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# Cbl-b, a member of the Sli-1/c-Cbl protein family, inhibits Vav-mediated c-Jun N-terminal kinase activation

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We have used the yeast two-hybrid system to identify proteins that interact with Vav, a GDP/GTP exchange factor for the Rac-1 GTPase that plays an important role in cell signaling and oncogenic transformation. This experimental approach resulted in the isolation of Cbl-b, a signal transduction molecule highly related to the mammalian c-cbl proto-oncogene product and to the C. elegans Sli-1 protein, a negative regulator of the EGF-receptor-like Let23 protein. The interaction between Vav and Cbl-b requires the entire SH3-SH2-SH3 carboxy-terminal domain of Vav and a long stretch of proline-rich sequences present in the central region of Cbl-b. Stimulation of quiescent rodent fibroblasts with either epidermal or platelet-derived growth factors induces an increased affinity of Vav for Cbl-b and results in the subsequent formation of a Vav-dependent trimeric complex with the ligand-stimulated tyrosine kinase receptors. During this process, Vav, but not Cbl-b, becomes highly phosphorylated on tyrosine residues. Overexpression of Cbl-b inhibits the signal transduction pathway of Vav that leads to the stimulation of c-Jun N-terminal kinase. By contrast, expression of truncated Cbl-b proteins and of missense mutants analogous to those found in inactive Sli-1 proteins have no detectable effect on Vav activity. These results indicate that Vav and Cbl-b act coordinately in the first steps of tyrosine protein kinase receptor-mediated signaling and suggest that members of the Sli-1/Cbl family are also negative regulators of signal transduction in mammalian cells.

Keywords: Cbl family; Vav; tyrosine phosphorylation; JNK activation

## Introduction

The vav gene was first identified by virtue of its oncogenic activation during the course of gene transfer assays (Katzav et al., 1989). Its normal allele, the vav proto-oncogene, encodes a 95 kDa polypeptide that harbors a complex array of structural domains, including leucine-rich, acidic, Dbl-homology (DH), pleckstrin-homology (PH), cysteine-rich, SH3 and SH2 domains (for a review, see Bustelo 1996). The important role of this gene during mitogenic and ontogenic processes was demonstrated by both cell biology and genetic experiments. Thus, it has been shown that Vav can induce high levels of morphological transformation in rodent fibroblasts after deletion of N-terminal sequences (Katzav et al., 1989; Coppola et al., 1991). Moreover, disruption of the vav locus by homologous recombination techniques results in severe signaling defects of primary antigen receptors, leading to abnormal lymphocyte proliferation and lymphocytopenia (Fisher et al., 1995; Tarakhovski et al., 1995; Zhang et al., 1995). In T-cells this phenotype appears to be mediated, at least in part, by insufficient IL-2 secretion after T-cell receptor cross-linking (Tarakhovski et al., 1995; Zhang et al., 1995).

At the biochemical level, Vav becomes tyrosine phosphorylated upon stimulation of hematopoietic receptors such as the T-cell receptor-CD4 complex (Bustelo et al., 1992; Margolis et al., 1992), the mast cell IgE high affinity receptor (Margolis et al., 1992), the B-cell IgM antigen complex (Bustelo and Barbacid, 1992), the c-Kit and Flk-2 tyrosine kinase receptors (Alai et al., 1992; Dosil et al., 1993) and a large panel of cytokine receptors (Bustelo, 1996), further suggesting that the function of Vav is required during the earliest steps of receptor-mediated signal transduction.

Recent experimental evidence indicates that Vav acts as a guanosine-nucleotide exchange factor for the GTP-binding protein Rac-1 (Crespo et al., 1997), a GTPase of the Rho subfamily with roles in cytoskeletal architecture, cell proliferation, and cellular transformation (for a review, see Ridley, 1995). This enzymatic activity is stimulated during cell signaling by the phosphorylation of Vav on tyrosine residues, leading to the transition of the Rac-1 GTPase from the inactive (GDP-loaded) to the active (GTP-loaded state) (Crespo et al., 1997). Rac-1 activation leads in turn to the stimulation of intracellular routes such as the c-Jun N-terminal kinase (JNK) pathway (Crespo et al., 1996, 1997). This biochemical activity appears to be mediated by the Vav DH domain, since mutants of Vav with a non-functional DH motif do not induce Rac-1 nucleotide exchange nor JNK activation (Crespo et al., 1996, 1997).

In addition to this catalytic role, other experimental evidence suggests that protein-protein interactions also take part in the regulation of Vav activity. In agreement with this notion, several reports have indicated that the Vav SH2 and SH3 domains work

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as autonomous heterodimerization motifs. As an example, the Vav SH2 domain binds to ligand-stimulated mitogenic receptors such as the EGF and PDGF receptors (Bustelo et al., 1992; Margolis et al., 1992) as well as to cytoplasmic tyrosine-phosphorylated proteins such as the B-cell specific Vap-1 protein (Bustelo and Barbacid, 1992), the T-cell specific Zap70 protein tyrosine kinase (Katzav et al., 1994), members of the Jak and Btk families (Machide et al., 1995; Matsuguchi et al., 1995) and the hematopoietic-specific SLP-76 protein (Wu et al., 1996). These interactions are likely to be mediated by the specific recognition of the tyrosine phosphorylated YXEP (where X=M, L or E) sequence motif by the Vav SH2 domain (Songyang et al., 1994). On the other hand, the Vav SH3 domains have been identified as targets for the association of a plethora of quite unrelated proteins such as Grb-2 (Ye and Baltimore, 1994), hnRNP-K (Hobert et al., 1994; Bustelo et al., 1995), the nuclear protein Ku-70 (Romero et al., 1996) and the cytoskeletal protein zyxin (Hobert et al., 1996). These interactions are mediated by the recognition of specific proline-rich sequences by the Vav SH3 domains. Since these interactions do not require post-translational modification of these proteins, they are likely to be constitutive and independent of the mitogenic status of cells. At present, the biological significance of these interactions remains to be determined.

In this study, we have utilized the SH3-SH2-SH3 domain of Vav as a probe to isolate novel Vav-binding proteins by the two hybrid system (Bartel et al., 1993). This approach resulted in the identification of two Vav-binding proteins, the already-described hnRNP-K (Hobert et al., 1994; Bustelo et al., 1995) and a second protein, designated Cbl-b (Keane et al., 1995), whose characterization is presented in this work. Cbl-b is a ubiquitously-expressed protein that binds to the carboxy-terminal region of Vav through an interaction mediated by a cluster of proline-rich sequences located in the middle of the Cbl-b molecule. In addition, we show that overexpression of full length Cbl-b results in the inhibition of the Vav signaling pathway that triggers JNK activation. Cbl-b is highly related to the mammalian c-Cbl and the nematode Sli-1 proteins (for a review, see Langdon, 1995). c-Cbl is the cellular homologue of v-n l, a retroviral oncogene implicated in the development of pre-B lymphomas and myelogenous leukemia in mice that also induces acute transformation of immortalized rodent fibroblasts in vit o (Langdon, 1995). The Sli-1 protein has been recently identified as the product of a dominant inhibitor of the eleoane let23 gene, a homologue of the mammalian EGF receptor (Langdon, 1995). Hence, Cbl-b appears to be a member of an evolutionarily conserved family of proteins implicated in downregulating signals derived from tyrosine kinase receptors andfor their associated substrates.

## Results

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To isolate cDNAs whose protein products interact with Vav protein, we fused the carboxy-terminal region of mouse vav gene to the Gal-4 DNA- inding domain (GBD) in a vector (pGBT) that directs low levels of expression of the GBD fusion proteins (Bustelo et al., 1995). As the tester strain, we used YPB2 derivatives containing the bait plasmid and two Gal-4-inducible markers, H S3 and lanZ (Bartel et al., 1993). This tester strain was re-transformed with a cDNA library derived from human B-lymphocytes constructed in the pACT plasmid (Bartel et al., 1993), a vector that directs the synthesis of fusion proteins containing the Gal-4 transcriptional activation domain (GAD). In those cases where a GAD fusion protein interacts with the bait protein, a functional Gal-4 activity is reconstituted leading to histidine prototrophy (H S3) and /)-galactosidase expression (lanZ) (Bartel et al., 1993).

A total of $2.9 \times 10^6$ transformants were placed in selective media containing 15 mM 3-amino-1,2,4-triazole. Transformants showing histidine prototrophy after one week of culture were subsequently screened for the ability to produce /)-galactosidase using a filter lift assay, resulting in a total of 30 positives. To test whether the phenotypes of those clones were dependent on the interaction with the GBD-Vav SH3-SH2-SH3 fusion protein, the plasmids of those colonies were selectively recovered in bacteria, grouped in three families by Southern blot hybridization, and representatives of each family re-introduced in YPB2 cells with vectors encoding the Gal-4 DNA binding domain either alone or fused to the Vav SH3-SH2-SH3 region. As additional control, we performed parallel experiments with a GBD fusion protein containing the SH3-SH2-SH3 region of Vav-2, a Vav-related protein with transforming potential that displays an ubiquitous distribution (Schuebel et al., 1996). As illustrated in Figure 1a, the co-transformation of one of the library plasmids (pXRB85) with either the Vav or Vav-2 C-terminal domains induced comparable levels of transcriptional activation of the lanZ gene. This transactivation was as strong as that observed with the interaction between the yeast SNF4 and SNF1 protein (Figure 1a). As a control for the specificity of the interaction, neither Vav-2 nor Vav could trigger detectable /)-galactosidase activity when co-transfected with the non-chimeric GAD protein (Figure 1a). Likewise, no transactivation was observed when pXRB85 was co-expressed with the DNA binding domain of Gal-4 protein (Figure 1a). Subsequent experiments indicated that Vav and the protein encoded by pXRB85 were capable of interacting in unrelated techniques such as GST-pull down experiments and farwestern blot analysis, further demonstrating the specificity of the initial two-hybrid association. By contrast, the members of the other families of putative positive clones did not show any binding in those two alternative systems and were consequently discounted as Vav-interacting partners (data not shown).

Nucleotide sequence analysis revealed that the cDNA present in the pXRB85 plasmid encoded a 456 amino acid-long polypeptide identical to residues 526–982 of Cbl-b (Keane et al., 1995) (Figure 1b), a molecule identified during the initial characterization of our clone that belongs to the c-CblfSli-1 family of signal transduction molecules (Langdon, 1995). Members of this protein family are characterized by the presence of a highly conserved amino-terminal region

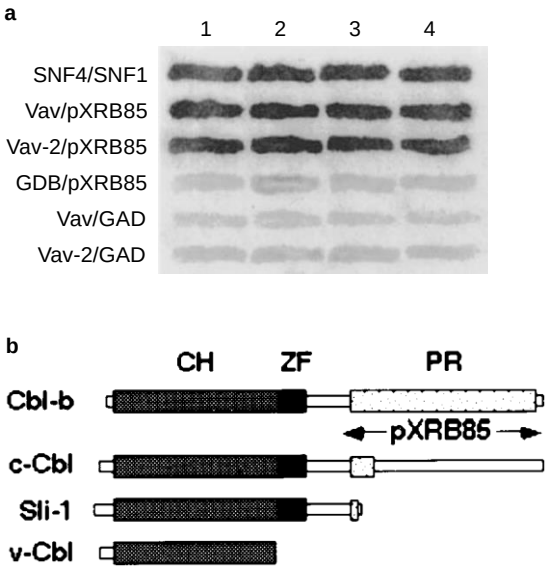


Figure 1 (a) Specific binding of the clone encoded in pXRB85 with the C-terminal regions of Vav and Vav-2. A total of 1 Jlg of pGBT-derived vectors containing the Vav and Vav-2 SH3-SH2-SH3 regions were co-transformed into yeast cells (YPB2 strain) with 1 Jlg of pGAD-GH or pACT vectors containing either no insert or cDNA sequences encoding the last 456 amino acids of the Cbl-b protein. Plasmids containing the yeast SFN4 and SNF1 proteins were used as positive controls. Four independent transfectants selected by LeufTrp prototrophy (labeled as 1 – 4) were assayed for /)-galactosidase activity. (b) Schematic representation of Cbl-b and related proteins. Highlighted domains include the bl-homology (CH) domain, the zinc finger (ZF), and the proline- ich (PR) region. The region of Cbl-b encoded by our two hybrid plasmid (pXRB85) is indicated

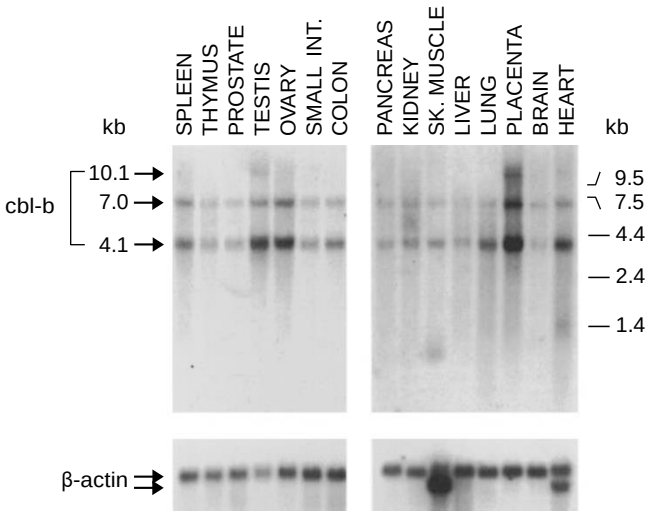


Figure 2 Distribution of n lo transcripts in adult human tissues. Filters containing 2 Jlg of polyA mRNA isolated from the indicated tissues (MTN blots, Clontech) were hybridized to either n lob (upper panels) or /)-antin (lower panels) cDNA probes as described in Materials and methods. Hybridized blots were exposed to autoradiography at −70°C with an intensifying screen for either 48 (upper panels) or 3 h (lower panels). The migration (n l-b and /)-antin) and size (n l-b) of the respective mRNAs are indicated by arrows. Migration of molecular weight markers is indicated on the right

that we will describe here as bl-homology (CH) region, a central zinc finger domain of the C3HC4f RING subclass, and a C-terminal proline-rich region of variable length that displays lower conservation (Figure 1b). Cloning of the full length n l-b cDNA from a human fetal brain cDNA library (see Materials and methods) resulted in the isolation of several cDNAs encoding a 982 amino acid-long protein that displayed only three amino acid changes (G210S, C266Y and T487A) with respect to the sequence originally reported by Keane et al. (1995). Whether these differences are due to polymorphic sequences remains to be determined.

To determine the pattern of expression of the n l-b gene, we performed Northern hybridization analysis of polyA mRNAs from various human tissues. To avoid cross-hybridization with the highly related c-n l mRNAs, we used as a probe the n l-b cDNA fragment contained in our two-hybrid isolate (pXRB85), a region which displays the lowest homology to the other members of the Cbl family. We observed n l-b mRNA expression in every tissue examined, with slightly higher levels in placenta, heart and tissues of the reproductive system (Figure 2, upper panel). The probe recognized three transcripts of 4.1, 7.0 and 10.1 kb. The relative abundance of the 4.1 and 7.0 kb mRNAs appears to be the same in every tissue tested (Figure 2, upper panel). In contrast, expression of the 10.1 kb transcript occurs at lower levels in a tissue-specific manner, with signals present only in placenta, testis, and ovary mRNAs (Figure 2, upper panel). Hybridization of the same filters with a /)-antin probe confirmed that all samples contained similar amounts of polyA-mRNA (Figure 2, lower panel).

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To characterize the structural elements implicated in the interaction between Vav and Cbl-b, we first incubated an in vit o translated $^{35}$S-labeled Cbl-b polypeptide (residues 476 – 982) with various GST fusion proteins corresponding to the carboxy-terminal motifs of Vav (Figure 3A). As a positive control, we used in vit o translated hnRNP-K, a protein previously shown to interact with the carboxy-terminal SH3 domain of Vav (Hobert et al., 1994; Bustelo et al., 1995). As illustrated in Figure 3B, the complete SH3-SH2-SH3 motif, but not its individual domains, was capable of interacting with the Cbl-b protein. This observation contrasts with our previous results obtained with the hnRNP-K protein (Bustelo et al., 1995), which requires only the most carboxy-terminal SH3 domain of Vav for efficient binding (Figure 3B, lane d).

To determine the Vav binding site in the Cbl-b molecule, we next analysed the interaction of a $^{32}$P-labeled GSTfVavSH3-SH2-SH3 protein to GST fusion proteins containing different regions of Cbl-b (Figure 4A). As a positive control, we used a GST protein containing the first Vav-binding site of hnRNP-K (Figure 4C) (Bustelo et al., 1995). Negative controls included GST alone as well as a chimeric GST fused to full length RhoA. Farwestern blot analysis indicated that the Vav SH3-SH2-SH3 probe recognized the GST fusion protein containing a stretch of proline-rich

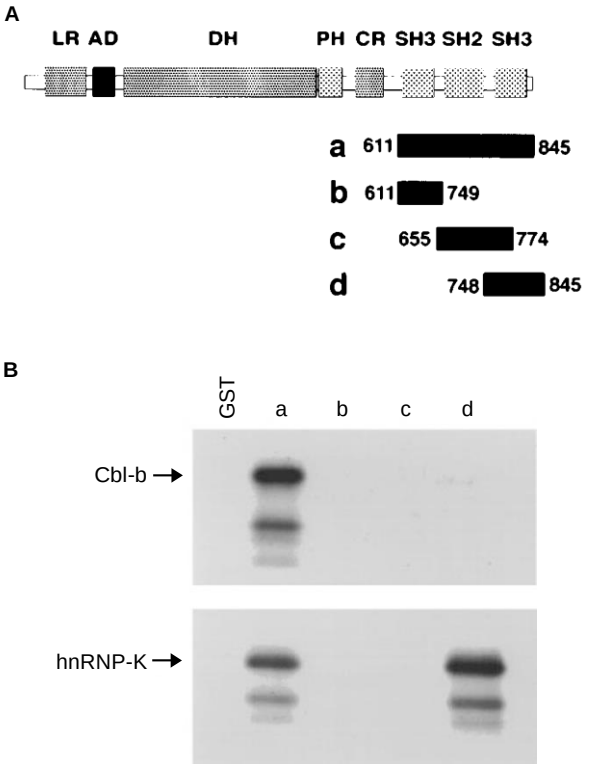


Figure 3 Cbl-b binds to the carboxy-terminal region of Vav. (A) Schematic representation of Vav and the GSTfVav fusion proteins used in this study. They include the Vav SH3-SH2-SH3 regions (a), the amino-terminal SH3 domain (b), the SH2 motif (c) and the carboxy-terminal SH3 domain (d). Other depicted Vav motifs include the leucine rich region (LR), acidic domain (AD), Dbl homology (DH) region, pleckstrin homology (PH) domain and cysteine-rich (CR) region. (B) Binding of GST fusion proteins to Cbl-b and hnRNP-K. The $^{35}$S-labeled proline-rich region (residues 476 – 982) of Cbl-b and the full length hnRNP-K protein were generated by in vit o translation and incubated at 4°C for 2 h with 5 Jlg of the above GST fusion proteins or GST alone. The resulting complexes were fractionated by SDS – PAGE (8%), fixed and soaked in Enlightning (NEN). Dried gels were exposed overnight (—70°C) to autoradiographic films. The migration of Cbl-b and hnRNP-K is indicated by arrows

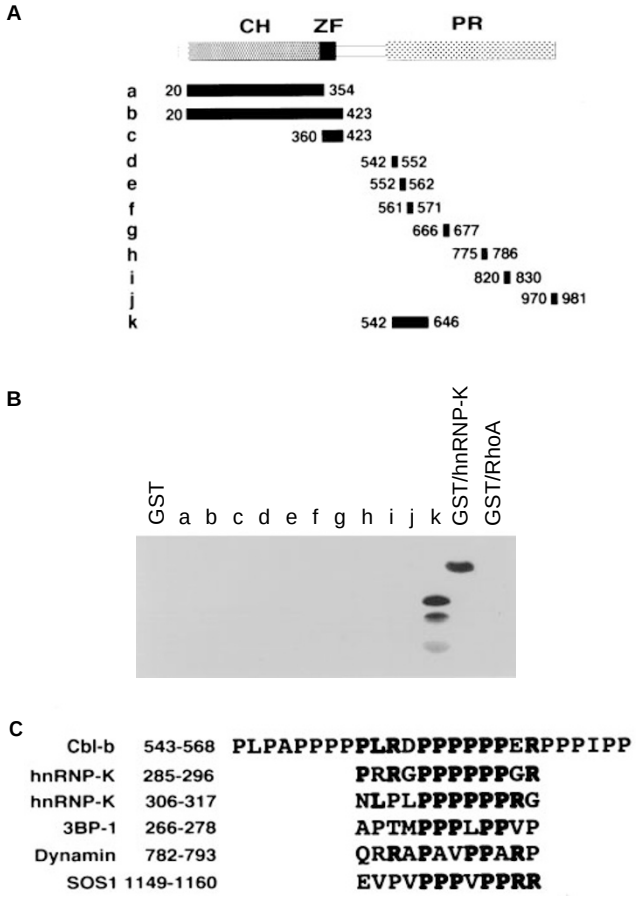


Figure 4 Vav binds to a proline rich region of Cbl-b. (A) Schematic representation of Cbl-b and the GSTfCbl-b fusion proteins used in this experiment. Cbl-b domains are those described in Figure 1b. (B) Farwestern blot analysis of the induced GST fusion proteins using $^{32}$P-labeled GSTfVavSH3-SH2-SH3 probe. (C) Alignment of the proline-rich regions present in the Vav binding sites of Cbl-b and hnRNP-K with those of other well-known SH3 binding proteins. Identical residues are shaded

sequences present in Cbl-b (Figure 4B, lane k), but not those carrying each of the individual proline-rich regions present in the carboxy-terminal region of Cbl-b (Figure 4B, lanes d – j). Likewise, no significant interaction was observed with either the CH or ZF domains of Cbl-b (Figure 4B, lanes a – c). These results indicate that the proline-rich sequences spanning residues 543 – 568 of Cbl-b are the minimal structural elements required for binding to Vav (Figure 4C). Interestingly, this region contains a motif, R-X-$P_{(6)}$-X-R, shared by one of the proline-rich regions of hnRNP-K that mediate its interaction with Vav (Figure 4C) (Bustelo et al., 1995). The second hnRNP-K proline-rich domain involved in Vav binding also contains a cluster of six prolines in the core of the binding site (Figure 4C).

### In vivo indino of Vav and lo p oteine

To determine whether the Cbl-b and Vav proteins associate in vivo, we conducted a series of co-immunoprecipitation experiments using NIH3T3 cell lines expressing Vav alone and in combination with the Cbl-b protein tagged at its amino-terminus with the FLAG epitope. As negative controls, we used NIH3T3 clones containing Cbl-b and Grb-2, a signal transduction protein whose structure closely resembles the SH3-SH2-SH3 carboxy-terminal region of Vav (Suen et al., 1993). The resulting Cbl-b immunoprecipitates were probed with anti-FLAG antibodies to determine expression levels of the Cbl-b protein and, subsequently, re-blotted with anti-Vav antibodies. As illustrated in Figure 5a (lower panel), Vav was readily detected in the Cbl-b immunocomplexes derived from exponentially growing cells, indicating that these proteins form heteromolecular complexes in vivo. Anti-FLAG immunoprecipitates derived from cell lines that express Vav, but not Cbl-b, did not show any significant levels of Vav. Notably, the amount of Vav in the anti-FLAG immunoprecipitates was proportional to the amount of Cbl-b present in these samples (Figure 5a, upper panel), suggesting that their co-immunoprecipitation is concentration-dependent.

To confirm the specificity of this interaction, we examined the presence of the Vav protein in anti-FLAG immunoprecipitates obtained either in the absence or presence of competing FLAG peptide (10 Jlgfml). As an additional control, we analyzed parallel immunoprecipitates obtained with an unrelated

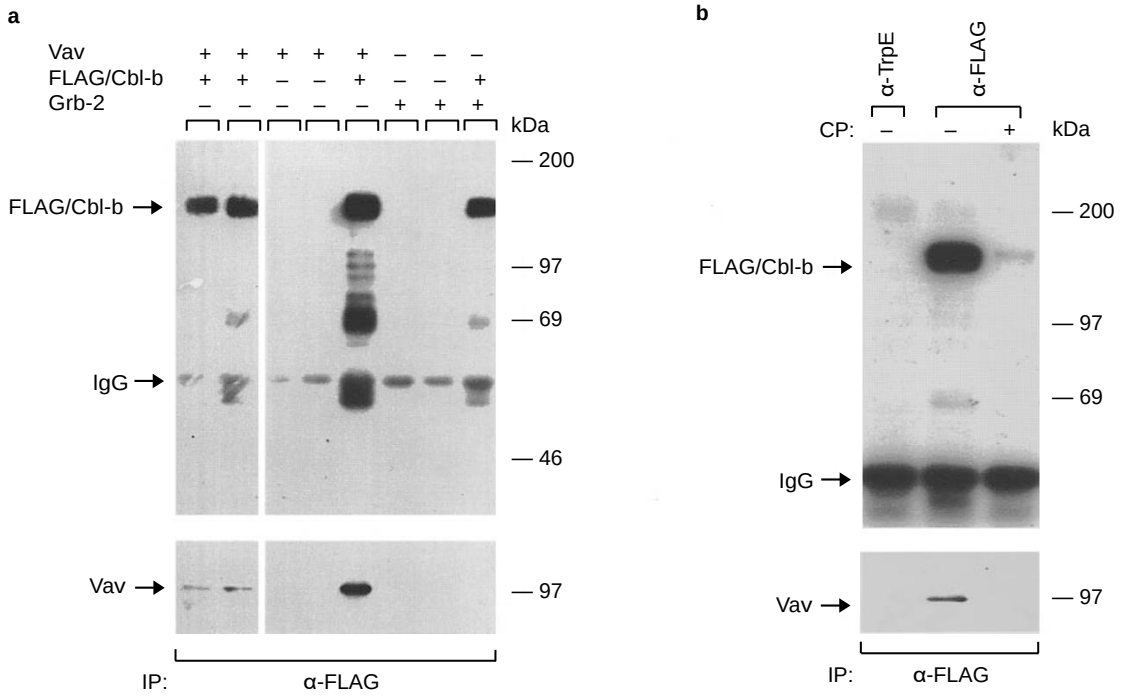


Figure 5 n vivo binding of Cbl-b and Vav proteins. (a) Exponentially growing cultures of cell lines expressing the indicated combinations of proteins were lysed in RIPA buffer and immunoprecipitated (IP) for 3 h at 4°C with anti-FLAG antibodies. The resulting immunocomplexes were washed, separated by SDS – PAGE, and submitted to Western blot analysis with either anti-FLAG (upper panel) or anti-Vav (lower panel) antibodies. Development of Cbl-b and Vav signals was performed by autoradiography using $^{125}$I-labeled goat anti-mouse IgG and by chemiluminescence (ECL system), respectively. (b) Cellular lysates from NIH3T3 cells co-expressing Cbl-b and Vav proteins were immunoprecipitated with anti-TrpE or anti-FLAG antibodies either in the absence (−) or presence (+) of competing FLAG peptide (CP) (10 Jlgfml). The resulting immunocomplexes were submitted to Western blot analysis using anti-FLAG (upper panel) or anti-Vav (lower panel) antibodies as described above. The migration of Cbl-b, Vav, and immunoglobulin heavy chains (IgG) is indicated by arrows. Co-electrophoresed molecular weight markers include myosin (200 000), phosphorylase b (97 000), bovine seroalbumin (69 000) and ovoalbumin (46 000)

monoclonal antibody (anti-TrpE). In all cases, only those immunocomplexes that contained the Cbl-b protein showed detectable precipitation of Vav (Figure 5b). These observations indicate that Cbl-b and Vav interact in vivo in a specific manner.

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Since c-Cbl and Vav participate in receptor-mediated signal transduction, we examined whether Cbl-b can act as a substrate of tyrosine protein kinase receptors. To this end, we determined the phosphorylation levels of Cbl-b in resting and mitogen-stimulated NIH3T3 cells by anti-phosphotyrosine immunoblot analysis. As expected, Vav became highly phosphorylated on tyrosine residues upon treatment of these cells with either EGF or PDGF (Bustelo et al., 1992; Margolis et al., 1992) (Figure 6). However, we could not detect significant tyrosine phosphorylation of Cbl-b under the same experimental conditions. Cbl-b immunoprecipitates contained tyrosine phosphorylated proteins of 95 and 180 kDa in EGF-stimulated cells and of 95 and 190 kDa in PDGF-stimulated cells (Figure 6). The mobility of these proteins is reminiscent of those of Vav and the autophosphorylated EGF- and PDGF-receptors that co-immunoprecipitated with Vav upon ligand stimulation (Bustelo et al., 1992). Western blot analysis using specific antibodies demonstrated that indeed these tyrosine-phosphorylated proteins were Vav (95 kDa), EGF receptor (180 kDa) and PDGF receptor (190 kDa) (data not shown). These results indicate that Cbl-b forms a trimeric complex with Vav and, depending on the mitogen used, either the EGF or PDGF receptor.

To determine whether the association between Vav and Cbl-b was affected by the mitogenic state of the cells, the same anti-FLAG immunoprecipitates were submitted to Western blot analysis using an anti-Vav antiserum (Figure 6, lower left panel). This analysis indicated that the association of Vav with Cbl-b, albeit detectable at low levels in non-stimulated cells, is significantly increased upon treatment with either EGF or PDGF in a time-dependent manner. Whereas the interaction of Cbl-b with Vav and EGF receptors is transient, its interaction with Vav and PDGF receptors appears to be more stable. Mitogenic stimulation of NIH3T3 cells with either EGF or PDGF does not result in increased expression of either Vav (Figure 6, lower right panel) or Cbl-b (data not shown). These results suggest that the affinity of Vav for Cbl-b is modulated in vivo by activation of tyrosine protein kinase receptors.

### lo ove exp eeeion inhi ite Vavo ediated 1NK eti tlation

Recent genetic analysis in nematodes has indicated that the product of the eli-1 gene down modulates the activity of Let23, the eleoane homologue of the mammalian EGF receptor (Yoon et al., 1995). These observations prompted us to analyse whether

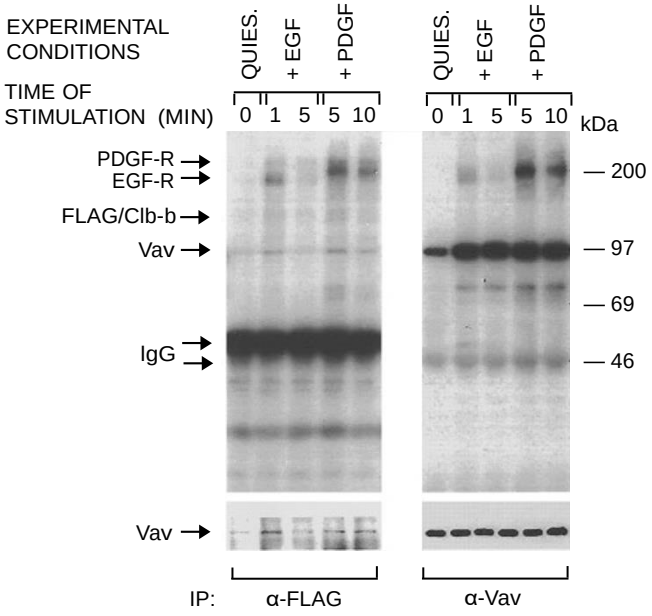


Figure 6 Mitogenic stimulation induces the association of Cbl-b with Vav and tyrosine protein kinase receptors. Cells co-expressing Vav and Cbl-b proteins were made quiescent by serum withdrawal for 96 h and then stimulated with either EGF (500 ngfml) or PDGF-BB (50 ngfml) for the indicated periods of time. After stimulation, cells were lysed and immunoprecipitated (IP) with either anti-FLAG (left panels) or anti-Vav (right panels) antibodies. Immunocomplexes were analysed by either anti-phosphotyrosine (upper panels) or anti-Vav (lower panels) immunoblots as described in the legend to Figure 5. The migration of Cbl-b, Vav, immunoglobulin heavy chains (IgG) and the EGF and PDGF receptors is indicated by arrows. Co-electrophoresed molecular weight markers are those described in the legend to Figure 5

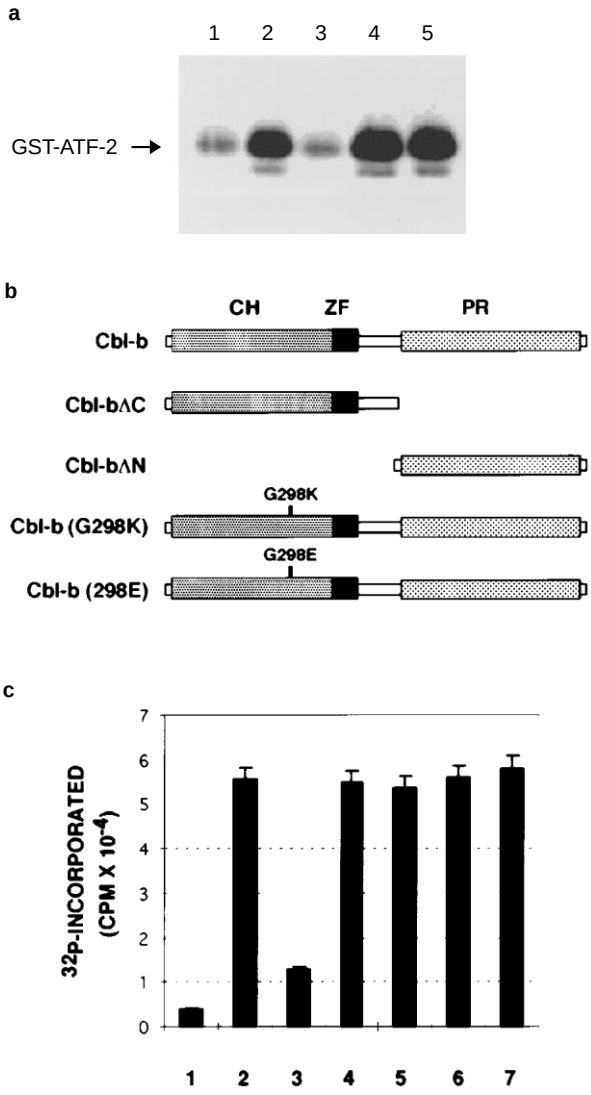


Figure 7 Cbl-b inhibits the activation of JNK mediated by the vav oncogene product. (a) Inhibition of Vav-mediated JNK activity by wild type Cbl-b. HA-tagged JNK was expressed in COS-7 cells either alone (lane 1) or in combination with the Vav oncoprotein (lane 2), Vav plus Cbl-b (lane 3), MEKK (lane 4) and MEKK plus Cbl-b (lane 5). After 48 h in culture, cells were lysed and the JNK activity in each co-transfection determined as indicated in Materials and methods. The mobility of the phosphorylated GST-ATF-2 is indicated by an arrow. (b) Cbl-b mutants used in this study. Cbl-b domains are those described in Figure 1b. (c) Biological activity of wild type and Cbl-b mutants. HA-tagged JNK was expressed in COS-7 cells either alone (lane 1) or in combination with the Vav oncoprotein (lane 2), Vav plus wild type Cbl-b (lane 3), Vav plus Cbl-b$\Delta$C (lane 4), Vav plus Cbl-b$\Delta$N (lane 5), Vav plus Cbl-b (G298K) (lane 6), or Vav plus Cbl-b (G298E) (lane 7). After culture for 48 h, cells were harvested and the activity of JNK determined as described in Materials and methods. Values in the histogram represent the mean and standard deviation of three independent determinations

Cbl-b could also act as a dominant inhibitor of the signal transduction pathway regulated by Vav. We investigated therefore the effect of Cbl-b on the activation of JNK, a downstream element of the Vav signaling pathway (Crespo et al., 1996, 1997). To this end, a hematoagglutinin-tagged version of JNK and Vav were co-expressed in COS-7 either alone or in combination with Cbl-b. As a control, HA-JNK and Cbl-b were expressed in the presence of MEKK, a well known upstream activator of JNK (Coso et al., 1995). After transfection, cells were lysed, HA-JNK immunoprecipitated using anti-HA antibodies, and its catalytic activity determined in an in vit o kinase reaction using [y-$^{32}$P]ATP and a GSTf ATF-2 (residues 1 – 96) fusion protein as exogenous substrate. Labeled proteins were separated by SDS – PAGE and the incorporation of $^{32}$P into the GSTf ATF-2 evaluated by autoradiography. In agreement with our previous observations (Crespo et al., 1996), the co-expression of Vav and JNK leads to a tenfold increase in the catalytic activity of this protein kinase (Figure 7a). Co-expression of Cbl-b abrogates this activation, leading to levels of JNK activity similar to those found in cells in which JNK is transfected alone (Figure 7a). In contrast, Cbl-b does not have any significant effect on the activation of JNK by MEKK (Figure 7a), a result that illustrates the specificity of the inhibitory action of Cbl-b on Vav signaling.

To further dissect the molecular events behind this biological effect, we determined the inhibitory activity of a number of Cbl-b mutants on Vav-mediated JNK activation (Figure 7b). These proteins included truncations of Cbl-b lacking either the N- (residues 1 – 463) or C-terminal (residues 482 – 982) regions and two missense mutations (G298E and G298K) similar to those found in loeeoofoftnntion mutants of the eli-1 gene (Yoon et al., 1995). As shown in Figure 7c, neither of these constructs were capable of affecting the activation of JNK mediated by Vav. This result suggested that inhibition of Vav activity requires the presence of a

full-length Cbl-b protein with a fully functional CH domain.

## Discussion

In this study, we report the identification of Cbl-b as a novel Vav-binding protein. The interaction between Cbl-b and Vav is mediated by the recognition of proline-rich regions in Cbl-b by the carboxy-terminal SH3 domains of Vav, the same structural elements previously described for the interaction of Vav with other proteins such as hnRNP-K. The proline-rich domain of Cbl-b contains a palindromic consensus motif composed of a hexaproline core flanked by two positively charged amino acids (R-X-$P_{(6)}$-X-R), a structure that closely resembles the class II SH3 binding sites of other signaling proteins. The presence of such a high content on proline residues is likely to contribute to the generation of left handed type II (PPII) helices, the optimal conformation for SH3 domain binding (Yu et al., 1994). Despite this apparent similarity, the interaction of Vav with Cbl-b and hnRNP-K reveals important structural differences. Whereas binding of Vav to hnRNP-K can be mediated by either of the two hnRNP-K hexaproline-containing regions, its association with Cbl-b requires multiple proline-rich regions. In addition, binding of Vav to Cbl-b needs the complete carboxy-terminal region instead of a single SH3 domain as in the case of hnRNP-K. These findings suggest that the two Vav SH3 domains act synergistically, perhaps by binding to cooperating proline-rich sequences in Cbl-b. The structural nature of the association between Vav and Cbl-b is further complicated by the observation that the affinity between these proteins increases upon tyrosine phosphorylation of Vav andfor SH2-mediated binding to tyrosine phosphorylated receptors, suggesting that other conformational andfor subcellular localization changes contribute to the establishment of a tight interaction between these two signaling molecules.

n vit o experiments indicate that Cbl-b also binds to Vav-2, a new oncoprotein that not only has a remarkable sequence homology with Vav (55% overall sequence identity) but also displays each of the known structural motifs of Vav in the same spatially related manner (Schuebel et al., 1996). However, unlike Vav, Vav-2 is expressed at very high levels during embryonic development and in the majority of adult mouse tissues (Schuebel 1996), thus suggesting that Vav-2 may play the same role as Vav in cell of non-hematopoietic lineage. Since Cbl-b is also ubiquitously distributed, it is likely that this protein plays a common regulatory role in all the cells in which it is expressed by interacting with either Vav (in hematopoietic cells) or Vav-2 (other cell types) proteins.

Cbl-b can also form complexes with the EGF and PDGF receptors in a ligand- and time-dependent manner. This association appears to be mediated by the formation of a trimeric complex with Vav, since such complexes could not be detected in the absence of Vav (unpublished observations). Interestingly, association of Cbl-b with these mitogenic receptors does not result in its phosphorylation on tyrosine residues. Since immunoprecipitations were conducted in the presence of sodium orthovanadate, this lack of phosphorylation cannot be attributed to dephosphorylation of Cbl-b by tyrosine phosphatases. These results indicate that Cbl-b behaves as some adaptor (Grb-2, p85) and effector (Sos) signal transduction molecules that are poor substrates for receptors with intrinsic protein tyrosine kinase activity. Unlike Cbl-b, the highly related c-Cbl protein becomes highly phosphorylated on tyrosine residues upon activation of a number of mitogenic and antigenic surface receptors (Langdon, 1995). To date, the role of this tyrosine phosphorylation in the regulation of c-Cbl biological activity is unknown. However, an interesting inference from the lack of phosphorylation of Cbl-b obtained in our experiments is that the relevant physiological outcome of the association of Cbl proteins with activated receptors may not reside in their phosphorylation but, rather, in their translocation to the plasma membrane or in their association with other receptor-bound signaling molecules.

Critical information regarding the physiological role of the SlifCbl family of proteins has recently emerged from genetic studies in eleoane aimed at identifying negative regulatory elements of the let23 signaling pathway (Yoon et al., 1995). Null mutations in the eli-1 gene fully restore the viability, posterior epidermal development, male spicule formation and vulva induction of eleoane mutants carrying reduction-of-function mutations in the let23 gene (Yoon et al., 1995). These mutants also rescue the vtlvaleee phenotype induced by reduction-of-function mutations of ee -5, the eleoane homologue of the mammalian Grb-2 protein (Yoon et al., 1995). These results suggest that Sli-1 plays an inhibitory role, hitherto uncharacterized, in the signaling pathway(s) that irradiate from the Let23 receptor (Yoon et al., 1995).

The inhibitory role of mammalian c-Cbl is also suggested by recent experiments showing that the overexpression of c-Cbl downmodulates the allergic response mediated by the mast cell FceRI receptor. This effect appears to be mediated by its specific association with the protein tyrosine kinase Syk (Ota and Samelson, 1997). Very recently, c-Cbl has been shown to become associated with Vav upon TCR activation in murine thymocytes and peripheral T cells (Marengere et al., 1997). Whether the interaction of Vav with c-Cbl and Cbl-b has the same or different physiological consequences remains to be established. Other studies have also shown that c-Cbl can associate in different cell lineages with cytoplasmic tyrosine kinases and signal transduction molecules such as phosphatidylinositol 3-kinase (Langdon, 1995). However, there is no evidence so far for an inhibitory role of c-Cbl on any of these enzymes.

Transduction of the n l gene by a murine retrovirus results in the induction of pre-B lymphoma and myelogenous leukemia (Langdon, 1995). It is possible that the c-Cbl protein acts as a negative regulator of mitogenic pathways in certain hematopoietic cells. If so, its oncogenic counterpart may act as a dominant negative mutant, liberating the infected cells from the proliferative constrains imposed by c-Cbl. Alternatively, c-Cbl may serve as a negative regulator of differentiating andfor commitment pathways. In this scenario, the product of the v-n l oncogene may

constitutively block such pathways resulting in the constitutive proliferation of lymphoid andfor myeloid precursors.

We have recently demonstrated that expression of the oncogenic Vav protein leads to the stimulation of Rac-1 guanosine nucleotide exchange and to the activation of the downstream elements of the Rac-1 pathway such as the SEKfJNK cascade (Crespo et al ., 1996, 1997). This stimulatory process appears to correlate to the signaling capabilities of Vav, since point mutations that eliminate the transforming activity of Vav also eliminate activation of Rac-1 and JNK (Crespo et al., 1996, 1997). In agreement with the genetic results obtained with eli-1 mutants of eleoane, our experiments have shown that Cbl-b blocks the activation of JNK induced by expression of the Vav oncoprotein. This effect is highly specific, since the activation of JNK by a different upstream activator, the MEK kinase, is not affected by Cbl-b. This inhibitory activity requires the full length Cbl-b protein, since expression of truncated versions of Cbl-b lacking either the CH or the proline-rich domains do not show any inhibitory effect. Likewise, point mutations in the Cbl-b CH domain affecting a residue also present in the nematode Sli-1 protein disrupt the inhibitory potential of the full length Cbl-b protein. These results strongly suggest a functional relationship between Vav and Cbl-b and provide, to our knowledge, the first biochemical evidence for an inihbitory role of a Sli-1-related protein towards protein tyrosine kinase substrates.

Taken together, our data is compatible with a model in which mitogenic stimulation of cells lead to the association of Vav with the autophosphorylated tyrosine kinase receptors via a SH2fphosphotyrosine interaction. This interaction would presumably result in the activation of Vav downstream elements, including Rac-1 and JNK. The subsequent binding of Cbl-b to Vav would result in the inhibition of the Vav signaling output, at least in the Rac-1fJNK pathway. The mechanism by which Cbl-b down modulates Vav activity remains to be determined. It is possible that formation of tight tertiary complexes with Vav and tyrosine kinase receptors blocks phosphorylation of critical Vav tyrosine residue(s). Cbl-b may control Vav signaling by regulating its GDPfGTP exchange activity towards Rac-1 protein. Alternatively, Cbl-b may simply induce the release of positive mediators of Vav signaling. Additional studies aimed at exploring some of these hypothesis should help to elucidate the molecular mechanisms controlling Vav signaling.

## Materials and methods

### elle

YPB2 yeast cells (MATa; t a3-52; hie3-200; ade2-101; lye2-801; t p1-901; let2-3,112; nan$^R$; oal4-542; oal 80-538; 3YS2 : : GA31-H S3, U A3 : : (GA317mers)-lanZ) were used for all the two-hybrid experiments. E noli MH4 strain (lanX74; oalU; oalK; he ; epL; letB600) was used to recover library plasmids from yeast cells. E noli DH5a cells were used for amplification of plasmids and for induction of GST fusion proteins.

### Anti odiee

A polyclonal rabbit antiserum raised against a MBP fusion protein containing the Dbl homology domain of the mouse Vav protein (residues 181 – 449) was used for immunoprecipitation assays (Bustelo et al., 1995). A rabbit polyclonal antiserum elicited against the cysteine rich region of Vav (Bustelo and Barbacid, 1992) was used for Western blot analysis. Monoclonal antibodies against FLAG and HA epitopes were purchased from Kodak and Babco, respectively. A monoclonal antibody against the bacterial TrpE protein was obtained from Oncogene Science. Other immunological reagents included monoclonal antibodies against the bacterial GST protein (Santa Cruz) and anti-phosphotyrosine residues (Upstate Biotechnology).

### Yeaet two hy id eyete

YPB2 cells were transformed to tryptophan prototrophy with pXRB26 (Bustelo et al., 1995) by the alkaline method (Ito et al., 1983). A single Trp$^+$ colony from this transfection was grown in SC-Trp medium and transformed with a human B-cell cDNA library, as previously described (Bustelo et al., 1995). After incubation for 1 week in the presence of 15 mM 3-AT, His$^+$ colonies were transferred onto Whatman-50 filters, permeabilized by freezing in liquid nitrogen (1 min) and thawed at room temperature. Filters were then overlaid on Whatman 3MM paper saturated with a lanZfX-gal solution (60 mM $Na_2HPO_4$, 60 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM /)-mercaptoethanol and 0.5 mgfml of 4-nitrophenyl /)-D-galactoside (Boehringer-Mannheim)) and incubated at 30°C. Positive colonies were patched onto a master plate and analysed further. Total DNA from yeast cells was prepared according to the method of Hoffman and Winston (1987) and used to transform E noli MH4 cells via electroporation using a Bio-Rad GenePulser. Transformants were plated on minimal medium supplemented with ampicillin. Primary positives were tested for target specificity by re-transformation into the test strain in conjunction with a number of unrelated fusion proteins to either the DNA binding domain or the activator region of Gal4. The specificity of the interaction of the positive clones was further assessed using two other independent assays, GST pull down and farwestern blot experiments, as previously described (Bustelo et al., 1995). Only those clones that were positive under the three independent experimental conditions were considered as truly positives and selected for further study.

### lonino of cbl-b cDNA

The cDNA insert of pXRB85 was liberated by XhoI digestion and used as a probe to screen $\blacklozenge 4.2 \times 10^5$ p.f.u. of a human embryonic brain cDNA library generated using random oligonucleotides (Stratagene). A total of nine phage cDNA clones were isolated, converted onto plasmid form by the addition of a helper phage, and characterized by restriction mapping. Six clones containing additional 5' (pXRB101, pXRB103, pXRB104 and pXRB105) and 3' sequences (pXRB85L and pXRB102) were further characterized by sequencing analysis. These analysis indicated that pXRB103 contained all the coding sequence of the Cbl-b protein whereas pXRB89L and pXRB104 contained two distinct forms of a differentially spliced 3' end (data not shown). pXRB101 and pXRB105 were identical to pXRB103 with the exception of the 5' end which contained junk DNA ssequences composed mostly of Alt repetitive sequences (data not shown). Complete sequence analysis was performed with overlapping oligonucleotides that annealed to both strands of the cDNAs using a Taq-polymerasefdye-terminator (Perkin-Elmer) PCR amplification technique.

Labeled fragments were separated by electrophoresis using a 373 DNA automatic sequencer (Applied Biosystems).

### Exp eeeion plae ide

Bacterial expression vectors included pKLS26, a pGEX-2TK derivative containing the Vav SH3-SH2-SH3 region (residues 611–845) (Bustelo et al., 1995); pKLS3, pGEX-2T derivative containing the Vav SH2 domain (residues 655–774); pKLS48, a pGEX-2TK derivative containing the N-terminal Vav SH3 domain (residues 611–749); pXRB14 is a pGEX-3X derivative containing the carboxy-terminal Vav SH3 motif (residues 748–845). pXRB96 is a pGEX-4T3 containing the full length RhoA protein. pGEX-5X1 derivatives included residues 171–298 of hnRNP-K (pXRB71) and residues 542–552 (pXRB106), 552–562 (pXRB107), 561–571 (pXRB108), 666–677 (pXRB109), 775–786 (pXRB111), 820–830 (pXRB112) and 970–981 (pXRB113) of Cbl-b protein. pGEX-5X2 derivatives included those containing the zinc finger domain (pXRB115), the CH domain (pXRB125) and the combination of both zinc finger and CH domains (pXRB126) of Cbl-b protein (see Figure 4A for amino acid residues). pKLS29, a pBMS-1 derivative (Bustelo et al., 1995) containing a GSTfVav SH3-SH2-SH3 fusion protein (residues 611–845) plus a carboxy-terminal protein kinase A site, was used for infecting insect Sf9 cells. A pMEX-derivative containing T3 and T7 promoters flanking the full length human hnRNP-K protein (pXRB75) was used for in vit o transcriptionftranslation studies. To generate epitope-tagged versions of the Cbl-b protein, we modified pXRB110, a pBluescript-SK derivative containing the full length human Cbl-b protein by inserting between the XbaI and PstI sites of the polylinker two annealed oligonucleotides (Flag-1F: 5'-CTAGCAGGCC AT G GACT- ACAAGGACGACGATGACAAGC-3'; Flag-1R: 5'-TGCA- G TTGT AT GT GT TTGTAGTC CATGGCCTG -3')
that contain an ATG (underlined) embedded in a Kozak's sequence (bolded) for optimal translation followed in frame by the FLAG epitope (in italics). The tagged nl 0 cDNA was then liberated by NotIfX aI digestion, filled-in and subcloned into S aI-linearized pMEX to generate pXRB118, a plasmid that allows the expression of polypeptides in mammalian cells under the regulation of the Moloney-MSV LTR. pAO8 and pAO9, two pcDNA derivatives containing the G298E and G298K point mutations of Cbl-b protein were generated using the Quickchange mutagenesis kit (Stratagene) according to the manufacturer's recommendations. To generate pAO3, the coding sequences coding for the N-terminal domains of Cbl-b (residues 1–481) were excised for the n lob cDNA by a HI digestion and cloned in the pcDNA3 vector. A similar vector (pXRB144) containing the C-terminal proline rich region of Cbl-b (residues 464–982) was generated by PCR using appropriate oligonucleotides. pMEX derivatives containing untagged versions of the wild type mouse vav proto-oncogene cDNA (pJC11) and the mouse (pJC12) and human (pJC7) version of the vav oncogene cDNA have been already described (Coppola et al., 1991). A pcDNA derivative containing the mouse vav oncogene (pcDNA3-oncovav) was described elsewhere (Crespo et al., 1996).

### No the n lot analyeie

n lo and /)-antin probes were labeled with a-$^{32}$P-dCTP using a random hexamer primer technique (Amersham). Probes were hybridized overnight at 65°C with two multiple tissue Northern blots (Clontech) in a solution containing 5×SSPE, 10×Denhardt's solution, 200 Jlgfml of denatured sheared salmon sperm DNA and 2% sodium sulfate (SDS). After hybridization, filters were washed with 2×SSC, 0.05% SDS three times at room temperature (15 min each), followed by four times (20 min each, 65°C) with 0.1% SSC, 0.1% SDS. Washed filters were submitted to autoradiography at −70°C in the presence of intensifying screens.

### Sti tlation of nelle and i tnop enipitation

Exponentially growing cells were made quiescent by serum withdrawal for 48–96 h. Quiescent cells were then stimulated with either EGF (500 ngfml, Upstate Biotechnology) or PDGF-BB (50 ngfml, GibcofBRL) for the indicated periods of time and quickly frozen on dry ice. Frozen cells were thawed and lysed in RIPA buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 150 mM NaCl, 1% aprotinin (Sigma), 250 JlM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride and 100 JlM sodium orthovanadate) and subsequently centrifuged at 11 000 r.p.m. for 20 min at 4°C. Clarified supernatants were incubated in the presence of the appropriate antibody for 2–4 h at 4°C. After incubation, immunocomplexes were collected using either protein A-sepharose beads (PharmaciafLKB) or protein G-agarose beads (Oncogene Sciences) for 30 min at 4°C under gentle agitation. Immunoprecipitates were then washed three times in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.1% Triton X-100, dissolved in 1×SDS-sample buffer, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE).

### In vitro t anen iption/t anelation GST 'ptll down' expe i ente and Weete n lot analyeie

Both Cbl-b and hnRNP-K proteins were produced in vit o using a TNT reticulocyte lysate (Promega) in the presence of [$^{35}$S]methionine according to the manufacturer's recommendations. GST fusion proteins were purified and used in pull down experiments as previously described (Bustelo et al., 1995). Associated proteins were separated by SDS–PAGE, fixed and exposed to autoradiography films at −70°C in the presence of intensifying screens. Western and farwestern blot analysis were performed as described by Bustelo et al. (1995).

### no1tn Note inal kinaee aeeaye

Subconfluent COS-7 cells were transfected with the indicated vectors by the DEAE-dextran technique. Total amount of plasmid DNA was adjusted to 5–10 Jlg per plate with vector DNA when necessary. After transfection, cells were cultured for 48 h, washed by PBS, and lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM /)-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 20 Jlgfml aprotinin and 20 Jlgfml leupeptin. Cellular lysates were then immunoprecipitated from the cleared lysates by incubation with anti-HA antibodies (12CA5, Babco) for 1 h at 4°C. Immunocomplexes were recovered with the aid of gamma-bind sepharose beads (Pharmacia). Washed immunocomplexes were resuspended in 30 Jll of kinase reaction buffer containing 1 JlCi of [y-$^{32}$P]ATP per reaction and 20 JlM unlabeled ATP, using 1 Jlg of GSTfATF-2(96) fusion protein as substrate, as previously described (Coso et al., 1995). After 20 min, reactions were terminated by addition of 10 Jll of 5×SDS–PAGE sample buffer. Samples were denatured by heating and analysed by SDS–PAGE (12%) gels. After fractionation, gels were fixed, dried and submitted to autoradiography with the aid of intensifying screens. Parallel anti-HA, anti-Vav, and anti-FLAG immunoprecipitates were processed by Western

blot analysis using the same antibodies to demonstrate the equal expression in all samples of JNK, Vav and Cbl-b, respectively.

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