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# New insights into Dok-4 PTB domain structure and function

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ABSTRACT

The seven members of the Dok adapter protein family share a highly conserved phosphotyrosine-binding (PTB) domain. In the case of Dok-1, 2 and 3, the PTB domain binds to the lipid phosphatase Ship1, a key component of their inhibitory signaling mechanisms in immune cells. In contrast to most other Dok family members, Dok-4 is expressed widely but is poorly understood, largely because of limited knowledge of its partner molecules. We previously showed that, in contrast to the Dok-1 PTB domain (defined as aa 107–260), the homologous sequence in Dok-4 (aa 100–233) bound very poorly to Ret, a known Dok-4 partner. In the current study, we show that binding of Dok-4 to Ret requires residues C-terminal to the previously defined PTB domain boundaries (up to aa 246). These residues are predicted to form an extension in a critical C-terminal α-helix. We show that the Dok-4 PTB domain also binds the phosphorylated NPXY motifs in Ship1 but not Ship2. Finally, we found that a rare human single nucleotide polymorphism causing a R186H substitution in the PTB domain abolishes tyrosine phosphorylation of Dok-4 by Ret. In addition to providing a clearer understanding of Dok-4 PTB domain structure and function, our findings point to a potential mechanism for Dok-4 inhibitory signaling in T-cells and to the possibility of a rare Dok-4-related phenotype in humans.



## 1. Introduction

Like the related IRS (insulin receptor substrate) family, the seven members of the Dok family of adapter proteins are characterized structurally by a tandem of N-terminal pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains followed by more divergent C-terminal sequences [1]. The first three members of the Dok family, Dok-1, 2 and 3, form a separate subfamily (Dok-A) that displays extensive homology and possess expression patterns mostly restricted to immune cells including monocyte/macrophages [1,2] where they inhibit antigen receptor signaling through multiple mechanisms, including PTB domain-mediated binding of the lipid phosphatase Ship-1 [2]. In contrast, Dok-4, 5 and 6 form a distinct sub-family (Dok-B) whose function is poorly understood. While expression of Dok-5 and 6 is essentially restricted to neural tissues [3,4], Dok-4 is expressed widely with a preference for neural, endothelial and epithelial tissues [5]. Dok-4 is also expressed in circulating T-cells where it participates in inhibitory signaling through a poorly defined mechanism [6]. Dok-7 is a more distant member of the Dok family with expression restricted to muscles, where it serves a critical role in neuromuscular junction formation [7]. In summary, Dok-4 – and to a lesser extent Dok-1 – is the only ubiquitous member of the Dok family but it remains one of the least understood.

Although its exact biological function remains unclear, Dok-4 has been reported to *enhance* Ret-mediated neurite outgrowth through enhanced activation of Erk [3]. Conversely, Dok-4 has also been shown to *inhibit* activation of the Erk target transcription factor Elk-1 downstream of multiple tyrosine kinases. These seemingly contradictory roles highlight how adapter protein function is dependent on an array of partner molecules to accomplish their tasks, a feature that renders them highly dependent on cellular context. Unfortunately, only two partner proteins to have so far been rigorously identified for Dok-4. The best studied of these is the receptor tyrosine kinase (RTK) Ret [3]. This interaction involves phosphorylated tyrosine residue 1062 of Ret presumably binding to the Dok-4 PTB domain since Y1062 represents a known site of Dok-1 and Shc PTB binding [8–10]. Another RTK, Tie2, also presumably binds Dok-4 through a PTB/phospho-Tyr dependent mechanism, though this has yet to be directly studied [3].

Despite often limited primary amino acid (aa) sequence homology, PTB domains share a highly conserved 3D structure. This consists of seven anti-parallel β-strands forming a sandwich of two orthogonal β-sheets capped on one side by a C-terminal α-helix of varying length. In most cases, peptide binding is mediated by residues from the β5 strand and the C-terminal α-helix of the PTB domain [11]. Although many PTB domains can bind their target proteins independently of a phosphotyrosine residue, the most conserved consensus target sequence for PTB domains is a phosphorylated NPXY motif.

To date, the boundaries of Dok family PTB domains have been deduced from their sequence homology to IRS-1. The validity of

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this approach has been confirmed by crystallographic analysis of the Dok-1 PTB domain bound to a phosphorylated Ret peptide [11]. However, we have previously shown that a similarly defined Dok-4 PTB domain displayed poor interaction with a known Dok-4 partner, Ret [5]. Unfortunately, the 3D structure of the Dok-4 PTB domain has not yet been described and crystallographic analysis of the highly homologous Dok-5 PTB domain using a peptide corresponding to aa 127–233 has been attempted but not completed [12].

In the current study, we have explored in more detail the structure and function of the Dok-4 PTB domain.

## 2. Materials and methods

### 2.1. Plasmids and cDNAs

The expression vectors pME18S-Fyn Y528F, pCDNA3.1-Ret C634R, pCDNA3.1-Dok-4-Myc/His were described previously [5]. Additional Dok-4 constructs (point mutants G207A and R186H; deletion mutant Δ130–233) were generated by PCR, cloned in frame in pCDNA3.1-Myc/His as previously described [5] and fully sequenced. The pEGFP-N1-Ship1 wild type and Y918/1021F mutant as well as pCDNA-Ship1 constructs were obtained from Dr. Gerry Krystal. The c-Abl cDNA was obtained from Addgene (pCX-EGFP-Abl) and subcloned into the pcDNA3.1 vector. The HA-Ship2 expression construct was obtained from Dr. Christina Mitchell.

### 2.2. Cells

293HEK and COS-1 cells were cultured in DMEM-high glucose containing pyridoxine–HCl and sodium pyruvate with 10% FBS.

### 2.3. Antibodies, immunoprecipitation (IP) and immunoblotting

Antibodies for Ret (C-19), Ship1 (P1C1), Myc (9E10), Fyn (Fyn3), GST (B-14), Abl (K-12) were from Santa Cruz. Anti-phosphotyrosine (4G10) was from Millipore. Dok-4 antisera were previously described [5]. Horseradish peroxidase-coupled secondary antibodies were from Jackson Immunoresearch. Cell lysis, IP and immunoblotting were performed according to standard protocol as detailed previously [5].

### 2.4. Yeast two-hybrid (Y2H) screening

Y2H screening was performed by Hybrigenics (Paris, France). A LexA-Dok-4 chimera was constructed by cloning full-length mouse Dok-4 sequence in frame with the LexA DNA-binding domain in vector pB27. To allow detection of phosphotyrosine-dependent interactions, a yeast strain expressing mammalian Lyn tyrosine kinase was used. After yeast transformation with pB27-Dok-4, titration with 3-aminotriazole (3-AT) was performed to minimize background transactivation. Yeast were then transformed with a mouse kidney library (cloned in the vector pP6) and screened for growth on His- plates with 20 mM of 3-AT.

### 2.5. Glutathione S-transferase (GST) pulldown assays

GST and GST-Dok-4 fusion proteins were created in the pGEX-2T vector (GE Healthcare) using the BamH1 and EcoRI cloning sites as described previously [5].GST-Dok-4(100–233) was described previously [5]. GST-Dok-4 (100–325) and GST-Dok-4(130–246) were generated by PCR and fully verified by sequencing. Recombinant proteins expression and pull downs assays were performed according to standard protocol as previously described [5]. Bead volume was identical for all pull-down conditions and comparable loading of GST fusion proteins was verified on blotted membranes by either Ponceau stain or anti-GST immunoblotting.

## 3. Results

### 3.1. Redefining the boundaries of the Dok-4 PTB domain

Ret, the receptor for GDNF, is one of only two confirmed partners of Dok-4, the other being Tie2, the receptor for angiopoietin [3]. Surprisingly, we had previously found that, compared to the Dok-1 PTB domain, the Dok-4 PTB domain bound Ret very poorly in GST pull-down assays [5]. We wondered if delineation of the Dok-4 PTB domain according to homology with Dok-1 and IRS-1 (i.e. aa 100–233) might have unknowingly excluded key structural elements. Indeed, a recombinant Dok-4 construct containing aa 100–325, which comprises the entire C-terminal region of Dok-4, was able to bind Ret much more efficiently in GST pull-down assays, whereas a construct limited to aa 100–233 did so only very weakly (Fig. 1A). This suggested that residues located C-terminally to aa 233 were required for proper PTB function. To clarify this, we analyzed the sequences of Dok-1 and Dok-4 with the 2D prediction algorithm PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) [13]. PSIPRED accurately predicted all of the secondary structures of the Dok-1 PTB domain defined crystallography [14] including the C-terminal α helix (α2) (Fig. 1B and data not shown) with the exception of the β5 strand. PSIPRED also predicted a similar arrangement of β-strands and α-helices for the Dok-4 PTB domain with boundaries matching those predicted by alignment with the resolved Dok-1 PTB domain [14]. However, it predicted that the α2 helix of the Dok-4 PTB domain extended to aa 246, 13 aa beyond the boundary predicted by homology to Dok-1 (Fig. 1C). Interestingly, while these 13 aa (i.e. aa 234–246) are not conserved in Dok-1, they are conserved in Dok-6 and to a lesser extent in Dok-5, where PSIPRED also predicted them to form an extended α-helix (data not shown). This suggested that, unlike Dok-1 and IRS-1, the PTB domain of Dok-4 (and perhaps other Dok-B family members) possesses an extended C-terminal α-helix, similar to that found in more distant PTB domains such as Shc and Dab1 [11,15]. Given the typical involvement of β5-strands and C-terminal α-helices of PTB domains in peptide binding [15], the integrity of this extended α-helix might be critical for binding of Dok-4 to its target proteins. To explore this possibility more directly, we created a new GST-Dok-4 PTB construct that comprised aa 131–246 of Dok-4 (including the putative extended α-helix) instead of our previously used PTB construct comprising aa 100–233 [5]. Amino acid 131 was used as the N-terminal boundary of this new construct based on homology to the resolved crystallographic structure of the Dok-1 PTB domain [14] as well as on PSIPRED analysis of Dok-4 PTB, which both pointed to aa 138 as the start of the first β-strand (data not shown). The ability of the GST-Dok-4 constructs to bind activated Ret (C634R) was then compared in pull-down assays. As shown in Fig. 1D, whereas the GST-Dok-4(100–233) fusion protein bound barely more Ret than did GST alone, the GST-Dok-4(131–246) chimera could bind Ret more strongly, similar to the larger GST-D4(100–325) chimera. These results confirmed that the Dok-4 PTB domain is contained within aa 131–246 and that the C-terminal extension of its boundary is necessary for binding to one of its target proteins.

### 3.2. Dok-4 interacts with Ship1 but not Ship2

In order to identify novel phosphotyrosine-dependent interactions involving Dok-4, we performed yeast two-hybrid (Y2H) screen using full-length Dok-4 as bait with co-expression of Lyn tyrosine kinase in a mouse kidney library (see Section 2). A total