Identification and kinetic analysis of the interaction between Nck-2 and DOCK180

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Abstract Nck-2 is a newly identified adapter protein comprising three N-terminal SH3 domains and one C-terminal SH2 domain. We have identified in a yeast two-hybrid screen DOCK180, a signaling protein implicated in the regulation of membrane ruffling and migration, as a binding protein for Nck-2. Surface plasmon resonance analyses reveal that the second and the third SH3 domains interact with the C-terminal region of DOCK180. The interactions mediated by the individual SH3 domains, however, are much weaker than that of the full length Nck-2. Furthermore, a point mutation that inactivates the second or the third SH3 domain dramatically reduced the interaction of Nck-2 with DOCK180, suggesting that both SH3 domains contribute to the DOCK180 binding. A major Nck-2 binding site, which is recognized primarily by the third SH3 domain, has been mapped to residues 1819–1836 of DOCK180. Two additional, albeit much weaker, Nck-2 SH3 binding sites are located to DOCK180 residues 1793–1810 and 1835–1852 respectively. Consistent with the mutational studies, kinetic analyses by surface plasmon resonance suggest that two binding events with equilibrium dissociation constants of $4.15 \pm 1.9 \times 10^{-7}$ M and $3.24 \pm 1.9 \times 10^{-9}$ M mediate the binding of GST-Nck-2 to GST fusion protein containing the C-terminal region of DOCK180. These studies identify a novel interaction between Nck-2 and DOCK180. Furthermore, they provide a detailed analysis of a protein complex formation mediated by multiple SH3 domains revealing that tandem SH3 domains significantly enhance the weak interactions mediated by each individual SH3 domain.

Key words: DOCK180; Nck-2; SH3 domain; Protein–protein interaction; Two-hybrid assay; Surface plasmon resonance

1. Introduction

Protein–protein interactions are critical events in signal transduction controlling cell migration, cytoskeleton organization, proliferation and differentiation. Many of the protein–protein interactions are mediated by structurally conserved protein binding modules such as Src homology-3 (SH3) and -2 (SH2) domains [1]. SH2 domains bind to phosphorylated tyrosine residues in specific sequence contexts and therefore protein–protein interactions mediated by SH2 domains are regulated by tyrosine phosphorylation. SH3 domains contain approximately 60 amino acid residues that fold into compact structures consisting of two small anti-parallel β sheets packed against each other [1,2]. SH3 domains typically recognize relatively short, proline-rich sequences and interactions mediated by individual SH3 domains are typically quite weak, with K_D values in the μM range [1–3].

Nck is a family of ubiquitously expressed adapter proteins comprising primarily three N-terminal SH3 domains and one C-terminal SH2 domain. The first member of the Nck family (Nck-1 or Nckα, previously known as Nck) was cloned from screening of a human melanoma cDNA library [4]. The second member of the Nck family, Nck-2 [5] (also known as Nckβ [6] or Grb4 [7]) was identified and cloned recently. Nck proteins are implicated in cellular signal transduction regulating cytoskeleton organization, growth and gene expression [8]. For example, genetic studies have demonstrated that mutations in the *Drosophila* Nck homolog dreadlocks (DOCK) disrupted growth-cone guidance and signaling [9,10], a process involving formation of filopodial and lamellipodial protrusions and coordination between signal transduction pathways mediated by cell adhesion molecules, small GTPases of Rho family, and receptor tyrosine kinases. The importance of the Nck proteins in regulation of fundamental cellular processes has attracted much attention on the molecular mechanisms whereby they function in signal transduction. One of the important structural features of Nck proteins is that they contain multiple SH3 domains in tandem. In this study, we have utilized yeast two-hybrid screens to identify proteins that interact with the Nck-2 SH3 domains. Our results show that DOCK180, a protein that has been implicated in integrin- and Rac-signaling, membrane ruffling and cell migration [11–13], specifically interacts with the second and the third SH3 domains of Nck-2. Furthermore, using a combination of mutagenesis and surface plasmon resonance methods, we have identified DOCK180 sequences that are recognized by Nck-2 SH3 domains and carried out a kinetic analysis of the Nck-2–DOCK180 interaction.

2. Materials and methods

2.1. Cells, antibodies and other reagents

Human 293 embryonal kidney cells were from American Type Culture Collection (Rockville, MD, USA). Human A431 epidermoid carcinoma cells were kindly provided by Dr. Jeffrey E. Kudlow (University of Alabama at Birmingham, AL, USA). Media for cell culture were from Gibco Laboratories (Grand Island, NY, USA) or Mediatech/Cellgro^R (Herndon, VA, USA). Anti-DOCK180 antibody was from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-GST antibody was from Sigma. Restriction enzymes, DNA modifying en-

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zymes, DNA molecular weight markers and dideoxyribonucleotide triphosphates (dNTPs) were purchased from Promega. Synthetic oligonucleotides were prepared by Gibco/BRL.

2.2. Yeast two-hybrid assays

A cDNA fragment encoding the third SH3 domain of human Nck-2 (amino acid residues 176–274) was amplified by PCR and inserted into the *Eco*RI/*Xho*I site in the pLexA vector (Clontech). The sequence of the bait construct (pLexA/SH3) was verified by DNA sequencing and introduced into EGY48[P8OP-lacZ] yeast cells by transformation. The transformants were used to screen a human lung MATCHMAKER LexA cDNA library ($>5.7\times10^6$ independent clones, Clontech) as previously described [5,14]. Briefly, the EGY48-[p8op-lacZ; pLexA/SH3] cells transformed by the library plasmids were selected by plating on the SD/-His/-Ura/-Trp/-Leu/X-gal medium (Clontech). The expression of proteins encoded by the pB42AD/libaray vectors was induced by growing the cells in the presence of galactose (SD/Gal/Raf/-His/-Ura/-Trp medium, Clontech). Forty positive clones, as indicated by activation of both reporter genes (*LEU2* and *lacZ*), were identified. pB42AD plasmids were isolated from the positive yeast clones and used to transform *Escherichia coli* KC8 cells. The KC8 cells containing the pB42AD vectors were selected by growing in medium lacking tryptophan. The pB42AD plasmids were isolated from the KC8 cells and analyzed by restriction digestion. Nineteen representative plasmids were selected based on the results from the restriction digestion analyses. The SH3 binding activity was further tested by yeast two-hybrid binding assays using purified pB42AD plasmids as described [5,14]. Sequences of the cDNA inserts in the confirmed plasmids were determined by DNA sequencing.

2.3. Site-directed mutagenesis

A QuickChange™ site-directed mutagenesis system (Stratagene) was used to change the highly conserved tryptophan residues (amino acid residues 148 and 234) in the second and third SH3 domains of Nck-2 to lysine. The point mutations were confirmed by DNA sequencing using Sequenase Version 2.0 kit (United States Biochemicals).

2.4. DNA sequencing

Sequences of DNA fragments were determined manually using Sequenase Version 2.0 kit (United States Biochemicals).

2.5. Expression of recombinant GST fusion proteins containing Nck-2 and DOCK180 sequences

To generate GST fusion proteins, full length or partial human Nck-2 and DOCK180 cDNA sequences (as specified in each experiment) were amplified by PCR and inserted into the *Eco*RI/*Xho*I site of a pGEX-5x-1 vector (Pharmacia). The recombinant vectors were then used to transform *E. coli* M20 cells. The expression of the GST fusion proteins was induced with IPTG, and the proteins were purified with glutathione-Sepharose 4B beads.

2.6. Co-precipitation assays

Human 293 embryonal kidney cells were cultured in Eagle's MEM medium supplemented with 10% FBS. Human A431 epidermoid carcinoma cells were grown in DMEM medium supplemented with 10% FBS. Cells were washed once with cold PBS and lysed with the lysis buffer (0.5% Triton X-100 in 50 mM HEPES buffer, pH 7.1, containing 100 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 200 μm sodium orthovanadate, 0.2 mM AEBSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin A and 5 μg/ml leupeptin). The lysates were clarified by centrifugation at $10\,000\times g$ for 15 min and pre-incubated with glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 0.5 h. The beads were removed by centrifugation at $3000\times g$ for 5 min and the clarified cell lysates were incubated with equal amounts (as specified in each experiment) of GST fusion proteins containing the wild-type or mutant forms of Nck-2, or GST alone as a negative control. At the end of the incubation, the solutions were mixed with glutathione-Sepharose 4B beads, incubated for 1 h or longer, and the GST fusion proteins were then precipitated with glutathione-Sepharose 4B beads by centrifugation. The precipitates were washed five times with washing buffer (0.2% Triton X-100 in 50 mM HEPES buffer, pH 7.1, containing 100 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 200 μm sodium orthovanadate, 0.2 mM AEBSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin A and 5 μg/ml leupeptin). After washing, DOCK180 that was associated with the GST-Nck-2 fusion proteins was detected by immunoblotting with a rabbit anti-DOCK180 antibody, a horseradish peroxidase-conjugated anti-rabbit IgG antibody (27 ng/ml) and the SuperSignal^R chemiluminescent substrate (Pierce).

2.7. Analyses of DOCK180–Nck-2 interaction by surface plasmon resonance

Surface plasmon resonance analyses were performed using a BIAcore system as previously described [15].

2.7.1. Interactions between Nck-2 and DOCK180 fusion proteins. 2135 resonance units (RU) of GST fusion protein containing DOCK180 residues 1754–1865 (2.1 ng/mm^2) were covalently coupled to one flow cell of a CM5 sensor chip (BIAcore, Inc., Piscataway, NJ, USA) using the BIAcore Amine Coupling Kit as described [16]. The surface was then blocked with ovalbumin (Sigma, St. Louis, MO, USA) and neutralized with ethanolamine. A second (control) flow cell was blocked with ovalbumin and ethanolamine, but was not exposed to the GST fusion protein containing the C-terminal region of DOCK180. GST fusion proteins containing the full length Nck-2 or its SH domains were injected over both flow cells at a rate of 20 μl/min for 1.5 min. Dissociation was then monitored for 3 min. The interaction of the C-terminal region of DOCK180 with Nck-2 (or Nck-2 SH domains) was disrupted with 5 μl of 20 mM NaOH between injections. In some experiments, use of NaOH was avoided by allowing the proteins to dissociate for 5 h between injections. To test the integrity of the DOCK180 surface, full length Nck-2 was injected last. The control (ovalbumin) binding curve was subtracted from the experimental (DOCK180) binding curve to remove 'bulk effects' resulting from refractive index changes due to the presence of the protein in the buffer.

2.7.2. Binding of Nck-2 fusion proteins to DOCK180 peptides. The following DOCK180 peptides were biotinylated at the N-termini to facilitate immobilization of the peptides to BIAcore sensor chips: peptide 1 containing DOCK180 residues 1758–1775 (SPSSPSSQQTPPPVTPRA), peptide 2 containing DOCK180 residues 1793–1810 (ADVADVPPPLPLKGSVAD), peptide 3 containing DOCK180 residues 1819–1836 (DLLGSPTPPPPPPHQRHL) and peptide 4 containing DOCK180 residues 1835–1852 (HLPPPLPSKTPPPPPPKT). Each biotinylated peptide was injected in HBS buffer, pH 7.3, over one of four flow cells of a streptavidin-coated BIAcore SA chip. This resulted in rapid and essentially irreversible binding. GST-Nck-2 wild-type and its mutants and fragments, or GST alone, were then injected over all four flow cells at a rate of 5 μl/min to determine binding to each peptide. Resulting sensorgrams were trimmed and scaled for presentation using BIAcore BIAevaluation software, version 3.0. The experiments were repeated using different amounts of immobilized peptides (to avoid the potential mass transfer effects). In experiments (Fig. 4C) where a smaller amount of the peptides was immobilized, the data represent the peptide 1 curve (in which binding was negligible) subtracted from the peptide 3 curve. This was required to normalize for refractive index changes due to differences in buffer composition, due to the smaller signal to noise ratio resulting from the small amount of ligand immobilized.

2.7.3. Kinetics experiments. GST fusion protein containing the C-terminal region of DOCK180 was covalently bound to a flow cell as described above, except in lower concentration to minimize 'mass transport' effects, i.e. binding limited by the rate of delivery of GST-Nck-2 to the DOCK180 surface. GST-Nck-2 solutions were injected at 50 μl/min at concentrations of 0.5, 1.0, 1.5, and 2.0 μM. Equilibrium and kinetic rate constants were determined using BIAevaluation version 2.1 software (BIAcore, Inc., Piscataway, NJ, USA) and a two-site binding model. K_D1 and K_D2 (equilibrium constants) were calculated from k_d1/k_a1 and k_d2/k_a2 (ratios of rate constants).

3. Results and discussion

3.1. Identification of DOCK180 as a Nck-2 binding protein by yeast two-hybrid screens

To identify proteins that interact with Nck-2 SH3 domains, we screened a human lung LexA cDNA library ($>5.7\times10^6$ independent clones) with a bait construct (pLexA/SH3^3) encoding the third SH3 domain (SH3^3) of Nck-2 (amino acid

residues 176–274). Forty positive clones were isolated. Plasmids (pB42AD) from the positive clones were purified and analyzed by restriction digestion. Nineteen representative pB42AD plasmids were selected based on restriction digestion results. Proteins encoded by the purified pB42AD plasmids were further tested for their ability to interact with Nck-2 $SH3^3$ in yeast two-hybrid binding assays. Inserts from six plasmids encoding fusion proteins that exhibited strong and reproducible Nck-2 SH3 binding activity were sequenced. One cDNA insert was found to encode the C-terminal region (residues 1754–1865) of DOCK180, a protein that has been implicated in integrin- and Rac-signaling [11–13]. In control experiments, elimination of either the DOCK180 sequence or the Nck-2 $SH3^3$ sequence abolished the interaction (Table 1). Furthermore, the first SH3 domain of Nck-2, unlike the third SH3 domain, was unable to interact with the DOCK180 fusion protein (Table 1). Taken together, these results suggest that DOCK180 is a specific binding target of the third SH3 domain of Nck-2.

3.2. Biochemical binding assays confirm the interaction of the full length Nck-2 with DOCK180

To confirm the yeast two-hybrid binding results, we tested the ability of Nck-2 to interact with DOCK180 in a biochemical GST fusion protein pull-down assay. To do this, we incubated mammalian cell lysates with GST-Nck-2, or GST as a negative control. GST-Nck-2 and GST were precipitated with glutathione-Sepharose 4B beads. Immunoblotting analyses of the GST-Nck-2 precipitates showed that DOCK180 was co-precipitated with GST-Nck-2 (Fig. 1, lane 3). No DOCK180 was detected in the GST precipitate (Fig. 1, lane 2). Thus, consistent with the yeast two-hybrid results obtained with the Nck-2 $SH3^3$ domain and the DOCK180 C-terminal sequence, the full length Nck-2 is capable of interacting with the full length DOCK180 expressed by mammalian cells.

3.3. Surface plasmon resonance detection of the Nck-2 binding to the DOCK180 C-terminal region

We next employed surface plasmon resonance, which is a powerful tool for real time measurement of direct protein–protein interactions [17–20], to further characterize the interaction between Nck-2 and the C-terminal region of DOCK180. To do this, we covalently coupled a recombinant GST fusion protein containing the DOCK180 sequence (residues 1754–1865) to a BIAcore sensor chip. GST-Nck-2, or a GST fusion protein containing Nck-2 SH2 domain (GST-SH2) as a control, was passed over it by injecting 30 μl at a concentration of 1 μM. Nck-2 bound to the recombinant DOCK180 C-terminal fragment specifically and reversibly (Fig. 2A, curve a). By marked contrast, GST-SH2 was unable to interact with the DOCK180 C-terminal fragment (Fig. 2A, curve b). These results are highly consistent with the data from the yeast two-hybrid and GST fusion protein pull-down assays and show that the Nck-2–DOCK180 interaction can be readily detected by surface plasmon resonance.

Table 1
Identification of DOCK180 as a Nck-2 binding protein by yeast two-hybrid binding assays

pLexA construct	pB42AD construct	Reporter gene	
		LEU2	*lacZ*
pLexA-$SH3^3$	pB42AD-DOCK180	+	+
pLexA-$SH3^3$	pB42AD	−	−
pLexA-lamin C	pB42AD-DOCK180	−	−
pLexA-$SH3^1$	pB42AD-DOCK180	−	−
pLexA-$SH3^3$ ($W_{234} \rightarrow K$)	pB42AD-DOCK180	−	−

pLexA-$SH3^3$, pLexA-$SH3^3$ ($W_{234} \rightarrow K$), pLexA-$SH3^1$ and pLexA-lamin C encode LexA fusion proteins containing the third SH3 domain of Nck-2 (residues 176–274), the Nck-2 $SH3^3$ point mutant in which the highly conserved tryptophan was substituted with a lysine residue, the first SH3 domain of Nck-2 (residues 1–76) and an irrelevant protein lamin C respectively. pB42AD-DOCK180 encodes an AD fusion protein containing the C-terminal region of DOCK180 (residues 1754–1865). Yeast two-hybrid binding assays were performed as described in Section 2.

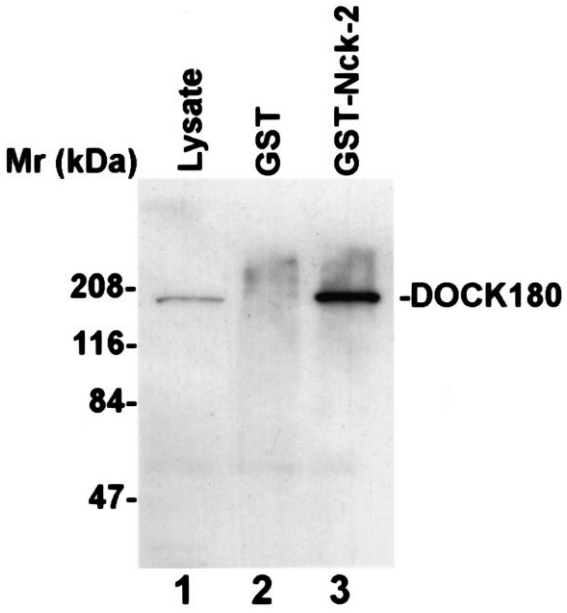


Fig. 1. Co-precipitation of DOCK180 with GST-Nck-2. A431 cell lysates (350 μg) were incubated with 10 μg of GST-Nck-2 (lane 3) or 10 μg of GST as a control (lane 2), for 2 h at 4°C. GST-Nck-2 and GST were precipitated and associated DOCK180 was detected by immunoblotting with an anti-DOCK180 antibody as described in Section 2. Lane 1 was loaded with 14 μg of A431 cell lysates. Similar results were obtained with 293 cell lysates (not shown in the figure).

3.4. Substitution of the highly conserved tryptophan with a lysine disrupts the binding of the Nck-2 SH3 domains to DOCK180

SH3 domains contain a highly conserved tryptophan residue located immediately C-terminal to the n-Src loop. Previous studies have shown that replacement of this highly conserved tryptophan with a lysine disrupts protein binding activities of several SH3 domains (e.g. the SH3 domains of Abl and Crk) [10,21,22]. To test whether this highly conserved tryptophan is also required for Nck-2 $SH3^3$ to interact with the C-terminal region of DOCK180, we substituted it with a lysine residue. The binding of the $W \rightarrow K$ point SH3 mutant was first tested in a yeast two-hybrid binding assay. The results showed that, unlike the wild-type Nck-2 $SH3^3$ domain, the $SH3^3$ $W \rightarrow K$ point mutant was unable to bind to the DOCK180 C-terminal fragment (Table 1). To further test this, we analyzed the DOCK180 binding activities of the wild-type and mutant forms of Nck-2 SH3 domains by surface plasmon resonance. The third SH3 domain, and to a less

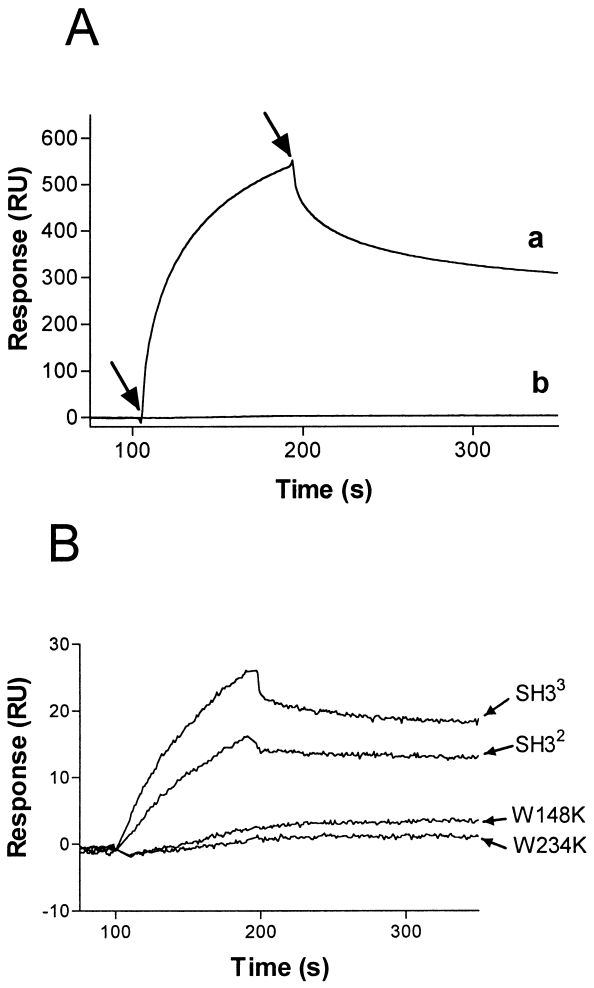


Fig. 2. Real time detection of the interaction between purified recombinant Nck-2 and DOCK180 proteins by surface plasmon resonance. A: Interaction of Nck-2 with DOCK180. Sensorgrams were obtained by injecting 30 μl of 1 μM GST-Nck-2 (a) or 1 μM GST-SH2 (b) over 2135 RU (2.1 ng/mm^2) of DOCK180 immobilized onto a flow cell of a BIAcore CM5 chip. Arrows indicate beginning and end of injections. Non-specific binding of the same injections over immobilized ovalbumin in a control flow cell of the same chip has been subtracted from the curves. B: Interactions of Nck-2 SH3 domains with DOCK180. Sensorgrams were obtained by injecting 30 μl of equal concentration (1 μM) of GST fusion proteins containing the second (SH3^2) or the third (SH3^3) SH3 domains of Nck-2 or their corresponding W→K point mutants (W148K and W234K) were injected over the same immobilized DOCK180 surface used in A. Non-specific binding to an ovalbumin surface has been subtracted.

extent the second SH3 domain, bound to the immobilized DOCK180 C-terminal fragment (Fig. 2B). However, the DOCK180 binding activities of the individual SH3 domains were markedly lower than that of the intact Nck-2 molecule (Fig. 2A and B). A point mutation at the highly conserved tryptophan in the second (position 148) or the third (position 234) SH3 domain almost completely abolished the DOCK180 binding mediated by the Nck-2 SH3 domains (Fig. 2B). Consistent with the yeast two-hybrid binding results (Table 1), the binding of the first SH3 domain to the DOCK180 C-terminal fragment was negligible (data not shown). Taken together, these results demonstrate that (1) the third SH3 domain, and to a less extent the second SH3 domain, can bind to the C-terminal region of DOCK180 and (2) the highly conserved tryptophan is critical for the binding of the Nck-2 SH3 domains to the C-terminal region of DOCK180. Additionally, the fact that the DOCK180 binding activities of the Nck-2 individual SH3 domains are much weaker than that of the intact Nck-2 suggests that the interaction of Nck-2 with the C-terminal region of DOCK180 involves multiple, cooperative bindings mediated by the two Nck-2 SH3 domains.

3.5. Multiple SH3 domains contribute to the Nck-2–DOCK180 interaction

To assess the contributions from the second and the third SH3 domains to the Nck-2–DOCK180 interaction, we generated Nck-2 point mutants in which the critical tryptophan residue located within the second (residue 148) or the third (residue 234) SH3 domain is substituted with a lysine. In addition, we generated a double mutant of Nck-2 in which the critical tryptophan residues located in the second (residue 148) and the third (residue 234) SH3 domain are substituted with lysine. GST-Nck-2 fusion proteins containing the single or the double W→K mutations were purified (Fig. 3A) and their DOCK180 binding activity was determined in a pull-down assay (Fig. 3B). As expected, the GST fusion proteins containing the wild-type Nck-2 readily interacted with DOCK180 (Fig. 3B, lane 2). The double mutations that inactivate both the second and the third SH3 domains almost completely eliminated the interaction of Nck-2 with DOCK180 (Fig. 3B, lane 5), confirming that the interaction with DOCK180 is mediated primarily by the second and the third SH3 domains. Noticeably, a single mutation that eliminates either the DOCK180 binding activity of the second SH3

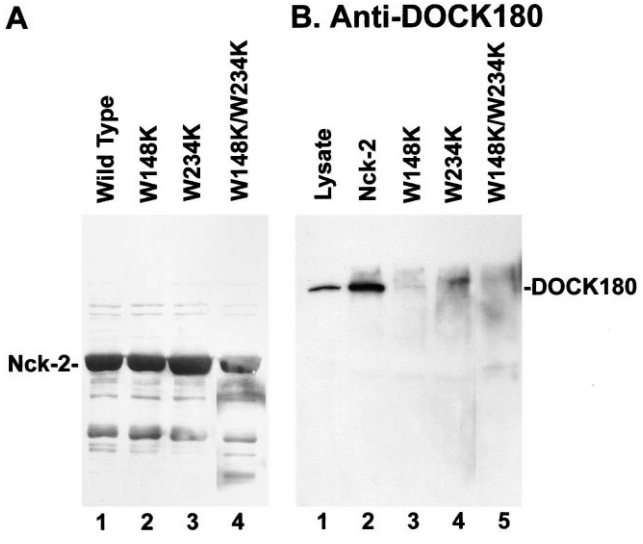


Fig. 3. Multiple SH3 domains mediate the interaction of Nck-2 with DOCK180. A: Coomassie Blue staining of GST fusion proteins containing the wild-type Nck-2 (lane 1) or mutant forms of Nck-2 in which residue W_{148} (lane 2), W_{234} (lane 3) or both (lane 4) were substituted with K. B: DOCK180 binding. Human 293 cell lysates (300 μg) were incubated with equal amount (10 μg) of GST fusion proteins containing the wild-type Nck-2 (lane 2), the W_{148}→K point mutant (lane 3), the W_{234}→K point mutant (lane 4) or the double W→K mutant (lane 5), for 2 h at 4°C. GST fusion proteins were precipitated and associated DOCK180 was detected by immunoblotting with an anti-DOCK180 antibody as described in Section 2. Lane 1 was loaded with 14 μg of 293 cell lysates.

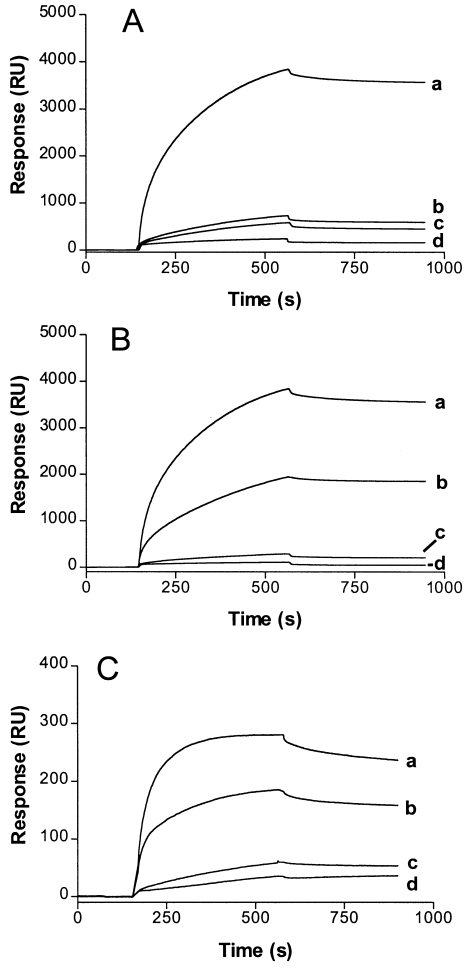


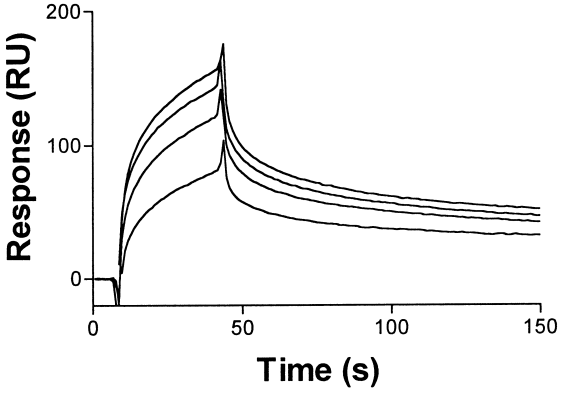
Fig. 4. Identification of DOCK180 sequences recognized by the Nck-2 SH3 domains. A: Binding of GST-Nck-2 to DOCK180 peptides. Equal amounts of biotinylated DOCK180 peptides, each representing one potential SH3 recognition site located in the C-terminal region of DOCK180, were immobilized on a BIAcore SA chip, each in a different flow cell. GST-Nck-2 was injected over all four peptides at a concentration of 1 μM as described in Section 2. a = peptide 3 (residues 1819–1836: DLLGSPTPPPPPPHQRHL), b = peptide 2 (residues 1793–1810: ADVADVPPPLPLKGSVAD), c = peptide 4 (residues 1835–1852: HLPPPLPSKTPPPPPPKT), d = peptide 1 (residues 1758–1775: SPSSPSSQQTPPPVTPRA). B: Binding of individual Nck-2 SH3 domains to peptide 3 of DOCK180. Equal concentrations (1 μM) of GST fusion proteins containing Nck-2, (curve a), $SH3^3$ (curve b), $SH3^2$ (curve c), or GST (curve d) were injected over sensor chip surfaces immobilized with peptide 3 of DOCK180. C: Binding of Nck-2 point mutants to peptide 3 of DOCK180. Equal concentrations (1 μM) of GST fusion proteins containing wild-type Nck-2 (curve a), Nck-2 point mutant (W148K) in which $SH3^2$ is inactivated (curve b), Nck-2 point mutant (W234K) in which $SH3^3$ is inactivated (curve c), or Nck-2 double mutant (W148K; W234K) in which both $SH3^2$ and $SH3^3$ are inactivated (curve d) were injected over sensor chip surfaces immobilized with peptide 3 of DOCK180. Note that the scale in panel C is different from panels A and B, due to a smaller amount of immobilized DOCK180 peptide 3 (approximately 8% of that in panels A and B).

domain (Fig. 3B, lane 3) or that of the third SH3 domain (Fig. 3B, lane 4) also dramatically reduced the interaction of Nck-2 with DOCK180, suggesting that both the second and the third SH3 domains contribute to the formation of a stable complex between Nck-2 and DOCK180.

3.6. DOCK180 sequences recognized by the Nck-2 SH3 domains

We next sought to identify DOCK180 sites that are recognized by the Nck-2 SH3 domains. Inspection of the DOCK180 protein sequence revealed that the C-terminal region of DOCK180 contains four potential SH3 recognition proline-rich sequences. To test experimentally whether these proline-rich sequences are recognized by Nck-2, we immobilized each of the peptides containing the DOCK180 proline-rich sequences on BIAcore sensor chips. GST-Nck-2, or GST as a control, was passed over the sensor chips at a concentration of 1 μM. One of the DOCK180 peptides (peptide 3, DLLGSPTPPPPPPHQRHL) was readily recognized by GST-Nck-2 (Fig. 4A, curve a). Two additional DOCK180 sequences (ADVADVPPPLPLKGSVAD and HLPPPLPSKTPPPPPPKT) exhibited much weaker but reproducible Nck-2 binding activity (Fig. 4A, curves b and c). Peptide 1, which contains DOCK180 sequence SPSSPSSQQTPPPVTPRA, failed to interact with GST-Nck-2 (Fig. 4A, curve d). In control experiments, none of the DOCK180 peptides interacted with GST (data not shown, also see below), confirming the specificity of the binding. These results suggest that (1) there exist multiple Nck-2 binding sites (one major site, two minor sites) in the C-terminal region of DOCK180 and (2) the DOCK180 sequence DLLGSPTPPPPPPHQRHL constitutes the major Nck-2 binding site.

Because both the second and the third SH3 domains of Nck-2 are utilized in the interaction with DOCK180 (Fig. 3B), we next analyzed the ability of the individual SH3 domains to interact with the major Nck-2 binding site. To do this, equal concentrations of GST fusion proteins containing the third SH3 domain or the second SH3 domain, and GST-Nck-2 as a positive control and GST as a negative control, were injected into BIAcore sensor chips immobilized with the peptide 3. As expected, GST-Nck-2 (Fig. 4B, curve a), but not GST (Fig. 4B, curve d), readily interacted with the DOCK180



k_a1 $2.4 \pm 1.8 \times 10^5$ k_a2 $2.0 \pm 3.9 \times 10^4$

k_d1 $7.4 \pm 0.3 \times 10^{-2}$ k_d2 $7.9 \pm 2.0 \times 10^{-5}$

K_D1 $4.1 \pm 2.0 \times 10^{-7}$ K_D2 $3.2 \pm 2.0 \times 10^{-9}$

Fig. 5. Kinetics of Nck-2 binding to DOCK180. GST-Nck-2 at concentrations of 0.5, 1.0, 1.5, and 2.0 μM was injected at 50 μl/min over a surface of immobilized DOCK180 at a density of 2 ng/mm^2 to generate these sensorgrams. Each curve was analyzed with BIAevaluation version 2.0 software using a multiple binding site model (Pharmacia). Analysis yielded $K_D1 = 4.15+1.9\times10^{-7}$ M and $K_D2 = 3.24+1.9\times10^{-9}$ M.

peptide. The third SH3 domain (Fig. 4B, curve b), and to a much less extent the second SH3 domain (Fig. 4B, curve c), interacted with the DOCK180 peptide. This result suggests that the DOCK180 sequence is primarily a target of the third SH3 domain. It is worth noting, however, that the full length Nck-2 (Fig. 4B, curve a) bound the DOCK180 peptide significantly better than the third SH3 domain (Fig. 4B, curve b), suggesting that the weak binding to the same DOCK180 sequence (presumably to a distinct peptide in the close vicinity) mediated by the second SH3 domain can significantly enhance the interaction of Nck-2 with the DOCK180 peptide.

To further assess the contributions of the second and the third SH3 domains, we analyzed the binding of the full length Nck-2 W→K mutants, in which either the second SH3 domain or the third SH3 domain or both were inactivated, to the major Nck-2 binding site of DOCK180 (Fig. 4C). Inactivation of the third SH3 domain eliminated most of the binding activity (Fig. 4C, curves a and c), confirming that the binding is mediated to a large extent by the third SH3 domain. Inactivation of the second SH3 domain significantly reduced, but did not eliminate, the binding activity (Fig. 4C, curve b). The remaining binding activity exhibited by the W→K point mutant in which the second SH3 domain is inactivated is clearly mediated by the third SH3 domain, as inactivation of both the second and the third SH3 domains (Fig. 4C, curve d) almost completely abolished the binding. These data confirm the results obtained with the individual SH3 domains. Taken together, they indicate that (1) the DLLGSPTPPPPPPHQRHL site of DOCK180 is preferentially recognized by the third SH3 domain and (2) the binding mediated by the second SH3 domain, although weak by its own, significantly enhances the binding of Nck-2 to the DLLGSPTPPPPPPHQRHL site. It is interesting to note that the major Nck-2 SH3 recognition site (DLLGSPTPPPPPPHQRHL) is different from those recognized by other SH3 domains. For example, CRK SH3 domain recognizes two different sites (PPPLPLK and PPPLPSK, which are located within the second and the fourth peptides used in this study) on DOCK180 [11]. These results clearly show the high degree of selectivity between different SH3 domains and suggest that DOCK180 may function as a scaffolding protein mediating formation of multi-protein complexes.

We have also analyzed the interactions of the second and the third SH3 domains of Nck-2 with DOCK180 peptide 2 (ADVADVPPPLPLKGSVAD) by surface plasmon resonance. Both the second and the third SH3 domains interacted specifically with peptide 2, although the binding activities were low (data not shown), which is consistent with the relative weak binding activity of Nck-2 towards this site (Fig. 4A).

3.7. Kinetics of the Nck-2–DOCK180 interaction

The foregoing mutational experiments show that the interaction of Nck-2 with DOCK180 is mediated by multiple sites from both binding partners, implying that multiple binding events could occur during the complex formation between Nck-2 and DOCK180. To test this, we analyzed the kinetics of the binding of Nck-2 to the C-terminal region of DOCK180 by surface plasmon resonance, as follows. We immobilized GST fusion protein containing the C-terminal region of DOCK180 on BIAcore sensor chips and then injected GST-Nck-2 over the DOCK180 surface at different concentrations (Fig. 5). In control experiments, no binding was detected when GST was passed over the GST-DOCK180 surface or when GST-Nck-2 was passed over the GST surface (data not shown), confirming the specificity of the binding. Kinetic analyses of the Nck-2–DOCK180 binding data with BIAevaluation 2.1 software suggest that two interactions with equilibrium dissociation constants (K_D1 and K_D2) of $4.15 \pm 1.9 \times 10^{-7}$ M and $3.24 \pm 1.9 \times 10^{-9}$ M, respectively, occurred during the interaction between GST-Nck-2 and GST fusion protein containing DOCK180 C-terminal fragment (Fig. 5). Although the affinity measurements are not absolute due to the presence of GST, the kinetic data are consistent with the results from the mutational analyses indicating that multiple sites from each binding partner are involved in the interaction. Based on the mutational and kinetic analyses, we propose a model for the complex formation between Nck-2 and DOCK180. In this model, a strong binding (with K_d in the nM range) between Nck-2 and DOCK180 is achieved through two cooperative interactions mediated by two tandem (the second and the third) SH3 domains, as inactivation of either SH3 domain significantly reduced the binding between Nck-2 and DOCK180 (Figs. 3 and 4). One of the two interactions is mediated by the third SH3 domain of Nck-2 and residues 1819–1836 (DLLGSPTPPPPPPHQRHL) of DOCK180. The other interaction most likely involves the second SH3 domain of Nck-2 and either a different Nck-2 binding site (e.g. residues 1793–1810, ADVADVPPPLPLKGSVAD) in the same DOCK180 molecule or residues 1819–1836 of another DOCK180 molecule that is in the same complex. The relative weak binding between Nck-2 and DOCK180 occurs when one of the SH3 domains or some of the Nck-2 binding sites of DOCK180 are not available (either due to binding to other proteins or due to mutations as in the mutational studies described in this paper). It is interesting to compare the interaction of Nck-2–DOCK180 with that of Grb2 and SOS, another SH3-mediated interaction. Similar to the interaction between Nck-2 and DOCK180, the SH3-mediated binding of Grb2 to SOS is also very tight, with K_d value in the 10^{-9} M range [23]. However, in contrast to the Nck-2–DOCK180 interaction described in this study, the tight SOS binding is mediated primarily through one (the N-terminal) of the two SH3 domains of Grb2 [23]. Thus, there are two general mechanisms by which a tight protein complex can be formed through interactions mediated by SH3 domains. The first one is through a tight interaction mediated by a single SH3 domain (one example is the formation of the Grb2–SOS complex). The second one is through relatively weak cooperative interactions mediated by multiple SH3 domains as exemplified by the Nck-2–DOCK180 interaction shown in this study. Because the interactions mediated by individual SH3 domains are typically quite weak (with the notable exception of the N-terminal SH3 domain of Grb2), the second mechanism is likely utilized by many proteins containing multiple SH3 domains during assembly of protein complexes. The formation of a stable protein complex through multiple binding events mediated by tandem SH3 domains provides cells with a versatile mechanism regulating signal transduction.

In summary, we have identified a novel interaction between Nck-2 and DOCK180, a component of the integrin- and Rac-signaling pathways involved in the regulation of membrane ruffling and cell migration [12,13]. In addition, we have carried out a detailed biochemical analysis of the interaction between Nck-2 and DOCK180 revealing a general mechanism

by which two relative weak interactions mediated by tandem SH3 domains result in the formation of a stable complex. Nck-2 is concentrated in membrane ruffles of spreading cells (Tu and Wu, unpublished observation), where DOCK180 is known to localize and function [12,13,24]. Recent studies by Chen et al. have demonstrated that Nck-2 regulates PDGF-stimulated membrane ruffling [25]. Given the well documented importance of DOCK180 in the regulation of membrane ruffling and cell migration [12,13,24], the interaction of Nck-2 with DOCK180 described in this paper suggests that the Nck-2–DOCK180 complex likely plays an important role in cellular control of cell migration.

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