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STRUCTURE NOTE

Solution structure of the SH3 domain of DOCK180

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ABSTRACT

DOCK180 family proteins are Rho guanine nucleotide exchange factors. DOCK1-5 contains an N-terminal SH3 domain implicated in their autoinhibition. Release of the closed conformation requires the interaction between SH3 and engulfment and cell motility (ELMO). Here, we solved the solution structure of DOCK180 SH3 domain, which shares similar target binding features with the SH3 domain of DOCK2. The conserved N-terminal extension packs with the SH3 core domain and forms a new target binding site distinct from the canonical "PxxP" site. Our results demonstrate that the bidentate target binding mode of DOCK180 SH3 domain might be a general feature in all DOCK proteins.

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Key words: atypical SH3; NMR spectroscopy; DOCK180

INTRODUCTION

DOCK180 (DOCK1) represents an evolutionarily conserved family of proteins functioning as guanine nucleotide exchange factors for Rho GTPases,[1–4] which contains an N-terminal Src homology (SH3), two central DOCK homology regions DHR-1 and DHR-2, and a PxxP region in the C-terminus. It has been suggested that the SH3 of DOCK180 binds directly to its DHR-2 to inhibit the activity of the protein in the steady state[5] and release of the autoinhibited state results from binding to engulfment and cell motility (ELMO) protein.[6–8] The binding interface between DOCK180 and ELMO was proposed to involve two sites: the SH3 domain of DOCK180 binds to a proline-rich (PxxP) motif at the C-terminus of ELMO,[7] and the adjoining helical region (aa 69-187) binds to the atypical PH domain of ELMO.[9] However, the structural basis of SH3-mediated DOCK180-ELMO interaction is unclear.

In this work, we solved the solution structure of DOCK180 SH3 domain, which adopts an atypical SH3 fold. A 12-residue conserved N-terminal extension packs with the SH3 core domain and thus forms a new target binding site distinct from the canonical "PxxP" site. Further structural analysis reveals

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that the DOCK180 atypical SH3 domain interacts with a bidentate ELMO1 peptide, and this binding mode is likely conserved in all SH3-containing DOCK proteins.

MATERIALS AND METHODS

DNA sequences encoding mouse DOCK180 SH3 domain (residues 1–74) and the N-terminal deletion mutant (residues 9–74) were individually cloned into a pGEX-4T-1 vector. The resulting SH3 domain constructs contained a Glutathione S-transferase (GST)-tag in the N-terminus. Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) host cells at 16°C and were purified by GSH-sepharose affinity chromatography followed by size-exclusion chromatography. The N-terminal GST-tagged peptide fragment was then cleaved by digesting the fusion protein with thrombin, and the proteins were purified by another step of size-exclusion chromatography. Uniformly ^{15}N or ^{15}N, ^{13}C-labeled SH3 proteins were prepared by growing bacteria in M9 minimal medium using $^{15}NH_4Cl$ as the sole nitrogen source or $^{15}NH_4Cl$ and $^{13}C_6$-glucose (Cambridge Isotope Laboratories) as the sole nitrogen and carbon sources, respectively.

NMR samples contained 0.1 m*M* or 0.4 m*M* of the SH3 proteins in 50 m*M* Phosphate Buffered Saline (PBS, pH 6.5), with 50 m*M* NaCl, and 1 m*M* ethylenediaminetetraacetic acid (EDTA) in 90% H_2O/10% D_2O or 99.9% D_2O. All NMR experiments were performed at 25°C on a Varian VNMRS 600 MHz spectrometer equipped with a 5-mm HCN cryoprobe. Backbone and side-chain resonance assignments of SH3 were achieved by the standard heteronuclear correlation experiments.[10] The side chains of aromatics were assigned using ^1H two-dimensional total correlation spectroscopy/NOESY experiments.[11] Approximate inter-proton distance restraints were derived from NOESY experiments (a 2D homonuclear ^1H NOESY, a 3D ^{15}N-separated-NOESY, and a 3D ^{13}C-separated NOESY). Hydrogen bonding restraints were generated from the standard secondary structure of the protein based on the NOE patterns and backbone secondary chemical shifts. Backbone dihedral angle restraints (Φ and Ψ angles) were derived from the chemical shift analysis program TALOS.[12] CYANA was used to calculate 20 structures from random starting conformers.[13] The conformer with the lowest penalty function value was then used as input for the refinement using the torsion angle-simulated annealing calculations in Crystallography & NMR System (CNS).[14] The final 20 structures out of total 200 structures computed were validated by PROCHECK[15] and displayed for further analysis by PyMOL (DeLano Scientific LLC). The atomic coordinates and assigned resonances of DOCK180 SH3 were deposited to the Protein Data Bank and the Biological Magnetic Resonance Bank under the accession codes 2m0y and 18832, respectively.

For paramagnetic pseudo-contact shifts perturbation assay, a total of 4 mL of fully reduced ^{15}N-labeled DOCK180 SH3 mutant proteins (50 μM protein concentration in 50 m*M* Tris buffer, pH 8.0 containing 100 m*M* NaCl) was incubated with 10-fold excess of *S*-(2-pyridylthione)-cysteaminyl-EDTA (Toronto Research Chemicals). The reaction was carried out for 3 h at room temperature. Excess reagent and any side products were removed by exchanging the reaction mixture into 50 m*M* PBS buffer (pH 6.5) containing 50 m*M* NaCl using a PD-10 column.

Fluorescence assays were performed on a PerkinElmer LS-55 fluorimeter equipped with an automated polarizer at 25°C. In a typical assay, a fluorescein isothiocyanate (Molecular Probes)-labeled peptide ($\sim$1 μM) was titrated with DOCK180 SH3 in 50 m*M* PBS pH 6.5 buffer containing 50 m*M* NaCl, 1 m*M* Dithiothreitol (DTT), and 1 m*M* EDTA. The K_d values were obtained by fitting the titration curves with the Langmuir binding model.[16]

Isothermal titration calorimetry (ITC) measurements were performed on an ITC200 Micro calorimeter (MicroCal) at 18°C. All protein samples were dissolved in 50 m*M* PBS pH 6.5 buffer containing 50 m*M* NaCl, 1 m*M* DTT, and 1 m*M* EDTA.

RESULTS AND DISCUSSION

The three-dimensional structure of the DOCK180 SH3 domain (aa 1–74) was determined to a high resolution using NMR spectroscopy [Fig. 1(A), and Table I]. Except for the N- (aa 1-12) and C- (aa 70-74) termini, all the other regions of the domain are well defined [Fig. 1(A)]. The SH3 core domain of DOCK180 shares the typical β-barrel fold described for other SH3 domains containing five anti-parallel β strands arranged into two orthogonal β sheets [Fig. 1(B)]. It is noted that an evolutionarily conserved short fragment (aa 1-12) at the extreme N-terminal end of SH3 [Supporting Information Fig. S1], which is predicted not to be a part of SH3 domain based on protein domain analysis databases including SMART, plays a role in stabilizing the domain in solution. Deletion of the entire N-terminal fragment led to misfolding/aggregation of the SH3 domain, as the protein formed inclusion bodies when expressed in bacteria (data not shown). Most of the resonance signals of residues 1–12 disappeared in HSQC or NOESY spectra of the SH3 domain (aa 1-74). Nevertheless, a series of NOEs could be observed between side chain protons of residues from the N-terminal fragment and the SH3 core domain [Supporting Information Fig. S2(A,B)], indicating that this fragment is not intrinsically flexible, but packs with the SH3 core domain and undergoes intermediate changes. The pack-

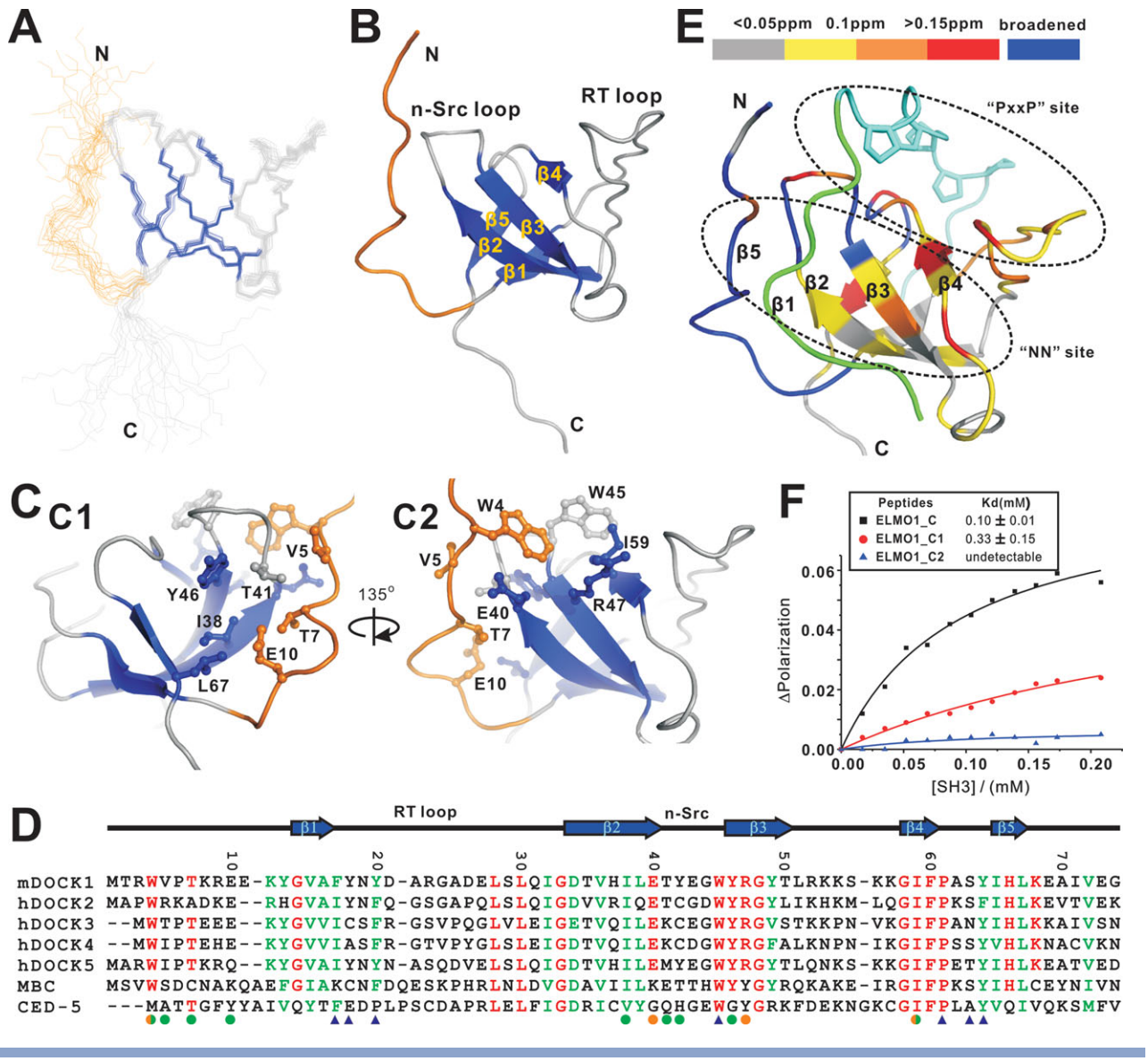

Figure 1

Solution structure of the atypical SH3 domain of DOCK180. **A**: Superposition of the backbone of the final 20 conformers. **B**: Ribbon diagram of a representative NMR structure of the DOCK180 SH3 domain. **C**: The N-terminal extension (aa 1-12) packs with the SH3 core domain. **D**: Sequence alignment of the SH3 domains of DOCK family members. In this alignment, the absolutely conserved amino acids are in red, and the highly conserved residues are in green. According to the structures of the DOCK180 SH3 (aa 1-74) and DOCK2-ELMO1 complex (PDB ID: 3A98),[17] the residues involved in the packing between the N-terminal extension and the SH3 core domain are indicated with green circles. The residues involved in direct binding to the "NN" motif are indicated with orange circles, whereas the ones involved in the binding to the "PxxP" motif are indicated with blue triangles. The secondary structures of SH3 domain are indicated at the top of the alignment. MBC (Myoblast city) and CED-5 are the *Drosophila melanogaster* and *Caenorhabditis elegans* orthologs of DOCK180. **E**: NMR chemical shift perturbation-based assay showing the bidentate recognition mode of DOCK180 SH3 toward the ELMO1 C-terminal peptide. The combined ^{1}H and ^{15}N chemical shift changes are defined as: $\Delta_{ppm} = [(\Delta\delta_{HN})^2 + (\Delta\delta_N \times \alpha_N)^2]^{1/2}$. $\Delta\delta_{HN}$ and $\Delta\delta_N$ represent chemical shift differences of amide proton and nitrogen chemical shifts of DOCK180 SH3 domain upon ELMO1 peptide binding. The scaling factor (α_N) used to normalize the ^{1}H and ^{15}N chemical shifts is 0.17. The coloring scheme is represented using a horizontal bar at the top. The DOCK180 SH3/ELMO1 complex was built as a model according to the DOCK2/ELMO1 complex (PDB ID: 3A98).[17] In this drawing, the "PxxP" motif is shown in cyan and the "NN" motif in green. **F**: Fluorescence polarization-based measurement of the binding affinities of the ELMO1 C-terminal peptide and its two fragments with DOCK180 SH3 domain.

ing force between the N-terminal fragment and the SH3 core domain is mainly mediated by hydrophobic interactions. Specifically, the bulky aromatic side chain of Trp4 packs with the side chains of Trp45, Arg47, and Ile59 [Fig. 1(C2)]. Val5 and Thr7 make extensive contacts with Ile38 and Thr41. The hydrophobic interactions between the aliphatic side chain of Glu10 and the side chains of Ile38 and Leu67 further strengthen the

Table I
Structural Statistics for the 20 Structures of the DOCK180 SH3 Domain[a]

Restraint statistics	
Distance restraints	
Intraresidue ($i - j = 0$)	341
Sequential ($\|i - j\| = 1$)	230
Medium range ($2 \leq \|i - j\| \leq 4$)	84
Long range ($\|i - j\| > 5$)	421
Hydrogen bonds	32
Total	1108
Dihedral angle restraints	
Φ	38
Ψ	39
Total	77
Structure statistics	
NMR assignment statistics (%)	
Backbone assignment	88.5
Side chain assignment	93.0
Aromatic assignment	86.8
Mean r.m.s. deviations from the experimental restraints	
Distance (Å)	0.004 ± 0.000
Dihedral angle (°)	0.221 ± 0.031
Mean r.m.s. deviations from idealized covalent geometry	
Bond (Å)	0.001 ± 0.000
Angle (°)	0.308 ± 0.005
Improper (°)	0.130 ± 0.010
Mean energies (kcal mol^{-1})	
E_{NOE}[b]	1.01 ± 0.14
E_{cdih}[b]	0.23 ± 0.07
$E_{L\text{-}J}$	−258 ± 12
Ramachandran plot[c] (%)	
Most favorable regions	72.2
Additional allowed regions	23.7
Generously allowed regions	3.0
Disallowed regions	1.1
Coordinate precision	
Atomic r.m.s. difference (Å)[d]	
Residues 13–69	
Backbone heavy atoms (N, Cα, and C′)	0.34
Heavy atoms	0.95

[a]None of the structures exhibits distance violations greater than 0.3 Å or dihedral angle violations greater than 4°.
[b]The final values of the square-well NOE and dihedral angle potentials were calculated with force constants of 50 kcal mol^{-1} $Å^{-2}$ and 200 kcal mol^{-1} rad^{-2}, respectively.
[c]The program Procheck[15] was used to assess the overall quality of the structures.
[d]The precision of the atomic coordinates is defined as the average r.m.s. difference between 20 final structures and the mean coordinates of the protein.

packing between the N-terminal fragment and the SH3 core domain [Fig. 1(C1)].

To further demonstrate the interaction between the N-terminal fragment and the SH3 core, the N-terminus was tagged with *S*-cysteaminyl-EDTA, an EDTA derivative used to detect long-range interactions in proteins using paramagnetic pseudo-contact shifts.[18,19] Cys was introduced into the protein at site Lys8 to achieve site-specific paramagnetic tagging. Comparison of the HSQC spectra of the mutant with that of the wild type protein indicated minimal mutation-induced structural changes of the protein [full data not shown, see Supporting Information Figs. S2(C) and S3(D)]. When compared with the ^{1}H, ^{15}N-HSQC spectrum of diamagnetic EDTA-derivatized K8C, addition of Co^{2+} led to chemical shift changes or peak broadening limited to the neighborhood of β2 [Supporting Information Fig. S2(C)]. It is noted that K8C mutation somehow slightly alters the local environment of β2, as peak doubling could be observed for residues 38–40 from β2 even in the absence of paramagnetic metal ions [Supporting Information Fig. S2(C1)], which in a way demonstrates the correlation between the N-terminal fragment and β2. Taken together, the paramagnetic pseudo-contact shift perturbation data further demonstrate that the N-terminal fragment (aa 1-12) physically packs along the β2 strand of the SH3 core domain.

While this manuscript was in preparation, Hanawa-Suetsugu *et al.* arrived to similar conclusions analyzing the structure of the DOCK2:ELMO1 complex.[17] An N-terminal extension (residues 1–11) packs with the β2 strand of the DOCK2 SH3 core domain [Supporting Information Fig. S3(A)], and thus, forms a new binding site accommodating the C-terminal region (Asn719-Asn727) downstream of the "PxxP" motif of ELMO1 ("NN" binding site hereafter). It is noteworthy that the residues involved in ELMO1 binding (including both "PxxP" and "NN" motifs), as well as those responsible for the packing between the N-terminal extension and the SH3 core domain, are highly conserved in DOCK180 and DOCK2, and other SH3-containing DOCK proteins [Fig. 1(D) and Supporting Information Fig. S3(A–C)].

To test whether the N-terminal extension of DOCK180 SH3 plays the same role in ELMO1 binding by forming the new "NN" binding site, we compared the ^{1}H, ^{15}N-HSQC spectra of DOCK180 SH3 domain in the absence and presence of the ELMO1 C-terminal bidentate peptide (ELMO1_C, Ile704-Asn727) [Supporting Information Fig. S3(D)]. A number of residues underwent significant chemical shift changes upon addition of ELMO1_C. We mapped the ELMO1_C-induced chemical shift changes of DOCK180 SH3 to the three-dimensional structure of the domain and found that ELMO1_C-binding requires two sites: one is the canonical "PxxP" site of SH3, and the other is the newly formed "NN" site [Fig. 1(E)], demonstrating that the atypical SH3 domain of DOCK180 binds to ELMO1_C in a bidentate mode as that of DOCK2. In line with this result, W4A mutation, which is supposed to impair the packing between the N-terminal fragment and the SH3 core, as well as the interaction of SH3 with the "NN" motif led to nearly three fold decrease of the binding affinity to ELMO1_C than that of the wild type protein (0.10 m*M* vs. 0.28 m*M*) [Supporting Information Fig. S3(E)].

Furthermore, to evaluate the contributions of the two packing regions to the interaction between SH3 and ELMO1_C, we divided the ELMO1_C peptide into two fragments (ELMO1_C1, Ile704-Ser718 and ELMO1_C2, Asn719-Asn724). Consistent with the fluorescence-based binding data between the SH3 W4A mutant and ELMO1_C [Supporting Information Fig. S3(E)], the

ELMO_C1 peptide binds specifically to the "PxxP" site, with threefold lower binding affinity to DOCK180 SH3 than that of the full-length peptide (0.10 m*M* vs. 0.33 m*M*) [Fig. 1(F) and Supporting Information Fig. S3(F)], whereas ELMO_C2 shows undetectable binding to DOCK180 SH3 [Fig. 1(F)], indicating that the two weak target binding sites of the atypical SH3 domain of DOCKs might function cooperatively in binding to a bidentate peptide. In contrast, although several "PxxP" motifs (e.g., "^{1796}xxxPPxLPxxx^{1806}") have been found in the C-terminal proline-rich region of DOCK180, neither of them has a subsequent "NN" motif [Supporting Information Fig. S4(A)]. Moreover, two critical proline residues (Pro707 and Pro717) that strengthen the "PxxP"-SH3 packing in the ELMO1-DOCK2 complex are missing in the DOCK180 C-terminal "PxxP" motifs (e.g., "^{1796}xxxPPxLPxxx^{1806}" vs. "^{707}PxxPPxIPxxP^{717}").[17] Thus, the DOCK180 C-terminal "PxxP" motifs display very weak binding to the SH3 domain [Supporting Information Fig. S4(B)], implying that the C-terminal proline-rich region might be constitutively free for CrkII sequestering.[20]

Taken together, our structural and biochemical data reveal that the bidentate target binding property of the atypical SH3 domain is a general feature of DOCK family proteins. Importantly, our solution structure of the DOCK180 SH3 (aa 1-74) clearly demonstrate that the new "NN" binding site is formed before ELMO1 binding through the extensive interaction between the N-terminal extension and the SH3 core domain. Consistent with this, the paramagnetic pseudo-contact shift perturbation patterns of K8C (located in close vicinity of one edge of the "NN" site) in the absence and presence of ELMO1 are similar [Supporting Information Fig. S2(C,D)]. Although the "NN" motif is highly conserved in most ELMO family proteins,[9,17] it needs further investigation to verify whether this motif is also involved in other DOCK SH3/protein interactions. Moreover, Ile32 which plays critical role in the autoinhibitory SH3:DHR-2 interaction is located beyond the "PxxP" and "NN"-binding sites,[5] implying the possible presence of the third target binding site in the DOCK180 atypical SH3.

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