The Wiskott-Aldrich Syndrome Protein-interacting Protein (WIP) Binds to the Adaptor Protein Nck*

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Nck is a ubiquitous adaptor molecule composed of three Src homology 3 (SH3) domains followed by a single SH2 domain. Nck links, via its SH2 domain, tyrosine-phosphorylated receptors to effector proteins that contain SH3-binding proline-rich sequences. In this report, we demonstrate that recombinant Nck precipitates endogenous WIP, a novel proline-rich protein that interacts with the Wiskott-Aldrich syndrome protein (WASP), from BJB cell lysates. Nck binds through its second SH3 domain to WIP, and Nck binds to WIP at a site (amino acids 321–415) that differs from the WASP-binding site (amino acids 416–488). WIP has been shown to associate with the actin polymerization regulatory protein profilin and to induce actin polymerization and cytoskeletal reorganization in lymphoid cells. We demonstrate the presence of profilin in Nck precipitates suggesting that Nck may couple extracellular signals to the cytoskeleton via its interaction with WIP and profilin.

Nck is a 47-kDa protein ubiquitously expressed in mammalian cells (1) and is composed of three tandem Src homology 3 (SH3)3 domains followed by a single SH2 domain. Nck has no intrinsic catalytic activity and acts as an adaptor molecule to couple upstream signals, usually those initiated by activation of receptor tyrosine kinases (RTKs), to downstream signal transducer molecules.

Ligand binding to RTK induces the receptor chains to dimerize and to transphosphorylate on specific tyrosine residues that provide docking sites for SH2 domains (2). Nck interacts via its SH2 domain with phosphotyrosine residues in RTKs such as the receptors for epidermal growth factor, platelet-derived growth factor, vascular endothelial cell growth factor, and ephrin receptors (EphB1 and EphB2) (3–7) or in protein substrates of RTKs such as insulin receptor substrate-1 (8). Nck interacts via its SH3 domains with effector molecules containing proline-rich sequences bringing them to the proximity of ligand-activated RTKs.

The three SH3 domains (SH3.1, SH3.2, and SH3.3) of Nck interact selectively with target proteins. The SH3.1 domain mediates Nck association with the Nck-associated protein 1 (Nap1) (9). The SH3.2 domain mediates Nck interaction with p21-associated kinase (10, 11), Sos, a guanine nucleotide exchange factor for Ras (12), the serine/threonine kinase PRK2/NAP (13, 14), and Nck, Ash-, and phospholipase Cγ-binding protein 4 (15). The SH3.3 domain mediates Nck interaction with the Wiskott-Aldrich syndrome protein (WASP) (16), and the γ isoform of the serine/threonine kinase casein kinase I (CKI-γ) (17). Other proteins that have been shown to interact with Nck but for which the specific SH3 domain that mediates binding has not been defined include c-Cbl (18), focal adhesion kinase (19), pp105, a lymphocyte-type CRK-associated substrate that binds to FAK and Crk (20), and Nck interacting kinase (21). Recently a nuclear protein, SAM 68, has been identified as a specific binding partner of nuclear Nck (22). The SH3 domains of Dock, the Drosophila homologue of Nck, have been shown to interact with the Drosophila protein-tyrosine-phosphatase dPTP61F (23).

Cytoskeletal rearrangement is triggered by a variety of external stimuli such as growth factors, stress, and adhesion through integrins (24) and is mediated by small GTPases. In mammalian cells, Rho family GTPases control the reorganization of the actin cytoskeleton in response to growth factors. For example, epidermal growth factor and platelet-derived growth factor activate Rac which induces ruffling of the cell membrane with lamellipodia formation (24). Polymerization of actin filaments in the cytosol is orchestrated by secondary messengers of signal transduction pathways and by proteins that interact with actin. Profilin is a 15-kDa G-actin-binding protein that regulates actin filament assembly. Profilin promotes actin polymerization byfavoring the exchange of ADP to ATP on actin (25) and by lowering the critical concentration of ATP-actin (26). Profilin can also contribute to the pool of unassembled actin when barbed ends are capped (27).

Recently, we have identified a novel WASP-interacting protein (WIP) (28). WIP is a widely expressed 503-amino acid long protein with homology in its amino-terminal sequence to the yeast protein verprolin which is involved in cytoskeleton organization (29). WIP overexpression increases the basal level of polymerized actin in human lymphoid cells and induces the formation of actin-rich cerebriform projections on the cell surface (28). WIP is proline-rich and contains potential SH3 domain-binding sequences. In an effort to investigate the role of WIP in signal transduction, we analyzed WIP binding to Nck. Our results show that Nck binds to WIP and suggest that WIP-Nck interaction may bridge cell-surface receptors to the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—Full-length Nck cDNA was cloned in-frame into the bait vector pGBT9 (CLONTECH). The sequence of the
WIP Interacts with Nck

TABLE I

| pGAD9 | pGAD424 |
|-------|---------|
| None  | WIP     | TRAF1   |
|       |         |         |
| No    | ND      | –       |
| Nck   | –       | –       |
| WASP  | –       | –       |
| Laminin | –   | ND      |

FIG. 1. In vivo binding of endogenous Nck to WIP. TAG-tagged WIP4 construct was transfected into human BJAB cells, and cell lysates were immunoprecipitated with anti-FLAG M2 antibody (αF, lane 1) or control MOPC 21 mAb (Ctr, lane 2). The immunoprecipitates were Western-blotted with anti-Nck mAb (upper panel) or anti-FLAG M2 antibody (lower panel) followed by horseradish peroxidase-conjugated goat anti-mouse and then developed with ECL immunoblotting detection system. As additional controls, total lysates of WIP4-transfected BJAB cells (lane 4) and anti-FLAG immunoprecipitates from untransfected BJAB cells (lane 3) were Western-blotted with anti-Nck antibody or M2 antibody. The heavy (denoted by *) and light (□) chains of the immunoprecipitating mAb are visualized because goat anti-mouse antibody was used as the second antibody in developing the Western blots. MOPC 21 heavy chain showed faster mobility in SDS-PAGE than M2 heavy chain.

RESULTS AND DISCUSSION

Nck Interacts with WIP in the Yeast Two-hybrid System— WIP contains several proline-rich sequences including three repeats of the sequence GRSGPXPPXP. This sequence is repeated twice in WASP and is involved in the binding of WASP to the SH3.3 domain of Nck (30). We therefore reasoned that WIP may be a candidate for binding to Nck. Since all the three GRSGPXPPXP sequences were present within WIP, a truncated version of WIP that contains amino acids 321–503, we tested the interaction of WIP4 with Nck by the yeast two-hybrid system. Table I shows that Nck interacts specifically with WIP4. Nck did not interact with human TRAF1 (tumor necrosis factor receptor-associated factor 1) used as a control, and WIP4 did not interact with laminin (Table I). As expected, WIP4 interacted with WASP (28).

Endogenous Nck Co-immunoprecipitates with WIP from

clone was confirmed by DNA sequence analysis, and the clone was designated Nck-GBT9.

WIP4 is a truncation of WIP cDNA that encodes the carboxy-terminal portion of WIP (amino acids 321–503) (28). WIP4 cDNA cloned in the yeast two-hybrid vector pGAD was used to construct the WIP4 deletion mutants. Deletions were obtained by digestion with the appropriate restriction enzymes followed by Klenow treatment and religation. pGAD-WIP-(321–415) was obtained by digestion with StuI and PstI, and pGAD-WIP-(321–376) was obtained by digestion with SfiI and PstI, and pGAD-WIP-(377–503) was obtained by SfiI digestion, Klenow digestion, and a second Klenow treatment. WIP inserts for pGAD-WIP-(416–503) and pGAD-WIP-(416–488) constructs were obtained by polymerase chain reaction. All constructs were confirmed by sequencing.

Yeast transformation and colony analysis were performed according to the manufacturer’s instructions (Matchmaker Two-Hybrid System Protocol, CLONTECH).

GST Fusion Proteins—Glutathione S-transferase (GST) fusion proteins of Nck and of each of its three SH3 domains were generated as described previously (11). All expression constructs were verified by DNA sequence analysis. Expression of fusion proteins in transformed Escherichia coli was induced for 2 h with 0.1 mM isopropyl-thio-β-D-galactosynoside. Fusion proteins were purified as described previously (28).

Generation of WIP Expressing BJAB Cells—WIP4 cDNA was cloned into a modified pCDNA3 vector that expresses cloned cDNA as an amino-terminal FLAG fusion protein and was transfected into the human B lymphoma cell line BJAB as described (28). The culture medium for BJAB-transfected cells was supplemented with 1.5 mg/ml G418 (Calcibiochem).

Affinity Precipitation of WIP by GST Fusion Proteins—Lysates of BJAB cells transfected with pCDNA3 or with pCDNA-WIP4 were obtained as described previously (28) and preclared for 1 h with 25 μl of GST-Sepharose (Amersham Pharmacia Biotech). Supernatants were tumbled for 16 h with 2 μg of GST or GST fusion proteins immobilized on GSH beads. The beads were washed, suspended in Laemmli loading buffer and subjected to PAGE on 4–15% gradient gels and Western blotting. The blots were developed with rabbit anti-WIP followed by goat anti-mouse conjugated to horseradish peroxidase or with anti-FLAG M2 antibodies. The blots were developed with horseradish peroxidase-conjugated goat anti-mouse and then developed with ECL immunoblotting detection system.

Immunoprecipitation of FLAG-WIP from BJAB Cells—BJAB cells or BJAB cells transfected with pCDNA-WIP were washed twice with phosphate-buffered saline and lysed (45 × 10^6 cells in 0.35 ml) in ice-cold lysis buffer (50 mM Tris, pH 7.4, containing 150 mM NaCl, 5 mM MgCl_2, 30% glycerol, 0.4 mM Na_3VO_4, 10 mM NaF, 10 mM Na_3P_2O_7, protease inhibitor mixture (Complete, Boehringer Mannheim) and 1% Brij 96) for 30 min. Lysates were centrifuged at 16,000 × g for 15 min at 4°C and preclared for 1 h at 4°C with 5 μl of normal mouse serum bound to protein G-Sepharose (Amersham Pharmacia Biotech) and then incubated overnight at 4°C with 8 μg of anti-FLAG M2 monoclonal antibody (anti-FLAG M2 Ab). After washing, the beads were resuspended on SDS-PAGE gel and transferred to nitrocellulose. The blots were developed with horseradish peroxidase-conjugated goat anti-mouse and then developed with ECL immunoblotting detection system.

Two-hybrid assay results for HF7c clones containing the Gal 4 binding (pGBT9) or activation (pGAD424) domain vectors with the indicated fusion protein insert are shown. WIP4 represents amino acids 321–503 of WIP. TRAF1 represents amino acids 62–416 of human TRAF1. A + indicates no growth on Leu/Tryptophan negative SD synthetic medium in the presence of 20 μM 3-amino-triazole. A ++ indicates growth both on the selective medium and β-galactosidase activity with color development in 2 h, and +++ indicates growth on the selective medium and color change in 30 min. ND, not done.

**FIG. 1.** In vivo binding of endogenous Nck to WIP. TAG-tagged WIP4 construct was transfected into human BJAB cells, and cell lysates were immunoprecipitated with anti-FLAG M2 antibody (αF, lane 1) or control MOPC 21 mAb (Ctr, lane 2). The immunoprecipitates were Western-blotted with anti-Nck mAb (upper panel) or anti-FLAG M2 antibody (lower panel) followed by horseradish peroxidase-conjugated goat anti-mouse and then developed with ECL immunoblotting detection system. As additional controls, total lysates of WIP4-transfected BJAB cells (lane 4) and anti-FLAG immunoprecipitates from untransfected BJAB cells (lane 3) were Western-blotted with anti-Nck antibody or M2 antibody. The heavy (denoted by *) and light (□) chains of the immunoprecipitating mAb are visualized because goat anti-mouse antibody was used as the second antibody in developing the Western blots. MOPC 21 heavy chain showed faster mobility in SDS-PAGE than M2 heavy chain.

BJAB Cells—To demonstrate the Nck-WIP association in vivo, we examined whether Nck and WIP co-immunoprecipitate from cells. To this purpose, we examined the presence of Nck in anti-FLAG immunoprecipitates of lysates from human B cells BJAB transfected with FLAG-tagged WIP4 cloned in pCDNA3. Fig. 1 shows the presence of Nck in anti-FLAG immunoprecipitates from FLAG-WIP4 transfected cells (lane 1). Nck was not detected in MOPC21 mAb immunoprecipitates of WIP4-transfected cells (lane 2) nor in M2 immunoprecipitates of untransfected BJAB cells (lane 3). To ascertain the presence of FLAG-tagged WIP in the immunoprecipitates, the membrane was stripped and rebalotted with anti-FLAG M2 mAb (Fig. 1, lower panel). FLAG-tagged WIP4 is detected in M2 immunoprecipitates from BJAB cells transfected with FLAG-WIP4 (lane 1) and, as expected, in total lysates from FLAG-WIP4-transfected cells (lane 4). FLAG-WIP4 was neither detected in MOPC21 immunoprecipitates from WIP4-transfected cells (lane 2) nor in M2 immunoprecipitates from untransfected cells (lane 3).
Treatment of cells with phorbol 12-myristate 13-acetate for 15, 30, or 60 min did not alter the capacity of Nck and WIP to co-immunoprecipitate (data not shown) suggesting that Nck phosphorylation induced by phorbol 12-myristate 13-acetate (31) does not regulate WIP-Nck interaction.

**WIP Binds to the Second SH3 Domain of Nck—**To confirm Nck interaction with full-length WIP, we used GST-Nck fusion protein to affinity precipitate endogenous WIP from BJAB cells. The precipitates were run on SDS-PAGE and Western-blotted with anti-WIP rabbit antibody. Fig. 2A shows that WIP is present in GST-Nck precipitates but not in control GST precipitates.

Since proteins that bind to Nck have a preference for one of its three SH3 domains, we sought to determine which of the three SH3 domains of Nck preferentially interacts with WIP. GST fusion proteins of Nck and of each of its individual SH3 domains were used to affinity precipitate WIP from BJAB cells. The precipitates were run on SDS-PAGE and Western-blotted with anti-WIP rabbit antibody. Fig. 2A shows that WIP is present in GST-Nck precipitates but not in control GST precipitates.

SH3 domains to bind WIP.

Two copies of the sequence GRSGPXXPPX, which has been implicated in the binding of WASP to SH3.3 of Nck are present in the shortest truncation of WIP that binds Nck (WIP-(321–415)). Yet WIP bound poorly to SH3.3 of Nck. This suggests that residues other than those in the above sequence determine binding to individual SH3 domains of Nck. The SH3.3 domain of Nck mediates its binding to the serine/threonine kinase CKI-γ2 (17). It would be important to determine if WIP, which binds to the SH3.2 of Nck, is a potential target for phosphorylation by CKI-γ2.

**Mapping of the Nck-binding Site of WIP—**We have previously shown that WASP binds to the carboxyl-terminal region of WIP, amino acids 377–503 (28). To determine whether the WASP- and Nck-binding sites on WIP overlap, we examined
the interaction of WIP deletion mutants with WASP and Nck using the yeast two-hybrid system. Fig. 3 shows that WIP-(416–488) binds to WASP but not to Nck. In contrast, the WIP deletion mutant WIP-(321–415) binds to Nck but not to WASP. Taken together, these results show that the WASP and Nck binding domains of WIP differ.

Since WIP and WASP bind preferentially to distinct SH3 domains of Nck, Nck may simultaneously engage WIP and WASP, thereby increasing the local concentration of both proteins and enhancing their interaction. Since different domains of Nck bind to WIP and WASP, different sites on WASP bind to WIP and Nck, and different sites on WIP bind to Nck and WASP; trimolecular complexes of Nck, WIP, and WASP may exist in which each of the proteins could contact the two others. Because each of WIP, WASP, and Nck has non-overlapping binding sites for the other two proteins, formal demonstration of a trimolecular complex of the three full-length proteins is not possible.

WIP May Bridge Nck to Profilin and the Cytoskeleton—WIP interacts with profilin (28). The two profilin binding consensus sequences in WIP (APPPPP) are located at positions 8–13 and 427–432 and are outside the Nck-binding site (amino acids 321–415). This raised the possibility that WIP may couple Nck to profilin. We therefore examined whether profilin co-precipitates with Nck. Fig. 4 shows that endogenous profilin from lysates of BJAB cells is bound to GST-Nck but not to GST. Nck lacks proline-rich sequences, including profilin binding consensus sequences (A, G, L, or S followed by PPPPP) (32) and fails to interact with profilin in the yeast two-hybrid system (data not shown). These results suggest that the binding of profilin to Nck is indirect and may be mediated by WIP, although we cannot rule out a role for other intermediaries.

The Drosophila homologue of Nck, Dock, has been shown to be involved in the photoreceptor cell (R cell) axon guidance, suggesting that it plays a role in cytoskeletal reorganization (33). In addition to binding profilin, WIP contains the actin-binding KLKK sequence, and its overexpression increases the cell content of F-actin. Furthermore, via its interaction with WASP (28) and N-WASP, WIP may modulate cytoskeletal reorganization. Therefore, WIP may link Nck to the cytoskeleton. Since Nck is recruited to RTKs following their tyrosine phosphorylation subsequent to ligand binding, the Nck-WIP interaction we describe may provide an important link between extracellular signaling via RTKs and reorganization of the cytoskeleton.

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