

# Nck and Phosphatidylinositol 4,5-Bisphosphate Synergistically Activate Actin Polymerization through the N-WASP-Arp2/3 Pathway\*

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**The Wiskott-Aldrich syndrome protein (WASP) and its relative neural WASP (N-WASP) regulate the nucleation of actin filaments through their interaction with the Arp2/3 complex and are regulated in turn by binding to GTP-bound Cdc42 and phosphatidylinositol 4,5-bisphosphate. The Nck Src homology (SH) 2/3 adaptor binds via its SH3 domains to a proline-rich region on WASP and N-WASP and has been implicated in recruitment of these proteins to sites of tyrosine phosphorylation. We show here that Nck SH3 domains dramatically stimulate the rate of nucleation of actin filaments by purified N-WASP in the presence of Arp2/3 *in vitro*. All three Nck SH3 domains are required for maximal activation. Nck-stimulated actin nucleation by N-WASP-Arp2/3 complexes is further stimulated by phosphatidylinositol 4,5-bisphosphate, but not by GTP-Cdc42, suggesting that Nck and Cdc42 activate N-WASP by redundant mechanisms. These results suggest the existence of an Nck-dependent, Cdc42-independent mechanism to induce actin polymerization at tyrosine-phosphorylated Nck binding sites.**

The Nck adapter protein consists of three Src homology (SH)<sup>1</sup> 3 domains and one SH2 domain and has been postulated to link changes in tyrosine phosphorylation to actin assembly. The SH2 domain of Nck associates with tyrosine-phosphorylated proteins on the cell surface, and its SH3 domains bind signaling proteins (such as WASP, WIP, and p21-activated kinase) that regulate processes such as membrane ruffling, vesicle motility, and axon guidance (1–9). Dock, the *Drosophila* homolog of Nck, is required for photoreceptor axon targeting

and is localized to the growth cone (10). In the actin-based motility of *Vaccinia* virus, Nck coordinates the assembly of an actin nucleation complex at the viral surface by binding to a tyrosine-phosphorylated viral protein through its SH2 domain and by recruiting neural Wiskott Aldrich syndrome protein (N-WASP) through its SH3 domains (11). We have previously demonstrated that N-WASP, when coordinately activated by Cdc42 and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), can stimulate actin nucleation through the Arp2/3 complex (12). Because *Vaccinia* motility is independent of Cdc42, an unanswered question is how this phosphotyrosine-Nck pathway activates N-WASP. In this study, we demonstrate that Nck can fully activate N-WASP in cooperation with PIP<sub>2</sub> (but not Cdc42). Maximal activity requires all three SH3 domains of Nck. These results suggest the presence of a phosphotyrosine-Nck/PIP<sub>2</sub> pathway that is distinct from the previously described Cdc42/PIP<sub>2</sub> pathway and provide support for the principle that WASP family proteins can integrate multiple upstream signals.

## EXPERIMENTAL PROCEDURES

**Protein Expression and Purification**—Glutathione *S*-transferase (GST) fusions of human Nck1 and the various Nck fragments shown in Fig. 4A were expressed in *Escherichia coli* and affinity purified on glutathione-Sepharose beads according to established protocols. For particular experiments, GST was cleaved from Nck using the PreScission protease (Amersham Pharmacia Biotech) following the manufacturer's recommendations. Purification of untagged full-length rat N-WASP and hexahistidine-tagged WGP and WG fragments produced in Sf9 cells, GST-VCA produced in *E. coli*, and the Arp2/3 complex purified from bovine brain extracts has been described previously (12, 13).

**Actin Polymerization Assays**—The pyrene actin assays used to monitor actin polymerization in the presence of various components, as well as the methods used for data analysis, have been described in detail previously (13). All polymerization reactions contained 1.0 μM unlabeled actin, 0.3 μM pyrene-actin (50% labeled), 0.2 mM ATP, 30 nM Arp2/3, 100 nM N-WASP, and various proteins at the indicated concentrations in 80 μl of XB buffer (20 mM Hepes, pH 7.7, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA). Because the activation of the Arp2/3 complex by N-WASP accelerates the kinetics of polymerization by catalyzing nucleation, the maximum slope of these polymerization curves is an indicator of the extent of N-WASP activation by added components.

**Binding Assays**—The Nck far-Western assays were performed with GST fusions labeled with glutathione-conjugated horseradish peroxidase (GSH-HRP; Sigma). Recombinant proteins (N-WASP, WGP, WG, or VCA) were separated on 12% or 8% SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and blocked with 10% non-fat dry milk in Tris-buffered saline with Tween (150 mM NaCl, 10 mM Tris 8.0, 0.05% Tween 20) at 4 °C overnight. GST fusions of Nck fragments, which served as probes in the binding assay, were preincubated for 30 min at room temperature with GSH-HRP (1 μg of SH3 domain + 0.2 μg of GSH-HRP). The PVDF strips with immobilized N-WASP mutants were incubated with the different GSH-HRP-loaded SH3 domains at 4 °C for 1 h at a final concentration of 1 μg of SH3 domain/ml

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<sup>1</sup> The abbreviations used are: SH, Src homology; WASP, Wiskott-Aldrich syndrome protein; N-WASP, neural WASP; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; GST, glutathione *S*-transferase; GSH-HRP, glutathione-conjugated horseradish peroxidase; PVDF, polyvinylidene fluoride; WIP, WASP-interacting protein.

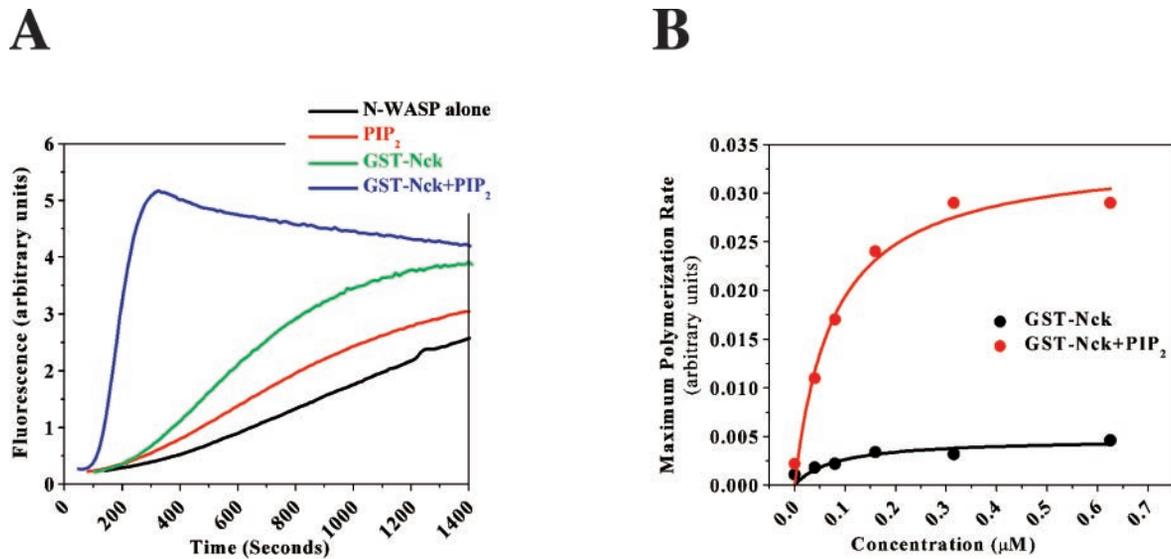


FIG. 1. **Nck and PIP<sub>2</sub> synergistically activate N-WASP.** *A*, the pyrene actin assay was used to compare the abilities of GST-Nck, PIP<sub>2</sub> vesicles (10 μM; PIP<sub>2</sub>:phosphatidylinositol:phosphatidylcholine = 10:45:45), or both together to activate actin polymerization (1 μM unlabeled actin + 0.3 μM pyrene-labeled actin) in the presence of Arp2/3 (30 nM) and N-WASP (100 nM). *B*, dose response curves showing the activation of N-WASP (100 nM) by increasing concentrations of GST-Nck in the presence or absence of 10 μM PIP<sub>2</sub> vesicles. The maximum polymerization rate, calculated from the linear phase of polymerization curves of the type shown in *A*, is taken as a measure of N-WASP activity. The curves are non-linear least squares fits of binding isotherms to the data and yield values of  $K_{act}$  and  $P_{max}$  of 77 nM and 0.0048/s for GST-Nck alone and 81 nM and 0.034/s for GST-Nck plus PIP<sub>2</sub>.

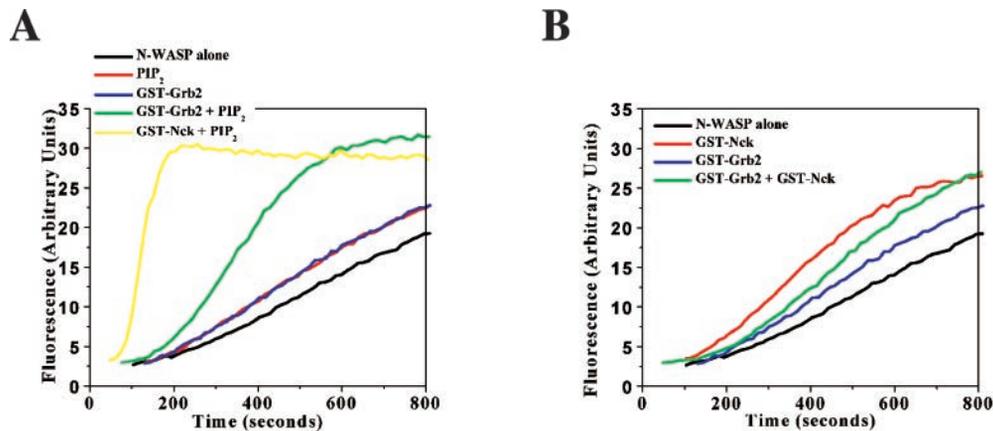


FIG. 2. **Nck activates N-WASP more effectively than Grb2.** *A*, the pyrene actin assay was used to compare the abilities of GST-Grb2 (500 nM), PIP<sub>2</sub> vesicles (10 μM), or both together to activate actin polymerization (1 μM unlabeled actin + 0.3 μM pyrene-labeled actin) in the presence of Arp2/3 (30 nM) and N-WASP (100 nM). Activation achieved by 200 nM Nck and 10 μM PIP<sub>2</sub> vesicles is shown for comparison. *B*, actin polymerization (at the same actin, Arp2/3, and N-WASP concentrations as in *A*) activated by GST-Nck (200 nM), GST-Grb2 (500 μM), or both together in the absence of PIP<sub>2</sub> vesicles.

in Tris-buffered saline with Tween. Strips were washed at 4 °C in Tris-buffered saline with Tween three times for 10 min each, and the bound probe was detected by enhanced chemiluminescence.

For solution binding assays, 293T cells were transfected with plasmid expressing rat N-WASP tagged at the N terminus with six tandem copies of the Myc epitope, and lysed 2 days post-transfection in TXB (1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol). Equal amounts of lysate were incubated at 4 °C with 10 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech) pre-bound to 5 μg of GST or the molar equivalent of GST-Nck fusion proteins. After 1 h the beads were washed 3 times in lysis buffer, resuspended in Laemmli sample buffer, split into two aliquots, and separated by SDS-polyacrylamide gel electrophoresis on duplicate 10% gels. One gel was stained with Coomassie Brilliant Blue, and the other was transferred to PVDF membrane and immunoblotted with monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology).

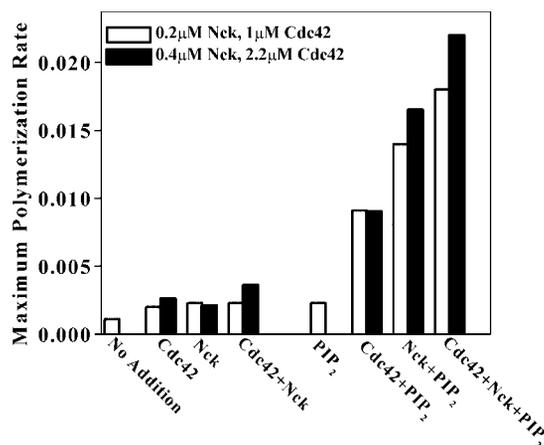
#### RESULTS AND DISCUSSION

Given the published observation that Nck can physically associate with WASP (and likely N-WASP) through its SH3 domains, we used an established pyrene-actin assay system to test whether recombinant GST-Nck could stimulate the ability

of N-WASP to activate the Arp2/3 complex (3, 14). GST-Nck alone could activate N-WASP in a dose-dependent, saturable fashion (Fig. 1). The concentration of GST-Nck required for half-maximal activation ( $K_{act}$  or potency) was ~80 nM, and the maximum polymerization rate ( $P_{max}$  or efficacy) at saturating concentrations of GST-Nck was ~4-fold higher than that for the unactivated reaction. The SH3-SH2-SH3 adapter protein Grb2 has also been shown to activate N-WASP with a similar  $K_{act}$  (100–200 nM) (15). In the presence of 10 μM PIP<sub>2</sub>-containing vesicles, which have minimal effects on N-WASP activity on their own, the  $P_{max}$  for GST-Nck increased dramatically to over 30-fold above the unactivated reaction whereas the  $K_{act}$  stayed at ~80 nM (Fig. 1). Thus, Nck and PIP<sub>2</sub> vesicles can synergistically activate N-WASP in a manner similar to that previously demonstrated for Cdc42 and PIP<sub>2</sub> (12). The requirement for two signals for N-WASP activation reinforces the emerging principle that WASP family proteins sit at integration points in signaling networks (13). We have confirmed that activation of N-WASP by Nck is not dependent on GST, because GST-fused

or -cleaved versions of Nck (and the Nck mutants described later) do not significantly differ in activity (data not shown).

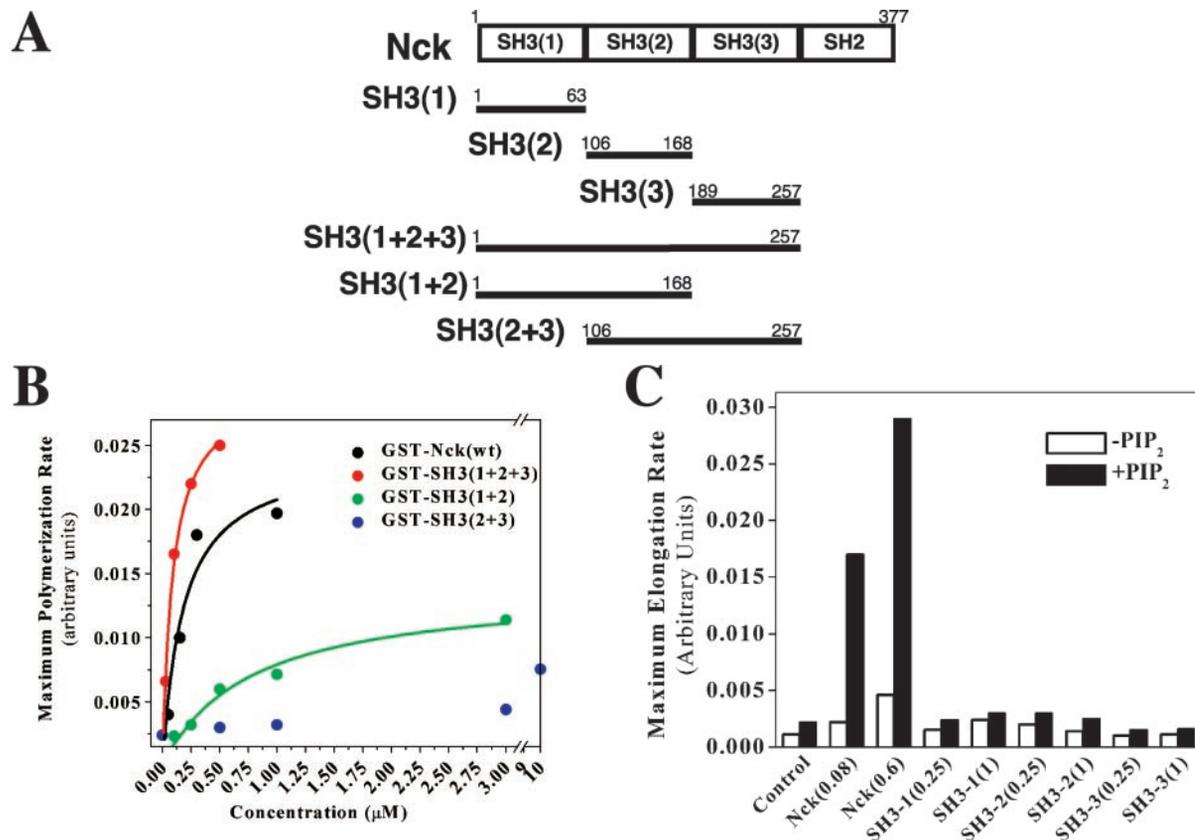
Because the Grb2 adaptor had been previously reported to stimulate N-WASP-mediated actin polymerization (15), we compared the efficacy of Nck and Grb2 in activating N-WASP. GST-Grb2 had only a slight effect on actin polymerization under these conditions, even when present at very high levels (500  $\mu\text{M}$ ), and the combination of GST-Grb2 and PIP<sub>2</sub>-containing vesicles resulted in only a modest further stimulation,



**FIG. 3. Nck and Cdc42 have redundant effects on N-WASP activity.** The maximum polymerization rate was used to quantitate N-WASP (100 nm) activity in the presence of Arp2/3 (30 nm) and the indicated combinations of Cdc42, Nck, and PIP<sub>2</sub> vesicles (10  $\mu\text{M}$ ). Both the Cdc42 and Nck used in this experiment have been cleaved from GST.

significantly lower than that seen with the combination of GST-Nck and PIP<sub>2</sub> (Fig. 2A). We also tested whether Nck and Grb2 had could synergistically activate N-WASP. As shown in Fig. 2B, the combination of GST-Nck and GST-Grb2 was actually less effective than GST-Nck alone, consistent with competition between Nck and Grb2 for binding to the same sites on N-WASP. Taken together our results suggest that Nck is a more potent activator of N-WASP than is Grb2 and that their modes of activation are qualitatively similar.

Because Cdc42 and SH3 domains likely bind to different sites on the N-WASP molecule, it has been proposed that these two classes of activators may also act synergistically (16). In fact, Grb2 and Cdc42 appear to have at least additive effects on the activity of N-WASP (15). However, we did not find evidence for synergy between Cdc42 and Nck in the activation of N-WASP (Fig. 3). In both the presence and absence of PIP<sub>2</sub> vesicles, the effects of Nck and Cdc42 together were significantly less than additive, indicating that they are functionally redundant. Despite this apparent redundancy, the fact that Cdc42 and Nck bind to different sites on N-WASP indicates that the specific mechanism by which they activate the molecule (that is, stabilize the active conformation of N-WASP relative to the inactive one) must be different. In addition, the functional redundancy of Cdc42 and Nck supports the notion that the Nck pathway is an independent signaling mechanism for the control of actin nucleation through the N-WASP-Arp2/3 module. This N-WASP-Arp2/3 module requires at least two inputs for maximal activation, a property that can allow it to amplify spatially and temporally coincident signals (17). In this regard, it will be interesting to examine the effects of SH3 domain



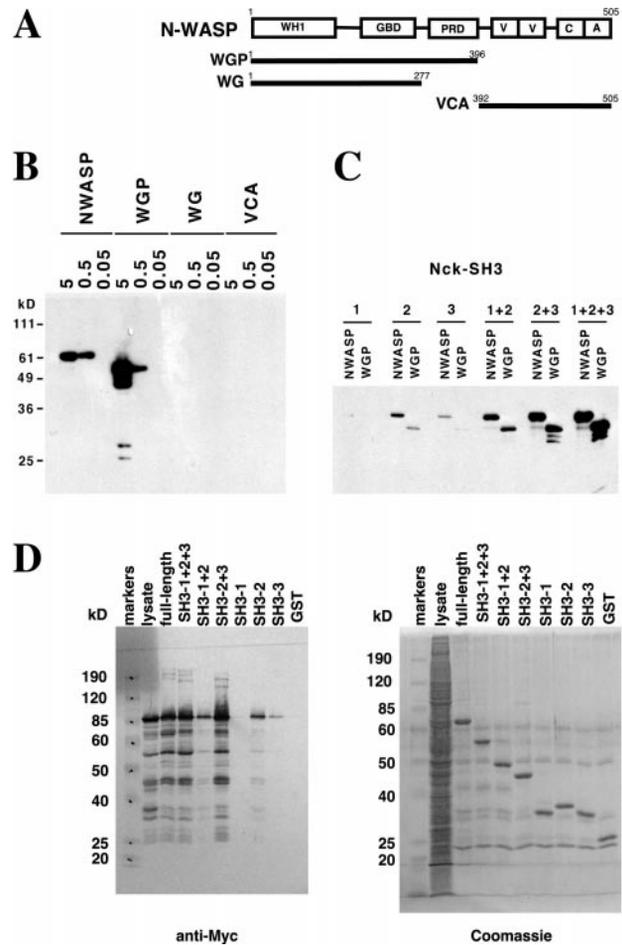
**FIG. 4. All three SH3 domains of Nck are required to maximally activate N-WASP.** A, a summary of the nomenclature and amino acid boundaries of the various domains and fragments of Nck described in this study. B, dose-response curves showing the activation of N-WASP (100 nm) by increasing concentrations of various Nck fragments (defined in A) in the presence of Arp2/3 (30 nm) and PIP<sub>2</sub> vesicles (10  $\mu\text{M}$ ). The curves are non-linear least squares fit of binding isotherms to the data and yield values of  $K_{\text{act}}$  and  $P_{\text{max}}$  of 160 nm and 0.025/s for GST-Nck, 100 nm and 0.029/s for GST-SH3(1+2+3), and 800 nm and 0.014/s for GST-SH3(1+2). The data for GST-SH3(2+3) could not be fit to a simple binding isotherm. C, N-WASP (100 nm) activity in the presence of Arp2/3 (30 nm), various fragments of Nck (concentrations given in parentheses in  $\mu\text{M}$ ), and PIP<sub>2</sub> (10  $\mu\text{M}$ ).

proteins such as Grb2 and Nck on the activity of the related WASP molecule, which differs from N-WASP in requiring only one input (PIP<sub>2</sub>) for high levels of activity (18).

To map the interaction between Nck and N-WASP in more detail, we investigated the abilities of several fragments of Nck to activate N-WASP in the presence of PIP<sub>2</sub> vesicles. The three SH3 domains of Nck are named 1, 2, and 3 moving from the N to the C terminus, and the nomenclature of the fragments is summarized in Fig. 4A. The SH2 domain of Nck is dispensable for the interaction with N-WASP, because GST-Nck displayed approximately the same  $K_{act}$  and  $P_{max}$  as a C-terminal truncation mutant carrying only the three SH3 domains (GST-SH3(1+2+3)) (Fig. 4B). To identify the specific SH3 domain(s) required for activation, we tested protein fragments containing SH3 domains 1 and 2 (GST-SH3(1+2)), SH3 domains 2 and 3 (GST-SH3(2+3)), or each individual SH3 domain (GST-SH3(1), GST-SH3(2), and GST-SH3(3)). GST-SH3(1+2) activated N-WASP with an ~7-fold higher  $K_{act}$  and a 2-fold lower  $P_{max}$  than GST-SH3(1+2+3) (Fig. 4B). In contrast, GST-SH3(2+3) did not significantly activate N-WASP until very high concentrations (~3–10  $\mu$ M) were added (Fig. 4B). None of the SH3 domains of Nck were capable of activating N-WASP when tested individually (Fig. 4C). Based on this domain analysis, optimal activation of N-WASP requires the presence of all three SH3 domains of Nck, consistent with the model in which multiple specific SH3 interactions are required to fix N-WASP in the activated conformation. This is quite distinct from the mode of interaction between Grb2 and N-WASP, where a single C-terminal SH3 domain from Grb2 is sufficient to achieve activation (15).

To examine the correlation between activation potency and binding affinity, we also mapped the physical interaction between N-WASP and Nck. Using a far-Western assay, several N-WASP truncation mutants (Fig. 5A) were tested for their abilities to bind to the GST-SH3(1+2+3) fragment of Nck, which fully activated N-WASP in the pyrene-actin assay. Full-length N-WASP and the WGP fragment (containing the WH1, G-protein-binding, and Poly-proline domains) bound to Nck, but the two fragments lacking the polyproline region (WG and VCA) did not (Fig. 5B). As expected, the polyproline region of N-WASP is required for the interaction with Nck. Next, the Nck truncation mutants shown in Fig. 4A were used to identify the SH3 domain(s) required for the interaction with N-WASP (Fig. 5C). The rank order of binding affinities for the Nck fragments is as follows: SH3(1+2+3)  $\geq$  SH3(2+3) > SH3(1+2) > SH3(2) > SH3(3) > SH3(1) (Fig. 5C). A similar rank order of binding (SH3(1+2+3) = SH3(2+3) > SH3(1+2) = SH3(2) > SH3(3) > SH3(1)) was observed when the Nck fragments were tested for their ability to pull down tagged N-WASP from lysates of transfected 293T cells (Fig. 5D), demonstrating that the far-Western assay accurately reflects the interaction between the proteins in solution. Based on these results, we conclude that multiple SH3 domains are required for the optimal interaction between Nck and N-WASP, with the second SH3 domain displaying the highest affinity for N-WASP. Although a previous report suggested that the third SH3 domain of Nck binds most tightly to WASP (3), we have found that although SH3-3 is indeed essential for optimal binding, SH3-2 binds more tightly than SH3-3 to both N-WASP and WASP (Fig. 5, and data not shown).

A surprising conclusion of our binding and activation data is that the sequence requirements for activation are more stringent than those for binding. For instance, although GST-SH3(2+3) binds quite tightly to N-WASP, it shows almost no ability to activate N-WASP. Instead, GST-SH3(1+2), which binds less tightly, can activate N-WASP to a moderate degree,



**FIG. 5. Mapping of the interaction between Nck and N-WASP.** A, a summary of the nomenclature and amino acid boundaries of the various domains and fragments of N-WASP described in this study. B, far-Western analysis of the interaction between GST-SH3(1+2+3) and various N-WASP fragments. 5, 0.5, or 0.05 pmol of each fragment was fractionated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and probed with soluble GST-SH3(1+2+3). C, the interaction of N-WASP or its WGP fragment (5 pmol each) with the indicated Nck SH3 domain fragments was tested using far-Western analysis. Identical membrane strips were incubated individually with different SH3 domain probes before being reassembled for imaging. D, association of N-WASP with Nck SH3 domains in solution. Equal amounts of lysate of 293T cells expressing full-length Myc-tagged N-WASP were incubated with beads bound to GST-Nck constructs as labeled, beads were washed, and bound fractions were analyzed by immunoblotting with anti-Myc antibody to detect N-WASP (left) or stained with Coomassie Blue to visualize GST fusion proteins (right).

and GST-SH3(1+2+3), which binds with about equal affinity, can fully activate N-WASP. This suggests that Nck activates N-WASP through multiple cooperative interactions, some of which contribute energy to binding (SH3(2+3)) and others that contribute energy to activation (SH3(1+2)). It is important to note that the total number of SH3 domains (independent of identity) is not the determinant of activity. Wild-type Nck cleaved from GST, with three SH3 domains, can fully activate N-WASP whereas GST-SH3(2+3), with four SH3 domains due to the dimerization of GST, cannot activate N-WASP (Fig. 3 and Fig. 4B, and data not shown).

Studies of *Vaccinia* in tissue culture cells have suggested that Nck recruits N-WASP indirectly to the viral surface through WIP (19). Our results show that Nck can also directly interact with N-WASP and dramatically influence its ability to nucleate actin assembly. Thus, recruitment and activation of N-WASP may be controlled by distinct mechanisms, perhaps providing tighter multilevel control over the signaling path-

way. Consistent with this idea, we have found that the addition of WIP has no effect on the ability of Nck and PIP<sub>2</sub> to activate N-WASP (data not shown). This negative result also shows that WIP (which has been shown to bind to the second SH3 domain of Nck, as well as the WASP homology domain 1 of WASP and N-WASP (4)) does not interfere with the interaction between Nck and N-WASP.

Another interesting question is how the interaction between N-WASP and Nck is regulated in cells. The SH3 domains of Nck have been shown to bind to several important signaling proteins that regulate the cytoskeleton, such as p21-activated kinase, Abl, and Cbl (1, 5, 20, 21). These proteins may regulate the Nck-N-WASP interaction by competing with N-WASP for binding to Nck or by the covalent modification of members of an actin nucleation complex containing Nck. In fact, phosphorylation of a conserved tyrosine residue in the regulatory domain of N-WASP has been predicted to stabilize the activated state (16). The presence or absence of these other proteins in complexes that assemble at the cell surface would modulate whether N-WASP is recruited and activated. The interaction of Nck with tyrosine-phosphorylated receptors would also serve to bring N-WASP into proximity with PIP<sub>2</sub> at the membrane surface.

In addition to studies with *Vaccinia* virus, PIP<sub>2</sub> and phosphotyrosine signals have been implicated in actin assembly stimulated by cytoplasmic vesicles rich in sphingolipid-cholesterol membrane microdomains or "rafts" (22). These rafts have been shown to stimulate actin assembly through the WASP-Arp2/3 pathway. Our results provide a plausible mechanism by which the phosphotyrosine and PIP<sub>2</sub> signals could be integrated by N-WASP through an adapter protein like Nck. Nck has also been implicated in the regulation of membrane ruffling downstream of the PDGF-R in NIH 3T3 cells (23). Interestingly, this pathway is dependent on Rac, which controls ruffling and lamellipodia formation, but not on Cdc42, which controls filopodia formation (24–26). Thus, the Nck pathway may serve as a general Cdc42-independent link between phosphotyrosine signals and the actin cytoskeleton.

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