorescence was selected with appropriate double fluorescence dichroic mirror and band pass filters and measures with blue-green-sensitive and red-sensitive side-one photomultipliers. All the data were registered at the same setting of the laser and multipliers. Focal sections were printed with a color video printer (Sony UP 7300 MDP).

RESULTS

Isolation of proteins interacting with the carboxy-terminal domains of human Vav. To identify proteins that interact with the C-terminal domains of human Vav, we used the yeast two-hybrid system with LexA recognition sites to regulate expression of both *his3* and *lacZ* (63). As bait, we fused SHVAV (residues 623 to 837 of Vav, including the SH2 and SH3 domains) to the C terminus of the LexA DNA-binding domain in pVJL10. Strain L40 was transformed with plasmid pLexA-SHVAV and tested for the absence of growth on selective medium and for the absence of β-galactosidase activity. This strain was retransformed with a Jurkat cell oligo(dT) cDNA library constructed in pGAD1318 to make fusion proteins with the Gal4 transcriptional activation domain. When a cDNA encodes a protein that interacts with SHVAV, the reporter strain is expected to grow in the absence of histidine and to produce β-galactosidase. We selected the blue colonies (using an X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] colony filter assay) which grew on yeast drop-out medium lacking Trp, Leu, His, Lys, and uracil. About 450 positive colonies were obtained out of 4×10^6 screened, and 72 of them were tested to find out whether their phenotypes were dependent on the interaction with LexA-SHVAV fusion protein. Plasmids were rescued and used to retransform the reporter strain in combination with the pLexA-SHVAV plasmid or with an unrelated gene fusion plasmid (pLexA-Ras^{V12} or pLexA-Lamin). The nucleotide sequences of 17 specific clones showed a 100% match to the C-terminal cDNA of human Ku-70 (Fig. 1A). v41, the largest clone, extended from residues 291 to 609 of Ku-70, and the shortest ones (v81, v86, v94, and v95) extended from residues 460 to 609. Figure 1B shows that the polypeptide encoded by v41 interacts with SHVAV but not with Ras^{V12} or with the LexA DNA-binding domain alone. Furthermore, the specificity of the SHVAV-v41 interaction was illustrated by the lack of reporter gene activation when the v41 polypeptide with the full-length Nck (an adaptor molecule with three SH3 domains and one SH2 domain) was assayed (data not shown).

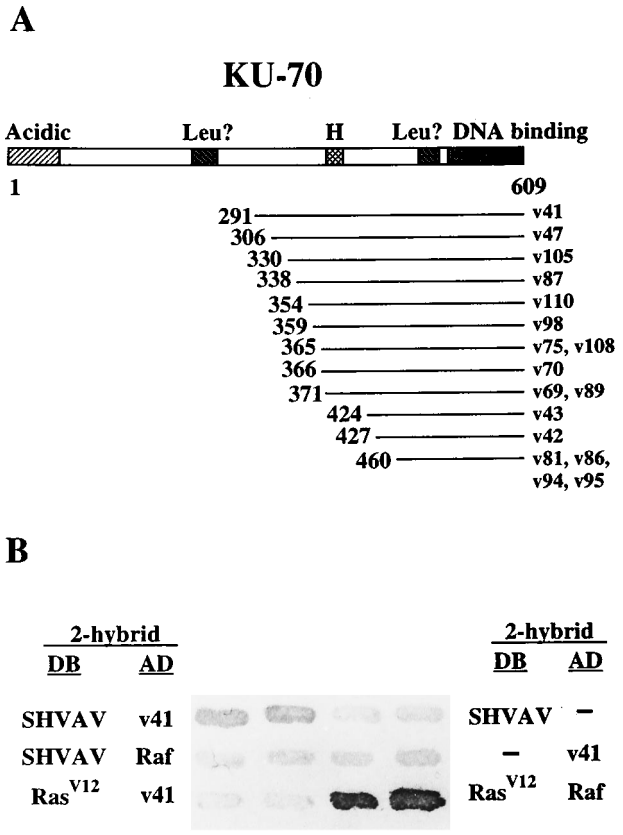

FIG. 1. Interaction of Ku-70 with p95^{vav} in the two-hybrid system. (A) Positive clones derived from Ku-70 obtained upon screening of a lymphoid cell cDNA library with SHVAV are shown. The different domains of Ku-70 are shown at the top. Leu?, potential leucine zipper; H, hydrophobic domain. (B) Interaction of polypeptide v41 with SHVAV. 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 grey patches). L40 carrying pLex-Ras^{V12} 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.

Several structural features with potential functional significance are apparent in Ku-70 (Fig. 1A) (55). The N terminus has an acidic domain similar to the acidic transcriptional activator domain of some transcription factors. Two regions with some similarity to leucine zipper motifs are present, and a hydrophobic domain reminiscent of a transmembrane sequence has also been suggested. Finally, a DNA-binding domain has been mapped to the C-terminal region. The shortest Ku-70 polypeptide interacting with Vav contains the 150 C-terminal residues of Ku-70 and includes one potential leucine zipper and the Ku-70 DNA-binding domain. Interestingly, this region of Ku-70 does not contain any stretch of proline residues. Since the Ku-70 fragment comprising the 150 C-terminal residues suffices for Vav interaction, to find out whether a shorter fragment of Ku-70 was still able to interact with SHVAV, we subcloned residues 484 to 609 (v41Cter) in pVJL10 (Fig. 2A). This clone includes one of the presumptive leucine zippers and the DNA-binding domain but lacks the 24 N-terminal residues of v94 (one of the smallest clones interacting with SHVAV). However, v41Cter did not interact with SHVAV (Fig. 2B). In the same way, we subcloned residues 291 to 483 of Ku-70 (v41Nter) in pVJL10. This clone does not interact with SHVAV. This suggests that the integrity of v94 is important in the interaction of SHVAV with Ku-70 in the two-hybrid system.

Vav interacts with Ku-70 in vitro. To validate the results obtained with the yeast two-hybrid system, the interaction of Ku-70 with Vav was studied by in vitro binding experiments. Two cDNAs encoding the C-terminal residues of Ku-70 detected in the initial screening (v41 and v94) were subcloned in a prokaryotic expression vector (pGEX-4-T2), and the bacterial lysates were purified on GST-agarose beads (see Materials and Methods). Lysates from a T lymphoma cell line (Jurkat) were incubated with the agarose beads, and after being washed, the proteins were resolved on gels, and the blots were developed with anti-Vav. Figure 3 shows clearly that Vav interacts with both of the GST-Ku-70-derived fusion proteins, whereas there is no interaction with either GST alone or with irrelevant GST fusion proteins [Nck-(SH3)$_3$/SH2, Csk-SH2/SH3, and Lck-SH2/SH3]. Similar results were obtained with lysates from the growth factor-dependent human megakaryoblastic cell line UT7-S Epo (data not shown). Vav interactions with GST-v41 and GST-v94 remained unchanged if nonspecific binding sites on the GST beads were blocked by preincubation with an excess of soluble GST. Therefore, by using fusion proteins, we show that recombinant Ku-70 (C-terminal portion) may directly or indirectly interact with Vav.

Localization of site of Vav binding to Ku-70. The domain of Vav responsible for the interaction with Ku-70 was studied. Three PCR fragments of *vav* were subcloned in fusion with the LexA DNA-binding domain (Fig. 4A): SH3-C Vav (residues 787 to 837), which contains the intact C-SH3 of Vav; SH3-C′

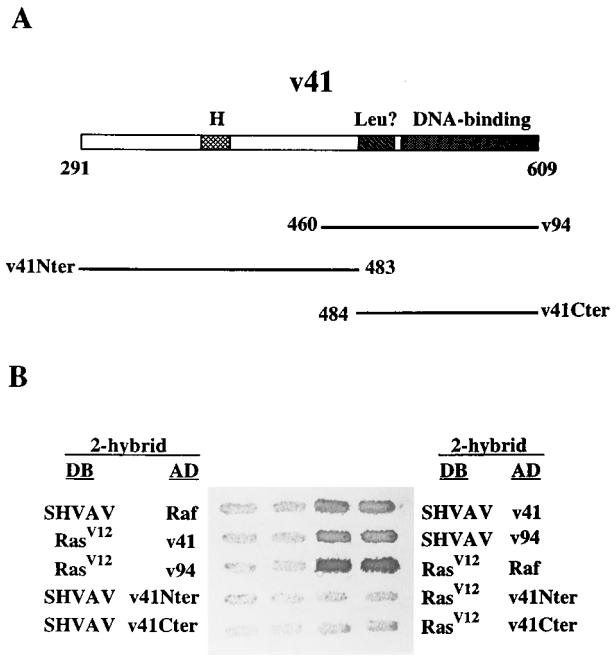

FIG. 2. Study of the minimum length of v41 fusion protein able to associate with SHVAV. (A) Diagram of the domains of the v41 polypeptide. H, hydrophobic domain; Leu?, potential leucine zipper. v94 is the minimum clone derived from Ku-70 detected in the SHVAV screen. v41Nter and v41Cter are subclones of v41. (B) Interaction of v94, v41Nter, and v41Cter polypeptides with SHVAV in the two-hybrid system. The L40 reporter strain was cotransformed with the indicated plasmids. Growth in the absence of histidine indicates the interaction between hybrid proteins. L40 carrying pLex-Ras^{V12} 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.

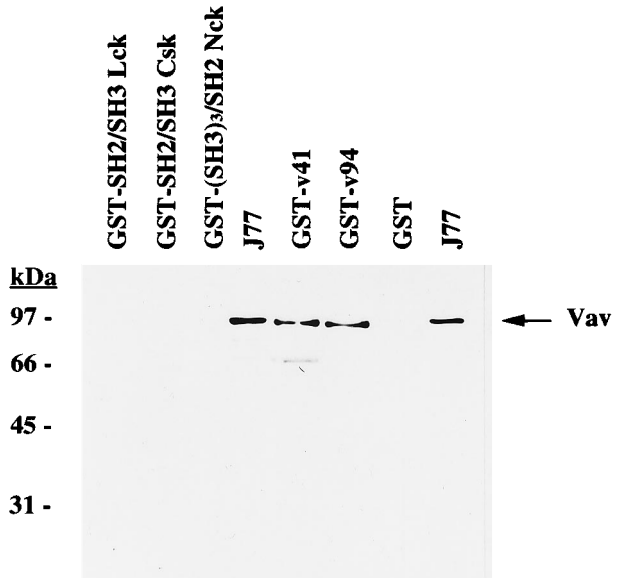

FIG. 3. Vav interacts with Ku-70 in vitro. Expression of the GST fusion proteins was induced by addition of IPTG, and proteins were isolated from bacterial lysates by affinity chromatography with glutathione-agarose beads and incubated with lysates from 5×10^6 Jurkat T cells. After being washed, proteins eluted in sample buffer were resolved by SDS-PAGE, blotted on nitrocellulose filters, and developed with monoclonal anti-Vav antibody. One microgram of each GST protein was used. J77, total lysate from 5×10^5 Jurkat cells.

Vav (residues 813 to 837), which comprises only the last 25 residues C-terminal of SHVAV; and SH3-N′/SH2 Vav (residues 623 to 812), which includes the whole SHVAV but lacks the 25 C-terminal residues of SH3-C′ Vav. By screening in the yeast two-hybrid system, we show that the C-SH3 domain of Vav is responsible for the interaction with the C-terminal part of Ku-70 (Fig. 4B). Moreover, the LexA-SH3-C′ Vav fusion protein, which contains only 25 residues from the C-SH3 domain of Vav, also interacts with Gal4-v41. This limits the interacting domain with Gal4-v41 and shows that the configuration is still maintained. However, SH3-N′/SH2 Vav missing these 25 residues was unable to interact with v41 (Fig. 4B).

SH3 domains are known to bind proteins through rather specific proline-rich sequences. However, the v41 polypeptide does not contain a consensus proline sequence (55). Nevertheless, to ascertain that the interaction between Vav and Ku-70 is not an SH3–proline-rich sequence interaction, we muted proline 833 of SHVAV to leucine to yield SHVAV P833L. This mutation is analogous to an SH3 mutation which abrogates the function of the Grb2 homolog Sem5 in the vulva differentiation pathway of *Caenorhabditis elegans* (12, 43) and inhibits the interaction between Vav and heterogeneous nuclear ribonucleoprotein particle K (34). Strain L40 was cotransformed with pLexA-SHVAV P833L and pGAD-v41, and the interaction between SHVAV and v41 polypeptide was still detected (Fig. 4C). However, SHVAV P833L was unable to interact with a poly(rC)-specific RNA-binding protein (data not shown). Therefore, the C-SH3 of Vav suffices for binding to Ku-70 independent of the presence of a proline motif.

Subcellular localization of Vav. Vav has been described as a cytoplasmic protein (35). As Ku-70 is localized exclusively in the nucleus (33, 54, 64), it was crucial to determine the subcellular distribution of Vav in order to evaluate the physiological relevance of the Vav/Ku-70 interaction. For this purpose, Western blot experiments with anti-Vav antibodies were performed on nuclear and cytoplasmic extracts of UT7-S Epo and Jurkat T cells, and the results showed the presence of Vav in both fractions (data not shown). As nucleus-cytoplasm fractionation procedures can lead to protein leakage or contamination from one compartment to the other, Vav localization was also investigated in intact cells by indirect fluorescence microscopy. UT7-S Epo cells were attached to poly-L-lysine-coated coverslips, fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with affinity-purified rabbit anti-Vav antibody followed by an FITC-conjugated donkey anti-rabbit IgG antibody. Coverslips were double-labeled with wheat germ agglutinin coupled to rhodamine in order to delineate the plasma membrane and Golgi apparatus plus endosomes. Labeled cells were observed with a confocal laser microscope. As shown in Fig. 5a and b, Vav was strongly expressed in the nucleus, and only a weak staining could be observed in the cytoplasm of UT7-S Epo cells. In order to study Vav localization in nonstimulated cells, UT7-S Epo cells were maintained for 20 h in medium without Epo or serum, which greatly decreases tyrosine phosphorylation of cellular proteins (17). In this condition, no difference in Vav distribution was observed (Fig. 5c and d). An identical localization pattern was obtained by the use of an alternative fixation procedure (glutaraldehyde plus paraformaldehyde). Similar results were obtained with Jurkat T cells (data not shown). These data therefore provided evidence for the interaction of Vav with Ku-70 in hematopoietic cells.

In vivo binding of Vav and Ku-70. Our next goal was to investigate whether Vav binds to Ku-70 in intact cells. UT7-S Epo cells and human thymocytes were lysed under mild con-

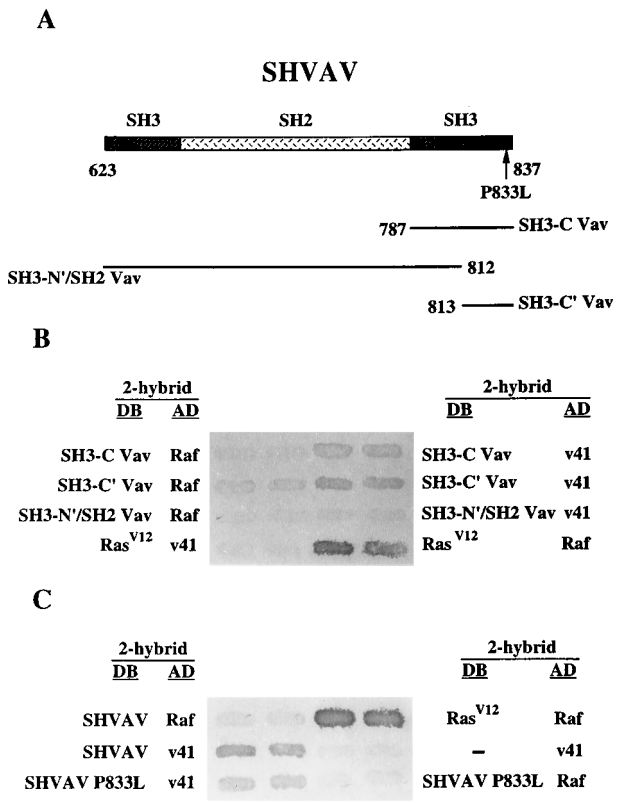

FIG. 4. Site of binding of Vav to Ku-70. (A) Different subclones and point mutants of SHVAV. (B and C) Interaction of Ku-70 with various subclones and point mutants of SHVAV in the two-hybrid system. See the legend to Fig. 1B for details.

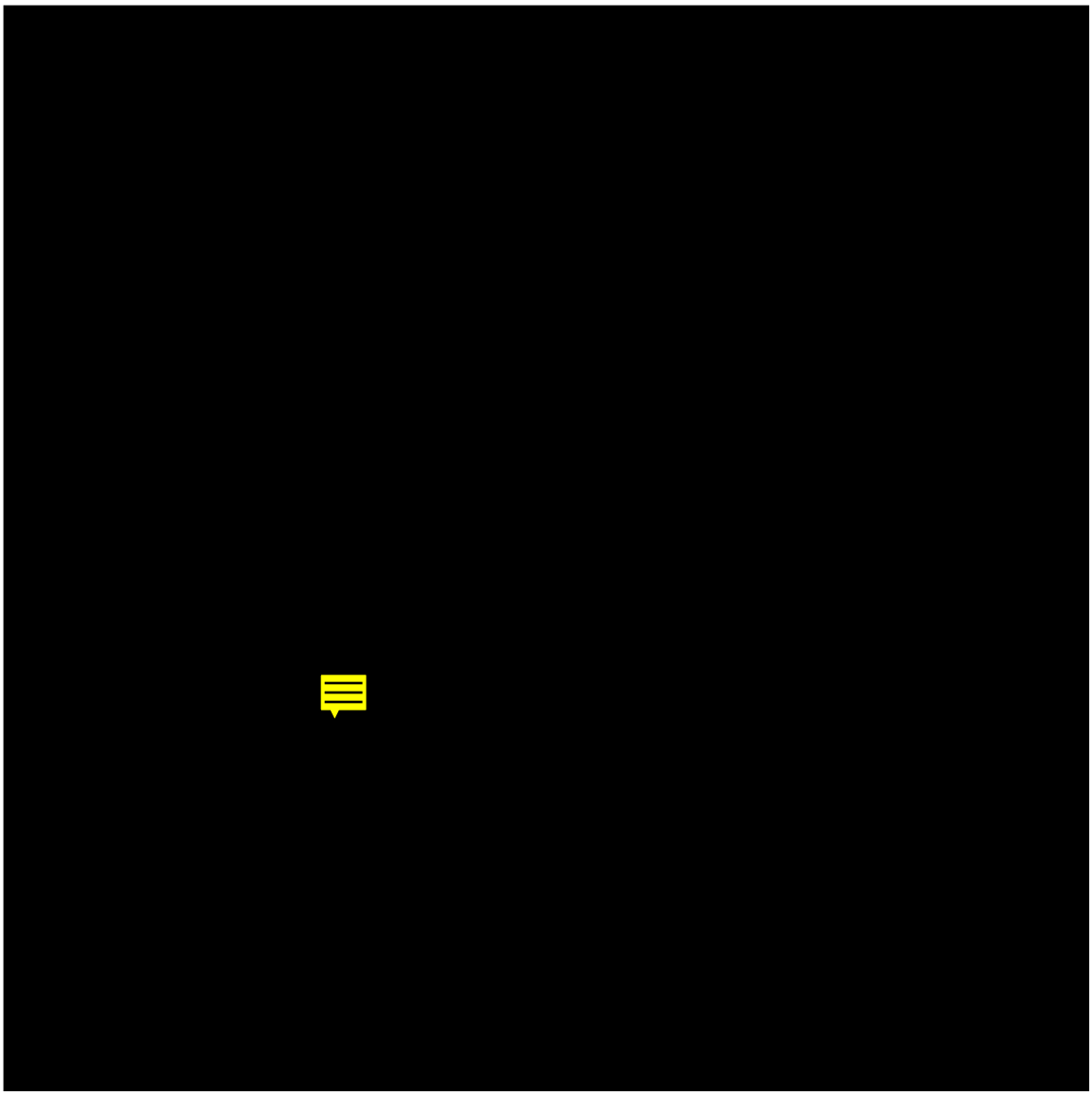

FIG. 5. Immunolocalization of Vav. Epo-stimulated (a and b) and nonstimulated (c and d) UT7-S cells were attached to poly-L-lysine-coated coverslips and stained with an affinity-purified rabbit anti-Vav antibody followed by a FITC-conjugated donkey anti-rabbit antibody and TRITC-conjugated wheat germ agglutinin. Single optical sections of cells obtained by confocal microscopy are shown. On the same cell section, anti-Vav staining in green is shown either alone (a and c) or together with TRITC-wheat germ agglutinin staining in red (b and d). Wheat germ agglutinin labeling delineates the plasma membrane and also the Golgi apparatus and endosomes.

ditions in Brij detergent, incubated with anti-Vav or irrelevant sera, washed with isotonic buffer, and resolved by SDS-PAGE. The blots were developed with anti-Ku-70-specific monoclonal antibodies (N3H10). As shown, Ku-70 is clearly detected in the anti-Vav immunoprecipitates with N3H10 but not with preimmune serum (Fig. 6). The stoichiometry of Ku-70 bound to Vav is not easy to evaluate. The amount of nuclear Vav, presumably the interacting molecule, has not been determined. Moreover, it is possible that part of Ku-70 interacting with Vav is detached during the washing steps. Overall, these findings show that Ku-70 complexed to Vav is recovered from intact cells, confirming by an in vivo approach the results obtained by the double-hybrid system and with the GST fusion proteins.

DISCUSSION

The p95 product of the *vav* proto-oncogene is expressed only in hematopoietic cells (14, 37) and plays an important role in at least some hematopoietic signaling pathways (8, 34, 65). Moreover, the recent development of $vav^{-/-}$ mice has confirmed a major role of Vav in the expansion of B- and T-cell precursors as well as of mature lymphoid cells (23, 61, 66). Vav contains multiple structural motifs used by intracellular signaling molecules, including a helix-loop-helix domain and a highly acidic amino acid region. Vav also contains nuclear localization signals, suggesting that the Vav protein has structural features for nuclear localization (36).

Using the yeast two-hybrid approach (23), we have shown that SHVAV (residues 623 to 837 of Vav, including the SH2

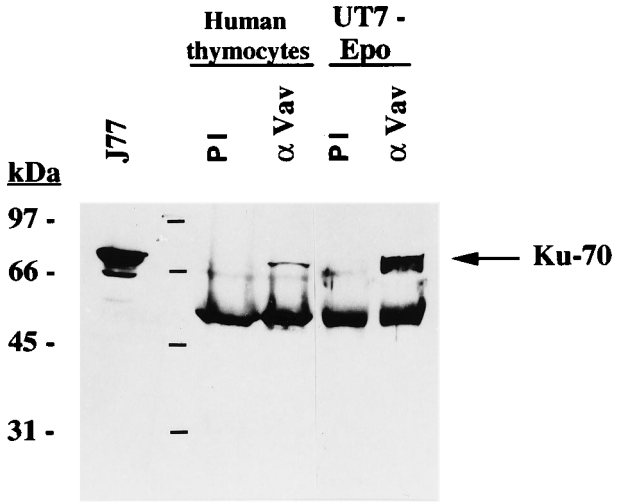

FIG. 6. Coimmunoprecipitation of Vav and Ku-70. Brij lysates from human thymocytes and UT7-S Epo cells (10^8 and 5×10^7, respectively) were incubated with anti-Vav or preimmune (PI) serum and, after washing, resolved by SDS-PAGE, transferred to nitrocellulose filters, and incubated with monoclonal antibody anti-Ku-70 (N3H10). The band at about 50 kDa is the IgG heavy chain.

and SH3 domains) interacts with Ku-70 (Fig. 1), a protein localized primarily or exclusively in the nucleus and nucleolus (33, 54, 64). In spite of the potential nuclear localization motifs, Vav is known as a cytoplasmic protein (35). However, our immunofluorescence and confocal microscopy studies (Fig. 5) show clearly that Vav is also localized in the nucleus of Epo-treated and nontreated UT7-S cells. Another clone of UT7 cells (granulocyte-macrophage colony-stimulating factor dependent) maintained with or without the cytokine revealed an identical localization of Vav (not shown). During the progression of this work, Clevenger et al. (13) reported that Vav is translocated to the nucleus during prolactin stimulation of the rat T lymphoid cell line Nb2. However, in the cells studied by us (UT7 and Jurkat), it is clear that Vav is detected in the nucleus in the absence of receptor triggering.

In this study, we show that Vav can bind to the ubiquitous nuclear DNA-binding protein Ku-70 (59). This protein, originally detected as an autoantigen (45), associates with Ku-80 to form a heterodimeric complex which in vitro binds to the ends of double-stranded DNA and to single- to double-strand DNA transitions and appears to be capable of diffusional movement along DNA (4, 15, 21). The Ku-70/Ku-80 heterodimer binds a catalytic 350-kDa component to constitute the DNA-dependent protein kinase (18). Some evidence shows that DNA-dependent protein kinase plays a role in DNA repair and in V(D)J recombination in mammalian cells (60). It also phosphorylates many substrates in vitro, including p53, c-Jun, c-Fos, c-Myc, Sp1, RNA polymerase II, Oct1, Oct2, Ku-70, and Ku-80 itself (55). Besides its ability to interact nonspecifically with DNA, specific binding sequences have been described in some cases (TREF, PSE1, and E1BF) (40, 48, 56).

Among the 72 random positive clones that interacted with SHVAV, 17 clones (24%) were 100% homologous to different fragments of the 3′ end of human Ku-70 cDNA (Fig. 1A). Efforts were made to delimit the region of interaction between Ku-70 and SHVAV. The finding that v41Cter, which lacks the 24 N-terminal residues of v94 (one of the smallest clones interacting with SHVAV, representing residues 460 to 609 of Ku-70), and v41Nter, which contains this region, no longer interacted with SHVAV (Fig. 2) suggested that a conformational motif, probably comprising residues 460 to 483 plus surrounding domains located in v94, is required for Ku-70/Vav interaction. A presumptive leucine zipper C-terminal to the 24 residues has been described, and such motifs have been implicated in protein-protein interactions (55). But in the absence of a specific configuration, this motif cannot by itself be responsible for Ku-70/Vav interaction, as judged by the finding that the v41Cter polypeptide, which possesses this domain, is unable to interact with SHVAV. However, other possibilities can also be envisaged.

On the other hand, using v41 (residues 209 to 609 of Ku-70) as bait and various deletions of SHVAV, we showed that the C-SH3 of Vav interacts with the v41 polypeptide (Fig. 4). The binding mechanism is not an expected interaction of SH3 domains and proline-rich regions. In fact, polypeptide v41 does not contain a proline-rich motif (55), and furthermore, the SHVAV P833L mutant, which does abolish interaction through proline domains (12, 34, 43), is still able to bind Ku-70. Therefore, the interaction must be of a different nature. Recently, through the analysis of the crystal structure of the SH2/SH3 fragment of Lck, it became apparent that intermolecular interactions between the SH2 and SH3 domains of Lck which do not involve a proline-rich region or tyrosine-phosphorylated residues are detected (19). We postulated that the Vav/Ku-70 interaction might entail a novel mode of protein-protein interaction.

The results obtained with the two-hybrid system were confirmed by both in vivo and in vitro experiments. We recovered Vav from lysates prepared with mild detergents by using Ku-70-derived fusion proteins (GST-v41 and GST-v94) (Fig. 3). Most important, it is possible that Ku-70 and Vav complexes exist in intact cells, because we coimmunoprecipitated Ku-70 with Vav from UT7-S Epo cells and from freshly isolated human thymocytes (Fig. 6). By reblotting the dehybridized nitrocellulose filter from anti-Vav immunoprecipitates with anti-Ku-80-specific monoclonal antibody 111, we detected Ku-80. Similarly, in vitro experiments with GST-v41 and GST-v94 showed that the binding of Ku-80 was detected in addition to the association of Vav with Ku-70-derived fusion proteins (results not shown). These results suggest that binding of Vav to Ku-70 does not modify the formation of the Ku-70/Ku-80 heterodimer. It will be of interest to see if the binding, activity, or substrate selectivity of the catalytic 350-kDa protein kinase is affected by Vav binding to Ku-70. However, the biological significance of the Vav/Ku-70 interaction remains to be determined. We and others have shown that Vav becomes rapidly tyrosine phosphorylated after activation of different surface receptors in hematopoietic cells, but the downstream elements remain largely unknown. Our finding of the association of Vav with Ku-70 is the first report of an interaction between a nucleus-specific protein and Vav.

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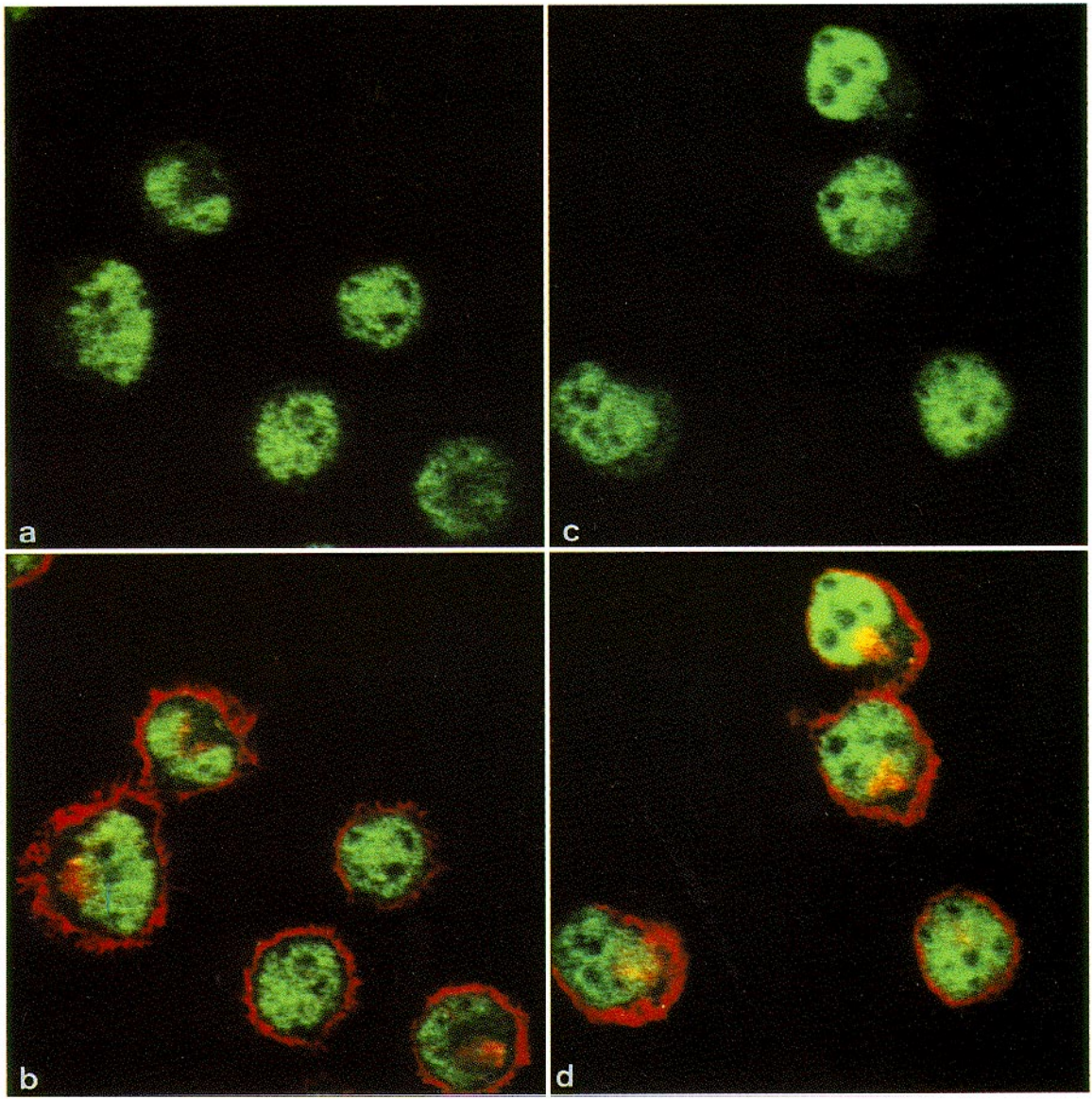
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