Independent SH2-binding Sites Mediate Interaction of Dok-related Protein with RasGTPase-activating Protein and Nck*

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A murine embryonic cDNA library was screened for potential substrates of the Src family kinase, Lyn, using a phosphorylation-screening strategy. One cDNA that we identified encodes Dok-related protein (DokR), a protein with homology to p62 dok (Dok), and members of the insulin receptor substrate-1 family of proteins. Analysis of murine tissue extracts with DokR-specific antisera revealed that DokR protein is expressed at highest levels in lymphoid tissues. Co-expression of a FLAG epitope-tagged form of DokR (FLAG-DokR) with Lyn in embryonic kidney 293T cells resulted in constitutive phosphorylation of FLAG-DokR on tyrosine residues and consequent physical association with RasGTPase-activating protein (GAP) and the Nck adaptor protein. Stimulation of BaF3 hematopoietic cells co-expressing the epidermal growth factor (EGF) receptor tyrosine kinase and FLAG-DokR with EGF also induced phosphorylation of FLAG-DokR and promoted its association with GAP. Immunoprecipitation experiments using DokR-specific antibodies revealed an interaction between endogenous DokR and a 150-kDa protein that is tyrosine-phosphorylated in EGF-stimulated BaF3 cells. The molecular basis of the interactions involving DokR with GAP and Nck was investigated using a novel glutathione S-transferase fusion protein binding assay and/or site-directed mutagenesis. Tandem SH2-binding sites containing Tyr-276 and Tyr-304 were shown to mediate binding of DokR to GAP, whereas Tyr-351 mediated the binding of DokR to Nck. These results suggest that DokR participates in numerous signaling pathways.

On the basis of their capacity for oncogenic transformation, Src family kinases have long been suspected to regulate cell growth and differentiation (1). In mammals, eight Src family kinases have been identified as follows: Src, Yes, Fgr, Fyn, Lyn, Hck, Lck, and Blk. Src family kinases have a conserved topology, consisting of a myristoylated amino terminus, a variable region, Src homology 3 (SH3)1 and SH2 domains, a linker region, a catalytic domain, and a regulatory tail (1–3). In unstimulated cells, catalytic activity is repressed by two intramolecular interactions, one involving the SH3 domain and a proline-containing motif in the linker region, and another involving the SH2 domain and a phosphorylated tyrosine in the tail region (Tyr-527 in chicken Src) (1, 3). Dephosphorylation or mutation of this tyrosine, or engagement of the SH3 or SH2 domains with specific ligands, results in enzymatic activation.

Src family kinases are associated with cell membranes via a myristoylated amino terminus and are physically or functionally coupled in different cell types to diverse cell surface molecules, including integrins, G-protein-coupled receptors, growth factor receptors, and antigen and antibody receptors (1). Numerous candidate substrates have been identified that are phosphorylated on tyrosine residues by Src family tyrosine kinases, including non-receptor tyrosine kinases (4–6), structural proteins implicated in cytoskeletal regulation (7–9), docking proteins (10–17), and cell surface receptors (18–21). Genetic evidence from analysis of mice lacking one or more Src family kinase indicates that members of this family have both redundant and non-redundant functions (2). This is thought to be due to the overlapping substrate specificities of different Src family kinases, such that different members of this family phosphorylate common targets in some tissues. Conversely, substrates that are unique to individual Src family kinases are also likely to exist.

The Lyn tyrosine kinase is expressed in several hematopoietic lineages including B lymphoid, myeloid, and erythroid cells and is implicated in signal transduction pathways that control cell proliferation (22–24), apoptosis (25, 26), differentiation (27), adhesion, and migration (28, 29). To identify potential components of Lyn-mediated signaling pathways, we have employed a cloning strategy to detect proteins by their capacity to act as substrates for Lyn kinase (30). We have identified both novel and known targets of Lyn or other Src family kinases. One potential Lyn substrate that we have detected, Dok-related protein (DokR) (31–33), contains an amino terminus that shares extensive similarity with the insulin receptor substrate-1 (IRS-1) family of proteins and, like the IRS proteins, contains multiple potential SH2-binding sites. Our results indicate that tyrosine phosphorylation of DokR by Lyn generates specific binding sites for RasGTPase-activating protein (GAP) and the Nck adaptor protein. We have determined the location of the critical sites underlying these interactions.

**EXPERIMENTAL PROCEDURES**

Cloning of cDNAs Encoding Lyn Substrates—Approximately $7 \times 10^5$ independent clones from a λExElx cDNA expression library generated...
from day 16 murine embryonic mRNA (Novagen) were screened for Lyn substrates essentially as described (30). Briefly, cDNA clones were immobilized on nitrocellulose filters (Micron Separations Incorporated) and the filters incubated in kinase buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 2 mM MnCl2, 0.1% Triton X-100, 250 mM ATP) with the kinase sequence of their 5′- and 3′-ends determined using the chain termination method and an automated DNA sequencer (Applied Biosystems). Sequences were scrutinized by reference to the non-redundant nucleic acid and protein data bases using the BLAST 2.0 program (35). Both strands of a 1615-base pair cDNA were sequenced and found to specify a novel protein that was designated Dok-related protein (DokR).

 Constructs—A FLAG-tagged DokR cDNA was generated by insertion of an EcoRI-Sacl-digested PCR fragment encoding an initiator methionine, the FLAG octapeptide (DYKDDDDK), and residues 2–44 of DokR, into a pEXlox plasmid (Novagen) containing the sequences encoding residues 45–412 of wild type DokR. Full-length FLAG-DokR cDNA was excised with EcoRI and HindIII, filled with Klenow enzyme, and subcloned using BstXI adaptors into the mammalian expression vector pEF-BOS. Site-directed mutagenesis was performed using the chain termination method and an automated DNA sequencer (Applied Biosystems). Sequences were scrutinized by reference to the non-redundant nucleic acid and protein data bases using the BLAST 2.0 program (35). Both strands of a 1615-base pair cDNA were sequenced and found to specify a novel protein that was designated Dok-related protein (DokR).
DokR Interactions with GAP and Nck

Tyrosine-phosphorylated FRS2 then interacts with the SH2 domain of growth factor receptor-binding protein 2, linking it to activation of the Ras pathway (44). Human FRS2/SNT-1 is identical with the product of an expressed sequence tag (est) designated h-dok-5 that was reported previously (31). Two predicted proteins (designated here as cORF1 and hORF1) that are encoded by an uncharacterized Caenorhabditis elegans gene (GenBankTM accession number AAB94991) and a human gene (GenBankTM accession number AAC24310), respectively, also possess DokR-like PTB domains and are therefore predicted to participate in tyrosine kinase-mediated signal transduction.

Expression Pattern of DokR Protein—We generated rabbit polyclonal antisera against different regions of DokR as follows: residues 260–341 (anti-DokR.1), residues 136–309 (anti-DokR.2), and residues 397–412 (anti-DokR.3). When tested in immunoprecipitation and Western blot analysis, the three DokR antisera, but not the corresponding preimmune sera, each recognized a FLAG epitope-tagged form of DokR (FLAG-DokR) when it was ectopically expressed in 293T cells (not shown). To determine the expression pattern of DokR protein, we prepared extracts from a range of adult mouse tissues and analyzed them by immunoprecipitation with the anti-DokR.2 antibody followed by immunoblotting with the anti-DokR.1 antibody. Extracts from 293T cells expressing FLAG-DokR and from BaF/3 cells, which were found to express endogenous DokR (not shown), were included in the analysis as positive controls. As shown in Fig. 2, the antibodies detected a 56-kDa protein in a subset of tissues, including thymus, lymph node, spleen, lung, and heart. This molecular mass is identical to that reported for DokR (31, 33) and less than that reported for Dok (60–62 kDa) (16, 17), suggesting that the protein detected in the immunoprecipitates is indeed DokR. The slightly greater molecular mass of FLAG-DokR relative to DokR isolated from tissues is attributed to the 8 additional residues that comprise the FLAG epitope tag. A weak band of approximately 56 kDa was detected in all samples and is due to reactivity of protein A-horseradish peroxidase (used to detect binding of anti-DokR.1) with the IgG heavy chain of the anti-DokR.2 antibody. A protein of approximately 66 kDa, the identity of which is not known, was detected in kidney. Significantly, the tissue distribution of DokR protein that we observed correlates closely with the mRNA expression profiles that we and others (31–33) have observed previously (not shown).

Phosphorylation of DokR by Src Family Kinases Induces Association of DokR with GAP—To test whether Src family kinases are capable of phosphorylating DokR in vivo, FLAG-DokR was transiently co-expressed in 293T cells with wild type Lyn or Hck or activated mutants of Lyn, Hck, or Src containing a phenylalanine substitution of their conserved regulatory tyrosine (Tyr-527 in Src). Cell lysates were immunoprecipitated with a FLAG antibody and analyzed by immunoblotting with a phosphotyrosine antibody. Fig. 3A shows that FLAG-DokR was strongly tyrosine-phosphorylated in cells that co-expressed either wild type or activated Src family kinases (upper panel). Perhaps surprisingly, the level of FLAG-DokR tyrosine phosphorylation was similar in the presence of activated or wild type Lyn and only moderately elevated in the presence of activated Hck compared with wild type Hck (Fig. 3A). Presumably this is due to a relative lack of catalytic repression of wild type Lyn (and to a lesser extent Hck) in 293T cells by endogenous regulatory kinases (e.g. carboxyl-terminal Src kinase (46), which are capable of phosphorylating the regulatory tyrosine residue of Src family kinases.

Since tyrosine phosphorylation of p62\text{rob} (Dok) by a variety of tyrosine kinases has been shown to induce Dok to associate

cortactin/EMS1 (7), hematopoietic lineage cell-specific protein 1/Lck-binding protein 1 (HS1/LckBP1) (13, 14), Crk-associated substrate (p130\text{crk}) (10), and embryonic Fyn substrate/Src-interacting protein (11, 12). We also identified insulin receptor substrate-2 (IRS-2) (39), a protein that is phosphorylated on tyrosine residues in response to stimulation of cells with insulin, insulin-like growth factor-1, and several cytokines (40). In addition, we identified SH3P7, a molecule that was initially cloned on the basis of its ability to bind to polyproline-containing peptides (41). Although not known to be tyrosine-phosphorylated in cells, SH3P7 nevertheless represents a strong candidate substrate of Src family kinases since it contains several consensus recognition sites for phosphorylation by v-Src (not shown). One protein that was identified shared extensive sequence similarity with p62\text{rob} (Dok) (33). In this report we will refer to the protein as DokR.

In agreement with published reports, nucleotide sequence analysis of the 1,615-base pair DokR cDNA indicated that it encoded a protein of 412 residues (not shown) including 13 possible tyrosine phosphorylation sites (31–33). Data base searches revealed that murine DokR shares 36% sequence identity with human DokR (not shown). Both proteins contain a putative pleckstrin homology (PH) domain at the amino terminus (residues 8–118 in mDokR) followed by a phosphotyrosine (PTB) domain (residues 148–254) (not shown) (32, 33). The carboxyl-terminal region of DokR (residues 255–412) contains 30 proline residues (Pro), representing 19% of amino acids, and contains multiple PXXP motifs that potentially interact with SH3 domain-containing proteins (not shown) (33).

Interestingly, data base searches also revealed that the amino terminus of DokR, including the PH and PTB domains, shares significant homology with the insulin receptor substrate (IRS) proteins, a family of cytosolic proteins that undergo cytokine- and growth factor-inducible tyrosine phosphorylation and interactions with specific SH2-containing proteins (40). Notably, no other proteins with PH domains other than IRS-1, IRS-2, and Dok were detected in these searches (not shown), perhaps an indication of the low sequence conservation among PH domains. A sequence alignment of the PH/PTB region of DokR, Dok, IRS-1, and IRS-2 is presented in Fig. 1A. The similarity between DokR and IRS proteins within the PTB domain was noted previously (33). However, the alignment shown in Fig. 1A reveals that the PH/PTB domain configuration has been conserved during evolution. These data strongly suggest that Dok, DokR, and the IRS proteins are members of two related subfamilies.

Several proteins with homology to the DokR PTB domain, but which lack a PH domain, were also identified. A sequence alignment showing these proteins together with members of the IRS family is presented in Fig. 1B. Included in this group, fibroblast growth factor (FGF) receptor substrate 2 (FRS2) (44) or sucl-associated neurotrophic factor target-1 (SNT-1) (45) and the related molecule, SNT-2 (45), are docking proteins that contain a myristoylated amino terminus. Both FRS2/SNT-1 and SNT-2 are rapidly phosphorylated on tyrosine residues following stimulation of cells with FGF or nerve growth factor.
with GAP (16, 17, 42), we tested whether phosphorylation of DokR by Src family kinases also promoted its association with GAP. Immunoblot analysis of FLAG immunoprecipitates with a GAP antibody revealed that endogenous GAP co-immunoprecipitated with FLAG-DokR in cells that co-expressed wild type or activated forms of Lyn, Hck, or Src (Fig. 3A, center panel).

Intriguingly, the amount of GAP associated with FLAG-DokR in cells co-expressing wild type and activated Lyn was higher than that observed in cells co-expressing wild type and activated Hck or activated Src. This enhanced capacity to associate with GAP was not due to higher total levels of phosphotyrosine (upper panel) but, conceivably, might be due to qualitative differences in tyrosine phosphorylation of FLAG-DokR. Indeed, immunoblot analysis of whole cell lysates of the transfected cells using a FLAG antibody revealed differences in the patterns of phosphorylated FLAG-DokR isoforms that were generated in the presence of Lyn, Hck, or Src (lower panel).

At least three electrophoretically distinct isoforms of FLAG-DokR were detected in cells expressing wild type or activated Lyn, whereas cells that expressed wild type or activated Hck or activated Src contained only two major isoforms. These data reveal a potential difference in the substrate specificities of Lyn, Hck, and Src and suggest that Lyn preferentially phosphorylates tyrosine residues that participate in binding to GAP.

To confirm the interaction between FLAG-DokR and GAP, we performed a reciprocal co-immunoprecipitation experiment with FLAG-DokR in cells that co-expressed wild type or activated forms of Lyn, Hck, or Src (Fig. 3A, center panel).

Intriguingly, the amount of GAP associated with FLAG-DokR in cells co-expressing wild type and activated Lyn was higher than that observed in cells co-expressing wild type and activated Hck or activated Src. This enhanced capacity to associate with GAP was not due to higher total levels of phosphotyrosine on FLAG-DokR than in cells co-expressing wild type and activated Hck or activated Src. Instead, the enhanced capacity to associate with GAP was likely due to qualitative differences in tyrosine phosphorylation of FLAG-DokR. Indeed, immunoblot analysis of whole cell lysates of the transfected cells using a FLAG antibody revealed differences in the patterns of phosphorylated FLAG-DokR isoforms that were generated in the presence of Lyn, Hck, or Src (lower panel).

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Associate with GAP but not Crk or CrkL. A, 293T cells were transfected with vector alone or plasmids encoding FLAG-DokR alone or FLAG-DokR plus Lyn wild type, Lyn508F, Hck WT, Hck499F, or Src527F. Cellular extracts containing 400 μg of protein were analyzed by immunoprecipitation (IP) with a FLAG antibody (anti-FLAG) and resolved by SDS-PAGE followed by Western blot analysis (WB) with anti-Tyr(p) (anti-pY) (upper panel) or GAP (center panel) antibody. Samples of crude extract (WCL) containing 20 μg of protein were analyzed in parallel by immunoblotting with anti-FLAG antibody (lower panel). B, 293T cells were transfected with vectors encoding Lyn WT, FLAG-DokR alone, or FLAG-DokR plus Lyn WT or Lyn508F. Anti-GAP immunoprecipitates were assayed for FLAG-DokR association by immunoblotting with anti-Tyr(p). C, plasmids encoding FLAG-DokR alone or FLAG-DokR plus Lyn WT were transfected in 293T cells. Whole cell lysates and anti-FLAG immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblotting with anti-CrkL (upper panel), anti-Crk (center panel), and anti-GAP (lower panel) antibodies.

Stimulation of BaF3/EGFR Cells with EGF Induces DokR to Associate with GAP and a 150-kDa Phosphoprotein—Previous studies showed that p62Dok (Dok) becomes rapidly tyrosine-phosphorylated and associated with GAP as a consequence of activation of diverse receptor tyrosine kinases, including the epidermal growth factor (EGF) receptor (42), the Eph family receptor, EphB2/Nuk (43), or the stem cell factor receptor (c-Kit) (16). Here, we have tested whether stimulation of the EGF receptor induces phosphorylation of DokR and promotes its association with GAP. BaF3 hematopoietic cells that ectopically expressed the EGF receptor (BaF3/EGFR cells) (37) were co-transfected with plasmids encoding FLAG-DokR and the puromycin resistance gene. Puromycin-resistant cell lines were isolated and tested for FLAG-DokR expression by immunoblotting with FLAG antibodies (not shown). A cell line that expressed intermediate levels of FLAG-DokR was identified (not shown). These cells were stimulated with EGF for a range of times, lysed, and subjected to immunoprecipitation with FLAG or GAP receptor antibodies. Immunoprecipitates were analyzed by immunoblotting with phosphotyrosine, FLAG, or GAP antibodies. As shown in Fig. 4, EGF stimulation induced rapid and transient tyrosine phosphorylation of FLAG-DokR (upper panel) in parallel with EGF receptor activation (lower panel). Phosphorylation of FLAG-DokR was negligible in control cells, increased to maximal levels after 1 min, and then declined to baseline levels by 1 h. Endogenous GAP associated with FLAG-DokR with similar kinetics to tyrosine phosphorylation of FLAG-DokR (third panel from top), suggesting that phosphorylation is necessary for this interaction. Immunoprecipitates of FLAG-DokR contained no detectable tyrosine-phosphorylated EGF receptor suggesting that DokR does not form a stable complex with the receptor (not shown). Our data suggest that DokR can act as a direct substrate of the EGF receptor or is phosphorylated by an endogenous kinase that becomes activated in BaF3 cells following EGF stimulation.

Since DokR is expressed in BaF3 cells (see Fig. 2), we also tested whether EGF could promote association of endogenous DokR with GAP. BaF3/EGFR cells were serum-starved for several hours and then treated or not treated with EGF for 2 min. DokR was immunoprecipitated from cellular extracts with
anti-DokR.1, anti-DokR.2, or anti-DokR.3 antibodies and then analyzed by immunoblotting with anti-phosphotyrosine or GAP antibodies. As shown in Fig. 5A, EGF rapidly induced tyrosine phosphorylation of endogenous DokR (upper panel) and promoted its association with GAP (lower panel). A background band that co-migrated with DokR (upper panel) corresponds to the IgG heavy chain of the DokR antisera. Interestingly, a tyrosine-phosphorylated protein of approximately 150 kDa (p150), the identity of which is unknown, was detected in all three anti-DokR immunoprecipitates from the EGF-stimulated cells but not from the unstimulated cells (Fig. 5A, center panel). Tyrosine-phosphorylated p150 was detected in DokR immunoprecipitates at least 7 min after stimulation with EGF (Fig. 5B, lower panel). These results suggest that p150 and DokR form a stable complex in the EGF-treated cells. At present we cannot distinguish whether the DokR-p150 complex is constitutive or induced by EGF. Long autoradiographic exposures were required to detect p150, suggesting that the phosphotyrosine content of p150 may be relatively low or, alternatively, that p150 associates with DokR with low stoichiometry.

Both GAP SH2 Domains Mediate Binding to Tyrosine-phosphorylated DokR—GAP contains a regulatory domain that consists of two SH2 domains (SH2N and SH2C) separated by an SH3 domain. To determine the role of these domains in binding to DokR, we generated glutathione S-transferase (GST) fusion proteins containing the SH2N, SH2C, and SH3 domains or the SH2,3,2 domain on the intrinsic potential of the SH2C domain to bind DokR, we generated GST fusion proteins containing the SH2N, SH2C, and SH3 domains or the SH2,3,2 domain. The relative amounts of FLAG-DokR protein that were used in the assay were assessed by immunoblotting with anti-FLAG, anti-GAP, and GAP antibodies (Fig. 6). The data show that both of the GAP SH2 domains and the SH3 domain with greater efficiency than to fusion proteins containing only the SH2N or SH2C domain, suggesting that the SH2 domains of GAP cooperate or synergize in binding to p190RhoGAP. To examine the individual contribution of each SH2 domain to binding DokR, we generated GST fusion proteins containing an inactivating mutation in either the SH2N or SH2C domain (R206A or R377A, respectively) (36). Equivalent amounts of these GST fusion proteins and a fusion protein with wild type SH2 domains were immobilized on glutathione-Sepharose and incubated with lysates from 293T cells that co-expressed Lyn and FLAG-DokR. The relative amounts of FLAG-DokR protein bound by the GST fusion proteins and the levels of GST fusion proteins that were used in the assay were assessed by immunoblotting with DokR or GST antisera. As shown in Fig. 6B, mutation of the SH2C domain (SH2,3,2*) had no significant effect on the ability of the SH2,3,2 region to bind FLAG-DokR. Intriguingly, however, mutation of the SH2N domain (SH2*3,2) abolished binding to FLAG-DokR. These results reveal a surprising inhibitory effect of the SH2 and/or SH3 domain on the intrinsic potential of the SH2C domain to bind to DokR (see Fig. 6A). Presumably, inhibition is mediated by an

Fig. 4. EGF stimulates tyrosine phosphorylation of FLAG-DokR and its association with GAP. A, BaF3 cells stably expressing the EGF receptor and FLAG-DokR were starved of serum and IL-3 or stimulated with EGF (100 ng/ml) for 6 h (−) or stimulated with EGF (100 ng/ml) for 1, 5, 10, 20, or 60 min. Equivalent amounts of extract from stimulated cells were analyzed by immunoprecipitation (IP) with anti-FLAG (upper three panels) or anti-EGFR receptor (lower panel) antibodies. Samples were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-FLAG (anti-pY) (upper and lower panels), anti-FLAG (2nd panel), or anti-GAP (3rd panel) antibodies. WB, Western blot.

Fig. 5. Endogenous DokR is associated with a 150-kDa tyrosine-phosphorylated protein in EGF-stimulated cells. A, BaF3/EGFR cells were serum- and IL-3-starved for 6 h (−) or stimulated for 2 min with 100 ng/ml EGF (→). Immunoprecipitates prepared with anti-DokR.1, anti-DokR.2, or anti-DokR.3 antibodies, and samples of whole cell lysate (WCL) were resolved by SDS-PAGE. After transfer to nitrocellulose, the blot was probed with phosphotyrosine (upper and center panels) or GAP antibodies (lower panel), and bands were detected using chemiluminescence. A 10-s (upper panel) and a 2-min exposure (center panel) of the anti-Tyr(P) (anti-pY) immunoblot are shown (center panel). B, BaF3/EGFR cells were serum- and IL-3-starved (−) or stimulated for 2 or 7 min with 100 ng/ml EGF. Extracts were immunoprecipitated with anti-DokR.2 serum and analyzed by SDS-PAGE and immunoblotting with anti-Tyr(P) followed by chemiluminescent detection. A 10-s (upper panel) and a 3-min exposure (lower panel) of the anti-Tyr(P) blot are shown. WB, Western blot.
in intramolecular mechanism and could involve steric hindrance of the SH2C domain or modification of its binding site.

Identification of a Minimal GAP Binding Region between DokR Residues 263 and 309—Since binding of DokR to GAP is predicted to be phosphorylation-dependent, we devised a binding assay to map the region of DokR that mediates this interaction. GST fusion proteins containing full-length or truncated forms of DokR (shown schematically in Fig. 7A) were expressed in bacteria and purified. Individual GST-DokR fusion proteins were then incubated with immunoprecipitates of Lyn in the presence of ATP in order to generate tyrosine-phosphorylated GST-DokR fusion proteins. The modified GST fusion proteins were immobilized on glutathione-Sepharose and tested for binding to GAP. These data define a region of DokR between residues 1 and 131 that lacks tyrosine phosphorylation sites, a region of DokR between amino residues 136 and 262 that contains at least one phosphorylation site that fails to bind GAP, and a minimal phosphotyrosine-dependent GAP-binding region between residues 263 and 309.

Independent SH2-binding Sites Mediate Association of DokR with GAP and Nck—The region of DokR between residues 263 and 309 contains two tyrosines as follows: Tyr-276 and Tyr-304, which are located within YXXPPX sequence motifs. This motif is conserved in the GAP-binding sites of p190RhoGAP (49) and platelet-derived growth factor receptor (50), suggesting that both sites in DokR might be involved in GAP recognition. The contribution of these residues and a third tyrosine, contained within a YXXPXG motif (Tyr-351), to binding GAP, was examined by generating FLAG-DokR mutants containing phenylalanine substitutions of Tyr-276 and/or Tyr-304 and/or Tyr-351. Wild type and mutant forms of FLAG-DokR (Fig. 8A) were co-expressed with Lyn in 293T cells. Cell lysates were analyzed by immunoblotting with a FLAG antibody followed by phosphotyrosine-dependent GAP-binding region between residues 263 and 309.

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both Tyr-276 and Tyr-304 were substituted for phenylalanine (Y276F/Y304F and Y276F/Y304F/Y351F). The relatively low level of the Y276F/Y304F mutant that was immunoprecipitated by the FLAG antibody does not account for the lack of detectable GAP, since GAP was readily detected in complex with similar levels of other mutants (e.g. Y304F and Y276F/Y351F). These results suggest that Tyr-276 and Tyr-304 represent separate GAP-docking sites, which presumably interact with the two GAP SH2 domains (see Fig. 6A).

Recently, Nck, an adaptor protein with three SH3 domains and an SH2 domain, was shown to associate with DokR in a phosphorylation-dependent manner (33). A synthetic peptide based on the sequence surrounding Tyr-351 was capable of binding to Nck only when phosphorylated, suggesting that this residue mediates this interaction (33). To test this prediction, Lyn was co-expressed with the wild type and mutant forms of FLAG-DokR shown in Fig. 8A, in 293T cells. Cell lysates were analyzed by immunoprecipitation with a FLAG antibody followed by immunoblotting with Nck or GAP antibodies. Consistent with the earlier prediction (33), all FLAG-DokR mutants in which Tyr-351 was substituted for phenylalanine failed to associate with Nck while retaining their capacity to interact with GAP (Fig. 8C). Conversely, the FLAG-DokR mutant, Y276F/Y304F, which did not associate with GAP, was capable of interacting with Nck. These results define a specific Nck-binding site within DokR that includes Tyr-351, which is distinct from the GAP-specific binding site containing Tyr-276 and Tyr-304.

**DISCUSSION**

We have identified DokR as a possible substrate of Lyn and other Src family kinases. This protein was also recently identified by others (31) as a substrate of Bcr-Abl tyrosine kinase and a protein that is capable of interacting in the yeast two-hybrid system with the intracellular domains of the IL-4 receptor α-chain (32) and the Tek/Tie2 receptor tyrosine kinase (33). A survey of a range of adult mouse tissues revealed that DokR protein is expressed primarily in lymphoid tissues including thymus, lymph node, and spleen (Fig. 2). In reconstitution experiments using transiently transfected cells, epitope-tagged DokR was phosphorylated on tyrosine residues when co-expressed with the Src family kinases, Lyn, Hck, or Src (Fig. 3), the Bcr-Abl tyrosine kinase (31) or an activated form of Tek/Tie2 (33). Moreover, stimulation of cell lines that stably expressed DokR with growth factors or cytokines, including EGF (Fig. 4), insulin, interleukin-2 (IL-2), IL-3 or IL-4, also resulted in rapid phosphorylation of DokR (32). Once phosphorylated, DokR was capable of interacting via specific binding sites with the SH2 domains of GAP (Figs. 6–8) (31–33) and with the adaptor protein, Nck (Fig. 8) (33). We have also presented evidence for an interaction between DokR and an unidentified 150-kDa phosphoprotein (Fig. 5). Together, these results provide evidence that DokR may participate in a variety of signal transduction pathways by coordinating the formation of one or more specific signaling complexes.

Structural similarities suggest that DokR and Dok are distinct relatives of a family of docking proteins that includes the IRS family of proteins (Fig. 1). These proteins each contain an amino-terminal PH domain and adjacent PTB domain and multiple phosphorylation sites that bind to specific SH2-containing proteins. PH domains are thought to have a role in regulating membrane association. The IRS-1 PH domain is required for efficient phosphorylation by the insulin receptor, suggesting that it promotes interaction of IRS-1 with the activated receptor (51). The PH domains of phospholipase C-γ, Bruton’s tyrosine kinase, dynamin, and the serine/threonine kinase, Akt, bind to the head groups of specific phosphoinositides, which are localized in cell membranes in vivo, and are thought to promote membrane targeting of these proteins (52-54). The DokR PTB domain was shown to mediate binding of DokR to the cytoplasmic domain of an activated form of the Tek/Tie2 receptor (33) and was proposed to mediate an interaction with a tyrosine-phosphorylated NPXY-containing motif in the IL-4 receptor α-chain (32). Conversely, mutation of the NPXY motif abrogated IL-4-dependent tyrosine phosphorylation of DokR. A model has been proposed in which DokR is recruited, via its PTB domain, to phosphorylated target sites within the cytoplasmic domains of specific receptors (32). DokR
is then phosphorylated, either by the intrinsic tyrosine kinase activity of the receptor or by a cytoplasmic tyrosine kinase associated with the receptor, thereby promoting subsequent interactions between DokR and specific SH2-containing proteins (32, 33).

Distinct phosphorylation-dependent binding sites for GAP and Nck were identified in DokR (Figs. 7 and 8). Since mutation of two potential phosphorylation sites, Tyr-276 and Tyr-304, but neither site alone abolished the interaction between DokR and GAP, it seems likely that both sites mediate binding to GAP in vivo. By contrast, substitution of Tyr-251 alone ablated the interaction between Nck and DokR. The simplest explanation for these observations is that interaction of GAP with DokR is mediated by the two GAP SH2 domains and the DokR phosphorylation sites, Tyr-276 and Tyr-304, whereas binding of DokR to Nck is mediated by the single SH2 domain of Nck and the phosphorylation site at Tyr-251. In support of the notion that two binding sites facilitate GAP binding, GST fusion proteins containing either of the GAP SH2 domains were capable of binding to DokR (Fig. 6). In an earlier study the GAP SH2C domain failed to bind to DokR (32). However, the GST fusion protein capable of binding to DokR (Fig. 6). In an earlier study the GAP fusion proteins containing either of the GAP SH2 domains were binding of DokR to Nck is mediated by the single SH2 domain of DokR phosphorylation sites, Tyr-276 and Tyr-304, whereas interactions between DokR and specific SH2-containing proteins are constitutively associated in unstimulated cells and p50 is only detectable after EGF stimulation because it becomes phosphorylated should also be considered.

What is the physiological relevance of DokR interactions involving GAP, Nck, and p150? Since GAP is capable of negatively regulating Ras by enhancing its intrinsic GTPase activity, one attractive model is that DokR links GAP to attenuation of Ras signaling, perhaps by recruiting GAP to cell membranes (32). Indeed, overexpression of DokR in 32D cells was shown to inhibit the activation of mitogen-activated protein kinases and expression of an AP-1 responsive reporter gene following stimulation with IL-2 (32), processes that are thought to be Ras-dependent. Conversely, primary T cells from the hairless (hr/hr) strain of mice, which were shown to express 3–5-fold lower levels of DokR than wild type mice, were shown to be hyper-responsive to stimulation with cytokines and concomitant T cell receptor activation (32). Although these results are consistent with a critical role for GAP, the contribution to negative regulation by Nck and other DokR-interacting proteins, such as p150, also needs to be investigated. We are now exploring the potential of the mutant forms of DokR that lacks the ability to interact with GAP, Nck, or both proteins to function as dominant negative mutants with which we should be able to address the relevance of these different pathways in DokR signaling.

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