Corrections

COLLOQUIUM PAPER. For the article “Synthetic zeolites and other microporous oxide molecular sieves” by John D. Sherman, which appeared in number 7, March 30, 1999, of Proc. Natl. Acad. Sci. USA (96, 3471–3478), the author notes the following corrections: (i) in Linear Paraffins for Biodegradable Detergents, OP ADS-34 should read UOP ADS-34; and (ii) in Impacts of Molecular Sieves on Human Welfare, the phrase, “From these numbers,” should be deleted.

Correction published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.110133597. Text and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.110133597

CELL BIOLOGY. For the article “Tyrosine phosphorylation of p62dok by p210bcr-abl inhibits RasGAP activity” by Nobuhiro Kashige, Nick Carpino, and Ryuji Kobayashi, which appeared in number 5, February 29, 2000, of Proc. Natl. Acad. Sci. USA (97, 2093–2098), the authors note that the image in lane 12 of Fig. 6a was mistakenly deleted in the printing process. The complete figure and its legend are shown below.

Correction published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.110137997. Text and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.110137997

GENETICS. In the article “Toward Anopheles transformation: Minos element activity in anopheline cells and embryos” by Flaminia Catteruccia, Tony Nolan, Claudia Blass, Hans-Michael Müller, Andrea Crisanti, Fotis C. Kafatos, and Thanasis G. Loukeris, which appeared in number 5, February 29, 2000, of Proc. Natl. Acad. Sci. USA (97, 2157–2162), the authors note that three mistakes were introduced inadvertently in assembling Fig. 4. The revised Fig. 4 printed below includes the correct photograph of E7 insertion at 21E, as well as the correct chromosomal locations of E4 at 25D (2L) and E5 at 36B (3R).

**Fig. 6.** Peptide competition analysis indicates that Tyr-296 and Tyr-315 play a critical role in the binding of p62dok to RasGAP. The ability of the diphosphopeptide to inhibit binding of Dok-1 to GAP suggests that the proper positioning of pTyr-296 and pTyr-315 in tandem is critical for the interaction of the two molecules. (a) Combinations of phosphopeptides corresponding to the regions surrounding individual tyrosines in p62dok fail to inhibit the binding of p62dok to RasGAP. However, a diphosphopeptide corresponding to residues 293–322 is able to inhibit binding of Dok-1 to the GAP SH2-SH3-SH2 region. Binding analysis was conducted as described. Synthetic phosphopeptides used for this experiment were: 1, SPPALpYAEPLDSLpYDSPLDST; 2, SQD-SlpYSDPDSL; 3, PKEDPlpYDEPEGL (pTyr-362); 4, VPPQPlpYDL-PREP (pTyr-409); and diphosphopeptide, PALpYAEPLDSLpYDSPLDST (pTyr-296 and pTyr-315). For control, unphosphorylated peptides were used. Each phosphopeptide was added in concentration of 50 μM. (b) Dose-dependent inhibition of p62dok binding to RasGAP by diphosphopeptide (Dok-1 aa 293–322). The diphosphopeptide was added in concentration of 0.5, 5, or 50 μM. The unphosphorylated peptide was added in concentration of 50 μM. (c) Dose-dependent inhibition of a truncated Dok-1 (the truncation construct 316: residues 316–481) binding to RasGAP by diphosphopeptide. The diphosphopeptide was added in concentration of 0.5, 5, or 50 μM.

**Fig. 4.** (A) Sequences of the Minos insertion sites in the genome of Sua 5.1* and Sua 4.0 cells. Chromosomal flanking sequences are represented with capital letters in italics. Small lettering represents the sequences of the Minos end. The expected TA dinucleotide of the insertion site is shown in bold. The chromosomal divisions and subdivisions from which the flanking sequences were derived are indicated with the chromosomal arm listed in parenthesis. (B) Typical results of determining the location of origin of the rescued genomic fragments by in situ localization to polytene chromosomes of the Suakoko mosquito strain.
PLANT BIOLOGY. For the article “Oryza sativa PSK gene encodes a precursor of phytosulfokine-α, a sulfated peptide growth factor found in plants” by Heping Yang, Yoshikatsu Matsubayashi, Kenzo Nakamura, and Youji Sakagami, which appeared in number 23, November 9, 1999, of *Proc. Natl. Acad. Sci. USA* (96, 13560–13565), the authors note the following correction. In line 18 of the first column on page 13565, “+5 positions” should read “+3 positions.”

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Tyrosine phosphorylation of p62\textit{dok} by p210\textit{bcr-abl} inhibits RasGAP activity

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The t(9;22) chromosomal translocation is found in almost all patients with chronic myelogenous leukemia. The resultant \textit{Bcr-Abl} fusion gene expresses a chimeric fusion protein p210\textit{bcr-abl} with increased tyrosine kinase activity. Hematopoietic progenitors isolated from chronic myelogenous leukemia patients in the chronic phase contain constitutively tyrosine-phosphorylated p62\textit{dok} protein. p62\textit{dok} associates with the Ras GTPase-activating protein (RasGAP), but only when p62\textit{dok} is tyrosine phosphorylated. Here we have investigated the interaction between p62\textit{dok} and RasGAP and the consequences of p62\textit{dok} tyrosine phosphorylation on the activity of RasGAP. We have found that p62\textit{dok} is directly tyrosine phosphorylated by p210\textit{bcr-abl}, and the sites of phosphorylation are located in the C-terminal half of the p62\textit{dok} molecule. We have identified five tyrosine residues that are involved in in vitro RasGAP binding and have found that tyrosine-phosphorylated p62\textit{dok} inhibits RasGAP activity. Our results suggest that p210\textit{bcr-abl} might lead to the activation of the Ras signaling pathway by inhibiting a key down-regulator of Ras signaling.

\begin{abstract}

**Keywords:** chronic myelogenous leukemia | Ras

Chronic myelogenous leukemia is a form of leukemia with distinct clinical and pathological features (1). A well-characterized chromosomal translocation within a single primitive myeloid stem cells produces the chimeric \textit{bcr-abl} gene, which in turn leads to the generation of a 210 kDa Bcr-Abl (p210\textit{bcr-abl}) fusion protein. Relative to normal c-Abl, p210\textit{bcr-abl} has increased tyrosine kinase activity and can transform a variety of fibroblastic and hematopoietic cell lines in culture (2, 3). Although c-Abl, as a nonreceptor tyrosine kinase, functions in both the nucleus and the cytoplasm, p210\textit{bcr-abl} is exclusively cytoplasmic. Both the elevated kinase activity and the subcellular localization of the oncoprotein are considered essential elements of its transforming abilities (4). Although the Ras pathway has been reported to be activated in cells containing p210\textit{bcr-abl} (5, 6), the mechanism by which p210\textit{bcr-abl} perturbs normal hematopoiesis has not yet been established.

One of the major targets of p210\textit{bcr-abl} in hematopoietic progenitors is p62\textit{dok}, the RasGAP-associated p62 protein (7). p62\textit{dok} (Dok-1) was first described as the major tyrosine-phosphorylated protein present within cells transformed by different activated tyrosine kinases (8). In addition, its state of hypotyrosine phosphorylation has consistently correlated with the transformed phenotype in cells expressing transforming oncogenes such as v-fps, v-src, v-abl, Ras, and mos (8, 9). Recently, the p62\textit{dok} protein was isolated both from a p210\textit{bcr-abl} transformed myeloid cell line and a v-Abl-transformed B cell line (10, 11). Cloning of the Dok-1 cDNA revealed the presence of a pleckstrin homology (PH) domain in the N-terminal portion of the protein molecule. PH domains are thought to mediate protein interactions with cellular membranes, possibly by binding to polyphosphoinositides (12–14). Dok-1 also contains numerous tyrosines in its C-terminal region that are potential targets of cytoplasmic tyrosine kinases. If phosphorylated, these tyrosines could serve as docking sites for proteins that contain SH2 domains. These structural features are reminiscent of the features found in such adaptor molecules as IRS-1 and IRS-2 (15), Gab1 (16–18), and Dos (19). Thus, the overall structure of Dok-1 suggests that it might serve as a docking molecule for different signaling molecules downstream of growth factor receptors.

Although at present the biological function of Dok-1 is unclear, several lines of evidence suggest that it does indeed play a role in distinct signal transduction networks, most likely as a component of a signaling cascade initiated by receptor or membrane-associated tyrosine kinases. The rapid tyrosine phosphorylation of a RasGAP-associated p62 in Rat-1 fibroblasts on epidermal growth factor (EGF) stimulation and in Rat-2 fibroblasts on platelet-derived growth factor stimulation was reported (8). Using a different system, Filvaroff et al. demonstrated the tyrosine phosphorylation of a RasGAP-associated p62 in primary mouse keratinocytes as one of the initial events in calcium-induced terminal differentiation (20). Other agents, such as EGF, type β transforming growth factor, and phorbol ester, did not induce tyrosine phosphorylation of the p62 within keratinocytes, although they did result in the tyrosine phosphorylation of the signaling molecules PLCY1 and P13 kinase. In yet a different biological context, application of the mitogen vascular endothelial cell growth factor (VEGF) to bovine endothelial cells resulted in tyrosine phosphorylation and RasGAP association of a p62 protein, most likely Dok-1 (21). The VEGF-stimulated tyrosine phosphorylation of p62 and the VEGF-promoted proliferation of endothelial cells were both inhibited by the tyrosine kinase inhibitor genistein. Thus, Dok-1 may function in signaling pathways downstream of a variety of receptors, in both transformed and nontransformed cells. However, the functional consequences of constitutive tyrosine phosphorylation of Dok-1 are still unclear.

Early studies revealed that when tyrosine phosphorylated, Dok-1 forms an in vivo protein–protein complex with the Ras regulatory protein p120 RasGAP (GAP). GAP interacts with p21ras and stimulates the latter’s intrinsic GTPase activity (22). Its catalytic activity is located in the C-terminal portion of the molecule. In cells transformed by tyrosine kinases, GAP can be found to be associated with both Dok-1 and a 190-kDa protein known as p190 RhoGAP. Both proteins interact with the N-terminal SH2-SH3-SH2 region of GAP (23). Unlike the C-terminal region of GAP, the N-terminal region of GAP has no known biochemical activity. However, the N-terminal noncatalytic domain of GAP is essential for its interaction with Dok-1, as revealed by the use of truncated GAP molecules (24). In addition, the N-terminal domain of GAP inhibits Dok-1 tyrosine phosphorylation in vitro (25). These results suggest that GAP is a negative regulator of Dok-1 activity.

The interaction between Dok-1 and RasGAP is of particular interest because it has been shown to be inhibited by the RasGAP-associated p62 (26). In this study, we have characterized the interaction between p62 and RasGAP in human fibroblasts and demonstrated that the interaction is mediated by tyrosine phosphorylation of p62. We have also shown that the interaction between p62 and RasGAP is inhibited by the RasGAP-associated p62 in Rat-1 fibroblasts on epidermal growth factor stimulation. Our results suggest that p210\textit{bcr-abl} might lead to the activation of the Ras signaling pathway by inhibiting a key down-regulator of Ras signaling.

### Abbreviations

- PH: pleckstrin homology
- Dok-1: p62\textit{dok}
- p210\textit{bcr-abl}: p210\textit{bcr-abl}
- RasGAP: Ras GTPase-activating protein
- GAP: p120 RasGAP
- IRS-1 and IRS-2: insulin receptor substrates
- Gab1: growth arrest and activation-binding protein 1
- Dos: docking protein
- PLCY1: phosphatidylinositol 4,5-bisphosphate 3′-kinase
- P13 kinase: protein kinase C

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lytic region of GAP (but not full-length GAP) has been shown to be active in several different biological assays, including cytoskeletal reorganization, adhesion to extracellular matrix (24), transcriptional activation (25), and inhibition of transformation induced by G protein-coupled receptors (26). The biological significance of the RasGAP-p62dok complex is unknown. In this report, we analyze the interaction between GAP and Dok-1, and we demonstrate that the activity of GAP toward Ras is inhibited by the binding of tyrosine-phosphorylated Dok-1 to GAP.

Materials and Methods

**Antibodies.** A monoclonal antibody against the C-terminal tag (RyYR8) was kindly provided by S. H. Lin (27) (Univ. of Texas, M. D. Anderson Cancer Center, Houston). Anti-p62dok rabbit polyclonal antibody was described previously (10), as was the use of anti-pTyr monoclonal 4G10 (Upstate Biotechnology). Monoclonal anti-Bcr antibody and antihemagglutinin antibody (12CA5) were from OSI (Uniondale, NY), and anti-GAP (B4F8) was from Santa Cruz Biotechnology.

**Cell Culture.** Human 293 cells and monkey COS cells, obtained from Cold Spring Harbor Laboratory Tissue Culture Facility, were maintained in DMEM containing antibiotics 22 units/ml penicillin (GIBCO/BRL), 100 μg/ml streptomycin (Sigma), and 10% calf serum (GIBCO/BRL) at 37°C and 5% CO2. All experiments utilized 293 cells between passages 1 and 20. Mammalian expression constructs were electroporated into cells at 200 V, 960 μF capacitance.

**Immunoprecipitation, Immunoblotting, and RasGAP-Binding Assays.** Immunoprecipitation and blotting were as described (10). GST-GAP proteins (constructs kindly provided by J. Schlessinger, New York University, New York) were incubated with lysate for 30 min at 4°C, washed extensively with lysis buffer, and bound proteins separated by SDS/PAGE.

**In Vitro Kinase Reactions.** Purified Dok-1 (250 ng), generated by overexpression in Escherichia coli, was incubated with 100 units Ab1 protein tyrosine kinase (NEB, Beverly, MA) in a buffer recommended by the manufacturer. The reaction was allowed to proceed at 30°C for the indicated time, after which it was stopped by the addition of SDS/PAGE sample buffer. For the reaction depicted in Fig. 1b, anti-Bcr antibodies were used to precipitate proteins from lysates of Mo7 or Mo7/p210 cells (10). Protein A-Sepharose beads (10 μl), to which the precipitated complexes were bound, were incubated with in vitro-translated p62dok according to instructions of the manufacturer. After a 30-min incubation at 30°C, the kinase reaction was stopped by addition of 500 μl precipitation buffer (10), and Dok-1 was precipitated by addition of anti-p62dok antibodies or antiphosphotyrosine antibody (4G10, Upstate Biotechnology).

**Truncations.** The PCR was used to create the tagged Dok-1 truncation products illustrated in Fig. 3a. Each construct was sequenced and subcloned into the pMT2 mammalian expression vector.

**Site-Directed Mutagenesis.** All Dok-1 Y→F point mutants were generated by site-directed mutagenesis of double-stranded DNA by using the QuickChange site-directed mutagenesis kit (Stratagene) according to manufacturer’s specifications. Mutagenesis was confirmed by sequence analysis.

**RasGAP Activity Assay.** Immunoprecipitated RasGAP activity was measured by analyzing the ratio of GTP and GDP bound to Ras as previously described (28), with some modification. Recombinant human H-Ras (Calbiochem) was loaded with [α-32P]GTP at 25°C for 10 min in 20 mM TrisHCl, pH 7.5, containing 5 mM EDTA. RasGAP was produced by transient transfection in 293 cells and obtained by precipitation with anti-RasGAP (10 μg) agarose conjugate (Santa Cruz Biotechnology). Enzyme activity was assayed by incubating bound RasGAP with 60 ng [α-32P]GTP-loaded Ras for 60 min or 90 min. In vitro-phosphorylated Dok-1 (0.05, 0.1, 0.5, and 1 μg) recombinant wild-type or 1 μg of mutant Dok-1 was added to the reaction. Reactions were terminated by adding ice-cold STOP buffer containing anti-H-Ras antibody agarose conjugate (Oncogene Science), as described (28). After rocking this mixture for 45 min at 4°C, the resin was washed four times with 0.5 ml of 0.5% (vol/vol) Nonidet P-40 in stop buffer and then once with stop buffer alone. The nucleotide was eluted off resin with 20 μl of 2 mM EDTA/2 mM DTT/0.2% SDS/0.5 mM GTP by heating to 65°C for 5 min and then separated on polyethyleneimine-cellulose TLC plate (J. T. Baker, Inc., Phillipsburg, NJ) developed with 0.75 M KH2PO4 (pH 3.4). The positions of GDP and GTP on the plates were visualized under UV light by using standard GTP and GDP. Ras-bound GDP and GTP were visualized by autoradiography and quantified by using NIH IMAGE 1.62 to calculate the GTP/GDP ratio.

**Results**

**Interaction Between p210bcr-abl, p62dok, and RasGAP.** The presence of tyrosine phosphorylation on p62dok in cells containing p210bcr-abl was confirmed by sequence analysis. A monoclonal antibody against the C-terminal tag (RyYR8) was kindly provided by S. H. Lin (27) (Univ. of Texas, M. D. Anderson Cancer Center, Houston). Anti-p62dok rabbit polyclonal antibody was described previously (10), as was the use of anti-pTyr monoclonal 4G10 (Upstate Biotechnology). Monoclonal anti-Bcr antibody and antihemagglutinin antibody (12CA5) were from OSI (Uniondale, NY), and anti-GAP (B4F8) was from Santa Cruz Biotechnology.
suggested that p62dok is a direct substrate of p210bcr-abl. Nonetheless, it is also possible that p210bcr-abl might activate other cytoplasmic tyrosine kinases that directly phosphorylate p62dok. The ability of Dok-1 to be phosphorylated by p210bcr-abl was tested in three separate assays. First, recombinant Dok-1 generated by overexpression in bacteria was tested as a substrate of the isolated kinase domain of Abl. Fig. 1a illustrates that Dok-1 was phosphorylated in a time-dependent manner by the kinase domain of Abl. This suggested that Dok-1 contains tyrosines whose surrounding amino acid residues comprise a motif that can be recognized by the Abl kinase. Because it is possible that p210bcr-abl might interact with possible substrates differently than the isolated Abl kinase domain, a second assay was used to test the ability of Dok-1 to be phosphorylated by p210bcr-abl. Fig. 1b illustrates that Dok-1, generated by in vitro translation of the Dok-1 cDNA in a rabbit reticulocyte lysate system, could be phosphorylated by p210bcr-abl isolated by immunoprecipitation from lysates of Mo7/p210 cells with anti-Bcr antibody (lane 6). That the incorporated phosphate was bonded to tyrosine was indicated by the ability of antiphosphotyrosine antibodies to precipitate Dok-1 (lane 8). Finally, an in vivo transfection assay was used to test whether p210bcr-abl could phosphorylate Dok-1. Dok-1 cDNA, cloned into a mammalian expression vector downstream of sequences encoding the hemagglutinin Tag, was cotransfected with the cDNA encoding p210bcr-abl into COS cells. Fig. 1c illustrates that Dok-1 was tyrosine phosphorylated in a p210bcr-abl-dependent fashion when overexpressed in COS cells. These results indicate that p62dok can be phosphorylated by p210bcr-abl, and that p62dok might be a substrate of p210bcr-abl within primitive blasts of patients with chronic myelogenous leukemia.

Numerous investigators working with a variety of transformed cell lines have observed the tyrosine phosphorylation of p62dok protein and its association with GAP (see for example refs. 8 and 29). The portion of the GAP molecule responsible for the interaction with p62dok has been mapped to the N-terminal region of GAP, comprising the SH2-SH3-SH2 domain (30). Therefore, the need for p62dok to be tyrosine phosphorylated to associate directly with GAP was tested. When recombinant Dok-1 was tested for the ability to bind in vitro to the N-terminal regions of GAP, only tyrosine-phosphorylated Dok-1 associated in vitro with the N-terminal region of GAP (see Fig. 2a). Similarly, when Dok-1 was overexpressed in 293 cells in the presence or absence of p210bcr-abl it was observed that only tyrosine-phosphorylated Dok-1 bound to GAP (see Fig. 2b). This confirms the long-held suspicion that tyrosine phosphorylation of Dok-1 is necessary for the interaction between Dok-1 and GAP.

p62dok Truncations and RasGAP Binding. To assess the region of p62dok, in particular the tyrosine(s), involved in the interaction with GAP, a series of truncation mutants of Dok-1 was constructed. The structures of the Dok-1 mutants are illustrated in Fig. 3a. Four different C-terminal truncations were constructed. In each case, in the resulting Dok-1 protein a different tyrosine and all amino acids C terminal to the selected tyrosine were substituted with a C-terminal antigenic tag (27). The tyrosines at which the different constructs were terminated were tyrosines 398, 362, 337, and 296. In addition to the C-terminal truncations, two N-terminal truncations were constructed by replacing sequences encoding N-terminal amino acids with a starting ATG. The resulting Dok-1 protein in one case lacked the PH domain and in the other case lacked all amino acids N terminal to Ala-294 (see Fig. 3a). When coexpressed with p210bcr-abl in 293 cells, each mutant Dok-1 protein became tyrosine phosphorylated, with the exception of the 295 Dok-1 mutant (see Fig. 3b).

Mutant Dok-1 proteins were also assessed for their ability to bind in vitro to the SH2-SH3-SH2 region of GAP. As illustrated in Fig. 3c, all mutant Dok-1 proteins with the exception of the 295 Dok-1 mutant were able to bind in vitro to the SH2-SH3-SH2 region of GAP. Thus, removal of all amino acid residues C terminal to Tyr-337 or N terminal to Ala-294 did not prevent the remaining portion of Dok-1 from binding in vitro to the SH2 region(s) of GAP. The mutant Dok-1 proteins were also assessed for their ability to interact with GAP in vivo. Mutant Dok-1 proteins were expressed in 293 cells in the presence or absence of p210bcr-abl. Then, antibodies directed against the C-terminal antigenic tag were used to precipitate the mutant Dok-1 proteins from extracts prepared from the transfected cells. As illustrated in Fig. 3d, GAP coprecipitated with each mutant Dok-1 protein in a p210bcr-abl-dependent fashion, with the exception of the 295 Dok-1 truncation mutant. These in vivo results complement the results obtained in the in vitro binding assays; specifically, the in vivo results point to the region between Dok-1 residues Ala-294 and Tyr-337 as involved in the association of Dok-1 with the SH2 domain(s) of GAP. Within the primary amino acid sequence of this region of Dok-1 are two tyrosine residues spaced 18 amino acids apart, Tyr-296 and Tyr-315. These tyrosines are implicated as involved in the association of p62dok with GAP. However, our data do not exclude the possibility that other tyrosines within the C-terminal region of Dok-1 are tyrosine phosphorylated and involved in GAP binding. In fact, Tyr-337, Tyr-362, and Tyr-398 of p62dok protein purified from p210bcr-abl expressing Mo7 cells were found phosphorylated by mass spectrometry and protein sequencing (data not shown).

Effect of p62dok Point Mutants on RasGAP Binding. The truncation experiment identified Tyr-296 and Tyr-315 as least involved in the association of Dok-1 to GAP. Indeed, it appeared likely that Tyr-296 and Tyr-315, when phosphorylated, could interact with the two SH2 domains of GAP, similar to the manner in which Tyr-1087 and Tyr-1105 of p190RhoGAP interacted with GAP (31). To test the role of Tyr-296 and Tyr-315 in GAP binding, we constructed a double Tyr→Phe point mutant Dok-1, in which both tyrosines were altered to phenylalanine. The tagged double

Fig. 2. Interaction between p62dok and RasGAP. (a) Tagged p62dok was expressed in E. coli, purified, and incubated in a kinase reaction in the presence of Abl kinase (+) or buffer only (−). After the kinase reaction, p62dok was incubated in an in vitro binding reaction with glutathione-Sepharose beads to which were bound GST(−), GST-GAP SH2-SH3-SH2 (232), GST-GAP N-SH2 (N), or GST-GAP C-SH2 (C). Proteins bound to GST fusion proteins were separated by SDS/PAGE, blotted to nitrocellulose, and detected by immunoblotting with antibodies to p62dok (α-p62dok) or phosphotyrosine (α-pTyr). (b) Mammalian-expressed p62dok associates with RasGAP in a phosphorylation-dependent manner. (Fig. 1c). C-terminally tagged Dok-1 cDNA was electroporated into 293 cell in the presence (+) or absence (−) of Bcr-Ab1. After a 24-h incubation, cells were lysed, and lysates were utilized in an in vitro binding reaction with glutathione-Sepharose beads to which were bound GST(→), GST-GAP SH2-SH3-SH2 (232), GST-GAP N-SH2 (N), or GST-GAP C-SH2 (C). Proteins bound to GST fusion proteins were separated by SDS/PAGE, blotted to nitrocellulose, and detected by immunoblotting with antibodies to the C-terminal tag (α-tag) or phosphotyrosine (α-pTyr).
Fig. 3. Removal of N- and C-terminal residues from p62\textsuperscript{295} does not prevent its \textit{in vitro} and \textit{in vivo} association with RasGAP. (a) Schematic diagram of p62\textsuperscript{295} truncations. Four C-terminal Dok-1 truncations were constructed. In each case, a different tyrosine (residues 398, 362, 337, and 296) and all amino acids C terminal to the selected tyrosine were replaced with a C-terminal alanine (Y296, Y315, Y362, Y398, Y409F). The vertical bars mark the location of the tyrosines within p62\textsuperscript{295}. The PH domain is indicated (PH). (b) Tyrosine phosphorylation of p62\textsuperscript{295} truncations. The indicated Dok-1 truncations and p210\textsuperscript{bcr-abl} were expressed in 293 cells. Antibody directed against the C-terminal tag was used to precipitate Dok-1 proteins. The resulting immunoblot was probed with antibodies to Dok-1 (Top) or antibody to phosphotyrosine (Middle), whereas the other portion was utilized in an \textit{in vitro} binding reaction to GST-GAP SH2-SH3-SH2 (Bottom). (c) Removal of N-terminal and C-terminal residues from p62\textsuperscript{295} does not prevent its \textit{in vitro} binding to GST-GAP. The indicated Dok-1 truncations and p210\textsuperscript{bcr-abl} were expressed in 293 cells as described above. Mutant Dok-1 protein was expressed in bacteria and phosphorylated \textit{in vitro} by Ab1 kinase. Then it was tested for its ability to bind the GAP SH2-SH3-SH2 domain, as described. Interestingly, this double-point mutant retained the ability to become tyrosine phosphorylated and bind to GAP (Fig. 4, lane 2). The GAP-SH2 domains bind preferentially to phosphorylated tyrosine residues within the context of pYxXp (11, 32). There are six tyrosine residues in p62\textsuperscript{295} that have proline in the +3 position: Tyr-296, Tyr-315, Tyr-362, Tyr-377, Tyr-398, and Tyr-409. Therefore, additional mutant proteins in which combinations of tyrosines were changed to phenylalanine were tested for their ability to bind GAP. The Y296F, Y315F, Y362F triple point mutant had reduced ability to bind GAP as compared with the Y296, 315F double point mutant (see Fig. 4, lane 3). Furthermore, when we mutated Tyr-377 to phenylalanine in the context of the Y296, 315, 362F triple mutant, the association of phosphorylated Dok-1 with GAP was significantly reduced (Fig. 4, lane 4). However, even when either Tyr-398 or Tyr-409 was additionally mutated to phenylalanine in the context of the Y296, 315, 362, 377F quadruple mutant, we still observed \textit{in vitro} association of the mutant protein with GAP (Fig. 4, lanes 5 and 6). Finally, when we mutated five specific tyrosines to phenylalanine (Y296, 315, 362, 398, 409F), we observed that Dok-1 was no longer able to bind \textit{in vitro} to the SH2-SH3-SH2 domain of GAP (Fig. 4, lane 7). This suggests that these five tyrosines contribute to the \textit{in vitro} association of Dok-1 with GAP.

The Effect of Tyrosine-Phosphorylated p62\textsuperscript{295} on RasGAP Activity. To test whether binding of Dok-1 to GAP had any effect on the activity of GAP, we used an \textit{in vitro} GAP activity assay (28). This assay measures the ability of GAP to increase the intrinsic GTPase rate of GTP-Ras. We analyzed the \textit{in vitro} activity of GAP in the presence of tyrosine-phosphorylated wild-type Dok-1 or in the presence of a five Tyr→Phe point mutant unable to associate with GAP (see Fig. 4, lane 7). The assays were carried out with Dok-1 proteins phosphorylated both \textit{in vivo} and \textit{in vitro}. In \textit{vivo} tyrosine-phosphorylated wild-type or mutant Dok-1 (Fig. 4, lane 7) was prepared by immunoprecipitation of

Lysates were prepared, and antibodies to the C-terminal tag were used to precipitate Dok-1 proteins. The resulting immunoblot was probed with antibody directed against GAP (Top) or against phosphotyrosine (Bottom).
Dok-1 from lysates of 293 cells coexpressing p62\textsubscript{dok} and p21\textsubscript{bcr-abl}. We observed a significant (approximately 35%) diminution of GAP activity when the assay was conducted in the presence of wild-type tyrosine-phosphorylated Dok-1 (see Fig. 5a, lane 5). In contrast, we observed no diminution of GAP activity when the assay was conducted in the presence of nontyrosine-phosphorylated Dok-1 (Fig. 5a, lane 6) or in the presence of a five Tyr→Phe point mutant Dok-1 unable to bind GAP (see Fig. 5a, lanes 7 and 8). In vitro tyrosine-phosphorylated wild-type or mutant Dok-1 was prepared by phosphorylation of bacterially expressed Dok-1 by c-Abl kinase. Again, we observed a significant diminution of GAP activity when the assay was conducted in the presence of phosphorylated wild-type Dok-1 as compared with GAP activity in the presence of nontyrosine-phosphorylated wild-type Dok-1 or a five Tyr→Phe point mutant Dok-1 unable to bind GAP (compare with Fig. 5b Top and Bottom). These results indicate that the association of tyrosine-phosphorylated Dok-1 with GAP can inhibit the GTPase-activating activity of GAP.

Peptide Competition Analysis. We synthesized tyrosine-phosphorylated peptides (15 mer) corresponding to the region surrounding each of tyrosines in YxxP motif (see Fig. 6 Legend). Then we attempted to inhibit the interaction between Dok-1 and the SH2-SH3-SH2 domain of GAP by including the peptides in the \textit{in vitro} binding reaction described in Fig. 2. Interestingly, any mixture of the singly tyrosine-phosphorylated peptides containing pYxxP motif, including the five GAP-binding tyrosines (Fig. 4, lane 7), failed to inhibit \textit{in vitro} association of Dok-1 with the SH2-SH3-SH2 domain of GAP (Fig. 6a, lanes 2–11, and data not shown).

Recently, Hu and Settleman demonstrated that the critical determinants of p190 RhoGAP mediating the association of p190 with GAP are two tyrosines spaced 17 residues apart, Tyr-1087 and Tyr-1105 (31). These tyrosines lie within the motif YxxPxD, and each appears to bind simultaneously to the two SH2 domains of GAP. There are two tyrosines of p62\textsubscript{dok} that lie within the motif YxxPxD, Tyr-296, and Tyr-315. Similar to the tyrosines within p190 RhoGAP, they are spaced 17 residues apart. We synthesized a diphosphopeptide of 30 amino acids corresponding to the residues surrounding Tyr-296 and Tyr-315 in tandem (Fig. 6b). These results indicate that the positioning of pTyr-296 and pTyr-315 in tandem is critical for the interaction of the two molecules. The diphosphopeptide completely inhibited the binding of p62\textsubscript{dok} to RasGAP. However, a diphosphopeptide corresponding to residues 293–322 is able to inhibit binding of Dok-1 to the GAP SH2-SH3-SH2 region. Binding analysis was conducted as described. Synthetic phosphopeptides used for this experiment were: 1, SPPALpYAEPLDS (pTyr-296); 2, SQD- SLpYSDPLDS (pTyr-315); 3, PKEDpYDEPEGL (pTyr-362); 4, VPPQG LpYDLPREPK (pTyr-377); 5, RVKEEYGpYPYNPATDD (pTyr-398); 6, NpATDD pYAVPPR (pTyr-409); and diphosphopeptide, PAPLaVAELDLSRIAPCPSQDS LpYSHPdLDST (pTyr-296 and pTyr-315). For control, unphosphorylated peptides were used. Each phosphopeptide was added in concentration of 50 \( \mu \)M.

\( \text{a} \) Dose-dependent inhibition of p62\textsubscript{dok} binding to RasGAP by diphosphopeptide (Dok-1 aa 293–322). The diphosphopeptide was added in concentration of 0.5, 5, or 50 \( \mu \)M. The unphosphorylated peptide was added in concentration of 50 \( \mu \)M. The diphosphopeptide was added in concentration of 50 \( \mu \)M.

\( \text{b} \) Dose-dependent inhibition of a truncated Dok-1 (the truncation construct 316: residues 316–481) binding to RasGAP by diphosphopeptide. The diphosphopeptide was added in concentration of 0.5, 5, or 50 \( \mu \)M. The unphosphorylated peptide was added in concentration of 50 \( \mu \)M.
Dok-1 can contribute to GAP binding, Tyr-296 and Tyr-315 double mutant may play the predominant role. Because a mixture of five singly phosphorylated peptides (including Tyr-296 and Tyr-315) was not able to completely inhibit the GAP binding (see Fig. 6a, lane 10), our results also suggest that a configuration that positions the two YxxPxD motifs (Tyr-296 and Tyr-315) is critical for the interaction of p62 dok with RasGAP.

Discussion

The GAP-Associated 62-kDa protein, p62 dok, was first described in vitro in vivo in GAP, it is possible that when two phosphotyrosines of p62 dok phosphorylated interact with other SH2 domain-containing proteins. We anticipate that the intracellular interaction of p210 bcr-abl, p62 dok, and GAP is complex. Further investigation is necessary to study tyrosine phosphorylation of p62 dok and its interacting proteins.

Finally and most importantly, we have demonstrated that Dok-1 binding to RasGAP negatively affects GAP’s activity in vitro toward the GTPase activity of Ras. Previously, Skorski et al. reported (33) that p210 bcr-abl negatively regulates the GTPase-promoting activity of GAP in chronic myelogenous leukemia cells. Our results are in agreement with their report. Pendergast et al. reported that Bcr-Abl forms a complex with Grb2 in vivo, and this interaction is required for Bcr-Abl-induced Ras activation (5). It is possible that there are several mechanisms for Bcr-Abl to activate Ras, including the following two scenarios: (i) by modulating the association and activity of the Grb2/Sos exchange factor complex; and (ii) by phosphorylating p62 dok, which in turn down-regulates Ras-GAP activity.

It is currently unknown whether phosphorylation of p62 dok affects the MAP kinase signaling pathway, or whether p62 dok plays a role in a signaling pathway parallel to a MAP kinase pathway. Both GAP and Dok-1 are predominantly cytosolic, although a portion of the GAP/Dok-1 complex translocates to the inner surface of the plasma membrane on growth factor stimulation (34).

Although at present the biological function of p62 dok is not known, our data suggest that one effect of p62 dok constitutive tyrosine phosphorylation is to disrupt the activity of a key down-regulator of the Ras signaling pathway. Thus, constitutive p62 dok tyrosine phosphorylation might directly contribute to the phenotypic and behavioral differences observed in chronic myelogenous leukemia hematopoietic progenitors (35).

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