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Characterization of a Novel Member of the DOK Family That Binds and Modulates Abl Signaling

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A novel member of the p62^{dok} family of proteins, termed DOKL, is described. DOKL contains features of intracellular signaling molecules, including an N-terminal PH (pleckstrin homology) domain, a central PTB (phosphotyrosine binding) domain, and a C-terminal domain with multiple potential tyrosine phosphorylation sites and proline-rich regions, which might serve as docking sites for SH2- and SH3-containing proteins. The DOKL gene is predominantly expressed in bone marrow, spleen, and lung, although low-level expression of the RNA can also be detected in other tissues. DOKL and p62^{dok} bind through their PTB domains to the Abelson tyrosine kinase in a kinase-dependent manner in both yeast and mammalian cells. DOKL is phosphorylated by the Abl tyrosine kinase in vivo. In contrast to p62^{dok}, DOKL lacks YxxP motifs in the C terminus and does not bind to Ras GTPase-activating protein (RasGAP) upon phosphorylation. Overexpression of DOKL, but not p62^{dok}, suppresses v-Abl-induced mitogen-activated protein (MAP) kinase activation but has no effect on constitutively activated Ras- and epidermal growth factor-induced MAP kinase activation. The inhibitory effect requires the PTB domain of DOKL. Finally, overexpression of DOKL in NIH 3T3 cells inhibits the transforming activity of v-Abl. These results suggest that DOKL may modulate Abl function.

The Abl oncogene, the v-Abl gene, was first identified in the genome of the Abelson murine leukemia virus, a potent transforming virus which specifically targets early B-cell lineages (for reviews see references 47 and 63). The v-Abl gene is derived by recombination between the viral Gag gene and the cellular c-Abl gene and encodes an activated Abl kinase. The c-Abl proto-oncogene encodes a ubiquitously expressed, nonreceptor protein tyrosine kinase which contains Src homology domains SH1, SH2, and SH3. The SH1 domain contains the kinase activity, the SH2 domain binds phosphotyrosine residues, and the SH3 domain binds proline-rich stretches. c-Abl has a unique carboxyl-terminal fragment containing multiple functional motifs. The exact physiological function of c-Abl is not yet known. Recent studies have shown that c-Abl kinase activity can be stimulated by DNA-damaging reagents (22, 28) and integrin engagement (27).

Although c-Abl kinase activity is normally tightly regulated in vivo (30, 42), oncogenic forms of Abl escape normal cellular regulation (19, 35). In v-Abl, the viral Gag replaces the SH3 domain of c-Abl, a negative regulatory domain of c-Abl, creating a fusion protein with unregulated high tyrosine kinase activity. The Bcr-Abl gene, a human Abl oncogene, encodes a protein in which the fusion of the Bcr region to the N terminus of Abl kinase also results in constitutively high kinase activity (32, 35). In addition, while c-Abl localizes to both the nucleus and cytoplasm, v-Abl and Bcr-Abl are predominantly cytoplasmic (32, 62). In particular, the myristoylation signal provided by the viral Gag sequence allows v-Abl to localize predominantly to the plasma membrane. Both the deregulated kinase activity and abnormal subcellular localization are thought to contribute to the transforming ability of v-Abl and Bcr-Abl.

Abl-interacting proteins can directly link Abl to critical signal transduction pathways. For example, the JAK-STAT pathway is constitutively activated by v-Abl and Bcr-Abl in hematopoietic cells; Jak1 and Jak3 were found to be associated with a proline-rich region in the C terminus of v-Abl (4, 7). Some molecules serve bridging roles. The adapter protein Shc was found to bind to the SH2 domain of the Abl oncoprotein in a phosphotyrosine-independent manner, and the phosphorylation of Shc by Abl kinase might provide a docking site for Grb2-Sos complexes and link Abl to the Ras pathway (44). CRKL and Cbl are tyrosine phosphorylated in v-Abl- and Bcr-Abl-transformed cells and are physically associated with Abl oncogenic proteins (3, 47). CRKL and Cbl can bridge the binding between Abl and other signaling proteins and facilitate the formation of signaling complexes. The binding of phosphatidylinositol 3-kinase to Bcr-Abl is believed to be bridged by CRKL and Cbl (50).

p62^{dok} is another Abl-associated adapter protein which has been cloned recently (5, 64). p62^{dok} was first noted for its ability to be phosphorylated by multiple tyrosine kinases and for its strong association with Ras GTPase-activating protein (RasGAP) upon phosphorylation (10). p62^{dok} is highly phosphorylated in cells transformed by v-Src, v-Abl, v-Fps, v-Fms, v-Src, and Bcr-Abl; phosphorylation levels of p62^{dok} correlate with the transforming activities of these oncogene products (8, 10, 34, 35, 40). p62^{dok} is also rapidly phosphorylated upon stimulation by various growth factors, including platelet-derived growth factor (21), insulin-like growth factor (49), insulin (17), vascular endothelial growth factor (12), and colony-stimulating factor 1 (15). p62^{dok} associates with both v-Abl and Bcr-Abl in vivo (2, 64) and is one of the most prominent tyrosine-phosphorylated proteins in v-Abl- and Bcr-Abl-transformed cells. The binding between p62^{dok} and Abl does not require the SH2 domain of Abl (2), but the exact mechanism has been elusive. The function of p62^{dok} in Abl-mediated signal transduction is not clear.

To further our understanding of the mechanisms by which v-Abl and Bcr-Abl transform cells, we have attempted to iden-

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tify more proteins that interact with Abl and which might direct Abl to various signal transduction pathways. We cloned a new gene, the DOKL (for p62^{dok}-like protein) gene, which we have named for the homology of DOKL to the p62^{dok} RasGAP-binding protein. We show that both DOKL and p62^{dok} bind directly to Abl in a kinase-dependent manner through their PTB domains. Both DOKL and p62^{dok} are heavily phosphorylated by the Abelson tyrosine kinase. The two are not equivalent, however: overexpression of DOKL strongly inhibited v-Abl-stimulated MAP kinase activation, while p62^{dok} had no effect. Furthermore, overexpression of DOKL in NIH 3T3 cells potently inhibited the transforming activity of v-Abl.

MATERIALS AND METHODS

Yeast two-hybrid assay and cDNA cloning. *Saccharomyces cerevisiae* CTY 10-5d was transformed with various pairs of plasmids, and possible interactions were tested by scoring for expression of β-galactosidase produced from the reporter gene by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of colony replicas on nitrocellulose. Nucleotide sequence analysis of cDNA inserts was performed with the standard dideoxy method. The sequence data were analyzed by BLAST search (National Library of Medicine). The 5′ region of DOKL cDNA was amplified by PCR with a mouse liver marathon 5′ rapid amplification of cDNA ends (RACE)-ready cDNA library (Clontech). The 5′ DNA sequences were then fused with the DNA sequences cloned from a yeast two-hybrid screen to form full-length DOKL cDNA. The degree of homology between DOKL and p62^{dok} in the carboxy-terminal region is very low. To exclude the possibility that the DOKL gene we cloned arose from an aberrant recombination during the construction of the cDNA library, we used two different mouse marathon 5′ RACE-ready cDNA libraries as the template and primers flanking the homology junction site for PCR. The fragments we cloned by PCR were identical in sequence to the DOKL clone from the yeast two-hybrid screening, suggesting that the original DOKL clone is indeed authentic.

Cell culture and antibodies. Both 293 cells and NIH 3T3 cells were obtained from the American Type Culture Collection. The ecotropic phoenix packaging cell line was a kind gift from G. Nolan (Stanford University). 293 cells and the ecotropic phoenix packaging cell line were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% antibiotic (penicillin and streptomycin). NIH 3T3 cells were maintained in DMEM supplemented with 10% bovine calf serum. Anti-Abl antibodies (K-12), anti-myc monoclonal antibodies (9E10), and anti-RasGAP antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine monoclonal antibodies (RC20) were purchased from Transduction Laboratory. Anti-hemagglutinin (HA) monoclonal antibodies (12CA5) were purchased from Boehringer Mannheim. Anti-active mitogen-activated protein (MAP) kinase antibodies were purchased from Promega.

Expression plasmids. Different portions of the Abl gene were cloned into the yeast two-hybrid vector pSH2-1 (14), in frame with the LexA DNA-binding domain, to form plasmids AGP3 (encoding amino acids [aa] 29 to 513), AGP4 (encoding aa 4 to 1091), AGP5 (encoding aa 4 to 1091), and AC (encoding aa 602 to 978). The insulin receptor yeast two-hybrid constructs IR-C, IR-CKR were kind gifts from T. Gustafson (University of Maryland) (13). p62^{dok} cDNA was a kind gift from Y. Yamanashi and D. Baltimore (California Institute of Technology) (64). DOKL and p62^{dok} cDNA sequences were cloned into the yeast two-hybrid vector pGAD (29), in frame with Gal4 activation domain to form pGAD-DOKL and pGAD-p62^{dok}. The coding sequences for the intracellular regions of Tyro3 and Axl were cloned in frame with the LexA DNA-binding domain to form plasmids pSH2-Tyro3 and pSH2-Axl. pGAD-Grb2 and pGAD-Vav p95 were obtained from the yeast two-hybrid screening with Tyro3 or Axl as a bait, respectively. The coding sequences for DOKL and p62^{dok} were cloned into the mammalian expression plasmid pMT21 in frame with the sequence encoding the myc epitope. c-Abl and c-Abl KR coding sequences were also cloned into pMT21, but without fusing with the myc epitope coding sequence. pGDN and pGD-v-Abl were kind gifts from D. Baltimore (California Institute of Technology) (41). pMSV-tk-BCR-Abl was a kind gift from C. Sawyers (University of California at Los Angeles) (51). pCMV-ERK2HA was a kind gift from A. Minden (Columbia University) (33). Mutations in AGP4, pGAD-DOKL, pGAD-p62^{dok}, pMT21-DOKL, and pGD-v-Abl were all introduced by site-directed mutagenesis.

Northern blot analysis. A mouse multiple-tissue Northern blot filter carrying 2 μg of poly(A)$^+$ RNA from each tissue (Clontech) was probed with DOKL cDNA under high-stringency conditions. DNA probes were prepared with [^{32}P]dCTP and a random-priming kit (Amersham). The filter was preincubated for 1 h at 65°C in hybridization solution (80 mM Tris-HCl [pH 8.0], 4 mM EDTA, 0.6 M NaCl, 0.1% sodium dodecyl sulfate (SDS), 10× Denhardt's solution, 100 μg of denatured salmon sperm DNA/ml). The filter was then incubated overnight at 65°C with the radiolabeled probe in hybridization solution. The filter was washed three times with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C and analyzed by autoradiography.

RNase protection assay. Two antisense probes corresponding to either a 5′ region (nucleotides 21 to 412) or a 3′ region (nucleotides 1057 to 1301) of DOKL cDNA were prepared by in vitro transcription reactions (Ambion). The RNA probes were purified by electrophoresis on a 5% polyacrylamide–urea gel. Total RNA was prepared from 100 mg of spleen, bone marrow, and thymus from a young adult mouse and from NIH 3T3 cells, by the RNAzol B isolation method (TEL-TEST). The amount of RNA in each sample was determined both by determining the optical density at 260 nm and by ethidium bromide staining of agarose gels. Total RNA (10 μg) from each sample was used to protect the probes from RNase A plus T1 digestion for the RPA III RNase protection assay (Ambion). The digestion products were resolved on a 5% polyacrylamide–urea gel and detected by autoradiography.

In situ immunofluorescence staining. NIH 3T3 cells were plated in six-well plates with coverglass at a density of 2×10^5 cells per well 24 h before transfection. Cells were transfected with 2 μg of pMT21-DOKL with Lipofectamine transfection reagents (Gibco-BRL) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were briefly rinsed with phosphate-buffered saline (PBS) and fixed with 100% methanol for 15 min at −20°C. Fixed cells were rinsed and blocked with 2% fetal bovine serum in PBS for 30 min. Cells were incubated with anti-myc antibodies (9E10) at a final concentration of 1 μg/ml for 1 h at 37°C. Cells were rinsed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma) for 1 h at 37°C. Nuclei were counterstained with 4′,6′-diamidino-2′-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim) at a final concentration of 1.5 μg/ml for 30 min at room temperature. Cells were then examined by immunofluorescence microscopy (Nikon).

Immunoprecipitation. 293 cells were transfected by the calcium phosphate method. Forty-eight hours after transfection, cells were lysed by EBC buffer (50 mM Tris [pH 7.6], 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM dithiothreitol, 10 mM NaF, 1 mM sodium vanadate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml). Cell lysates were clarified by centrifugation at 10,000 × *g* for 15 min at 4°C. For immunoprecipitation, the cell lysates were incubated with 1 μg of the appropriate antibodies at 4°C for 1 to 2 h. The immunocomplexes were collected with protein A or protein G agarose beads (Santa Cruz Biotechnology) and washed five times with lysis buffer. The bound proteins were eluted with Laemmli sample buffer. For measurement of the level of active MAP kinase, 293 cells were lysed with radioimmunoprecipitation assay buffer (10 mM sodium phosphate [pH 7.4], 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM sodium vanadate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml).

Western blot analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with TBST (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature and incubated with appropriate primary antibodies in TBST for 1 h. Membranes were then washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin G secondary antibodies for 1 h. Membranes were washed extensively with TBST and developed with an ECL kit (Amersham). To measure phosphotyrosine levels, membranes were blocked in 1% bovine serum albumin in TBST for 30 min at 37°C and incubated with HRP-conjugated anti-phosphotyrosine antibodies (RC20) for 30 min at 37°C. Membranes were then washed and developed with an ECL kit.

Establishing DOKL-overexpressing cell lines. NIH 3T3 cells were cotransfected with 18 μg of pMT21-DOKL and 2 μg of pBJ-puro, which carries a puromycin resistance gene, by the calcium phosphate precipitation method. Forty-eight hours after transfection, cells were plated into medium containing 10 μg of puromycin/ml. After 10 days of selection, puromycin-resistant clones were picked and expanded. Expression levels of DOKL were determined by immunoblot analysis with anti-HA antibodies.

Retrovirus infection and transforming assay. Ecotropic phoenix packaging cells were transiently transfected with pGDN or pGD-v-Abl by the calcium phosphate precipitation method (41). Culture supernatants were collected 2 days after transfection and filtered through 0.45-μm-pore-size filters. Viruses were then serially diluted, mixed with 8 μg of Polybrene/ml, and used to infect fresh NIH 3T3 cells for 4 h at 37°C. Two days after infection, cells were either selected in complete medium containing 800 μg of G418/ml or maintained in DMEM supplemented with 4% calf bovine serum. After 2 weeks of incubation, drug-resistant clones and transformed foci were scored.

Nucleotide sequence accession number. The GenBank accession number for the cDNA sequence reported in this paper is AF179242.

RESULTS

Identification of a novel Abl-binding protein. We used the yeast two-hybrid system to individually test candidate products of cDNA clones recovered in our laboratory in a number of unrelated library screens for their ability to interact with Abl. The product of one clone, initially recovered via its interaction with the murine retroviral Gag protein, showed significant

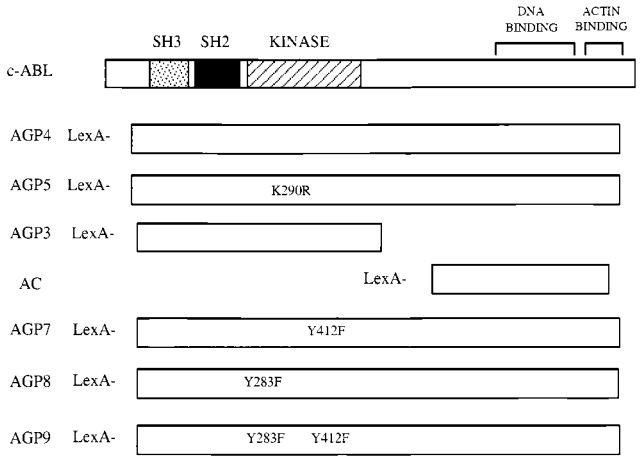


FIG. 1. Schematic representation of Abl baits used in the yeast two-hybrid assays. The positions of the SH3, SH2, tyrosine kinase, DNA-binding, and actin-binding domains on Abl are indicated. Yeast two-hybrid constructs AGP3, AGP4, AGP5, and AC were made by cloning different Abl gene fragments into the yeast two-hybrid vector SH2-1 in frame with the LexA DNA binding domain. The K290R mutation renders AGP5 kinase inactive. The sequences in AGP4 coding for two previously identified Abl autophosphorylation sites, Tyr283 and Tyr412, were mutated to generate yeast two-hybrid constructs AGP7, AGP8, and AGP9. The stable expression of all these yeast two-hybrid constructs was confirmed by Western blotting.

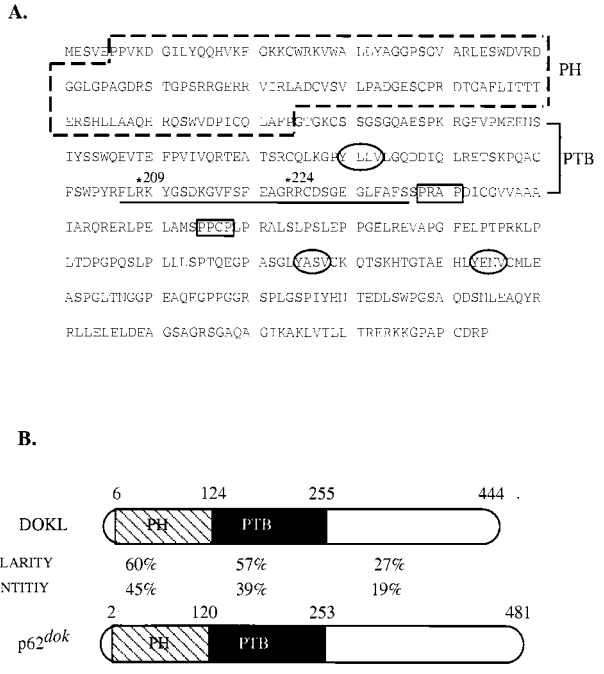


FIG. 2. Sequence analysis of Abl-interacting protein DOKL. (A) Deduced amino acid sequence of DOKL. The potential PH domain is boxed with dashed lines. The region with homology to the IRS-1 PTB domain is underlined. Arg209 and Arg224, two Arg residues that are conserved in IRS-1 and that directly interact with phosphotyrosine residues of other molecules are indicated with asterisks. Three potential SH2 domain binding sites (YxxV motif) are circled. Two potential SH3 binding sites (PxxP motif) are boxed with solid lines. (B) Amino acid sequence homology between DOKL and p62^{dok}. The similarities and identities between homologous regions are indicated. The degree of similarity between DOKL and p62^{dok} is high at the N-terminal halves of the molecules but low at the C-terminal halves.

sequence similarity to p62^{dok}, the most prominent tyrosine-phosphorylated protein in v-Abl- and Bcr-Abl-transformed cells and known to associate with v-Abl in vivo. We termed this protein DOKL (for p62^{dok}-like protein). To test for its interaction with Abl, a construct (AGP4) expressing a LexA-Abl fusion protein in yeast was used as bait (Fig. 1). The encoded protein was stable as judged by Western blotting, did not activate reporter gene expression by itself, was autophosphorylated, and exhibited potent tyrosine kinase activity in vivo (data not shown). When yeast strain CTY 10-5d was cotransformed with AGP4 and pGAD-DOKL, a strong activation of the β-galactosidase reporter gene was observed (Table 1).

Nucleotide sequence analysis showed that pGAD-DOKL contained a single long open reading frame fused to the Gal4 activation domain. The 5′ portion of the gene was cloned by 5′ RACE and was used to reconstruct a full-length cDNA. The first ATG in the DNA sequence matches well with consensus sequence (GCCATGG) (25) and is likely to be the authentic translation initiation codon. The conceptual translation predicts a 444-aa protein (Fig. 2A).

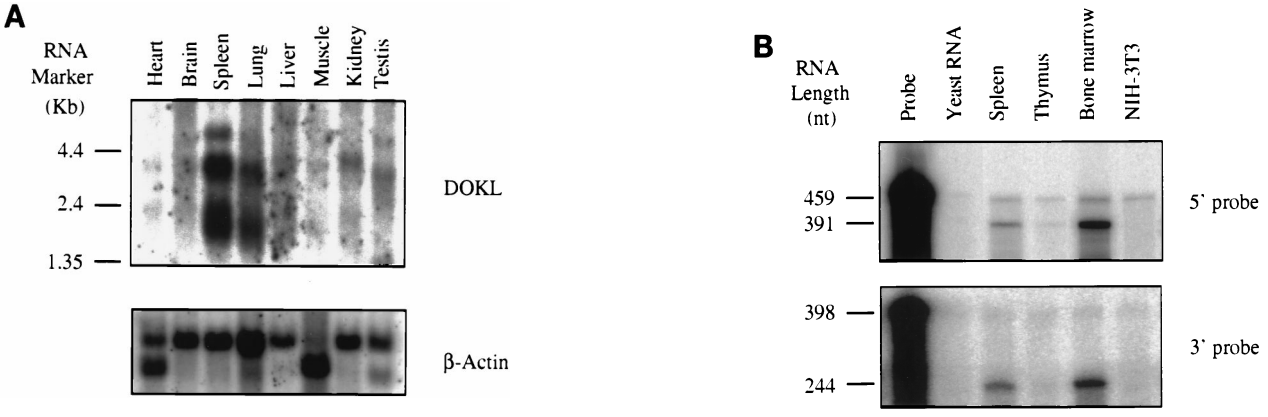
TABLE 1. Analysis of the interaction between DOK proteins and Abl in the yeast two-hybrid system[a]

Plasmid	β-Galactosidase activity when cotransformed with plasmid:						
	AGP3	AGP4	AGP5	AC	AGP7	AGP8	AGP9
pGD-DOKL	+++	+++	−	−	+++	+++	+++
pGD-DOKL^{R209A}	+/−	+/−	−	−	+/−	+/−	+/−
pGD-DOKL^{R224A}	++	++	−	−	++	++	++
pGD-DOKL$^{R209, 224A}$	+/−	+/−	−	−	+/−	+/−	+/−
pGD-p62^{dok}	+++	+++	−	−	+++	+++	+++
pGD-p62$^{dok\ R207A}$	+/−	+/−	−	−	+/−	+/−	+/−

[a] Plasmids in the heading contained different DNA fragments in frame with the LexA DNA-binding domain. Plasmids in the left column contained different DNA fragments in frame with the Gal4 activation domain. Yeast strain CTY 10-5d was transformed with various pairs of plasmids, followed by an X-Gal staining assay. The strength of interaction was indicated by the timing and intensity of blue color. +++, dark blue visible in 1 h; ++, dark blue visible in 4 h; +/−, pale blue visible overnight; −, white overnight.

DOKL contains multiple features of signaling proteins. Analysis of the predicted amino acid sequence of DOKL revealed several features (Fig. 2). The N-terminal part of the protein contains a pleckstrin homology (PH) domain thought to be involved in the membrane localization of proteins (26, 36). There is a potential phosphotyrosine binding domain (PTB domain) near the central region. DOKL and p62^{dok} have a high degree of homology in this region, and the same region is also weakly homologous to a portion of the PTB domain of IRS-1 (64). The PH and PTB domains of DOKL have the greatest sequence similarity with those of p62^{dok}, at 60 and 57%, respectively (Fig. 2B). However, the similarity between DOKL and p62^{dok} in the carboxy-terminal region is very low. The sequence diversity between C-terminal parts of DOKL and p62^{dok} suggests that these two proteins might function as adapters for different sets of signal-transducing molecules and as parts of different signaling complexes.

Preferred peptide sequences serving as substrates for receptor tyrosine kinases and nonreceptor tyrosine kinases have been defined (66). Cytosolic tyrosine kinases generally prefer sites with consensus sequence (I/V/L)-Y-(G/A/S/E/D), while receptor tyrosine kinases prefer sites with sequences such as (E/D)-Y-(G/V/I/M). In DOKL, there are several potential target sites for cytosolic tyrosine kinases. Three DOKL tyrosine residues are in the context of YxxV, the proposed (57) SH2



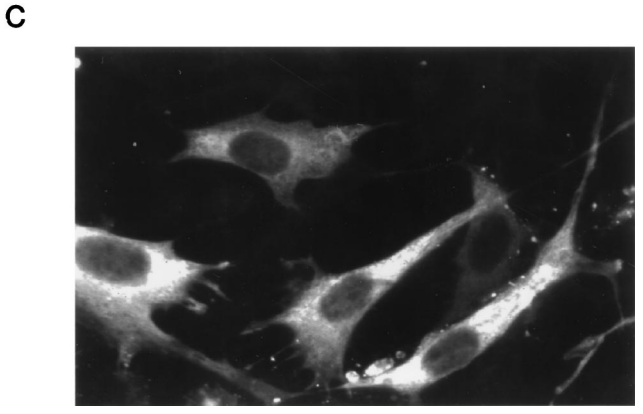


FIG. 3. Distribution of DOKL mRNA expression in tissue and subcellular localization of DOKL protein. (A) Northern blot analysis of DOKL gene expression. A filter containing poly(A)$^+$-selected RNA prepared from multiple mouse tissues was hybridized with a radiolabeled DOKL probe (top) or actin probe (bottom). The positions of migration of RNA molecular weight markers are indicated at left. (B) RNase protection analysis of DOKL gene expression. Two antisense probes corresponding to either a 5′ region (nucleotides 21 to 412) (top) or a 3′ region (nucleotides 1057 to 1301) (bottom) of DOKL cDNA were used in RNase protection assays. Equal amounts of total RNAs from the indicated mouse tissues and cells were used to protect DOKL antisense RNA probes from RNase digestion; products were analyzed by electrophoresis and autoradiography. Similar results were obtained when different preparations of total RNA were used in RNase protection assays. Sizes of RNA probes and protected RNA fragments are indicated at left. Probe, ^{32}P-labeled RNA without RNase; yeast RNA, negative control RNA. (C) DOKL is localized in the cytoplasm. NIH 3T3 cells were transiently transfected with myc-tagged DOKL, and the expressed proteins were localized by indirect immunofluorescence with anti-myc antibodies. Cells were examined with a Nikon immunofluorescence microscope. Cells with different DOKL expression levels are shown in this field. Many cells in this field were not transfected and are not visible; the background staining is very faint.

recognition motifs of Src family tyrosine kinases and SHPTP2 (Fig. 2A). The RasGAP-SH2 domain binding site YxxP, of which p62^{dok} has six, is absent in DOKL. Like that of p62^{dok}, the C terminus of DOKL is relatively proline rich (12%). DOKL contains two PxxP motifs, the most conserved sequence motif within known SH3 domain ligands (65), suggesting that DOKL might bind other SH3-containing proteins.

Overall, DOKL is relatively rich in serine and threonine residues (15%). There are several potential phosphorylation sites for Ser/Thr protein kinases such as protein kinase C (S63, T100, S138, T171, T194, S237, T295, T232), casein kinase (T98, S113, S154, S203), and cdc2 kinase (S138, T295).

Tissue-specific expression and subcellular localization of DOKL. The distribution of DOKL mRNA expression in tissue was examined by Northern blot and RNase protection experiments. Northern blot analysis of poly(A)$^+$ RNA from a variety of mouse tissues showed that DOKL was expressed at high levels in spleen and lung and only at low levels in other tissues (Fig. 3A), a pattern more restricted than that for p62^{dok} (64). Several splicing forms of DOKL mRNA were observed: one major species approximately 1.6 kb in size, another approximately 4 kb in size, and one minor species about 6 kb in size. Faint bands in kidney and testes were approximately 4 kb but migrated at slightly different positions. The size of our full-length cDNA clone corresponded to that of the shortest spliced form. The structures of other alternatively spliced forms are still unclear.

We then performed RNase protection assays to examine the expression of DOKL in hemapoietic tissues such as spleen, bone marrow, and thymus, by using two probes spanning either a 5′ or a 3′ region of the DOKL cDNA. Similar results were obtained with the two probes (Fig. 3B): the level of expression of DOKL was very high in bone marrow and in spleen; it was very low in thymus and undetectable in NIH 3T3 cells. These results suggest that DOKL may be selectively expressed in hematopoietic cells and expressed at particularly high levels in cells of the B-cell lineage. This specificity may be noteworthy in light of the selective transforming activity of the v-Abl oncogene for pre-B cells.

To test the intracellular localization of the DOKL protein, DOKL cDNA was cloned into the mammalian expression vector pMT21, fusing a myc epitope to the C terminus of the protein. NIH 3T3 cells were transiently transfected with this DOKL expression vector, and indirect immunofluorescence was performed with anti-myc (9E10) antibodies to localize the protein. DOKL exhibited a clear cytoplasmic staining (Fig. 3C), consistent with its potential role as an adapter protein in signal transduction.

DOKL and p62^{dok} bind to Abl through their PTB domains in yeast. To test which part of Abl is responsible for the interaction between Abl and DOKL, we fused different parts of Abl to the LexA DNA-binding domain and examined their abilities to interact with DOKL in the yeast two-hybrid system. The structures of these different Abl baits are shown in Fig. 1. DOKL bound to the full-length Abl and to an amino-terminal fragment which retained kinase activity but did not bind to a carboxy-terminal Abl fragment without kinase activity (Table 1). Furthermore, DOKL did not bind to a kinase-deficient mutant of Abl carrying a single K298R substitution in the ATP

binding site. The results suggest that DOKL interacts with Abl in a kinase-dependent manner.

One possible explanation for the kinase dependence of DOKL binding is that the Abl kinase causes the phosphorylation of residues in Abl itself and these residues are then recognized by DOKL. As noted above, DOKL may have a PTB domain; Yamanashi and Baltimore first noted that there is a limited homology between the central region of p62^{dok} and the PTB domain of IRS-1, but the fact that only the C-terminal three β sheets of the IRS-1 PH domain are evident in p62^{dok} makes the function of this region uncertain (64). Two Arg residues in the IRS-1 PTB domain believed to directly bind to phosphotyrosine are conserved in both p62^{dok} and DOKL and correspond to Arg207 and Arg222 in p62^{dok} and Arg209 and Arg224 in DOKL (Fig. 2A). To test the notion that these residues might be important for binding, we mutated Arg209 and Arg224 in DOKL and tested the binding between the DOKL mutants and Abl baits in yeast. The results showed that the binding of DOKL^{R209A} to Abl was drastically reduced and that the binding of DOKL^{R224A} to Abl was also attenuated. The DOKL$^{R209,\ 224A}$ double mutant has even less ability to interact with Abl than either single mutant (Table 1). Similar expression levels of wild-type and mutant forms of the Gal4-DOKL fusion protein in yeast were confirmed by Western blotting (data not shown). These data suggest that DOKL binds to phosphotyrosine(s) on Abl via its PTB domain.

Since the N-terminal part of DOKL has a close sequence similarity to that of p62^{dok}, we suspected that p62^{dok} might also bind to Abl in a similar manner. To test this notion, we fused p62^{dok} with the Gal4 DNA-binding domain and tested its binding to Abl; p62^{dok} also bound to Abl in a kinase-dependent manner. Mutation of Arg207 of p62^{dok}, corresponding to Arg209 of DOKL, greatly reduced the binding between Abl and p62^{dok} (Table 1). Taken together, our data provide evidence that Abl directly interacts with DOK proteins and that the potential PTB domains in both DOKL and p62^{dok} are actually functional and important for binding to Abl. However, even the DOKL$^{R209,\ 224A}$ double mutant retained some residual binding activity to Abl (Table 1), suggesting that the PTB domain is not the only domain involved in the interaction.

Since DOKL and p62^{dok} contain PTB domains similar to that of IRS-1 and since p62^{dok} is a direct substrate of the insulin receptor (17), we tested the possibility that DOKL and p62^{dok} could bind to the insulin receptor. Yeast cells were transformed with a yeast expression construct expressing a LexA-insulin receptor intracellular domain fusion protein (13) and pGAD-DOKL or pGAD-p62^{dok}, followed by β-galactosidase assays. Indeed, in the yeast two-hybrid system, both p62^{dok} and DOKL bound weakly to the insulin receptor in a kinase-dependent manner. Mutation of a conserved Arg in the PTB domain disrupted the binding, indicating that DOKL and p62^{dok}, like IRS-1, might bind to the insulin receptor through their PTB domains (Table 2).

We performed several controls to test the specificity of the binding between the SH2 or PTB domain and autophosphorylated tyrosine kinases. In the yeast two-hybrid system, we found that Grb2 and Vav p95 strongly interacted with the carboxy termini of two different receptor tyrosine kinases, Tyro3 and Axl, in a kinase-dependent manner (Table 2 and data not shown). These two SH2 domain-containing proteins, however, failed to associate with Abl or the insulin receptor. In contrast, DOKL and p62^{dok} bound to Abl and the insulin receptor but not to Axl or Tyro3 (Table 2). The differential bindings indicate that the recognition of phosphotyrosine motifs on tyrosine kinases by SH2 and PTB domains is quite specific in yeast.

TABLE 2. Analysis of the interaction between DOK proteins and the insulin receptor in the yeast two-hybrid system[a]

Plasmid	β-Galactosidase activity when cotransformed with plasmid:				
	IR-C	IR-CKR	pSH2-Tyro3	pSH2-Axl	AGP4
pGD-DOKL	+	−	−	−	+++
pGD-DOKL^{R209A}	−	−	−	−	+/−
pGD-p62^{dok}	+	−	−	−	+++
pGD-p62$^{dok\ R207A}$	−	−	−	−	+/−
pGD-Grb2	−	−	+++	+++	−
pGD-Vav p95	−	−	+++	+++	−

[a] Plasmids in the heading contained different DNA fragments in frame with the LexA DNA-binding domain. Plasmids in the left column contained different DNA fragments in frame with the Gal4 activation domain. Yeast strain CTY 10-5d was transformed with various pairs of plasmids, followed by an X-Gal staining assay. The strength of interaction was indicated by the timing and intensity of blue color. +++, dark blue visible in 1 h; +, blue visible in 6 h; +/−, pale blue visible overnight; −, white overnight. IR-C represents the intracellular region of the insulin receptor, and IR-CKR represents the intracellular region of the insulin receptor with the defective kinase activity.

In principle, Abl might also bind to proline-rich motifs on DOKL through its SH3 domain. However, the fact that the kinase-deficient AGP5 protein did not bind to DOKL makes this possibility unlikely.

Phosphorylation of Tyr514 and Tyr385 is not required for the binding between Abl and DOKL. Both v-Abl and Bcr-Abl contain a high level of phosphotyrosine in vivo; this phosphorylation is strongly dependent on the kinase activity of the Abl protein. In v-Abl, Tyr514 is thought to be a major phosphorylation site, while Tyr385 is a minor phosphorylation site (23, 24). Since the binding between Abl and DOK proteins seemed to involve the association of the PTB domain of DOKL with phosphotyrosine sites on Abl, we examined whether phosphorylation of these two tyrosine residues was important for the interaction. Individual or double mutations causing a change of tyrosine to phenylalanine in the ABL protein were introduced at these sites in the AGP4 plasmid, and the mutants were tested for binding to DOK proteins. Surprisingly, the binding between Abl and DOK proteins was not affected by any of these mutations (Table 1).

To test the contribution of Tyr385 and Tyr514 to the phosphotyrosine level of v-Abl in vivo, nucleotide changes reflecting the same tyrosine-to-phenylalanine mutations were introduced into pGD-vAbl, a retroviral vector carrying the v-Abl gene and a neomycin resistance gene. Retroviruses were generated by transfection of the ecotropic phoenix packaging cell line. NIH 3T3 cells were infected with the retroviruses and selected with G418-containing medium. The phosphotyrosine levels of v-Abl in cells transduced with the v-Abl mutant genes were examined. To our surprise, v-Abl phosphotyrosine levels in cells transduced with v-Abl^{Y514F}, v-Abl^{Y385F}, and v-Abl$^{Y514,\ 385F}$ were not significantly reduced compared with that in cells transduced with wild-type v-Abl (Fig. 4). This suggests that other tyrosine residues on v-Abl must be phosphorylated in vivo and might contribute to the interaction between Abl and DOK proteins. The identity of these sites is not known. As a control, experiments were also performed with a mutant v-Abl construct containing a Lys392-to-Arg mutation, which disrupts kinase activity. As expected, v-Abl proteins in cells transduced with v-Abl^{K392R} contained no phosphotyrosine, indicating that Abl phosphorylation strongly depends on Abl kinase activity.

DOKL binds to v-Abl and c-Abl in mammalian cells. To examine the interaction between DOKL and Abl in mammalian cells, we performed transient expression assays after trans-

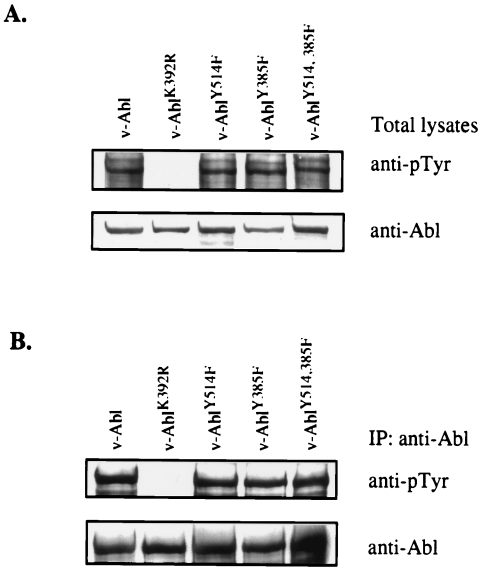


FIG. 4. Tyrosine phosphorylation levels of v-Abl mutants. v-Abl mutant viruses were generated by transiently transfecting the packaging cell line. NIH 3T3 cells were infected with v-Abl viruses and selected in G418-containing medium. Cell lysates were directly separated by SDS-PAGE (A) or they were immunoprecipitated (IP) with anti-Abl antibodies and the immunoprecipitates were separated by SDS-PAGE (B). Blots were probed with anti-phosphotyrosine (anti-pTyr) antibodies (top sections) and reprobed with anti-Abl antibodies (bottom sections). The v-Abl mutant with both previously identified autophosphorylation sites mutated still contains a significant level of phosphotyrosine.

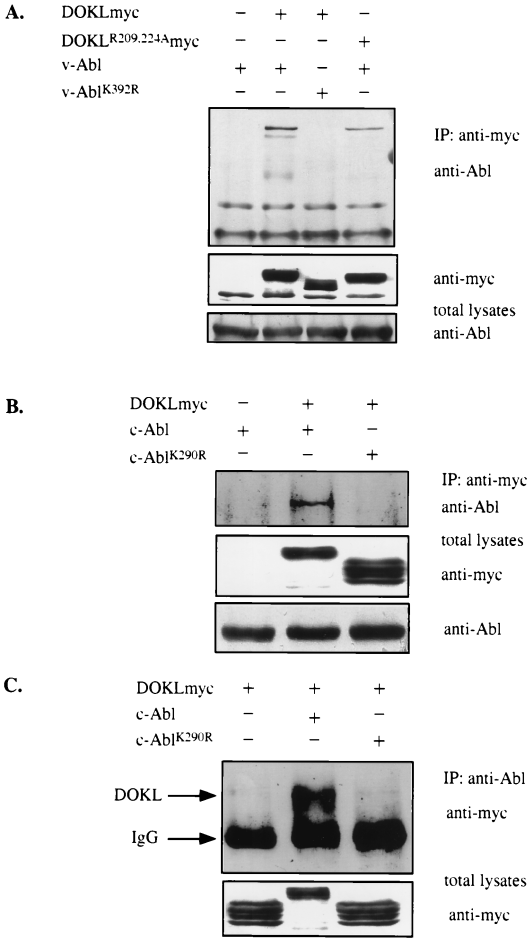


FIG. 5. DOKL and Abl form a complex in vivo. (A) DOKL binding to v-Abl requires Abl kinase activity and the DOKL PTB domain. 293 cells were transfected with the indicated expression constructs, and cell lysates were immunoprecipitated (IP) with anti-myc antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-Abl antibodies (top). The membrane was reprobed with anti-myc antibodies to examine the expression of DOKL (middle). The expression levels of Abl were examined by probing total lysates with anti-Abl antibodies (bottom). That a lower amount of v-Abl is associated with DOKL$^{R209, 224A}$ than with the wild-type DOKL has been repeatedly observed. DOKL migration becomes slower with coexpression of wild-type v-Abl. (B) DOKL binding to c-Abl requires Abl kinase activity. 293 cells were transfected with the indicated expression constructs. Cell lysates were immunoprecipitated with anti-myc antibodies, and immunoprecipitates were fractionated and probed with anti-Abl antibodies (top). The expression levels of DOKL and Abl in total lysates were examined with anti-myc (middle) and anti-Abl antibodies (bottom). (C) DOKL binding to c-Abl can be detected with anti-Abl antisera. 293 cells were transfected with the indicated expression constructs. Cell lysates were immunoprecipitated with anti-Abl antibodies, and immunoprecipitates were fractionated and probed with anti-myc antibodies (top). The expression levels of DOKL and Abl in total lysates were examined with anti-myc (middle) and anti-Abl antibodies (bottom). IgG, immunoglobulin G.

formation of 293 cells. The wild-type v-Abl, but not the v-Abl kinase-deficient mutant, was found to coimmunoprecipitate with myc-tagged DOKL (Fig. 5A). Further, we consistently observed that smaller amounts of v-Abl coimmunoprecipitated with DOKL$^{R209, 224A}$ than with the wild-type DOKL (Fig. 5A). These data confirmed our finding in yeast that the binding between Abl and DOKL absolutely requires Abl kinase activity and partially depends on the PTB domain of DOKL. A similar kinase-dependent interaction between v-Abl and p62^{dok} was observed (data not shown). The binding between v-Abl and DOKL in 293 cells could be detected under a wide range of experimental conditions; the binding was detected with buffers containing up to 1% Triton X-100 and 225 mM NaCl (data not shown). The interaction, however, was reduced in higher salt concentrations.

In these experiments we also examined the migration of the myc-tagged DOKL proteins in the same blots (Fig. 5A, middle panel). The wild-type DOKL and the DOKL$^{R209, 224A}$ mutant proteins both migrated more slowly when coexpressed with a kinase-active v-Abl than with the kinase-deficient v-Abl mutant. This result suggests that v-Abl kinase can lead to the phosphorylation of both DOKL and DOKL$^{R209, 224A}$.

We also tested the ability of DOKL to interact with c-Abl. Under normal physiological conditions, c-Abl kinase activity is tightly regulated. Overexpression of c-Abl in 293 cells, however, can overcome the negative regulation and results in activation of c-Abl. We found that overexpressed c-Abl and DOKL formed a stable complex in 293 cells (Fig. 5B and C). This binding was also dependent on c-Abl kinase activity; DOKL binds only to the wild-type c-Abl and not to a kinase-deficient c-Abl mutant.

p62^{dok} has been shown to interact strongly with RasGAP (64). The consensus binding site for the SH2 domain of RasGAP is YxxP (56), and p62^{dok} contains six such YxxP motifs. In contrast, there is no YxxP motif in DOKL. To test the binding of p62^{dok} and DOKL to RasGAP, 293 cells were cotransfected with myc-tagged p62^{dok} or DOKL with or without v-Abl. Immunoprecipitation assays were performed with anti-RasGAP antibodies. As expected, p62^{dok} bound to RasGAP in a v-Abl-dependent manner, but DOKL did not bind to RasGAP even in the presence of coexpressed v-Abl (Fig. 6). This finding is consistent with the lack of YxxP motifs in DOKL and suggests that DOKL and p62^{dok} bind to overlapping but different sets of signaling proteins in vivo.

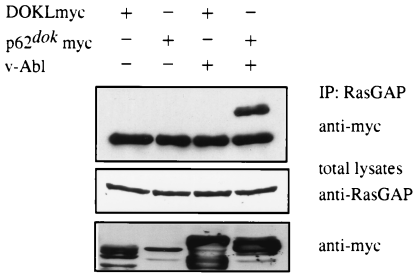


FIG. 6. $p62^{dok}$, but not DOKL, binds to RasGAP upon phosphorylation. 293 cells were transfected with the indicated expression constructs, and cell lysates were immunoprecipitated (IP) with anti-RasGAP antibodies. Immunoprecipitates were fractionated and blotted with anti-myc antibodies (top). The expression levels of RasGAP and DOK proteins in total lysates were examined with anti-RasGAP (middle) and anti-myc antibodies (bottom).

DOKL is a substrate for v-Abl, c-Abl, and Bcr-Abl in vivo. The association between DOKL and Abl suggests that DOKL might be a substrate for Abl tyrosine kinase activity. To test this possibility, 293 cells were transformed with DNAs expressing DOKL and wild-type v-Abl or a v-Abl kinase mutant and lysates were prepared. DOKL proteins were immunoprecipitated, fractionated on gels, blotted, and probed with anti-phosphotyrosine antibodies (Fig. 7). Overexpression of myc-tagged DOKL in 293 cells produced multiple bands, possibly arising from different degrees of phosphorylation, since these bands were collapsed by treatment with calf intestine phosphatase (data not shown). DOKL contained a low level of phosphotyrosine in the absence of v-Abl, and coexpression of v-Abl dramatically increased the DOKL tyrosine phosphorylation level (Fig. 7A). This phosphorylation could also be detected as a mobility shift of the protein bands. Coexpression of DOKL with wild-type c-Abl or Bcr-Abl also resulted in a remarkable increase in DOKL phosphorylation level (Fig. 7B). These data show that DOKL can be phosphorylated by activated Abl tyrosine kinases in vivo.

DOKL was phosphorylated on tyrosine residues at low levels when overexpressed in NIH 3T3 cells even without the coexpression of an exogenous kinase. It should be noted that $DOKL^{R209,\ 224A}$ was phosphorylated on tyrosine residues at a much lower level than wild-type DOKL (Fig. 7C), suggesting that a functional PTB domain is important for the phosphorylation of DOKL by endogenous tyrosine kinases.

Overexpression of DOKL inhibits v-Abl-dependent MAP kinase activation. v-Abl is known to potently stimulate the Ras-MAP kinase pathway (45, 46), and activation of this pathway is important for its transformation activity (51, 55). However, the exact molecular mechanism by which v-Abl activates the Ras-MAP kinase pathway is not clear. We showed that v-Abl was able to activate the Ras-MAP kinase pathway in a transient transfection assay. 293 cells were cotransfected with constructs expressing v-Abl and an HA-tagged version of ERK2, one of the MAP kinases known to be activated by v-Abl. Forty hours after transfection, transfected cells were starved in 0.2% fetal calf serum for another 18 h. To measure the levels of active MAP kinase, ERK2HA was immunoprecipitated with anti-HA antibodies and the immunoprecipitates were resolved by SDS-gel electrophoresis and probed with anti-active-MAP kinase antibodies. v-Abl strongly stimulated MAP kinase in this assay (Fig. 8A, lane 3), and as expected the Ras dominant-interfering mutant RasN17 (58) blocked this stimulation (Fig. 8A, lane 6). We also checked the effect of Bcr-Abl on MAP kinase activation, and, consistent with published reports (45), we did not observe any stimulation of MAP

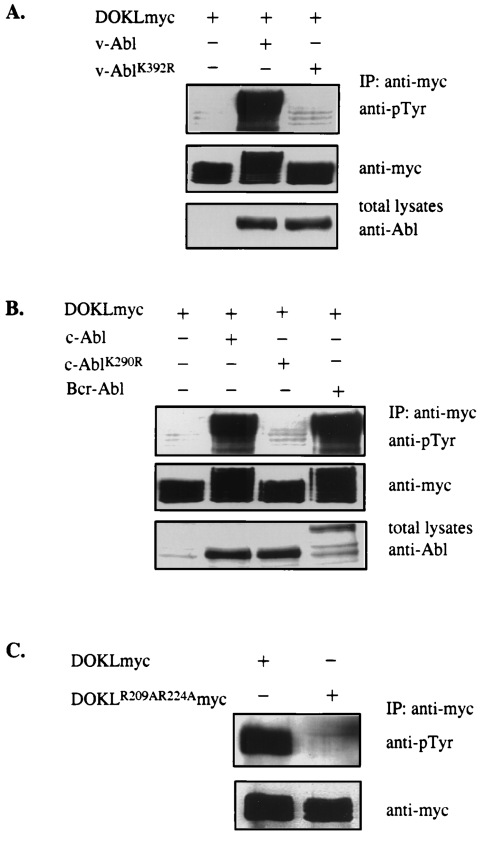


FIG. 7. DOKL is a substrate of activated Abl tyrosine kinase. (A and B) Phosphorylation of DOKL by Abl kinases. 293 cells were transfected with the indicated expression constructs. Cell lysates were immunoprecipitated (IP) with anti-myc antibodies, and immunoprecipitates were fractionated and probed with anti-phosphotyrosine (anti-pTyr) antibodies (top sections). The expression levels of DOKL were examined with anti-myc antibodies (middle sections). The expression of Abl kinases were examined by blotting total lysates with anti-Abl antibodies (bottom sections). DOKL migration becomes slower due to phosphorylation upon coexpression of active Abl kinases. (C) Phosphorylation of DOKL by endogenous tyrosine kinases. NIH 3T3 cells were transfected with the indicated expression constructs. Cell lysates were immunoprecipitated with anti-myc antibodies, and immunoprecipitates were fractionated and probed with anti-pTyr antibodies (top). The expression levels of DOKL were examined with anti-myc antibodies (bottom). The reason that tyrosine phosphorylation of DOKL in the absence of exogenous kinases appears greater in panel C than in panels A and B is that the blot in panel C was exposed for a much longer time.

kinase by Bcr-Abl (data not shown). We observed that overexpression of DOKL greatly reduced the activation of MAPK by v-Abl (Fig. 8A, lane 4). Abi-1, an Abl binding protein and a substrate of Abl kinase (53), did not inhibit this MAP kinase stimulation (Fig. 8A, lane 5). $p62^{dok}$, also a substrate of v-Abl and closely related to DOKL except for the carboxyl terminus, had no effect on the v-Abl-dependent MAP kinase activation (Fig. 8B). These experiments suggest that the inhibitory effect of DOKL on MAP kinase activation is not due to a simple competition with other endogenous substrates of v-Abl. As described earlier, DOKL bound to v-Abl predominantly through its PTB domain. Therefore, we tested the $DOKL^{R209,\ 224A}$ mutant and found that this mutant only slightly inhibited v-Abl-dependent MAP kinase activation, suggesting that a functional PTB domain is required for the inhibition (Fig. 8C).

Since v-Abl activates MAP kinase through Ras, we tested the effect of overexpression of DOKL on the activation of MAP kinase by a constitutively active Ras, RasV12 (6). Overexpression of DOKL had no effect on RasV12-dependent

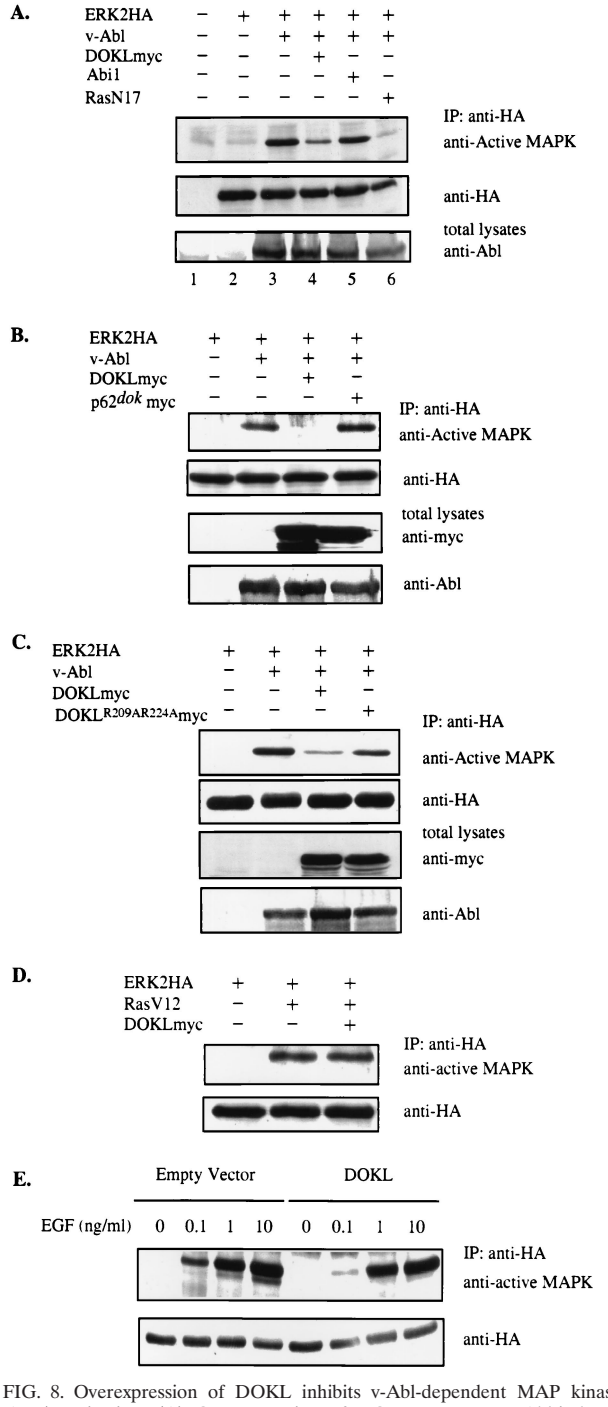


FIG. 8. Overexpression of DOKL inhibits v-Abl-dependent MAP kinase (MAPK) activation. (A) Overexpression of DOKL represses v-Abl-induced MAP kinase activation. 293 cells were transfected with the indicated plasmids. Forty hours after transfection, cells were starved with 0.2% serum for 18 h. After starvation, cells were lysed with RIPA buffer and cell lysates were immunoprecipitated (IP) with anti-HA antibodies. Immunoprecipitates were fractionated, transferred, and probed with anti-active MAP kinase antibodies. The membrane was stripped and reprobed with anti-HA antibodies. The expression levels of v-Abl were checked with anti-Abl antibodies. (B) Overexpression of $p62^{dok}$ does not inhibit v-Abl-induced MAP kinase activation. Cells and lysates were prepared as for panel A; antisera used are indicated. (C) The PTB domain is required for inhibiting v-Abl-induced MAP kinase activation by DOKL. Cells and lysates were prepared as for panel A; antisera used are indicated. (D) Overexpression of DOKL does not affect constitutively active Ras-induced MAP kinase activation. Cells and lysates were prepared as for panel A; antisera used are indicated. (E) Overexpression of DOKL does not affect EGF-induced MAP kinase activation. 293 cells were cotransfected with the indicated plasmids. Forty hours after transfection, cells were starved in 0.2% serum for 18 h. Cells were then restimulated with different amounts of EGF for 10 min. Cell lysates were immunoprecipitated with anti-HA antibodies. Immunoprecipitates were fractionated, probed with anti-active MAP kinase antibodies (top) and reprobed with anti-HA antibodies (bottom).

MAP kinase activation (Fig. 8D). This experiment suggests that the step blocked by overexpression of DOKL lies between v-Abl and Ras. Furthermore, as a control we tested the effect of DOKL overexpression on epidermal growth factor (EGF)-induced MAP kinase activation. The pathway leading to activation of the Ras pathway by engagement of the EGF receptor is well characterized; Grb2 is believed to be the major adapter protein that links the autophosphorylated receptor to the Ras pathway. 293 cells transfected with DOKL were serum starved and restimulated with EGF at various concentrations. We found that overexpression of DOKL had no significant effect on EGF-dependent MAP kinase activation (Fig. 8E).

In summary, we find that overexpression of DOKL inhibits v-Abl-dependent MAP kinase activation, that a functional PTB domain is required for this inhibition, and that this inhibition is not a consequence of broad substrate competition or general toxicity.

Overexpressing DOKL inhibits v-Abl transforming ability. Since overexpression of DOKL inhibits v-Abl-induced Ras pathway activation and since the activation of the Ras pathway is critical for the transforming activity of v-Abl (51, 55), we tested the effect of DOKL overexpression on v-Abl transforming activity. The DOKL gene was cloned into the expression vector pCGN with the coding sequence for an HA epitope fused to the 5′ end of the gene. NIH 3T3 cells were cotransfected with pCGN-DOKL and a puromycin selection marker. Puromycin-resistant clones were picked and expanded in puromycin-containing medium. The expression of DOKL was tested by immunoblotting with anti-HA antibodies. Five of ten randomly picked clones were found to overexpress DOKL (Fig. 9A). As was observed for 293 cells, overexpressed DOKL in NIH 3T3 cells also existed as multiple species, presumably resulting from multiple levels of phosphorylation. We did not detect any significant changes in cell morphology, doubling time, or cell density upon reaching confluence in cell lines overexpressing DOKL. Furthermore, these cell lines could be passaged in puromycin-free medium for up to 1 month without loss of DOKL expression, suggesting that overexpression of DOKL does not have any general toxicity.

Helper-free v-Abl virus was generated by transfecting an ecotropic phoenix packaging cell line with pGD-v-Abl. The virus was used to infect the DOKL-expressing cell lines and their parental NIH 3T3 cell line. All clones expressing or not expressing DOKL were analyzed in parallel. Two days after infection, medium with 4% serum was added to cells to select the outgrowth of transformed clones. Fourteen days after infection, the number of transformed foci on the lawn of confluent cells was determined. v-Abl virus preparations typically exhibited titers of about 10^5 focus-forming units/ml on parental NIH 3T3 and DOKL-negative cell lines. Interestingly, all five DOKL-overexpressing cell lines showed significant inhibition of virus-transforming activity, producing 2- to 14-times-fewer transformed foci than parental NIH 3T3 cells (Fig. 9B).

In principle, the inhibition of virus-transforming activity in DOKL-overexpressing cell lines could result from low infectivity of virus for DOKL-overexpressing cells or general toxicity of overexpressed DOKL protein. To rule out these possibilities, a control virus expressing a neomycin resistance gene was used to infect these same lines. All the cell lines tested

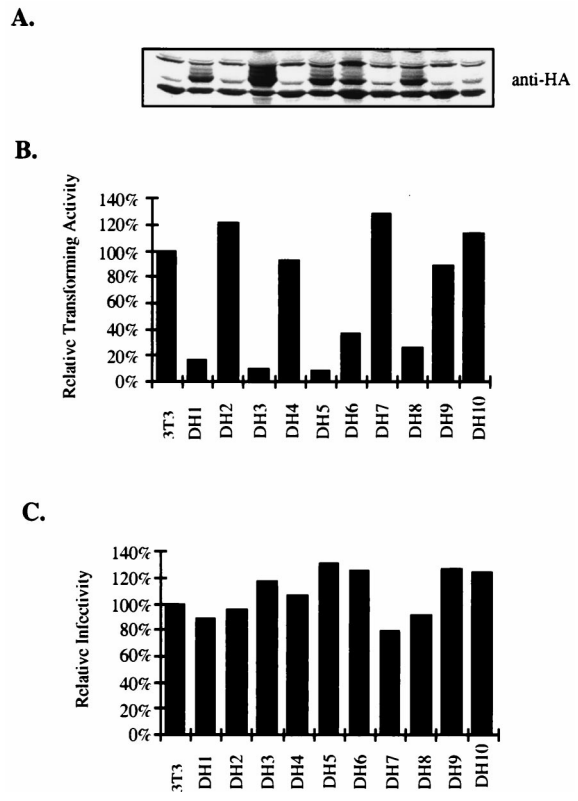


FIG. 9. Overexpression of DOKL in NIH 3T3 cells represses v-Abl transforming activity. (A) Western blot analysis of DOKL expression. NIH 3T3 cells were cotransfected with pCGN-DOKL and a puromycin selection marker. Puromycin-resistant clones were picked, expanded, and analyzed for DOKL expression with anti-HA antibodies. Extracts from parental NIH 3T3 cells are also shown. Several lines expressing HA-tagged DOKL protein were identified. Lane designations match those for bars in panels B and C. (B) Transforming efficiencies of v-Abl on DOKL-overexpressing cell lines. Cell lines were infected with serially diluted v-Abl virus and plated in low-concentration serum, and the number of transformed foci on plates were scored. The titer of v-Abl virus, in focus-forming units (FFU) per milliliter, was determined for each cell line. The apparent viral titers for the cell lines relative to the apparent viral titer for parental NIH 3T3 cells (100% corresponds to 1.1×10^5 FFU/ml) are indicated. (C) Infectivities of pGDN virus on DOKL-overexpressing cell lines. Cell lines were infected with serially diluted pGDN virus and selected in G418-containing medium, and G418-resistant clones were scored. The apparent viral titers for the cell lines relative to the apparent viral titer for parental NIH 3T3 cells (100% corresponds to 7.7×10^5 FFU/ml) are indicated.

produced roughly the same number of drug-resistant clones, indicating that the DOKL-expressing cell lines were equally susceptible to infection (Fig. 9C).

DISCUSSION

We have identified a novel DOK family protein that interacts directly with Abl. We have named the protein DOKL since the N-terminal part of the protein bears a significant similarity to p62^{dok}. DOKL contains an N-terminal PH domain, a PTB domain, and a relatively proline-rich C-terminal sequence. DOKL and p62^{dok} bound to Abl in a kinase-dependent manner in both yeast and mammalian cells. Mutations of the two conserved Arg residues in the PTB domains significantly reduced the binding of DOK to Abl, suggesting that DOK associates with Abl via the PTB domain. Overexpression of DOKL, but not its close relative p62^{dok}, greatly inhibited v-Abl-dependent MAP kinase activation. The inhibition required the PTB domain of DOKL. Finally, overexpression of DOKL suppressed v-Abl transforming activity in NIH 3T3 cells. Our study suggests that DOKL is a novel binding partner of the Abl oncoprotein and that it can modulate Abl transforming activity in vivo.

DOKL is a new adapter protein. DOKL belongs to a growing family of docking (adapter) proteins, which includes p62^{dok}, IRS-1, IRS-2 (59), Gab1 (16), p130CAS (48), Fes/Sin (1, 18), and Cbl (39, 60). These docking proteins do not have extensive sequence homology, but all contain protein-protein interaction motifs. Through the actions of SH2, PTB, and SH3 domains, as well as serine/threonine-rich and proline-rich regions, adapter proteins facilitate the formation of a multimolecular signaling complex. Many of these proteins also contain PH domains, which associate with different inositol phosphate components of the lipid bilayer and facilitate localization to the cell membrane (26). The most striking feature of all these docking proteins is the presence of multiple tyrosine residues, which become substrates for phosphorylation upon activation of a wide variety of tyrosine kinases. The phosphotyrosine residues then serve as docking sites for SH2 or PTB domain-containing proteins. In this way more signal transducers are recruited and the signal is propagated.

DOKL is a member of a rapidly emerging subfamily of docking proteins, of which p62^{dok} is the prototype. DOKL is highly homologous to p62^{dok} at its N terminus. However, the C-terminal half of the molecule is very divergent from p62^{dok}. p62^{dok} has been noted for its strong association with RasGAP. The preferential target of the SH2 domain of RasGAP has been shown to be YxxP, and there are six such motifs in p62^{dok} (5, 64). In contrast, DOKL has three YxxV motifs, but no YxxP motifs. Accordingly, we show that p62^{dok}, but not DOKL, can bind to RasGAP upon phosphorylation by v-Abl. The divergence in the carboxy termini of p62^{dok} and DOKL suggests that they might bind to different sets of adapter proteins, resulting in different signal outputs.

Besides DOKL, other p62^{dok}-like gene products, including DOK-R (20) and dok-2/FRIP (9, 37), have also been cloned. Unlike DOKL, the three other known DOK family members all contain multiple YxxP motifs and have been shown to bind to RasGAP upon phosphorylation. Therefore, DOKL is unique in the DOK family in this aspect. All members of the DOK family have a high degree of homology to each other in their central regions, denoted as DOK homology regions by Di Cristofano et al. (9). Our results, together with data of others (20, 37), suggest that this region of DOK proteins is a functional PTB domain.

The two-way binding model. How p62^{dok} interacts with v-Abl and Bcr-Abl is still not clear. In agreement with the progressive phosphorylation model (31), the p62^{dok} tyrosine phosphorylation level is decreased in cells harboring a Bcr-Abl mutant lacking the SH2 domain (2, 38). However, Bhat et al. have found that deletion of the SH2 domain of Bcr-Abl does not abolish binding to p62^{dok} (2). The question arises as to how Abl kinases interact with p62^{dok} in an SH2-independent manner. Our study suggests that this binding may be mediated by the direct interaction between the PTB domain on p62^{dok} and phosphotyrosine(s) on Abl kinase. In our yeast two-hybrid system, both p62^{dok} and DOKL bound to Abl in a kinase-dependent fashion; mutation of one critical Arg in the PTB domain of the DOK proteins nearly abolished the binding. We believe that the residual binding is mediated by the SH2 domain of Abl and phosphotyrosine residues on DOK proteins. Bhat et al. could not detect binding between Bcr-Abl and p62^{dok} in their yeast two-hybrid system, so they suggest that the binding is indirect and mediated by a third unknown protein (2).

We propose a direct two-way binding model for the association between Abl and the DOK protein. Initially, the PTB domain of DOK directly recognizes a phosphotyrosine on Abl that mediates the first interaction between kinase and substrate. Phosphorylation of DOK by Abl provides binding sites for the Abl SH2 domain, resulting in tighter binding and further phosphorylation. Mutating either the PTB domain on DOK or the SH2 domain on Abl results in reduced binding affinity and lower phosphorylation levels. One result in apparent contradiction to this model is that upon cotransfection of DOKL and v-Abl into 293 cells, the phosphorylation level of DOKL with its PTB domain mutated is only slightly lower than that of the wild-type DOKL (data not shown; Fig. 5A). The problem might result from the fact that both DOKL and v-Abl are overexpressed in this case.

The role of phosphotyrosine residues on Abl in transformation. The exact phosphotyrosine residue(s) on Abl kinase which DOK proteins bind is still unknown. We find that two previously proposed autophosphorylation sites on v-Abl are not important for the binding between Abl and DOK proteins and that there are other unidentified tyrosine phosphorylation sites on Abl. The importance of phosphotyrosine residues on Abl to its transforming activity is not clear. Pendergast et al. showed that phosphorylation on Tyr177 in the Bcr region of Bcr-Abl can serve as a docking site for Grb2, leading to activation of the Ras pathway. Bcr-Abl with this site mutated loses its ability to transform Rat1 fibroblasts (43). It is not clear, however, whether phosphotyrosine residues on Abl can serve as docking sites. The high tyrosine phosphorylation level of Abl kinase is generally correlated with a high level of transforming activity. Although at this time we cannot identify any phosphotyrosine residues serving as docking sites for DOK proteins that are also important for Abl transformation activity, our data suggest that phosphotyrosine sites on Abl kinase and a functional phosphotyrosine binding motif can be important for the binding of some Abl-interacting proteins, such as the DOK proteins.

The inhibition of the Ras pathway by DOKL. Many mechanisms by which v-Abl and Bcr-Abl might activate the Ras pathway have been proposed. For Bcr-Abl, there are several potential routes that might lead to Ras activation: (i) a phosphotyrosine residue in Bcr may recruit Grb2 (43); (ii) Bcr-Abl might also recruit Shc (11, 61), which subsequently recruits the Grb2-Sos complexes; and (iii) Bcr-Abl binds to CRKL, and this might lead to Ras activation (52). For v-Abl, the mechanism of Ras activation is even less clear. Lacking the phosphotyrosine residue which recruits Grb2 to Bcr-Abl, v-Abl does not directly bind to Grb2. The only known potential mechanism is through binding of Shc to the SH2 domain of Abl in a phosphotyrosine-independent manner, direct phosphorylation of Shc, and subsequent recruitment of Grb2-Sos complexes (44). In principle, Shc might also bind to the phosphotyrosine residues on Abl through its PTB domain. Some indirect evidence suggests that $p62^{dok}$ and RasGAP might serve as a link between v-Abl and the Ras pathway (40, 54). Moreover, CRKL was shown to directly bind to $p62^{dok}$ in a phosphorylation-dependent manner (2). Thus, $p62^{dok}$ provides a new link between v-Abl and CRKL, and possibly the Ras pathway.

DOKL blocks the MAP kinase activation induced by v-Abl but not that induced by constitutively active Ras, suggesting that DOKL blocks a step between v-Abl and Ras. Since the PTB domain regions of DOKL and $p62^{dok}$ are highly related and since both bind to Abl in a PTB domain-dependent manner, overexpressed DOKL might compete with endogenous $p62^{dok}$ for the same phosphotyrosine residues on v-Abl and inhibit v-Abl-induced Ras activation mediated by $p62^{dok}$. It is also possible that DOKL competes with other PTB domain-containing proteins, such as Shc.

The Ras pathway is crucial for the transforming activity of both v-Abl and Bcr-Abl (51, 55). Consistent with the idea that DOKL inhibits a pathway critical for v-Abl transforming activity, overexpression of DOKL in NIH 3T3 cells potently suppresses the transforming activity of v-Abl. It should be emphasized that the suppression of Abl transforming activity by DOKL is only observed in the setting of overexpression of DOKL. In the physiological condition, endogenous DOKL might serve either as a positive or negative effector for Abl kinase. Nevertheless, it will be interesting to find out whether overexpression of DOKL in vivo can render mice more resistant to Abelson virus transformation, whether loss of DOKL expression can render mice more prone to Abelson virus transformation, and whether the loss of DOKL expression correlates with the B-cell clonal selection during Abelson virus transformation and with the progression of human chronic myeloid leukemia.

In conclusion, we identified a new DOK family adapter protein. This protein can inhibit the v-Abl-induced Ras pathway and v-Abl transforming activity upon overexpression. Our results suggest that different adapter proteins may compete with each other for binding to Abl and that the transforming activity of the Abl oncogene may depend on the balance of these different adapter proteins. Perturbing this balance will affect the transforming activity of Abl kinase.

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