Recruitment of Dok-R to the EGF receptor through its PTB domain is required for attenuation of Erk MAP kinase activation

Nina Jones and Daniel J. Dumont

Dok (for downstream of tyrosine kinases) proteins are a newly identified family of docking molecules that are characterized by the presence of an amino-terminal pleckstrin homology (PH) domain, a central putative phosphotyrosine-binding (PTB) domain and numerous potential sites of tyrosine phosphorylation [1–6]. Here, we explore the potential role of the Dok family member Dok-R (also known as p56^{Dok2} or FRIP) in signaling pathways mediated by the epidermal growth factor (EGF) receptor. An intact PTB domain in Dok-R was critical for its association with two PTB-binding consensus sites on the EGF receptor and the PH domain further contributed to stable *in vivo* binding and tyrosine phosphorylation of Dok-R. Multiple sites on Dok-R were tyrosine-phosphorylated following EGF stimulation; phosphorylated Tyr276 and Tyr304 are proposed to dock the tandem Src homology 2 (SH2) domains of the p21^{Ras} GTPase-activating protein rasGAP and Tyr351 mediates an association with the SH2 domain of the adapter protein Nck. Interestingly, we have found that Dok-R could attenuate EGF-stimulated mitogen-activated protein (MAP) kinase activation independently of its association with rasGAP. Together, these results suggest that Dok-R has an important role downstream of growth factor receptors as a potential negative regulator of signal transduction.

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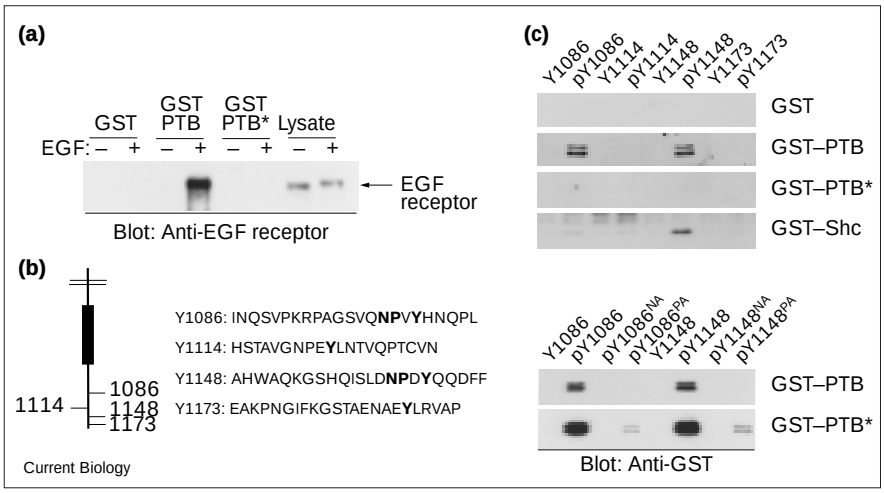
Results and discussion

Tyrosine phosphorylation of Dok in response to EGF stimulation has previously been reported [7,8]. Given the similar expression profiles of Dok and Dok-R, we reasoned that Dok-R might also function in EGF-mediated signaling pathways. To test this, we used the COS1 cell line, which expresses high levels of EGF receptor [9] and does not contain endogenous Dok-R (data not shown); there is thus no interference with the analysis of signaling mediated by exogenously expressed forms. In COS1 cells transiently expressing full-length Dok-R, we found that EGF stimulation resulted in complex formation between the EGF receptor and Dok-R and in tyrosine phosphorylation of both proteins (see Supplementary material). A region of Dok-R similar to the PTB domain of insulin receptor substrate-1 (IRS-1) has previously been shown to mediate binding of Dok-R to the phosphorylated Tek/Tie2 receptor [3]. As the sequence similarity between PTB domains is limited and as the Tek/Tie2 receptor does not contain the consensus binding site for PTB domains, we wanted to ascertain whether this region of Dok-R represented a true PTB domain. Purified fusion proteins of glutathione-S-transferase (GST) with the putative Dok-R PTB domain (GST–PTB) or a mutant domain lacking two conserved arginine residues (GST–PTB*) [10,11] were used to precipitate the EGF receptor from lysates of non-transfected unstimulated or EGF-stimulated COS1 cells. Figure 1a shows that the PTB domain of Dok-R could precipitate the EGF receptor following EGF stimulation and that the PTB* domain could not.

To determine which tyrosines of the EGF receptor could mediate the interaction with Dok-R, we designed a series of synthetic peptides corresponding to the sequences surrounding the autophosphorylation sites of the EGF receptor (Figure 1b) [12] and mixed them *in vitro* with purified GST–PTB fusion proteins. The GST–PTB domain of Dok-R could be precipitated by phosphopeptides representing Tyr1086 (pY1086) and Tyr1148 (pY1148) but not by the unphosphorylated counterparts (Y1086 and Y1148; Figure 1c). In contrast, neither GST–PTB* nor GST alone could be precipitated by either of these phosphopeptides and the PTB domain of Shc (GST–Shc) associated almost exclusively with pY1148 (Figure 1c). Tyr1086 and Tyr1148 are found within the predicted autophosphorylated Asn-Pro-X-Tyr (NPXY in the single-letter amino acid code, where X is any amino acid), so to investigate the contribution of the asparagine and proline residues in target binding, we designed synthetic phosphopeptides in which either of these residues was mutated to alanine. Mutation of either of these residues resulted in abrogation of the interactions, although weak binding to phosphopeptides lacking the proline residues (pY1086^{PA} and pY1148^{PA}) could still be seen in longer exposures of the immunoblot (Figure 1c). Estimates of the dissociation constants (K_d) between pY1086 and pY1148 phosphopeptides and the soluble PTB domain of

Figure 1



Dok-R contains a functional PTB domain that mediates the association with two NPXY motifs of the EGF receptor. (a) Lysates from unstimulated (–) or EGF-stimulated (+) COS1 cells were incubated with immobilized GST, Dok-R GST–PTB or Dok-R GST–PTB*. Bound proteins were detected with anti-EGF receptor antibodies (Blot). EGF receptor could be detected in both unstimulated and EGF-stimulated non-precipitated lysates (lysate). (b) Schematic representation of the intracellular domain of the EGF receptor showing three tyrosine autophosphorylation sites (Tyr1086, Tyr1148 and Tyr1173) and one non-phosphorylated site (Tyr1114). The remaining autophosphorylation sites (Tyr992 and Tyr1068) were not studied here. The synthetic biotinylated peptides corresponding to these sites that were used in the mapping studies are shown with the asparagine, proline and tyrosine residues that were altered in similar peptides in bold. (c) Associations between the various EGF receptor peptides and purified GST-fusion proteins were allowed to form *in vitro* and resultant complexes were recovered using streptavidin–agarose beads. Bound fusion proteins were visualized with anti-GST antibodies. The lower two panels show different exposures of the same immunoblot.

Dok-R revealed that the PTB domain could bind to both phosphopeptides with similar relative affinities (Table 1).

Our results to this point have demonstrated that the PTB domain of Dok-R is required for binding of Dok-R to the phosphorylated EGF receptor *in vitro*. As Dok-R also contains a PH domain, however, we wanted to examine the contribution of each domain to the association *in vivo*. We therefore generated a series of mutations in full-length Dok-R that would disrupt the functions of the individual domains, introduced the mutants into COS1 cells and examined their ability to associate with the EGF receptor and undergo tyrosine phosphorylation in response to EGF. Disruption of the PTB domain (PTB*) appeared to have a more dramatic effect on EGF receptor binding and Dok-R phosphorylation than deletion of the PH domain (ΔPH), although the disruption of both domains completely abrogated Dok-R phosphorylation (PTB*/ΔPH) (Figure 2). Phosphorylation of Dok-R has previously been shown to establish docking sites for downstream SH2-domain-containing signaling proteins including rasGAP and Nck [3–5]. We found that rasGAP and Nck could associate with Dok-R following EGF stimulation and that phosphorylation at both Tyr276 and Tyr304 was required for rasGAP binding whereas Tyr351 was required for Nck binding (see Supplementary material). These results are in agreement with those recently reported by Lock *et al.* [6], who showed that independent tyrosine residues mediate the interactions of rasGAP and Nck with Dok-R.

Recruitment of Dok-R to the EGF receptor and its association with rasGAP suggested that signaling through Dok-R might negatively regulate the Ras–MAP kinase pathway. To test this, we examined the effect of Dok-R expression on activation of the MAP kinase extracellular-signal-regulated kinase 2 (Erk2) by immunoblotting with phospho-specific anti-Erk antibodies and *in vitro* phosphorylation of the substrate myelin basic protein (MBP). Expression of wild-type Dok-R in COS1 cells resulted in a marked reduction in phosphorylation of Erk2 following EGF stimulation when compared with cells expressing the vector control (Figure 3a). Interestingly, this suppression of Erk2 activation was not abrogated by the mutant that cannot bind rasGAP (DM; Figure 3a) or a mutant that cannot bind rasGAP or Nck (data not shown). As expected, the PTB*/ΔPH mutant did not reduce Erk2 phosphorylation (data not shown).

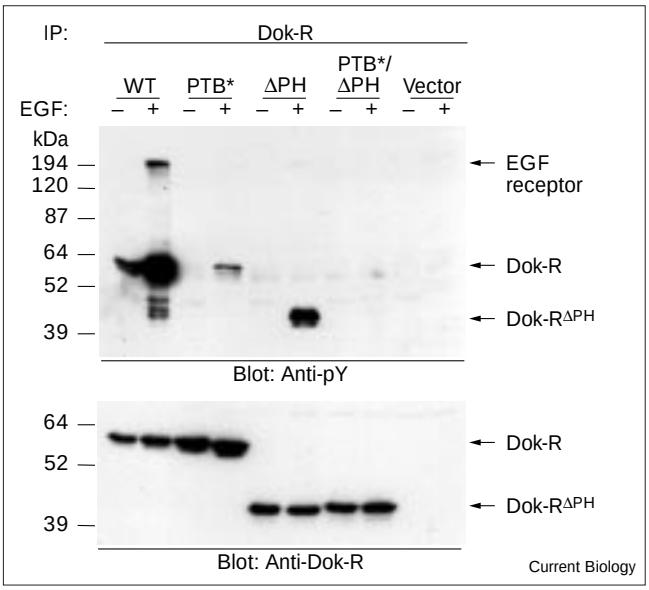
Table 1

Relative affinities of the PTB domain of Dok-R for phosphopeptides representing EGF receptor Tyr1086 and Tyr1148.

EGF receptor phosphopeptide	Binding constant (K_d)
Tyr1086	132 nM
Tyr1148	117 nM

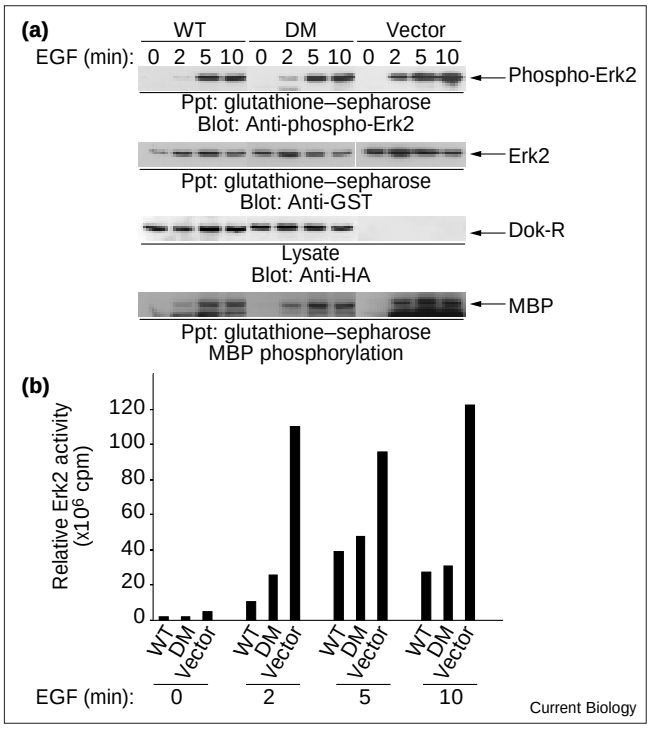
The PTB domain of Dok-R binds EGF receptor phosphopeptides with similar relative affinities. Phosphopeptides representing Tyr1086 and Tyr1148 were immobilized on sensor chips and the dissociation constants (K_d) between each of these phosphopeptides and the soluble PTB domain of Dok-R were determined using real-time biosensor (BIAcore) analysis [15]. This analysis provides an estimate of the relative affinity of the PTB domain for each of the two phosphopeptides. These values are likely to be overestimates because of the dimeric nature of GST in these experiments [16].

Figure 2



The PTB domain of Dok-R is required for binding to the EGF receptor *in vivo* and for tyrosine phosphorylation. Full-length wild-type Dok-R (WT) or mutant forms of Dok-R containing inactivating mutations in the PTB domain (PTB*), a deletion of the entire PH domain (ΔPH) or both mutations (PTB*/ΔPH) were introduced into COS1 cells. Unstimulated or EGF-stimulated lysates from these cells were immunoprecipitated (IP) with anti-Dok-R antibodies and immunoblotted with anti-phosphotyrosine (pY) antibodies. Immunoblotting with anti-Dok-R antibodies shows approximately equal expression of all Dok-R mutants and no expression of Dok-R is detected in cells expressing the vector alone.

Figure 3



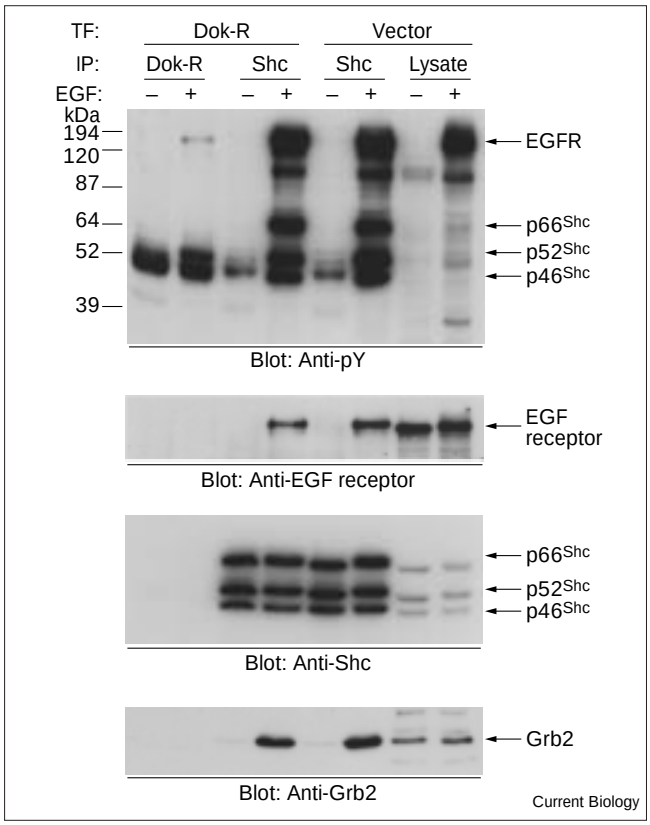
Dok-R expression attenuates EGF-induced Erk2 activation independently of rasGAP. (a) GST-tagged Erk2 (0.1 μg of plasmid) was coexpressed in COS1 cells with wild-type or mutant Dok-R (10 μg) and cells were left unstimulated or stimulated with EGF for the indicated time. Lysates were precipitated (Ppt) with glutathione–sepharose and subjected to an *in vitro* kinase assay using MBP as substrate. Samples were analyzed by SDS–PAGE and autoradiography or immunoblotting with anti-phospho-Erk2 antibodies. The immunoblot was reprobed with anti-GST antibodies to show approximately equal expression of Erk2 in all lanes. Non-immunoprecipitated lysates were immunoblotted with anti-HA antibodies to demonstrate equal expression of Dok-R mutants. (b) The MBP kinase activity assay was quantitated by phosphorimager analysis using the ImageQuaNT program (Molecular Dynamics); levels of MBP phosphorylation were normalized for relative expression of both Erk2 and Dok-R. Data shown are representative of a single experiment, which has been independently reproduced several times.

The degree of Erk2-mediated phosphorylation of MBP mirrored the observed attenuation of Erk2 phosphorylation (Figure 3a). Quantitation of the levels of MBP phosphorylation revealed that, in cells expressing the vector control, phosphorylation of Erk2 was induced within 2 minutes following EGF stimulation and persisted for at least 10 minutes (Figure 3b). In contrast, in cells expressing wild-type Dok-R, there was 10-fold less induction of Erk2 following 2 minutes of EGF stimulation and the level of activation did not reach vector control levels after 10 minutes of stimulation (Figure 3b). Interestingly, cells expressing the DM mutant induced Erk2 only marginally more than wild-type Dok-R, suggesting that the association between Dok-R and rasGAP is not required for this attenuation of Erk activity.

As the PTB domain of Dok-R can associate *in vitro* with the same phosphorylated tyrosine residue on the EGF receptor as Shc, we wanted to confirm that attenuation of Erk activation as a result of Dok-R expression was not due to displacement of Shc from the EGF receptor by Dok-R. We thus isolated Shc from unstimulated or EGF-stimulated COS1 cells that were either untransfected or transfected with Dok-R. Immunoprecipitated Shc isoforms became tyrosine-phosphorylated to the same extent with or without Dok-R; activated EGF receptor and Grb2 were readily detectable in these immunoprecipitates (Figure 4). Tyrosine phosphorylation of Dok-R and the associated EGF receptor could also be observed, and Dok-R did not associate with Grb2 (Figure 4). These experiments demonstrate that Shc signaling complexes are unaffected by the expression of Dok-R and the attenuation of Erk activation by Dok-R was not simply due to a loss of recruitment of the Shc–Grb2 complex to the receptor.

Our findings are consistent with those of Nelms *et al.* [4], which demonstrate that overexpression of FRIP in 32D cells inhibits the activation of MAP kinase following interleukin 2 stimulation. As rasGAP negative regulates Ras [13], the binding of rasGAP to Dok-R is an attractive mechanism

Figure 4



Shc signaling is not impaired in cells expressing Dok-R. COS1 cells were transfected (TF) with wild-type Dok-R or the control vector, stimulated with EGF (+) or left unstimulated (–), immunoprecipitated (IP) with anti-Dok-R or anti-Shc antibodies and immunoblotted with anti-phosphotyrosine (pY) antibodies. The immunoblot was subsequently reprobed with anti-EGF receptor, anti-Shc and anti-Grb2 antibodies and non-immunoprecipitated lysates (lysate) indicate the position of all proteins.

for suppression of Erk activation by Dok-R. Both Dok and Dok-R can bind rasGAP, but overexpression of Dok has no effect on MAP kinase activation in response to insulin [14], and a mutant of Dok-R that could not bind rasGAP was unable to rescue Erk activation levels in our studies. These observations suggest that the functional differences in MAP kinase activation between Dok and Dok-R are probably due to unidentified binding partners that are not conserved between the two Dok family members and the physiological consequence of rasGAP binding remains to be determined. The identification of additional Dok-family signaling partners will further illuminate the divergent functions of these docking proteins in growth factor signaling.

Supplementary material

Supplementary material showing the association of Dok-R with rasGAP and Nck and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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Supplementary material

Recruitment of Dok-R to the EGF receptor through its PTB domain is required for attenuation of Erk MAP kinase activation

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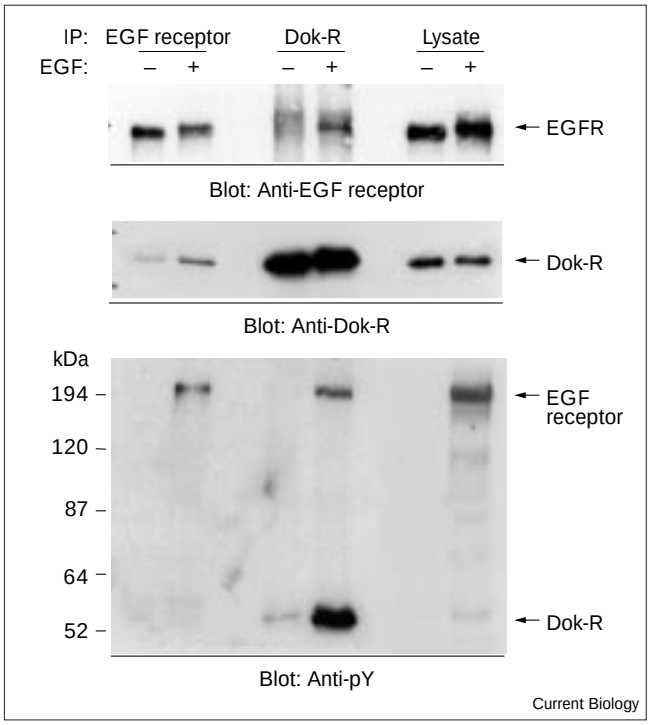
Results

Dok-R can associate with rasGAP and Nck through Tyr276, Tyr304 and Tyr351 following EGF stimulation

In this report, we have examined the ability of Dok-R to associate with the EGF receptor. In COS1 cells transiently expressing Dok-R, co-immunoprecipitation of Dok-R and EGF receptor and tyrosine phosphorylation of Dok-R could be observed following EGF stimulation (Figure S1). This phosphorylation established docking sites for the SH2-domain-containing signaling proteins rasGAP and Nck (Figure S2a). The consensus binding motif for both rasGAP and Nck is Tyr–X–X–Pro (YXXP) [S1–S3]; this motif is found four times in the Dok-R amino acid sequence at Tyr194, Tyr276, Tyr304 and Tyr351. Synthetic phosphopeptides corresponding to the last three YXXP motifs were generated; the amino-terminal SH2 domain of rasGAP (GAP-N) was found to associate with all three YXXP phosphopeptides *in vitro*, whereas the SH2 domain of Nck could associate only with phosphopeptides corresponding to Tyr304 and Tyr351 (Figure S2b). The presence of a putative Grb2-binding site was also observed in the extreme carboxy-terminal region of Dok-R (Tyr402), but we did not observe any association between the SH2 domain of Grb2 and a phosphopeptide corresponding to this tyrosine (Figure S2b).

Figure S1

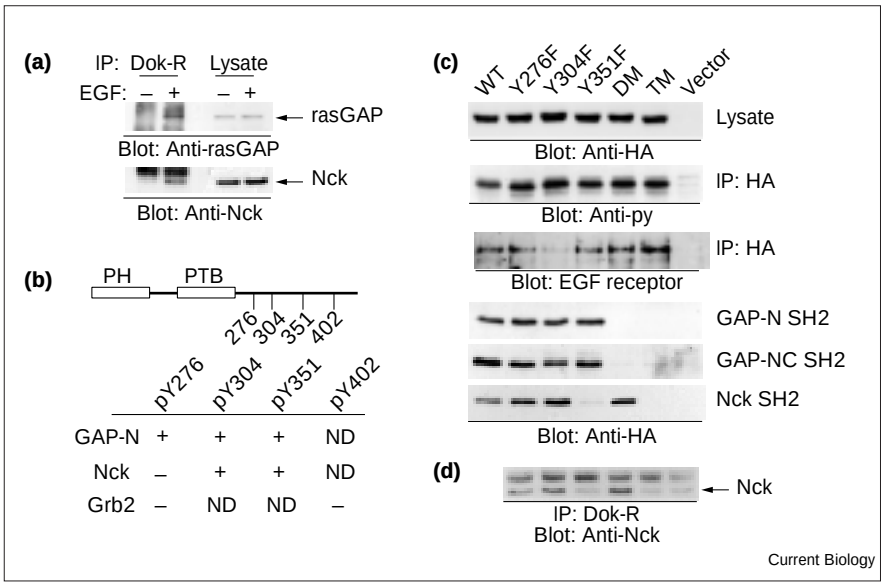


Dok-R is a novel substrate of the EGF receptor. Lysates from unstimulated (–) or EGF-stimulated (+) COS1 cells transiently expressing full-length Dok-R were immunoprecipitated (IP) with anti-EGF receptor or anti-Dok-R antibodies and immunoblotted as indicated. Non-immunoprecipitated lysates (lysate) show equal amounts of EGF receptor and Dok-R in the initial lysates.

The tyrosine residues on Dok-R that are phosphorylated in response to growth factor stimulation have not been defined, so the phosphopeptide analysis represented an initial approach to define the binding sites of rasGAP and Nck on Dok-R. To determine which sites could become tyrosine phosphorylated upon EGF stimulation *in vivo*, we generated HA-tagged tyrosine to phenylalanine point mutations in full-length Dok-R at Tyr276 (Y276F), Tyr304 (Y304F) and Tyr351 (Y351F). We also generated compound mutations in which Tyr276 and Tyr304 were doubly mutated (DM) or all three tyrosine residues were mutated (TM). All mutants were expressed at levels equivalent to wild-type Dok-R in COS1 cells and upon EGF stimulation, all mutants displayed similar levels of tyrosine phosphorylation and association with the EGF receptor (Figure S2c). Because the triple mutant appeared to be phosphorylated to the same extent as wild-type Dok-R, it is likely that there are additional sites of tyrosine phosphorylation throughout the protein.

Lysates from these cells were then incubated with immobilized GST–SH2 domains of rasGAP and Nck and bound proteins were visualized by anti-HA immunoblotting. Both the amino-terminal (GAP-N) and the tandem SH2–SH3–SH2 (GAP–NC) domains of rasGAP associated with all three single point mutants (Figure S2c), confirming the peptide data that showed that rasGAP can bind more than one site on Dok-R. The compound double mutant that lacks both Tyr276 and Tyr304 was no longer able to interact with the SH2 domains of rasGAP, however (Figure S2c), suggesting that rasGAP associates with Dok-R through a bisphosphorylated target motif comprising Tyr276 and Tyr304. The SH2 domain of Nck could associate with a phosphopeptide representing tyrosine residue 304, but mutation of this site in full-length Dok-R had no effect on Nck binding (Figure S2c). In contrast, mutation of Tyr351 in full-length Dok-R completely abrogated binding of Nck to Dok-R (Figure S2c), suggesting that Nck interacted with Dok-R exclusively through

Figure S2



Dok-R functions as a docking molecule for SH2-domain-containing proteins through phosphorylation at Tyr276, Tyr304 and Tyr351. (a) Lysates from unstimulated or EGF-stimulated COS1 cells expressing full-length wild-type Dok-R were immunoprecipitated with anti-Dok-R antibodies. Non-immunoprecipitated lysates (lysate) show the expression levels of rasGAP and Nck. (b) The position of three tyrosine residues found within YXXP motifs in the carboxy-terminal region of Dok-R (276, 304, 351) as well as one YXNX motif (402) are shown. Phosphopeptides were used for *in vitro* mixing experiments as described in Figure 1c and the results are presented with relative binding indicated as + or –. Interactions that were not determined are denoted ND. (c) Tyrosine to phenylalanine mutations were introduced in full-length HA-tagged Dok-R at the three indicated YXXP motifs and mutants were expressed in COS1 cells and stimulated with EGF. DM harbors mutations at both Tyr276 and Tyr304; TM has mutations at all three sites. Lysates from these cells were incubated with immobilized GST–SH2 fusion proteins and bound proteins were visualized by anti-HA immunoblotting. (d) Lysates from these cells were also immunoprecipitated with anti-Dok-R antibodies followed by anti-Nck immunoblotting to confirm the *in vivo* binding of Nck to Tyr351.

this phosphorylation site. These predictions were confirmed when the compound mutations were analyzed, as the DM mutant was still able to bind Nck but the TM mutant could no longer associate with Nck (Figure S2c). *In vivo*, Nck was coimmunoprecipitated with Y276F, Y304F and DM but not with Y351F or TM (Figure S2d). In summary, these results demonstrate that upon EGF stimulation, Tyr276, Tyr304 and Tyr351 of Dok-R are phosphorylated — 276 and 304 are required for rasGAP binding and Tyr351 is required for Nck binding.

The SH2 domain of Nck appears to bind exclusively to phosphorylated Tyr351 of Dok-R, which conforms to the predicted YDEP optimal binding motif for Nck [S1]. This tyrosine residue also represents a putative rasGAP-binding site, but the SH2 domains of rasGAP do not appear to bind this site in the context of full-length Dok-R. They associate instead with two different phosphorylated YXXP motifs at Tyr276 and Tyr304. The SH2 domains of rasGAP have been shown to bind simultaneously to two closely spaced phosphotyrosine residues in p190 rhoGAP that are both contained within the motif *YXXPXD* [S3]. This extended motif is conserved in Dok-R at Tyr276 and Tyr304 and mutagenesis of these closely linked tyrosine residues in Dok-R completely abrogates the interaction between rasGAP and Dok-R. The simultaneous interaction of both SH2 domains with bisphosphorylated targets is thought to promote a conformational change in rasGAP that exposes the target binding surface of the SH3 domain [S3]. We speculate that a similar mechanism occurs upon interaction of rasGAP with Dok-R following EGF stimulation.

Recently, the Nck protein has been shown to exist as Nckα/Nck1 and Nckβ/Nck2 isoforms [S4,S5]. Although neither isoform could interact directly with the EGF receptor, the Nckβ isoform was shown to associate with Dok more strongly than the α isoform [S4]. Furthermore, the β isoform was shown to inhibit EGF-stimulated DNA synthesis in an SH2-domain-dependent manner [S4]. Although we did not determine the identity of the Nck isoform in our immunoprecipitates because of antibody limitations, it is interesting to speculate that the effect of Dok-R expression on Erk activation may in fact be mediated by Nckβ. This notwithstanding, the inability of the mutant that cannot bind rasGAP or Nck to rescue Erk activation levels suggests that additional effector molecules are involved in this downregulation.

Supplementary materials and methods

Plasmids

The cDNA encoding full-length Dok-R [S6] was subcloned into pcDNA3.1(+) (Invitrogen) or pcDNA3.1(+) containing a hemagglutinin (HA) tag (HA-pcDNA3.1; a kind gift of Bryan Snow, AMGEN Institute, Toronto, Canada). Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) and all mutations were confirmed by sequencing. To generate Dok-R^{PTB*} and Dok-R GST–PTB*, Arg202 and Arg217 were altered to glutamine. Dok-R$^{\Delta PH}$ (amino acids 142–412) was constructed using PCR with appropriate primers containing restriction sites suitable for in-frame insertion into HA-pcDNA3.1. The Dok-R GST–PTB construct was

described previously [S6] and pEBG-Erk2 was a gift of Jim Woodgett (Ontario Cancer Institute, Toronto, Canada).

Cell culture, transient transfection and binding assays

COS1 cells were maintained and processed as described in [S6] except that cells were serum-starved for 24 h in DMEM containing 0.1% fetal bovine serum (Gibco BRL) before stimulation with 100 ng/ml EGF (Upstate Biotechnology). MBP phosphorylation assays were performed essentially as described in [S7].

GST fusion proteins and antibodies

Production of GST fusion proteins has been described in [S6]. The SH2-SH3-SH2 domain fusion protein of rasGAP was purchased from Santa Cruz. Peptide association assays are described in [S8] and the sequences corresponding to the phosphorylated Dok-R peptides are as follows: Y276, RPESPpYSRPHDSL; Y304, APEGEpYAVPFDTV; Y351, DHlpYDEPEGVA; Y402, PQATEpYDNVlLKK. Affinity-purified polyclonal anti-Dok-R antibodies have been described previously [S6]. Polyclonal anti-Shc antibodies were a gift of Jane McGlade (Hospital for Sick Children, Toronto, Canada). Monoclonal anti-EGF receptor antibodies (E12020, Transduction Labs) in conjunction with rabbit anti-mouse IgG (Upstate Biotechnology) were used for immunoprecipitation and polyclonal anti-EGF receptor antibodies (1005; Santa Cruz) were used for western blotting. Monoclonal anti-HA clone 12CA5 (Boehringer Mannheim) was used in immunoprecipitations and as a horseradish peroxidase conjugate for western blotting. The remaining antibodies used for western blotting were obtained as follows: monoclonal anti-phosphotyrosine 4G10 (Upstate Biotechnology); monoclonal anti-Grb2, anti-rasGAP and anti-Nck (G16720, G12920 and N15920, Transduction Laboratories); monoclonal phospho-MAP kinase p44/p42 (E10, New England Biolabs); and polyclonal anti-GST (Z-5, Santa Cruz).

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