The Phosphotyrosine Peptide Binding Specificity of Nck1 and Nck2 Src Homology 2 Domains*§

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Nck proteins are essential Src homology (SH) 2 and SH3 domain-bearing adapters that modulate actin cytoskeleton dynamics by linking proline-rich effector molecules to tyrosine kinases or phosphorylated signaling intermediates. Two mammalian pathogens, enteropathogenic Escherichia coli and vaccinia virus, exploit Nck as part of their infection strategy. Conflicting data indicate potential differences in the recognition specificities of the SH2 domains of the isoproteins Nck1 (Nckα) and Nck2 (Nckβ and Grb4). We have characterized the binding specificities of both SH2 domains and find them to be essentially indistinguishable. Crystal structures of both domains in complex with phosphopeptides derived from the enteropathogenic E. coli protein Tir concur in identifying highly conserved, specific recognition of the phosphopeptide. Differential peptide recognition can therefore not account for the preference of either Nck in particular signaling pathways. Binding studies using sequentially mutated, high affinity phosphopeptides establish the sequence variability tolerated in peptide recognition. Based on this binding motif, we identify potential new binding partners of Nck1 and Nck2 and confirm this experimentally for the Arf-GAP GIT1.

Dynamic processes in eukaryotic cells, such as cellular movement, changes in cell shape, and transport of vesicles, rely on constant remodeling of the actin cytoskeleton. Adapter proteins, essential in transmitting and modulating corresponding stimuli, frequently contain SH23 domains to recognize and bind tyrosine-phosphorylated motifs. Nck1 (Nckα) and Nck2 (Nckβ or Grb4) are two such adapter proteins (1–3), both bearing three SH3 domains and a C-terminal SH2 domain (4). Mice lacking both Nck genes are not viable, underscoring the importance of these adapters (1). A high sequence identity (68% overall and 82% for the SH2 domains) and single gene knockouts of Nck1 and Nck2 (1) indicate that the function of the proteins may substantially overlap. Both bind receptor tyrosine kinases such as the PDGFR (5) and other tyrosine-phosphorylated proteins via their SH2 domains (3). However, Nck1 or Nck2 has also been reported to bind distinct targets. Exclusive Nck2 binders include EphrinB1 (6, 7), EphrinB2 (8), andDisabled-1 (Dab-1) (9), all involved in neuronal signaling. In the case of the PDGFR, Tyr(P)1009 is reported to be Nck1-specific (5), whereas Tyr(P)1009 is Nck2-specific (10).

Furthermore, Nck1 and Nck2 have both been implicated in the infection process of enteropathogenic Escherichia coli (EPEC) (11), a frequent cause of severe infant diarrhea (12). EPEC adheres tightly to the membrane of intestinal enterocytes inducing massive remodeling of the microfilament system and suppression of microvilli (13, 14). This involves the “translocated intimin receptor” (Tir), introduced into the host cell by a type III secretion system (11). Insertion of Tir into the host cell membrane (15) provides a binding site to the bacterial outer membrane protein intimin (16). Tir clustering induces phosphorylation of its cytosolic C-terminal Tyr(74) by an Src family kinase (17, 18) and hence recruitment of Nck1 and/or Nck2 through their SH2 domains (11). By recruiting and activating N-WASP and hence the Arp2/3 complex through their SH3 domains (11, 19), Nck1 or Nck2 in turn induce actin polymerization producing dynamic bacteria-presenting protrusions of the plasma membrane known as pedestals (20).

The vaccinia virus similarly exploits Nck. Following its intracellular replication, the virus is transported to the cell periphery in a microtubule-dependent process (21), where the viral envelope protein A36R is tyrosine-phosphorylated at Tyr(112) recruiting Nck through its SH2 domain. Again, N-WASP/Arp2/3-induced actin polymerization leads to the formation of motile plasma membrane projections beneath the virus (21–23).

Here we have investigated the extent to which the SH2 domains of Nck1 and Nck2 differ in phosphotyrosine signaling. By surface plasmon resonance (SPR) spectroscopy, we identify a Tir-derived phosphopeptide as the strongest natural ligand of both Nck1 and Nck2 SH2 domains. By epitope scan, based on the sequential mutation of the Tir sequence, we derive the optimal peptide profile for both SH2 domains. Furthermore, we present the crystal structure of Nck1-SH2 without ligand and that of both Nck1- and Nck2-SH2 in complex with Tir-derived phosphopeptides. Based on our results, we predict new potential interaction partners for the SH2 domains of Nck1 and Nck2 and demonstrate the tyrosine phosphorylation-dependent interaction for one of these, GIT1. Overall, our analyses do not support distinct phosphotyrosine peptide affinities of the SH2 domains to explain distinguishing characteristics of Nck1 and Nck2. Differences must therefore involve regions outside the peptide-binding groove of the SH2 domains.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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4 The abbreviations used are: SH, Src homology; EPEC, enteropathogenic E. coli; PBS, phosphate-buffered saline; GST, glutathione S-transferase; MES, 4-morpholineethanesulfonic acid; PDGFR, platelet-derived growth factor receptor; r.m.s.d., root mean square deviation; SPR, surface plasmon resonance.
EXPERIMENTAL PROCEDURES

Production of GST Fusion Proteins—The cDNA for Nck and Grb2 proteins was a kind gift from Michael Way (Cancer Research UK, Lincoln’s Inn Fields Laboratories, London, UK) and Ottmar Janssen (Institute of Immunology, Kiel, Germany). Coding sequences of the SH2 domains of human Nck1 (amino acids 281–377) and Nck2 (amino acids 284–380) were amplified by PCR and ligated into the vector pGEX-6P-1 (Amersham Biosciences) using primers 5’-GTCGGATCCCTTTTGTTATATTGCGAAGTCACTGATATGCATGACAAGATATAAAATGTCAC-3’ (5’-primer, BamHI restriction site) and 5’-GGCGCGTTCAGTCGATCTGATATGCATGACAAGATATAAAATGTCAC-3’ (5’-primer, XhoI restriction site) for Nck1-SH2, and 5’-GGCTCGATCCGGATCCCTTTTGTTATATTGCGAAGTCACTGATATGCATGACAAGATATAAAATGTCAC-3’ (5’-primer, BamHI restriction site) and 5’-GCGCTCGAGTGGTACTACGGGAACGTG-3’ (3’-primer, XhoI restriction site) for Nck2-SH2. GST fusions of Nck and Grb2 SH2 domains were produced in E. coli strain BL21-CodonPlus (Stratagene) at 22 °C overnight. Cells were centrifuged, resuspended in 50 ml of PBS, and disrupted by a French press. The supernatants were incubated with glutathione-Sepharose (Amersham Biosciences) and washed with PBS. For pulldown assays, PBS plus 10% glycerol allowed snap-freezing in liquid nitrogen. For all other assays, the GST/Nck-SH2 fusion protein was eluted using 10 mM glutathione in PBS (Nck1) or 20 mM Tris/HCl, pH 8.0, 200 mM NaCl (Nck2) and either used as such in peptide overlay studies or cleaved using PreScission protease (Amersham Biosciences). Further purification included Mono Q anion exchange (20 mM citrate buffer, pH 6.0, Nck1) and gel filtration chromatography (20 mM citrate buffer, pH 6, 200 mM NaCl for Nck1 and 20 mM Tris/HCl, 200 mM NaCl for Nck2).

Crystallization and Data Collection—Nck1-SH2 was crystallized by hanging drop vapor diffusion at 4 °C by using 6–10 mg/ml protein in 20 mM Tris/HCl, pH 8.0, and 25% PEG5000 MME, 0.1 M MES, pH 6.5, 0.2 M (NH4)2SO4, 0.1 M guanidine HCl as precipitant. 20% MPD in mother liquor served as cryoprotectant.

For co-crystallization, protein and peptide were mixed in a ratio of 1:1.1. Nck1-SH2:Tir12, where Tir12 is a 12-residue, chemically synthesized peptide derived from the EPEC-protein Tir. The complex was crystallized in 2.4 M (NH4)2HPO4, 0.1 M Tris, pH 8.5; Nck2-SH2:Tir18 in 50% MPD, 15% ethanol, and 0.01 M Na acetate. Diffraction data were collected at 100K at beamlines BL2 (Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung mbH, Berlin, Germany) and BW6 (Max-Planck-Gesellschaft, Deutsches Elektronen-Synchrotron, Hamburg, Germany). Data collection statistics are summarized in Table 1.

Structure Determination—Data were processed using the HKL (24) and CCP4 (25) suites. Structures were solved by molecular replacement using EMPIR (26) and Grb2 as a search model for Nck1-SH2 and the latter for both protein-peptide complexes. The structures were refined by CNS using rigid-body and simulated annealing protocols (27) and REFMAC5 (28). Matching subsets of diffraction data (5%) were set aside for Rfree calculation (29). The programs O (30) and Coot (31) were used for manual model building and structural analysis. Structures were validated using PROCHECK (32) and WHATIF (33). Figs. 1 and 2 were prepared using MOLSCRIPT (34), GRASP (35), and POV-Ray and Fig. 3 using PYMOL.

Epitope Scanning—233 Tir-derived dodecapeptides (11 positions × 20 amino acids, 11 negative control peptides with unphosphorylated Tyr, plus 1 positive and 1 negative control peptide; see supplemental material) were generated by spot synthesis on cellulose as solid support. The membrane was blocked by overnight incubation with 10 ml of MBS (2 ml of blocking reagent (Sigma-Genosys), 0.5 g of saccharose, pH 7, and 8 ml of T-TBS (8.0 g/liter NaCl, 0.2 g/liter KCl, 6.1 g/liter Tris base, 0.05% Tween, pH 8.0)). GST-Nck1- or GST-Nck2-SH2 was incubated with the peptide membrane (5 μg/ml in MBS) for 2 h. After a brief T-TBS rinse, the membrane was incubated with a horseradish peroxidase-conjugated GST antibody for 1 h. After two washing steps, bound antibody was detected via development with the ECL kit (Roche Applied Science). The experiment was performed in triplicate. Intensity of spots was quantified by luminometry employing a CCD camera (Fujix) and analyzed using AIDA software (Raytest, Germany). Spot intensities of each experiment were normalized by division through the means of control experiments (original Tir12–peptides). Normalized intensities were set to percentages (mean of Tir12–peptides: 100%) and averaged over all three experiments.

Surface Plasmon Resonance—Surface plasmon resonance experiments were performed using a BIACore 2000 (BIAcore AB, Uppsala, Sweden) at 25 °C, and a flow rate of 10 μl/min. Biotinylated decapetides were passed over and allowed to bind to the surface of a streptavidin-coated sensor chip (SA, Pharmacia Biosensor). A dilution series of ~1 mM to 10 nM Nck1- and Nck2-SH2 was prepared in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20). Protein association and dissociation were each recorded for 2 min for each concentration. The surface was regenerated between injections using 1 M NaCl. All experiments were performed in triplicate. Sensorgrams for each protein concentration were aligned to the same base line after subtraction of the reference. The sensorgram data were quantified by plotting the resonance units at equilibrium (R eq) against the protein concentration. The binding constant Kd was determined by curve fitting to a 1:1 binding isotherm as shown in Equation 1,

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\text{R}_{\text{eq}}[\text{Nck}] = \frac{R_{\text{max}}[\text{Nck}]}{K_{\text{D}} + [\text{Nck}]}
\]

where R eq indicates the resonance units at equilibrium; Kd is the dissociation constant; [Nck] is the molar concentration of Nck, and R max is the maximal resonance signal.

Pulldown Assays—A431 cells (ATCC, CRL 1555) and HeLa S3 cells (CCL-2.2) were maintained as recommended by the supplier (ATCC, LGC Promochem, Wesel, Germany). 10-cm diameter dishes of 70% confluent cells were serum-deprived for 24 h and treated with Dulbecco’s modified Eagle’s medium in the presence or absence of 2 μM phorbol 12-myristate 13-acetate for 10 min at 37 °C to stimulate protein phosphorylation. Cells were lysed in 500 μl of ice-cold lysis buffer (12 mM Tris, 16 mM HEPES, pH 7.4, 50 mM NaCl, 15 mM KCl, 20 mM NaF, 1 mM NaVO4) for 10 min on ice and harvested with a cell scraper. The lysate was cleared at 15,000 × g for 15 min at 4 °C and incubated for 1 h at 4 °C on a rotary wheel with 25 μl of glutathione-Sepharose loaded with GST or GST-SH2 fusions of Grb2, Nck1, or Nck2. Precipitates were washed twice, resolved by SDS-PAGE, and analyzed by immunoblotting. Anti-GIT1 was purchased from Santa Cruz Biotechnology.

RESULTS

Binding Affinities of Nck1- and Nck2-SH2 for Target-derived Phosphopeptides—We have determined the binding affinities of the sequentially related SH2 domains of Nck1 and Nck2 (Fig. 1A) for reported binding partner peptides by surface plasmon resonance spectroscopy using chemically synthesized phosphopeptides, purified by high pressure liquid chromatography and verified by mass spectrometry. Peptides include those of the exogenous virulence factors Tir (EPEC) and A36R (vaccinia) and the endogenous interaction partners PDGFR, Dab-1, EphrinB1, and EphrinB2. Dab-1 and PDGFR each contain two phosphorylation sites, Tyr220 and Tyr232 for Dab-1, Tyr731 and
Tyr1009 for PDGFR, and one for both EphrinB1 and -B2 (Tyr298 and Tyr304).

Dissociation constants ($K_D$) for various peptides were determined by analyzing the association and dissociation of Nck1- or Nck2-SH2 at various concentrations to peptide-coated chips (Table 1). With a $K_D$ of 60 nM and 370 nM for Nck1 and Nck2, both SH2 domains bind the Tir-derived phosphopeptide with high affinity. This is similar to the 313 nM observed for Nck1-SH2/Tir-phosphopeptide determined in an earlier study using fluorescence polarization (11). As expected, neither SH2 domain binds to the nonphosphorylated Tir peptide, a negative control. Binding to the A36R-derived peptide is 10011011-fold weaker ($K_D$ of 5926/22M, 150 M for Nck1/Nck2) than to the Tir-derived phosphopeptide but similar to those for PDGFR Tyr(P)1009 (27 M/230 M) and EphrinB1/2 (22 M/130 M) peptides. Unexpectedly, binding of Nck1- or Nck2-SH2 to Dab-1 Tyr(P)751 and Tyr(P)232 and PDGFR Tyr(P)751 peptides was not observed, contradicting their identification as Nck1 and Nck2 SH2 ligands.

Overall, the isolated Nck2-SH2 displays a slightly weaker affinity (2–9-fold) than Nck1-SH2. Nevertheless, both proteins bind to the same peptides, and the trend in binding affinities is similar.

Crystal Structures, Ligand-free SH2 Domain of Nck1 — We started to crystallize both domains alone and in complex with tightly binding Tir-derived phosphopeptide to identify potential differences in phosphopeptide binding of Nck1 and Nck2 SH2 domains.

The ligand-free structure of Nck1-SH2 at 1.8 Å resolution (Fig. 1B and Table 2) (Protein Data Bank code 2CI8) is broadly similar to that of other SH2 domains (36). It consists of a central $\beta$-sheet ($\beta$A, $\beta$B, and $\beta$C) flanked by two $\alpha$-helices ($\alpha$A and $\alpha$B). Compared with other SH2 domains, Nck1-SH2 has a second $\beta$-sheet ($\beta$F) near the domain surface, also observed in the recent NMR structure of Nck2-SH2 (37). In our crystal, we find that Nck1-SH2 forms a domain-swapped dimer. The loop D’E that connects $\beta$-strands D’ and E adopts an extended conformation separating N- and C-terminal halves of the domain and allowing two domains to form a symmetrical dimer. The domain

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swapped dimeric form of Nck1-SH2 was not detected in solution by gel filtration, dynamic light scattering, and analytical ultracentrifugation, indicating that this was probably caused by 0.1 M guanidine hydrochloride in the crystallization solution, and thus is not biologically relevant (also see supplemental material).

The Mode of Ligand Binding Is Conserved between Nck1 and Nck2 SH2 Domains—Nck1- and Nck2-SH2 were co-crystallized with Tir-derived phosphopeptides of different length (Table 2), 12 residues (Tir12, EEHY*DEVAADP) for Nck1 (Fig. 2A) (Protein Data Bank code 2C1I) and 9 residues (Tir8, HIY*DEVAA) for Nck2 (Fig. 2B) (Protein Data Bank code 2CIA). Peptide binding does not significantly affect the conformation of the SH2 domains. A relatively high root mean square deviation (r.m.s.d.) of 0.9 Å for C-α atoms between ligand-free Nck1-SH2 and Tir12-bound Nck1-SH2 is primarily caused by adjustments in the C terminus as well as flexible loops ββ-BC, BC-βD, and βF-βG not involved in peptide binding. Similarly, the SH2 domains of peptide-bound Nck1- and Nck2-SH2 are again highly similar despite an r.m.s.d. of 0.7 Å caused by deviations in the loops described above (Fig. 3). Residues involved in phosphopeptide binding are strictly conserved. As a result, interactions between the peptides and the Nck peptide-binding site are mostly equivalent (Figs. 2C and 3A) corroborating the finding that both SH2 domains prefer the same substrates. Nonconserved residues are confined to loops (Fig. 3B) not involved in phosphopeptide binding.

As observed previously for other SH2 domains (36), the phosphate group of Tyr(P) is tightly bound in a hydrophilic pocket (Fig. 2). Two conserved arginines (Arg-αA2, Arg-βB5), three serines (Ser-βB7, Ser-βC2, and Ser-βC3) and the amide N of Glu-βC1 ensure tight coordination (Fig. 2C). Apart from the phosphate group, Tyr(P) is also held in position by hydrophobic interactions of the aromatic ring with Lys-βD6 and Arg-αA2 (cation-π interaction and stacking). Other phosphopeptide residues involved in binding include Valα-5, Alaα-4, Ileα-1, and Hisα-2, recognized by specific interactions including salt bridges, hydrogen bonds, and van der Waals interactions. Specific interactions for residues outside the eight central amino acids include a salt bridge of Argα-2, recognized by specific interactions including salt bridges, hydrogen bonds, and van der Waals interactions. Specific interactions for residues outside the eight central amino acids include a salt bridge of Argα-2, recognized by specific interactions including salt bridges, hydrogen bonds, and van der Waals interactions.

Table 2 shows the data collection and refinement statistics for Nck1-SH2 and Nck2-SH2 with Tir peptides. The binding specificity of Nck SH2 domains is summarized in Table 1, which lists the binding affinities for various phosphopeptides.

### Table 1: Binding Specificity of Nck SH2 Domains

| Peptide sequence | Nck1-SH2 Binding Constant | Nck2-SH2 Binding Constant |
|------------------|---------------------------|---------------------------|
| Tir10            | 0.06 (±0.01)              | 0.37 (±0.04)              |
| Tir10 (unphosphorylated) | No binding              | No binding              |
| PDGFR751         | 27 (±1)                   | 230 (±60)                 |
| PDGFR1009        | 22 (±3)                   | 130 (±50)                 |
| EphrinB1/2       | No binding                | No binding                |
| Dab-1 (220)      | No binding                | No binding                |
| Dab-1 (232)      | 59 (±9)                   | 150 (±70)                 |
| A36R             |                           |                           |

### Table 2: Data Collection and Refinement Statistics

| Data Set       | Nck1-SH2 | Nck1-SH2/Tir12 | Nck2-SH2/Tir8 |
|----------------|----------|---------------|--------------|
| Space group    | C222     | P212,21       | P212,21      |
| Unit cell (a, b, and c in Å) | 62.9, 82.5, 44.5 | 2 + 2 | 1 + 1 |
| Monomers/asymmetric unit | 1 | 1 | 1 |
| Wavelength (Å) | 0.92 | 1.05 | 1.05 |
| Resolution range (Å) | 50–1.80 (1.83–1.80) | 30–1.40 (1.42–1.40) | 50–1.45 (1.48–1.45) |
| Rmerge (%)     | 2.6 (2.0) | 4.37 (3.1) | 4.3 (3.1) |
| Completeness (%) | 94.9 (94.5) | 96.4 (86.5) | 98.4 (96.3) |
| Redundancy (%) | 6.6 (4.6) | 4.0 (3.7) | 3.3 (2.9) |
| Unique reflections | 11,119 (490) | 28,419 (2052) | 33,053 (1088) |

### Notes
- Values in parentheses indicate shell of highest resolution.
- Favored/generous/additional/disallowed regions.

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4 S. Frese, W.-D. Schubert, A. C. Findeis, T. Marquardt, Y. S. Roske, T. E. B. Stradal, and D. W. Heinz, unpublished data.
detection (supplemental Fig. 2A). The experiment was conducted in triplicate, and values for each peptide were averaged following normalization according to the signal derived from the original Tir peptide (red letters or boxes in supplemental Fig. 2, A and B).

The peptide scans for Nck1- and Nck2-SH2 are summarized in Fig. 4, A and B. For each position of the phosphopeptide, those amino acid residues that retain detectable binding of the SH2 domain are listed. The height of each letter is proportional to the binding affinity of this peptide for the SH2 domain, where the sum of all affinities at this peptide position amounts to 100%. The numerical values are listed in Fig. 4C. For example, aspartate or glutamate are strongly favored in position +1 and position −4 essentially does not discriminate between amino acids.

**Prediction of Potential Interaction Partners and Proof of Principle**—Based on the results of the peptide scan, we searched the TrEMBL protein databases with the derived consensus peptide pYDXY(AYST)X- (DEC). Only proteins known to be involved in signaling to the actin cytoskeleton were retained as potential new interaction partners for Nck1- and Nck2-SH2. The identified proteins include the following: (i) GIT1 (Tyr(P)383), the G-protein coupled receptor kinase-interacting protein 1, and (ii) GIT2 (Tyr(P)392), a close relative, both presumably involved in signaling pathway integration for cytoskeletal organization, trafficking and adhesion (38, 39); GIT1 and Nck proteins co-localize in focal adhesions (40–42); (iii) Nephrin (Tyr(P)1217), a cell-cell adhesion protein in the glomerular ultrafiltration barrier of the kidney (43); and (iv) Ack1 (Tyr(P)635), a nonreceptor tyrosine kinase and effector of the Rho family GTPase Cdc42 (44).

To substantiate our prediction, we tested the ability of Nck1- and Nck2-SH2 to target GIT1 by a pulldown assay from cell lysates using SH2 domains of Nck1 and Nck2 as bait. Endogenously GIT1-expressing A431 human skin carcinoma cells (Fig. 5) and HeLa human cervix carcinoma cells (supplemental Fig. 3) were starved for 24 h to reduce phosphorylation to basal levels, or were starved and stimulated with full...
medium containing phorbol 12-myristate 13-acetate to induce hyperphosphorylation. Nck1 and Nck2 SH2 domains were able to precipitate GIT1 from stimulated but not from starved cell lysates (Fig. 5). We thus confirm that phorbol 12-myristate 13-acetate-induced phosphorylation (45) includes phosphorylation of GIT1, allowing Nck1 and Nck2 SH2 domains to bind, presumably through Tyr383. This is corroborated by the very recent finding that GIT2, which we also predict to bind Nck-SH2 domains, does indeed associate with Nck in an Src phosphorylation- and adhesion-dependent manner (46).

These findings confirm the specificity and similarity of the binding properties of Nck1 and Nck2 SH2 domains and substantiate the identified binding profile, proving it to be useful in identifying potential interaction partners of Nck1 and Nck2.

**DISCUSSION**

The Optimal Binding Sequence for Nck1 and Nck2 SH2 Domains—We have scrutinized the binding affinity and specificity of the SH2 domains of the related proteins Nck1 and Nck2 by using three independent biophysical detection methods. In line with the high degree of sequence conservation, all methods agree that the optimal binding sequence for Nck1 and Nck2 is largely equivalent, allowing only for subtle differences between the two.

Three positions dominate phosphopeptide binding (Fig. 4, A and B). Tyr(P) (position 0), and positions +3 and +1, in this order. Nonphosphorylation of Tyr(P) in itself is sufficient to incur complete loss of peptide binding (black boxes in supplemental Fig. 2A). Valine is similarly critical for high affinity binding at position +3. Proline, although tolerated, causes a significant reduction in affinity, although isoleucine and alanine reduce binding to the detection limit. All other residues are essentially disallowed in this position. Position +1 strongly favors aspartic acid. Despite its negative charge, glutamate is significantly discriminated against. Structurally an additional methylene group would appear possible, but clearly even this small structural change strains the interaction to such an extent that the complex is significantly destabilized. The only other amino acids that do not eradicate binding include serine, threonine, and asparagine.

A larger range of amino acids is tolerated at peptide residue positions +6 and +4. These positions, however, remain decisive for peptide recognition. Position +6 overwhelmingly favors negatively charged residues such as aspartate and glutamate, indicating the ionic interaction with Arg-BF1 to be crucial. Both are clearly superior to tyrosine, glycine, and serine. Arginine and lysine abolish peptide binding completely. In position +4, the best binders are alanine, threonine, serine, and tyrosine. The significantly larger size of the latter indicates that its C-β methylene group may fill the small hydrophobic alanine-binding pocket. However, its side chain would need to face away and could be stabilized by other interactions possibly stacking on Phe-BF1.

Interestingly, further peptide positions also reveal distinct preferences. Thus, both Nck1 and Nck2 SH2 domains favor a histidine at position −2, whereas proline, alanine, and valine also appear suitable. Positions −1 and +2 do not involve a direct interaction of the side chain with the SH2 domain in the crystal structure. Nevertheless, the SH2 domains still discriminate the residues at these positions. Thus hydrophobic residues such as isoleucine, leucine, and to a lesser extent valine, alanine, threonine, methionine, and phenylalanine are tolerated by position −1, and aromatic amino acids abrogate binding at position +2. Therefore, in total, as many as eight of the residues of the phosphopeptide affect the recognition by Nck SH2 domains (Fig. 4C).

Comparison to Published Binding Data—In a seminal study, Sonyang et al. (47) studied the sequence selectivity of more than 10 SH2 domains by an affinity purification approach using a peptide library with more than 5,800 peptides of the consensus GDGPYXXXSPPLL. The preferred phosphopeptide sequence for Nck1-SH2 was accordingly described to be pYDE(P/D/V). The specificity of the Nck2 SH2 domain has not yet been analyzed.
FIGURE 4. Phosphopeptide-binding motif of Nck1- and Nck2-SH2. A, schematic residue profile for Nck1-SH2. For each peptide position (from left to right), amino acids are listed vertically by the one-letter code. The size of each letter depicts the degree to which this residue is preferred over all others. Colors represent residues of similar properties, red, acidic; blue, basic; Mac, polar; yellow, aromatic; green, hydrophobic. B, identical profile for Nck2-SH2. C, the numerical values of A and B.
We observe that aspartate at position +1 is crucial for peptide binding for both Nck1-SH2 (as described) and Nck2-SH2. At position +3, valine is the optimal residue rather than proline, whereas aspartate is disfavored. Glutamate, although preferred at position +2, may be replaced by most other residues. Moreover, our results add significant detail to this motif by extending it to include additional adjacent residues.

Hence, limiting the Nck-SH2-binding motif to Tyr(P) and positions +1 to +3 may be acceptable as a shorthand but understates the case for the neighboring residues. Instead, all positions −2 to +6 (except +5) participate in creating a suitable interaction. Most importantly, unsuitable residues at any of these positions can individually abrogate binding.

The dramatic drop in affinity between the related Tir and A36R peptides may thus be rationalized based on our analyses. Although both share seven identical residues, including most crucial positions (−3 to +1 and +3 to +4), they differ in five. These five substitutions, including the salt bridge forming Asp+6, combine to reduce binding affinity by 3 orders of magnitude.

Phosphopeptides that we confirm as targets for Nck1- and Nck2-SH2 in SPR experiments (Tir Tyr474, A36R Tyr112, PDGFR Tyr1009, EphrinB1 Tyr298, and EphrinB2 Tyr273; see Fig. 6, names in green) all contain several residues that we find to be advantageous in the epitope scan (marked by green boxes) and no disadvantageous residues. By comparison, those phosphopeptides that we found not to recruit Nck in SPR experiments (PDGFR Tyr751, Dab-1 Tyr220, and Tyr232) contain at least one residue that prevents peptide binding in the epitope scan (Fig. 6, red boxes).

The good agreement between SPR, epitope scan, and structural analyses allows us to re-evaluate binding sites of other reported interaction partners (Fig. 6). Thus the binding sites of Flt-1 (Tyr433), Dok1 (Tyr362), and both SLP-76 sites (Tyr113 and Tyr128) comply with our peptide profile allowing us to classify them as “probable” Nck-binding partners (Fig. 6, underlined in green). The phosphopeptides derived from EphB1 (Tyr1009), FAK (Tyr397), FAK (Tyr211), PDGF (Tyr235), and two C-terminal EphrinB2 peptides (Tyr267 and Tyr328), by contrast, contain residues at the key positions +1 or +3 that abrogate binding in the epitope scan, indicating that all of these are unlikely to be interaction sites for Nck1- and Nck2-SH2 (Fig. 6, underlined in red). The PDGFR was reported to harbor differential binding sites, Tyr1009 for Nck1 and Tyr1009 for Nck2 (5, 10). According to our binding studies, however, both Nck1- and Nck2-SH2 would bind Tyr1009, whereas neither would bind Tyr251 (Table 2 and Fig. 6). Similarly, we do not confirm two of three binding sites for Nck SH2 domains in EphrinB2 peptides as recently analyzed by NMR (37). Although the neighboring residues of Tyr251 constitute a suitable Nck-SH2 domain ligand, those of Tyr316 and Tyr330 do not.

Do Nck1 and Nck2 SH2 Domain Specificities Differ?—Presently, published data on the extent to which the spectrum of SH2 domain-specific targets of Nck1 and Nck2 overlap or diverge are inconsistent. The reason for this is partly historical, as only Nck1 had initially been identified, and it is not always clear in retrospect whether this

**Figure 5. Potential interaction partners for Nck-SH2.** GIT1 was identified as a potential novel interaction partner of Nck1- and Nck2-SH2 in silico (see text). The interaction is confirmed in vivo by precipitating endogenous GIT1 from stimulated human A311 (but not from starved) cellular lysates using recombinant SH2 domains of human Grb2, Nck1, or Nck2. Note the interaction of Nck1- and Nck2-SH2 with GIT1 after phorbol ester stimulation of the cells. For a Coomassie-stained gel of the pull constructs and the pulldowns from HeLaS3-derived cellular lysates, see the supplemental material.

**Figure 6. Binding motifs of reported and predicted binding partners.** A, proteins bearing peptides that recruit Nck1- and Nck2-SH2 in SPR experiments are marked in green. Their sequences reveal optimal residues (green boxes) at positions identified by peptide scan to be crucial for binding. Peptides bearing suitable motifs and hence expected to recruit Nck1- and Nck2-SH2 are underlined in green. Peptides found not to interact with Nck1- and Nck2-SH2 in SPR experiments are marked in red. Their sequences reveal at least one residue that abrogates binding in the epitope scan (red boxes). These are poor ligands for Nck1 and Nck2, at best. Peptides predicted not to bind Nck-SH2 because of disadvantageous residues are underlined in red. B, potential new binding partners were identified by searching the human genome for the refined binding profile of Nck1- and Nck2-SH2 and limiting the search to proteins associated with the actin cytoskeleton. Corresponding phospho-tyrosine peptides are evaluated as described. For GIT1 binding was demonstrated experimentally (underlined in blue; compare Fig. 5).
Binding Specificity of Nck SH2 Domains

Nck is exclusively equivalent to Nck1. In addition, only the binding preference for Nck1 has been investigated in detail (47). The high sequence identity between Nck1- and Nck2-SH2 domains would have implied the binding profile for both to be similar. Incomplete data on Nck2-SH2 has, however, allowed ambiguous data to be ascribed to potential differences between the related domains. We now demonstrate that the peptide recognition pattern does not only hold for the SH2 domain of Nck1 but also for its close relative Nck2. In all of the positions involved in target binding, Nck1 and Nck2 are virtually identical (Fig. 3). Subtle variations in the preferred motif do not substantiate differential specificities of the two domains, although the isolated Nck2-SH2 generally appears to bind with somewhat reduced affinity compared with Nck1-SH2.

Toward a More Complete Set of Nck1- and Nck2-SH2 Domain Interaction Partners—The binding specificities of Nck1- and Nck2-SH2 having been established in detail allow new interaction partners to be predicted and help to confirm binding sites in new and established interaction partners.

We have identified a favorable Nck-binding motif in GIT1 and demonstrate that both Nck1- and Nck2-SH2 bind GIT1 in a phosphorylation-dependent manner. During manuscript preparation, an interaction of Nck1-SH2 with GIT2, a close relative of GIT1, was reported (46). Of three tyrosine phosphorylation sites located within the GIT2 sequence, one (Tyr392) corresponds to a potential Nck-binding site predicted by our data base search (Fig. 6). The remaining two sites (Tyr286 and Tyr399) are unlikely binding sites for Nck as one has an arginine at position +6 and the other a threonine at position +3, both of which abrogate binding in our assays (Fig. 4).

Constraints on SH2 Binding: the Bigger Picture—In addition to the tyrosine-phosphorylated peptides, the affinity of SH2 domain-bearing proteins for their target is known to be modulated by factors, such as neighboring domains. For example, the phosphorylated, closed, and hence inactive conformation of the protein kinase c-Src does not only depend on the interaction of the N-terminal SH2 domain with a phosphorylated C-terminal tyrosine. Instead, the crystal structure revealed a second stabilizing interaction, involving the central SH3 domain and a proline-rich motif (connecting the SH3 domain to the kinase domain), explaining why mutations in the SH3 domain impede autoinhibition of these kinases in vivo (48–50). Intermolecular interactions supporting SH2-mediated target recognition have also been found to alter the affinity between GADS/SLP76 and LAT (51). A 5–10-fold increase in affinity was observed for larger fragments of LAT compared with isolated domains, and only one of three peptides identified in vitro was found to be accessible in vivo (51).

Interactions of Nck1- and Nck2-SH2 may thus similarly be modulated both by SH3 domains and especially by the much more variable loops connecting individual domains. Also by folding around the SH2 domain, phosphorylated proteins could, in principle, interact with regions of the SH2 domains of Nck1 and Nck2 not addressed in our experiments. Here the 17 surface-exposed residues not conserved between Nck1- and Nck2-SH2 (Fig. 3B) could contribute to differential interactions with the target proteins. Such interactions would, however, merely modulate the interaction. They would not convert a nonbinding into a high affinity interaction.

Post-translational modifications of SH2 domains constitute yet an additional factor that potentially influences the affinity of the domain for target proteins either by extending interaction surfaces or by creating novel interaction sites. Both Nck1 and Nck2 are themselves known to be phosphorylated (52, 53), and computational analysis of potential phosphorylation sites indicates that Nck1- and Nck2-SH2 each bear unique phosphorylation sites, again allowing for the potential differentiation of Nck1 and Nck2.

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