

## Domain-dependent Function of the rasGAP-binding Protein p62Dok in Cell Signaling\*

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Zhou Songyang<sup>‡§</sup>, Yuji Yamanashi<sup>¶</sup>, Dan Liu<sup>‡</sup>, and David Baltimore<sup>||</sup>

From the <sup>‡</sup>Verna and Marrs Mclean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, <sup>¶</sup>Department of Oncology, Institute of Medical Science, University of Tokyo, Tokyo, Japan, and <sup>||</sup>California Institute of Technology, Pasadena, California 91125

**p62Dok, the rasGAP-binding protein, is a common target of protein-tyrosine kinases. It is one of the major tyrosine-phosphorylated molecules in v-Src-transformed cells. Dok consists of an amino-terminal Pleckstrin homology domain, a putative phosphotyrosine binding domain, and a carboxyl-terminal tail containing multiple tyrosine phosphorylation sites. The importance and function of these sequences in Dok signaling remain largely unknown. We have demonstrated here that the expression of Dok can inhibit cellular transformation by the Src tyrosine kinase. Both the phosphotyrosine binding domain and the carboxyl-terminal tail of Dok (in particular residues 336–363) are necessary for such activity. Using a combinatorial peptide library approach, we have shown that the Dok phosphotyrosine binding domain binds phosphopeptides with the consensus motif of Y/MXXNXL-phosphotyrosine. Furthermore, Dok can homodimerize through its phosphotyrosine binding domain and Tyr<sup>146</sup> at the amino-terminal region. Mutations of this domain or Tyr<sup>146</sup> that block homodimerization significantly reduce the ability of Dok to inhibit Src transformation. Our results suggest that Dok oligomerization through its multiple domains plays a critical role in Dok signaling in response to tyrosine kinase activation.**

p62Dok, the rasGAP-binding protein, is a common target of protein-tyrosine kinases (PTKs).<sup>1</sup> It is one of the major tyrosine-phosphorylated molecules in v-Src-, v-Abl-, and v-Fps-transformed cells and is also rapidly tyrosine-phosphorylated in response to receptor tyrosine kinase activation in various cell systems (1–5). These findings have underlined the importance of p62Dok in cellular signaling.

Recent molecular cloning of Dok has shed more light on the mechanism by which this protein may interact with PTK signaling pathways (4, 6). Dok consists of an amino-terminal Pleckstrin homology (PH) domain, a putative phosphotyrosine binding (PTB) domain, and a carboxyl-terminal tail harboring at least seven potential tyrosine phosphorylation sites (4, 6).

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§ To whom correspondence should be addressed: Verna and Marrs Mclean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-5220; Fax: 713-796-9438; E-mail: songyang@bcm.tmc.edu.

<sup>1</sup> The abbreviations used are: PTK, protein-tyrosine kinase; PH, Pleckstrin homology; PTB, phosphotyrosine binding; pY, phosphotyrosine; HA, hemagglutinin; MSCV, murine stem cell retroviral vector; PCR, polymerase chain reaction; GST, glutathione S-transferase.

The PH domain is likely to be necessary for targeting Dok to the membrane, because PH domains preferentially bind phospholipids (7). The putative PTB domain is most homologous to the IRS-1 and FRS2/SNT-1 PTB domains, which recognize phosphotyrosine (pY)-containing sequences (8–11). In the case of SNT-1, its PTB domain can also bind distinct unphosphorylated sequences (11). It remains to be determined whether the Dok PTB domain can bind phosphopeptides and, if it does, whether it recognizes sequences distinct from those recognized by the IRS-1 and SNT-1 PTB domains. The multiple tyrosine residues in the Dok carboxyl-terminal tail are candidate sites for tyrosine kinases. When phosphorylated, they become potential docking sites for Src homology 2-containing proteins such as p120 rasGAP and Nck (5, 12). Consistent with this notion, both rasGAP and Nck have been shown to bind tyrosine-phosphorylated Dok (4, 13). Therefore, the carboxyl-terminal tail of Dok likely functions as a molecular platform for signal complex assembly induced by activated PTKs. However, the functional significance of the Dok PTB domain and the carboxyl-terminal tail has yet to be addressed.

Additional Dok homologues such as Dok-L (or Dok-3) and Dok-R (or Dok-2 and FRIP) have been identified recently (14–18), indicating that Dok and its homologues may constitute a growing family of proteins involved in a range of signaling pathways downstream of PTKs. However, the physiological roles of Dok and its homologues remain to be elucidated. Despite their structural similarities to the IRS-1 family molecules, Dok family proteins have different PTB domains and carboxyl-terminal tails that potentially mediate different signal responses by recruiting distinct sets of Src homology 2-containing signaling molecules. The mechanism by which Dok is phosphorylated and primed to form specific signaling complexes thus becomes a key issue in understanding Dok signaling. We have demonstrated here that the Dok PTB domain is a functional phosphotyrosine binding module that facilitates tyrosine phosphorylation and rasGAP binding of Dok. We have also found that Dok can inhibit Src-induced cellular transformation. This inhibitory effect depends on both the PTB domain and the carboxyl-terminal tail of Dok. Furthermore, we have shown that Dok can oligomerize via its PTB domain and Tyr<sup>146</sup>. This oligomerization appears critical for the inhibition of v-Src-induced transformation. These results suggest that the multiple domains of Dok are required for Dok signaling.

### EXPERIMENTAL PROCEDURES

*Cell Lines and Antibodies*—NIH3T3 cells were maintained in Dulbecco's modified Eagle's media containing 10% calf serum (Hyclone). The anti-hemagglutinin (HA) monoclonal antibody was purchased from Berkeley Antibody Company. The anti-His-tag, anti-rasGAP, and anti-phosphotyrosine monoclonal antibodies (4G10 and PY20) were purchased from Santa Cruz Biotechnology. Anti-Ras monoclonal antibody was from Upstate Biotechnology.

**DNA Constructs**—The coding region of the murine Dok cDNA (4) tagged with the HA epitope was cloned into the *Hpa*I site of the murine stem cell retroviral vector MSCVpuro (19). The HA epitope was joined to the 5' end of Dok cDNA by polymerase chain reaction (PCR) using the *pfu* polymerase (4). The carboxyl-terminal truncated forms of Dok were similarly cloned. The Dok 277, 313, 336, and 363 constructs encode amino acids 1–277, 313, 336, and 363, respectively. The PTB domain mutant Dok-AA (Arg<sup>207</sup> and Arg<sup>208</sup> to Ala) and the Y146F mutants (DokN-Y146F, Dok313-Y146F, and Dok363-Y146F) were generated by site-directed mutagenesis using PCR with the *pfu* polymerase. All constructs were confirmed by DNA sequencing. Sequences encoding wild-type (chicken c-Src) or activated (Src527F) Src were cloned into the MSCV-neo vector as described previously (20).

**Generation of Cell Lines Expressing Src and Dok Variants**—The MSCV-based constructs encoding Src and HA-Dok and their variants were transfected into the retrovirus packaging cell line BOSC23 (21). Retroviruses were harvested 2 days after transfection and used to infect dividing NIH3T3 cells. Two days after infection, Dok- or Src-expressing cells were selected in puromycin- or G418-containing media. Cells that coexpressed Dok and Src were generated by infecting Dok-expressing cells again with Src viruses followed by selection in G418-containing media.

**Transformation Assay**—The ability of Src to induce cellular transformation was scored by the focus formation assay in NIH3T3 cells. Parental and Dok variant-expressing cells were infected by low titer ( $1 \times 10^4$ /ml) MSCV-c-Src or MSCV-Src527F viruses. These cells were maintained in Dulbecco's modified Eagle's media containing 10% calf serum for 7–10 days for focus formation. The number of foci were then quantitated to determine transformation activities. Three replicate dishes were plated for each sample, and each experiment was repeated three times.

**GST Fusion Proteins and Phosphopeptide Library**—To generate the glutathione S-transferase (GST) fusion protein construct containing only the amino-terminal PH and PTB domains of Dok (GST-DokN), the DNA fragment encompassing residues 1–277 of murine Dok was PCR-amplified and cloned into the *Sma*I site of the pGEX4T-1 vector (Amersham Pharmacia Biotech). Similarly, to generate the GST-PTB and GST-PTB-AA constructs, the DNA fragment encoding residues 125–264 was PCR-amplified using wild-type Dok or Dok-AA as a template. These fragments were subsequently cloned into the *Bam*HI site of pGEX4T-1. These constructs were used to transform *Escherichia coli* to produce GST fusion proteins that were purified using glutathione-agarose beads.

To study the binding specificity of the Dok PTB domain, 100–200  $\mu$ g of fusion proteins that were immobilized on glutathione-agarose beads were incubated with 1 mg of peptide library. The peptide library had a sequence of MAXXXNXXpYXAKKK, where X indicates any amino acid except Cys and Trp. This particular library was designed to examine PTB domain specificities, because PTB domains prefer turn-forming sequences near pY and hydrophobic residues at 5–8 positions amino-terminal to pY (22). The mixture of peptides bound to the fusion proteins was eluted with acid after washing. This peptide mixture was then sequenced on an ABI477 machine. The specificity of binding was then determined by comparing the sequence of bound mixture with that of the mock experiment using GST alone (12).

**His-tagged Fusion Proteins and Src Phosphorylation**—To generate poly-His-tagged Dok (His-DokN) fusion proteins, sequences encoding residues 1–264 of Dok were PCR-amplified and cloned into the *Bam*HI site of the pRSET vector (Invitrogen). His-DokN-Y146F was constructed in the same manner, except that Tyr<sup>146</sup> was mutated to Phe. His-tagged proteins were purified from BL21 cells using nitrilotriacetic acid-agarose (Qiagen) and eluted with imidazole. To phosphorylate these fusion proteins, they were incubated with 2  $\mu$ g of purified recombinant c-Src (a generous gift from Dr. Wenqing Xu, University of Washington) in the presence of 1 mM ATP and 20 mM MgCl<sub>2</sub> for 1 h at 30 °C.

**Immunoprecipitation and Western Blot Analysis**—Cells were washed once with PBS, lysed in the lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M pepstatin A, 1  $\mu$ M aprotinin, 1  $\mu$ M leupeptin, 2 mM  $\beta$ -glycerolphosphate, 500  $\mu$ M sodium vanadate, and 1 mM dithiothreitol), and centrifuged at 10,000  $\times$  g at 4 °C. The supernatants were subsequently collected for immunoblotting and immunoprecipitation. For immunoblotting, 1/10 of the supernatants was mixed with SDS-loading buffer, boiled, and analyzed by SDS-polyacrylamide gel electrophoresis. For immunoprecipitation, the supernatants were incubated with antibodies at 4 °C for 90 min. Protein A/G-agarose beads were then added, and the mixture was incubated at 4 °C for another 90 min. The beads were subsequently washed four times with lysis buffer, resus-

ended in SDS-loading buffer, and boiled, and the eluents were analyzed by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis and Western blot analyses were performed as described previously (4).

**Ras Activation Assay**—For analyzing the effect of Dok expression on Ras GTP loading in Src-transformed cells, GST-Raf Ras binding domain-agarose beads (Ras Activation Assay kit, Upstate Biotechnology) were used to precipitate GTP-bound Ras as described in the manufacturer's manual. A monoclonal antibody against Ras was also supplied in the kit and used to probe for endogenous Ras.

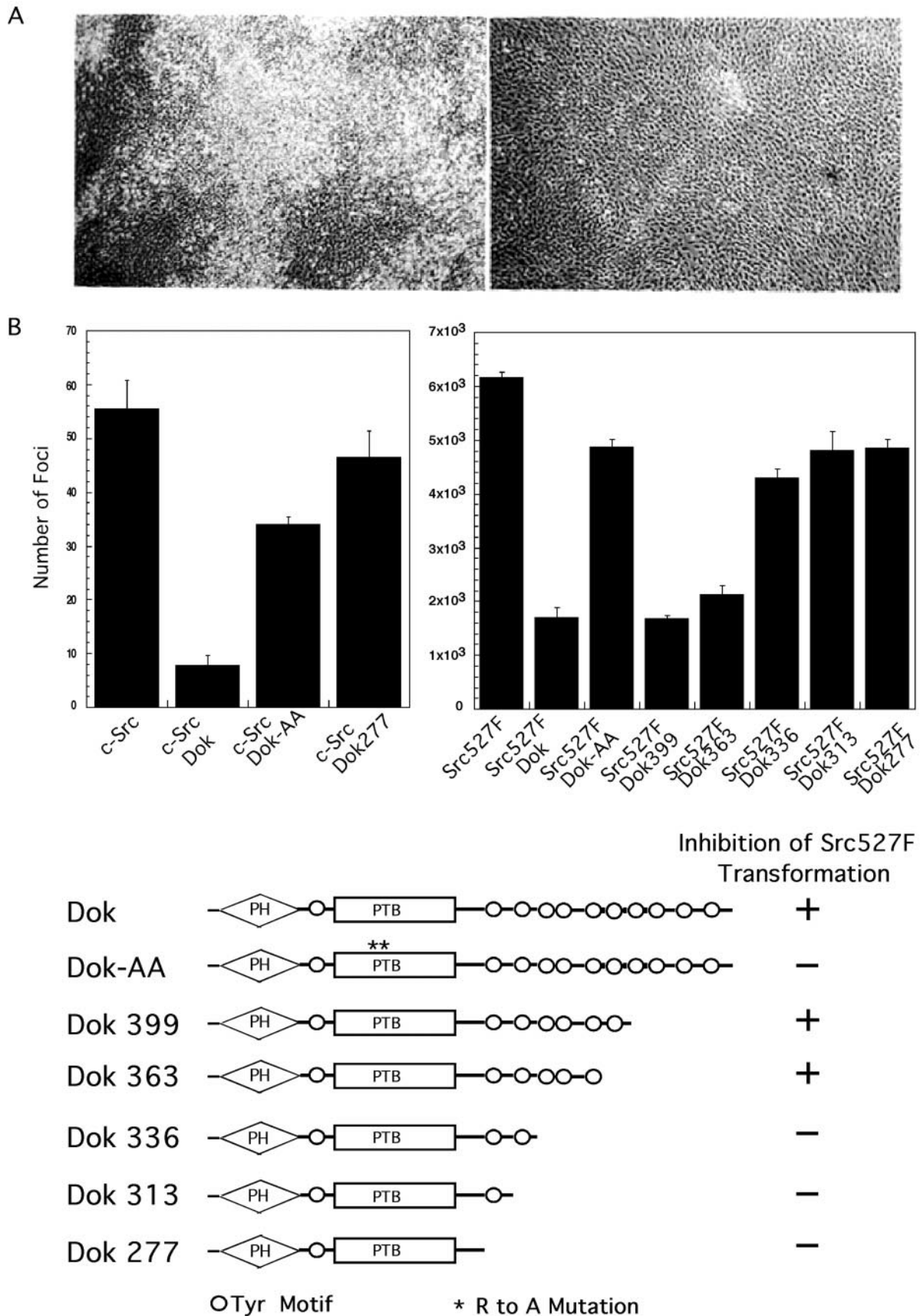
## RESULTS

**Dok Can Inhibit Src-induced Cellular Transformation**—Although tyrosine phosphorylation of Dok occurs concurrently with PTK activation, the exact role of Dok in cell signaling remains ambiguous. To address the question of whether Dok facilitates or inhibits Src transformation, we examined the effect of Dok expression on Src-induced transformation in NIH3T3 cells. Dok and Src retroviruses (see "Experimental Procedures") were used to infect NIH3T3 cells. As shown in Fig. 1, A and B, expression of c-Src or activated Src (Src527F) readily induced focus formation within 1 week. However, coexpression of Dok and Src strongly inhibited focus formation induced by Src. Although Dok is commonly phosphorylated by oncogenic tyrosine kinases, this result indicates that the physiological effect of DOK is to inhibit cellular transformation by the Src-tyrosine kinase.

**The Dok PTB Domain and Carboxyl-terminal Region Are Necessary for Its Inhibitory Effects**—Because Dok consists of multiple domains, we went on to map the functional domains in Dok that are responsible for its inhibitory effects. Mutant Dok molecules that were HA epitope-tagged were generated and coexpressed with Src in NIH3T3 cells. We postulated that the multiple potential tyrosine phosphorylation sites within the Dok carboxyl terminus might contribute to Dok inhibition of Src transformation. Consistent with this notion, deletion of the carboxyl-terminal tail (Dok277) abolished the inhibitory activity of Dok (Fig. 1B). Using a panel of carboxyl-terminal deletion constructs, we further defined the regions within the Dok carboxyl terminus necessary for transformation inhibition. Although Dok363 still blocked Src transformation, Dok313 and Dok336 no longer retained the inhibitory abilities (Fig. 1B). These data indicate that the residues between 336 and 363 constitute a functional domain for the inhibitory action of Dok.

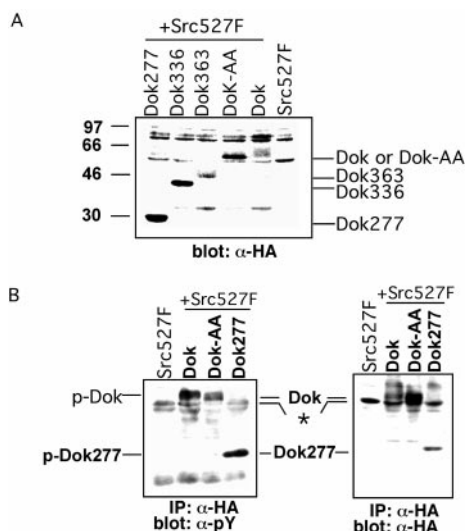
To determine the role of the amino-terminal portion, mutations were made in the putative PTB domain of Dok. On the basis of sequence homology with the IRS-1 PTB domain (4), amino acids Arg<sup>207</sup> and Arg<sup>222</sup> of Dok are predicted to coordinate phosphotyrosine binding. We therefore reasoned that mutation of Arg<sup>207</sup> might block phosphotyrosine binding and thereby affect Dok function. Supporting our hypothesis, mutation of Arg<sup>207</sup> and Arg<sup>208</sup> to Ala residues (Dok-AA) eliminated the inhibitory function of Dok (Fig. 1B). These results strongly suggest that the Dok amino-terminal PTB domain represents a distinct regulatory domain of Dok that may associate with tyrosine-phosphorylated proteins.

**Tyrosine Phosphorylation of Dok Mutant Proteins**—Because tyrosine phosphorylation of Dok may be critical for its *in vivo* activities, we examined tyrosine phosphorylation of various HA-tagged Dok mutants in Src527F-transformed NIH3T3 fibroblasts. The different Dok proteins including the PTB domain mutant (Dok-AA) and all carboxyl-terminal deletion mutants (Dok277, -313, -336, and -363) were expressed (Fig. 2, A and B). Western blots of whole cell lysates indicated that they were all tyrosine-phosphorylated (data not shown). Surprisingly, Western blots of anti-HA immunoprecipitates that were probed with anti-phosphotyrosine antibodies showed that even Dok277 (which lacks the carboxyl-terminal region with its mul-



**FIG. 1. Expression of Dok can inhibit Src transformation.** Parental and HA-tagged Dok-expressing NIH3T3 fibroblast cells were infected with c-Src or Src527F retroviruses. The effect of various Dok mutants on Src transformation was studied using a focus formation assay in these cells. *A*, culture morphology of cells expressing c-Src alone (*left*) or both c-Src and Dok (*right*). *B*, comparison of the focus-forming abilities of parental NIH3T3 cells or those that coexpressed Dok variants with either c-Src (*left*) or Src527F (*right*). The numbers indicate the numbers of foci formed per  $10^4$  Src viral particles. *C*, diagram of different Dok deletion mutants and mapping of domains that are required for Dok inhibitory function. +, inhibition; -, no inhibition.





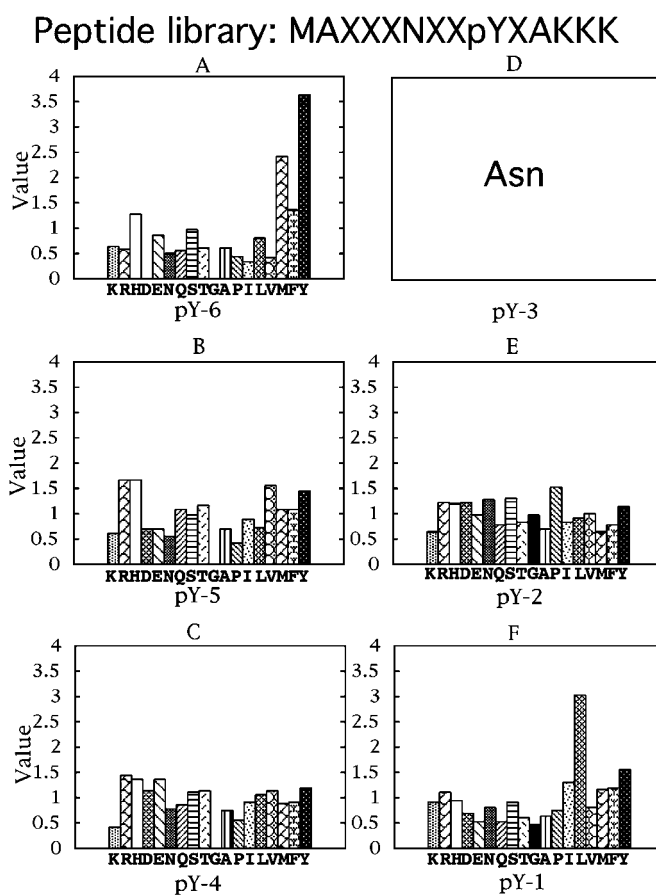
**FIG. 2. Expression and tyrosine phosphorylation of wild-type and mutant Dok proteins.** Lysates from cells that coexpressed various HA-tagged Dok constructs with Src527F were immunoprecipitated and Western blotted. *A*, expression of wild-type and mutant HA-tagged Dok proteins in Src527F-transfected cells. Equal amounts of whole cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were probed with anti-HA antibodies. *B*, comparison of tyrosine phosphorylation of HA-Dok, Dok-AA, and Dok277 proteins. Dok proteins were immunoprecipitated (IP) using anti-HA antibodies and probed with an anti-phosphotyrosine antibody. *p-Dok*, phosphorylated Dok; *p-Dok277*, phosphorylated Dok277.

multiple potential phosphorylation sites) was still tyrosine-phosphorylated (Fig. 2*B*), suggesting that a major tyrosine phosphorylation site is located in the amino-terminal domain of Dok. Therefore, changes in the gross tyrosine phosphorylation levels of various Dok mutants induced by the Src PTK may not account for the differences in their inhibitory abilities. However, phosphorylation of specific tyrosine sites on Dok may be necessary for inhibition of Src-mediated transformation.

**Dok PTB Domain Binds to Specific Phosphopeptide Sequences**—We have shown here that the Dok PTB domain (residues 125–264) is functional and necessary for Dok to inhibit Src-induced transformation, possibly through its interaction with phosphotyrosine-containing proteins. However, whether the Dok PTB domain does indeed bind specific phosphotyrosine-containing sequences remains to be determined. To this end, we used a combinatorial peptide library approach (see “Experimental Procedures”).

Briefly, GST fusion proteins containing either the Dok amino-terminal PH and PTB domains (GST-DokN) or the Dok PTB domain alone (GST-PTB) were purified and captured on GSH beads. The beads were then incubated with a soluble degenerate phosphopeptide library mixture to select for specific peptides that would bind to the PTB domains. The peptide library used had a sequence of MAXXXNXXpYXAKKK. The amino acid at position –3 to the phosphotyrosine was fixed (Asn), because PTB domains prefer turn-forming sequences (9, 22). The phosphopeptide mixtures that bound specifically to the Dok PTB domain were then isolated and sequenced by Edman degradation. A comparison of these sequences to those obtained using GST alone revealed that the Dok PTB domain recognizes phosphopeptides with the unique motif of Y/MXXNXLpY (Fig. 3). At position pY-1, Leu was exclusively selected, indicating the importance of this residue. At position pY-6, hydrophobic amino acids Tyr, Met, and Phe were strongly selected. Similar preferences for hydrophobic residues at positions pY-5 to pY-8 have been reported for other PTB domains as well (8, 22).

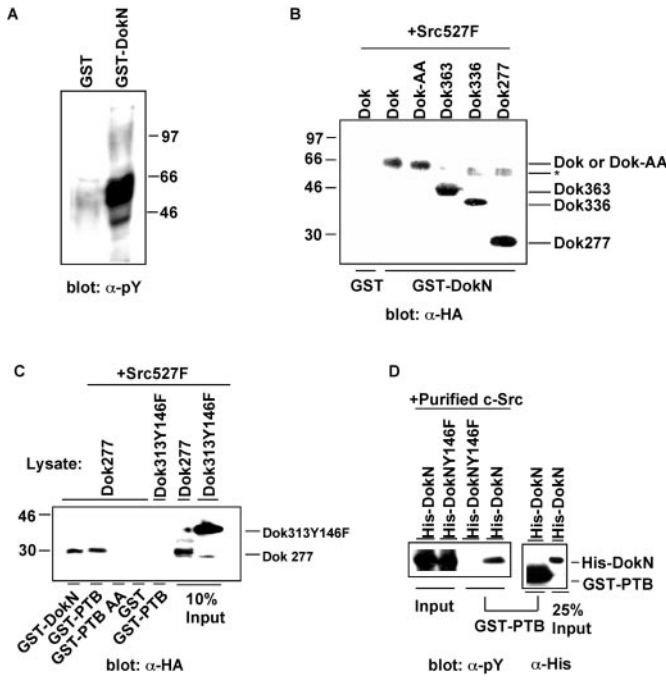
A region within the Dok PTB domain (amino acids 204–232)



**FIG. 3. The Dok PTB domain binds phosphopeptides with distinct sequences.** The binding specificity of the Dok PTB domain was examined using the phosphopeptide library MAXXXNXXpYXAKK. The relative preference of amino acids at each degenerate position (pY as position 0) was plotted. A value of  $\geq 1$  higher indicates a preferred amino acid. Selections are shown at position -6 (*A*), position -5 (*B*), position -4 (*C*), position -3 (*D*), position -2 (*E*), and position -1 (*F*).

is 41 and 52% identical to those of IRS-1 and SNT-1, respectively. The latter two PTB domains bind phosphopeptides with the consensus motif of NPXpY (8–11). Importantly, two critical Arg residues (Arg<sup>207</sup> and Arg<sup>222</sup>) that are known to mediate phosphotyrosine recognition in the IRS-1 PTB domain are conserved in the Dok PTB domain as well (Arg<sup>207</sup> and Arg<sup>222</sup>). To test whether Arg<sup>207</sup> also mediates the interaction between the Dok PTB domain and the phosphotyrosine moiety, we examined the Dok-AA mutant in which Arg<sup>207</sup> was replaced with Ala. As predicted, this mutation eliminated the ability of the Dok PTB domain to bind phosphopeptides (data not shown). Combined with the observation that Dok-AA could no longer inhibit Src transformation, these data support the model that Dok function depends on the PTB domain. Furthermore, despite their similarities in the structural basis for phosphotyrosine recognition, the Dok PTB domain recognizes distinct sequences (NXLpY) compared with the IRS-1 PTB domain (NPXpY). Therefore, the Dok PTB domain may associate *in vivo* with a set of tyrosine-phosphorylated proteins distinct from that bound by IRS-1.

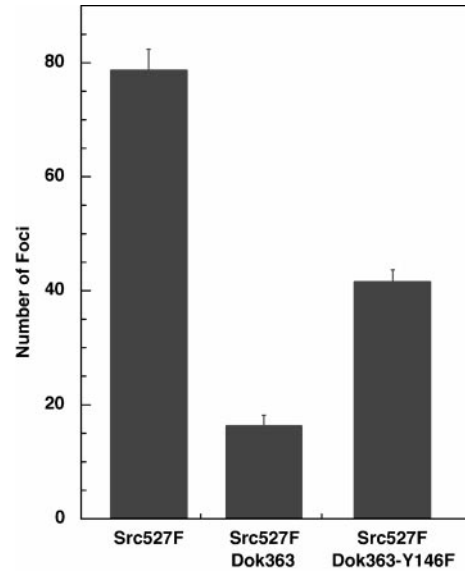
**The Dok PTB Domain Mediates Phosphotyrosine-dependent, Homotypic Interactions of Dok**—We have shown that the PTB domain recognizes specific tyrosine-phosphorylated sequences and is required for the inhibitory activity of Dok. It is likely that Dok may exert its inhibitory function by binding to specific phosphoproteins through the PTB domain. To further investigate the phosphoproteins that might associate with the PTB domain and the mechanism by which the PTB domain might



**FIG. 4. The Dok PTB domain mediates oligomerization of Dok proteins.** A, the amino-terminal domain of Dok binds to phosphoproteins at ~60 kDa. The Dok amino-terminal domain fusion protein (*GST-DokN*) or GST alone was incubated with lysates from Src527F-expressing NIH3T3 cells. The associated proteins were then detected by anti-phosphotyrosine antibodies and enhanced chemiluminescence. B, the amino-terminal domain of Dok interacts with different Dok mutant proteins. The *GST-DokN* fusion protein was incubated with lysates from cells expressing various HA-tagged Dok mutants. As a negative control, GST proteins were incubated with cell lysates from HA-Dok- and Src527-coexpressing cells. Proteins that bound to GST or *GST-DokN* were visualized using an anti-HA monoclonal antibody and enhanced chemiluminescence. \*, cross-reactive bands. C, the Dok PTB domain mediates homotypic interaction through Tyr<sup>146</sup> of Dok. GST fusion proteins of DokN (*GST-DokN*), Dok PTB (*GST-PTB*), and Dok PTB mutant (*GST-PTB AA*) were incubated with lysates from cells coexpressing Src527F and HA-tagged Dok277 or Dok313 with and Tyr<sup>146</sup> to Phe mutation (*Dok313Y146F*). Proteins that bound to GST fusion proteins were visualized using an anti-HA monoclonal antibody and enhanced chemiluminescence. D, the Dok PTB domain binds directly to the phosphorylated Tyr<sup>146</sup> site. *GST-PTB* fusion proteins were incubated with purified His-tagged DokN (*right*) or c-Src-phosphorylated, His-tagged DokN (*His-DokN*) and DokN-Y146F (*His-DokNY146F*; *left*). Proteins that bound to the GST PTB domain were visualized using anti-His-tag or anti-phosphotyrosine antibodies and enhanced chemiluminescence.

regulate Dok function, the *GST-DokN* fusion protein containing both PH and PTB domains was used to precipitate proteins from Src-transformed cells. As shown in Fig. 4A, multiple tyrosine-phosphorylated bands were specifically copurified with the Dok amino-terminal domain, including major proteins at ~60 kDa, which is similar to the molecular mass of Dok. We therefore speculated that some of these proteins might be Dok family members. To test this idea, *GST-DokN* fusion proteins were incubated with cell lysates from Src-transformed cells that also expressed various Dok mutants. Although GST alone did not precipitate any Dok molecules, *GST-DokN* bound specifically to HA-tagged, wild-type Dok and various mutant Dok proteins (Fig. 4B). The carboxyl-terminal tail of Dok is not required for this interaction, because Dok277 appeared to bind *GST-DokN* with the same efficiency as wild-type Dok. In addition, the *GST-PTB* domain alone was able to associate with HA-tagged Dok277, indicating that the PTB domain may mediate homotypic interactions of Dok (Fig. 4C).

On the basis of our combinatorial peptide library mapping of the consensus substrate motif (Y/MXXNXLpY) of the Dok PTB



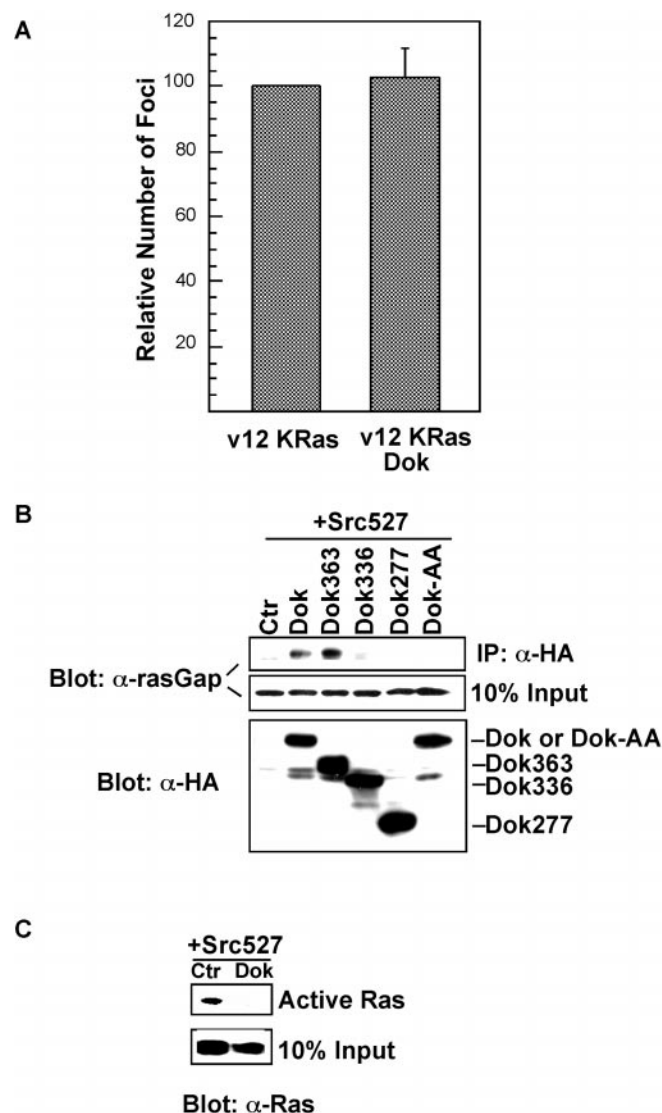
**FIG. 5. Tyr<sup>146</sup> is important for the inhibitory activity of Dok.** Parental NIH3T3 fibroblast cells and those that expressed HA-tagged Dok363 or Dok363-Y146F were infected with low-titer Src527F viruses. Focus formation was then assayed to compare the effect of various Dok mutants on Src transformation in these cells. Error bars indicate S.E. from three independent experiments.

domain, sequences surrounding residue Tyr<sup>146</sup> (LEMLEN-SLYS) on Dok constitute a perfect binding site for the Dok PTB domain when this tyrosine is phosphorylated. Tyr<sup>146</sup>, located between the Dok PH and PTB domains, is also a potential tyrosine phosphorylation site for the Src family PTKs (24). We hypothesized that Tyr<sup>146</sup> might be necessary for the homotypic interactions mediated by the Dok PTB domain. Consistent with our hypothesis, the HA-tagged Dok mutant with its Tyr<sup>146</sup> mutated to Phe (Dok313-Y146F) failed to copurify with the *GST-PTB* domain, even though the *GST-PTB* domain fusion proteins were able to pull down the Dok amino-terminal region (Fig. 4C). These data strongly indicate a PTB domain-mediated, direct interaction between Dok molecules.

PTB domains have been shown to mediate both phosphotyrosine-independent and -dependent interactions. To confirm that Dok-Dok interactions through the PTB domain were direct and tyrosine phosphorylation-dependent, we generated the His-tagged Dok amino-terminal domain (*His-DokN*) and *His-DokN* with the Y146F mutation (*His-DokN-Y146F*) in *E. coli*. These fusion proteins were *in vitro*-phosphorylated using recombinant c-Src and incubated with *GST-Dok PTB*. As shown in Fig. 4D, only *His-DokN* was able to bind *GST-Dok PTB*, although *His-DokN* and *His-DokN-Y146F* were equally phosphorylated by c-Src. In addition, the *GST-PTB* domain failed to bind *His-DokN* in the absence of c-Src. These data demonstrate that the Dok PTB domain mediates phosphotyrosine-dependent homotypic interactions through residue Tyr<sup>146</sup>.

**Tyr<sup>146</sup> Is Important for Regulating Dok Activity**—We showed that Dok363 was the shortest mutant to still retain the inhibitory activity (Fig. 1). To determine the role of Tyr<sup>146</sup> on Dok function, Dok363 with the Tyr<sup>146</sup> to Phe mutation (Dok363-Y146F) was generated and compared with Dok363 for its effect on Src-induced transformation in NIH3T3 cells. Notably, the Y146F mutation significantly reduced the inhibitory activity of Dok363 (Fig. 5). Therefore, mutations (in either the PTB domain or Tyr<sup>146</sup>) that prevent Dok oligomerization also abrogated its inhibitory activity. These results indicate that the homotypic interaction through Tyr<sup>146</sup> and the Dok PTB domain is necessary for Dok function.

**Dok Interacts with the Ras Pathway**—Src is known to acti-



**FIG. 6. Dok binds rasGAP and interacts with the Ras signaling pathway.** *A*, Dok does not inhibit V12-K-Ras-induced transformation. Focus-forming abilities of NIH3T3 cells expressing V12-K-Ras alone or with Dok were compared. *B*, mutant Dok proteins are impaired in their abilities to bind endogenous rasGAP. Lysates from cells that coexpressed Src527F and various HA-tagged Dok constructs were immunoprecipitated with anti-HA antibodies and Western blotted with anti-rasGAP antibodies. Whole-cell lysates were also probed with anti-HA monoclonal antibodies. *C*, Dok inhibits Ras GTP loading in Src-transformed cells. Active Ras (GTP-bound) was affinity-precipitated with Raf-1 RBD agarose conjugate from NIH3T3 cell lysates that expressed Src527 alone or with Dok and Western blotted using anti-Ras monoclonal antibodies. *Ctrl*, control.

vate the Ras pathway, and Dok is a rasGAP-binding protein. We therefore reasoned that Dok might inhibit Src transformation by recruiting rasGAP, thereby regulating Ras activity in the cell. V12Ras (activated Ras) is locked in the GTP-bound state and therefore should be unaffected by rasGAP activity (25). We first tested how Dok might affect V12Ras-mediated transformation in NIH3T3 cells. Consistent with its rasGAP binding ability, Dok expression did not affect focus formation induced by activated V12-K-Ras (Fig. 6A), suggesting that Dok likely acts upstream of Ras to block Ras activation.

We postulated that Dok might function by increasing the local concentration of rasGAP. To test this hypothesis, anti-HA immunoprecipitates from Src-transformed cells that also expressed full-length HA-tagged Dok, Dok363, Dok336, Dok277, or Dok-AA were probed with anti-rasGAP antibodies (Fig. 6B).

Full-length Dok and Dok363 were able to associate with rasGAP; however, Dok336, Dok277, and the PTB domain mutant Dok-AA were impaired in their abilities to bind rasGAP. The inability to bind rasGAP may explain the failure of Dok336, Dok277, and Dok-AA to inhibit Src transformation (Fig. 1B). These results further imply that the PTB domain together with the carboxyl-terminal region of Dok may function in clustering and recruiting rasGAP to the site of action. We further speculated that the recruitment of rasGAP by Dok may lead to inhibition of Ras GTP loading. Consistent with this idea, the amount of Ras GTP (active Ras) was found to be significantly reduced in Src-transformed cells coexpressing Dok compared with Src-transformed cells (Fig. 6C).

#### DISCUSSION

We have shown that expression of Dok can block c-Src-induced transformation in NIH3T3 fibroblasts, indicating that Dok may negatively regulate signal pathways that are activated by PTKs. It is possible that Dok functions to recruit negative regulators of PTK cascades. For example, Csk family kinases are known to down-regulate c-Src activity by phosphorylating Tyr<sup>527</sup> on c-Src (26, 27). Furthermore, Dok family proteins have been reported to associate directly with Csk (18, 28, 29). These data suggest that Dok may attenuate Src signaling by regulating Csk. However, this model was ruled out because Dok also inhibits transformation by activated Src (527F).

Alternatively, Dok may exert its inhibitory effects via the GTPase-activating protein rasGAP. Several lines of evidence support this hypothesis. First, Dok is a rasGAP-binding protein. Association of Dok with rasGAP can be readily detected during activation of PTKs such as Src, Abl, and the Eph receptor kinase (1–5). Among the seven potential tyrosine phosphorylation sites of Dok, five are predicted docking sites for the Src homology 2 domains of rasGAP (4, 5). The presence of multiple rasGAP binding sites on Dok suggests that Dok may provide the molecular platform necessary for high local rasGAP activity. In addition, the Ras pathway is activated by Src and required for Src transformation of fibroblast cells (30, 31). Dok may block Src transforming activity by interfering with Ras GTP loading and mitogen-activated protein kinase activation. Consistent with this model, we showed that Dok reduced Ras GTP loading and did not affect cellular transformation triggered by V12Ras, which is resistant to rasGAP activity. Furthermore, in correlation with their inability to inhibit Src-mediated transformation, Dok336, Dok277, and Dok-AA mutants failed to associate with rasGAP in Src527F-transformed cells. These observations suggest that one major function of Dok is to cluster rasGAP and thereby negatively regulate the Ras signal pathways. Increased PTK activity can result in hyperphosphorylation of Dok. In turn, more negative regulators such as rasGAP are recruited to the site of PTK activation to prevent Ras activation. Consistent with this model, it was demonstrated that Dok inhibits Ras activity in 293 cells (32). However, this model may not be universal, because Dok could inhibit rather than enhance rasGAP activity in some cells (33). One potential target of Dok is the mitogen-activated protein kinase pathway. We have shown recently that mitogen-activated protein kinase activity is up-regulated in B lymphocytes from Dok<sup>-/-</sup> mice (34). In addition, Dok is required to mediate the inhibitory effect of Fc $\gamma$ RIIB on Erk activation (35). Furthermore, recent evidence has indicated that the Dok homologues Dok-R and Dok-L inhibit mitogen-activated protein kinase activation induced by the epidermal growth factor receptor and v-Abl (17, 36).

Our results have indicated that there are at least two independent functional domains in Dok, the carboxyl-terminal tail



and the amino-terminal PH and PTB domains. We have shown that the carboxyl-terminal tail of Dok is necessary for Dok *in vivo* activity. Dok relies on its carboxyl-terminal tail to recruit Src homology 2-containing molecules such as rasGAP and Nck (5, 12). Deletion of residues 278–481, which encompass the cluster of potential tyrosine phosphorylation sites, was found to impair the inhibitory ability of Dok. Deletional analysis has located a minimum region (residues 336–363) on Dok that is essential for its function. Interestingly, the sequence DPIY<sup>361</sup>DEPE within this region is conserved among the Dok family proteins. Furthermore, Tyr<sup>361</sup> is also the major docking site for Nck and rasGAP (4, 13). A recent study has demonstrated that Tyr<sup>361</sup> plays a central role in Dok-mediated cell migration on insulin stimulation (13). It is therefore possible that Tyr<sup>361</sup> is important for the inhibitory activity of Dok.

How does the amino-terminal domain of Dok modulate Dok activities? The PTB and PH domains of Dok-R were shown to be necessary for Dok-R phosphorylation by the epidermal growth factor receptor (36). Therefore, the amino-terminal PH and PTB domains may be important for efficient phosphorylation by protein-tyrosine kinases. In addition, phosphorylated Dok-AA proteins had significantly decreased binding with endogenous rasGAP, suggesting that an intact PTB domain may be required to phosphorylate the rasGAP binding sites on Dok or to recruit Dok to where rasGAP is localized.

Importantly, the amino-terminal region of Dok may be needed for oligomerization of Dok and may recruit other signaling proteins. Our data on the Dok PTB domain and Tyr<sup>146</sup> support this model. We have shown that the Dok PTB domain is capable of binding to phosphotyrosine-containing sequences. Such binding is required for Dok function, because the Dok PTB domain mutant that failed to bind phosphopeptides also lost its ability to inhibit Src-mediated transformation. Furthermore, we have demonstrated that the PTB domain also mediates Dok oligomerization by binding to the phosphorylated Tyr<sup>146</sup> site (located between the PH and PTB domains). The Dok homotypic interaction may cluster Dok molecules at sites of PTK activation. Consistent with the importance of oligomerization, the Y146F mutation significantly decreased Dok inhibitory activity. Alternatively, the Dok PTB domain may bind negative regulators such as phosphatase SHIP1 (23). Therefore, the Dok amino-terminal domain may not only facilitate tyrosine phosphorylation of Dok but also cluster Dok and its associated proteins at the location for negative signaling.

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