Differential Regulation of WASP and N-WASP by Cdc42, Rac1, Nck, and PI(4,5)P2

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ABSTRACT: The Wiskott−Aldrich syndrome protein (WASP) and neural WASP (N-WASP) are key players in regulating actin cytoskeleton via the Arp2/3 complex. It has been widely reported that the WASP proteins are activated by Rho family small GTPase Cdc42 and that Rac1 acts through SCAR/WAVE proteins. However, a systematic study of the specificity of different GTPases for different Arp2/3 activators has not been conducted. In this study, we have expressed, purified, and characterized completely soluble, highly active, and autoinhibited full-length human WASP and N-WASP from mammalian cells. We show a novel N-WASP activation by Rho family small GTPase Rac1. This GTPase exclusively stimulates N-WASP and has no effects on WASP. Rac1 is a significantly more potent N-WASP activator than Cdc42. In contrast, Cdc42 is a more effective activator of WASP than N-WASP. Lipid vesicles containing PIP2 significantly improve actin nucleation by the Arp2/3 complex and N-WASP in the presence of Rac1 or Cdc42. PIP2 vesicles have no effect on WASP activity alone. Moreover, the inhibition of WASP-stimulated actin nucleation in the presence of Cdc42 and PIP2 vesicles has been observed. We found that adaptor proteins Nck1 or Nck2 are the most potent WASP and N-WASP activators with distinct effects on the WASP family members. Our *in vitro* data demonstrates differential regulation of full-length WASP and N-WASP by cellular activators that highlights fundamental differences of response at the protein−protein level.

Various cellular processes, such as cell motility, exocytosis, endocytosis, and cytokinesis, are dependent upon the rapid turnover of actin filaments. Diverse actin binding proteins are involved in the regulation of actin filament rearrangement. One of the most intensively studied actin-interacting proteins that initiates *de novo* actin filament assembly is the Arp2/3 complex (*1*). The Arp2/3 complex is intrinsically inactive and can be activated by members of the Wiskott−Aldrich syndrome protein (WASP[1])/Scar family known as nucleation promoting factors (*2*, *3*). WASP is expressed exclusively in hematopoietic cells, whereas its closely related homologue, N-WASP, is more widely expressed (*4*). Both WASP and N-WASP possess the same functional domains and motifs including a C-terminal WA (WH2 (WASP homology 2) and A (acidic)) domain, poly proline region, GTPase binding domain GBD/CRIB motif, highly basic region, and N-terminal WH1 domain (*4*). The three human Scar/WAVE proteins contain the Scar homology domain (SHD) at the N-terminus instead of the WH1 domain (*4*). The WH1 domain of WASP and N-WASP interacts with proteins from the WASP-interacting protein (WIP) family, such as WIP, CR16, and WICH (*5*−*7*). In addition, the WH1 domain of N-WASP binds F-actin (*8*). The basic region of N-WASP adjacent to the GBD binds phosphatidylinositol 4,5-biphosphate (PIP2) vesicles, unlike the similar domain of WASP that does not respond to PIP2 (*9*, *10*). It was shown that the GBD domain of both WASP and N-WASP interacts with Rho family small GTPase Cdc42 (*8*, *9*, *11*, *12*). The poly proline region binds Src family tyrosine kinases and Src-homology 2 and 3 (SH2/SH3) domain-containing adaptor proteins Grb2 and Nck. The autoinhibited conformation of WASP and N-WASP is secured by intramolecular interaction of the WA and the GBD domains. It has been shown that Cdc42-, PIP2-, and SH3-containing proteins can relieve this autoinhibition, enabling WASP's WA domain to interact with both the Arp2/3 complex and actin (*9*, *11*, *13*). The WA domain is the minimum essential domain for the activation of the Arp2/3 complex to nucleate actin polymerization (*14*−*16*).

Numerous reports have shown that both Rac and Cdc42 induce actin polymerization *in vivo* while conducting cellular signals from the corresponding receptors (*17*, *18*). So far, *in vitro* data have clearly demonstrated that both WASP and N-WASP preferentially interact with Cdc42 over Rac1 and that Rac1 controls Scar/WAVE activation (*9*, *14*, *19*, *20*). Although it is generally accepted that Rac1 is primarily implicated in Scar/WAVE protein regulation, there are also reports, based on *in vivo* data, that suggest the involvement of Rac1 in the Arp2/3 complex-mediated actin polymerization stimulated by N-WASP (*21*−*23*). The molecular mechanism of N-WASP regulation by Rac1 is not clear and has not been extensively studied.

In addition to Rho family small GTPases, proteins of the Nck family have an important role in the organization of

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[1] Abbrevations: GBD, GTPase binding domain; PIP2, phosphatidylinositol 4,5 bisphosphate; WASP, Wiskott−Aldrich syndrome protein.

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the actin cytoskeleton, cell movement, and axon guidance. This family has two known members in human cells, Nck1 and Nck2, sharing 68% amino acid identity (*24*, *25*). The SH2 domain of Nck associates with tyrosine-phosphorylated proteins on the cell surface, and its SH3 domains bind the poly proline domains of signaling proteins (*25*). It was shown that Nck1 interacts with N-WASP *in vitro*, stimulating actin polymerization (*26*). Nck2 has been mostly associated with the regulation of focal adhesions (*27*).

So far, there have been no reports of recombinant autoinhibited full-length WASP (*9*, *28*). The lack of a recombinant WASP has impeded detailed characterization of the protein, hampering understanding of the WASP's regulation. It is generally anticipated that WASP and N-WASP are similarly regulated by the same set of interacting proteins. In this study, we have isolated and characterized highly active, autoinhibited, recombinant full-length human WASP and N-WASP expressed in human cells. By using a pyrene−actin polymerization assay and estimating concentrations of generated barbed ends, we have examined the ability of different WASP and N-WASP regulators to activate them. To rank the activators, two parameters were taken into consideration: the ability to fully activate WASP and N-WASP, that is, to produce maximal theoretical number of barbed ends for the present concentration of the Arp2/3 complex, as well as the concentration at the half-effects (EC50). The results in this article show for the first time differential regulation of WASP and N-WASP *in vitro* and clarify a rather complex signaling network that regulates actin dynamics *in vivo*.

MATERIALS AND METHODS

Antibodies and Reagents. Mouse anti-Myc monoclonal antibodies were obtained from Sigma. Pyrenyliodoacetamide was obtained from Molecular Probes. Cell culture medium and antibiotics were obtained from Invitrogen. Cocktail Inhibitors were from Roche. Lipids, egg phosphatidylcholine (PC), brain phosphatidylserine (PS), and brain PIP2 were obtained from Avanti Polar Lipids. IGG Sepharose was purchased from Amersham and Calmodulin Agarose was obtained from Upstate. Anti c-myc antibodies coupled to agarose were obtained from Sigma.

Cell Culture and Protein Production. Recombinant, tagged proteins were produced using the Freestyle 293 expression system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, suspension Freestyle 293-FS cells were grown in defined serum-free media in shaker flasks at 37 °C with 8% CO_2. Cells were transfected on a 1.2 L scale with 1.2 mg of DNA and 1.6 mL of 293Fectin at a cell density of 1.1×10^6/mL. After 48 h, the cells were pelleted by centrifugation at 1000*g* for 15 min and frozen on liquid nitrogen for protein purification.

Full-Length WASP and N-WASP Purification. After transfection, 293 cells ($2 \cdot 10^8$) were lysed on ice for 30 min with lysis buffer (RIPA): 20 mM TRIS at pH 8.0, 1 mM EDTA, 1 mM EGTA, 10% Glycerol, 150 mM NaCl, 1% Nonident P-40, 0.125% Deoxycholate, 1 mM PMSF, cocktail protease inhibitors tablet (1 tablet for 50 mL), 1 mM sodium orthovanadate, 1 mM NaF, and 20 mM beta-glycerophosphate. Lysates were centrifuged at 163,000*g* for 13 min at 4 °C. TAP-WASP and TAP-N-WASP were purified according to the published method (*29*). Purified proteins were stored in 30% glycerol v/v at −80 °C.

Recombinant Protein Production in E. coli. GST-tagged proteins (Cdc42, Rac1, Nck1, Nck2, WASP fragment WA, and 105WASP) were produced in BL21star *Escherichia coli* (Invitrogen) as a GST fusion protein in GATEWAY pDEST15 and purified using glutathione−Sepharose 4B beads (Amersham Biosciences).

Actin Purification and Labeling. Actin was purified from acetone powder prepared from frozen chicken breast tissue on the basis of the published method (*30*). Actin was labeled with pyrene iodoacetamide (Molecular Probes) according to the published method (*31*). Both labeled and unlabeled actin were separated from polymerization nuclei and other contaminants by gel filtration using either Sephadex G-100 or Sephacryl S-200 (*32*). Column fractions with low basal polymerization were pooled, supplemented with sucrose to 10% (w/v), and drop frozen in liquid nitrogen prior to storage at −80 °C.

Purification of the Arp2/3 Complex. We have developed an efficient protocol for the purification of the Arp2/3 complex from human outdated platelets. Platelets were spun at 400*g* for 15 min at room temperature to remove red blood cells. The supernatant was spun again at 1200*g* for 15 min at room temperature. The pellet was resuspended in buffer containing 50 mM TRIS at pH 7.5, 30 mM NaCl, 10 mM EDTA, 1 mM DTT and a cocktail protease inhibitor tablet. Platelets were lysed with a microfluidizer by running two passes, 7−8 cycles each at 80 psi. The lysates were cleared by spinning at 125,000*g* for 2 h at 4 °C.

Supernatants (HSS) were either drop frozen in liquid nitrogen or loaded on the DEAE column. HSS (100 mL) was loaded onto 250 mL of DEAE Sepharose (Amersham) that was equilibrated with buffer A containing 10 mM TRIS at pH 8.0, 1 mM DTT, 1 mM MgCl, 30 mM KCl, 0.2 mM ATP, 1 mM EGTA·KOH, 2% Glycerol, 2 tablets of cocktail protease inhibitors per liter, and 1 mM PMSF. The Arp2/3 complex was eluted in flowthrough and 2 column volume washes with buffer A. A mixture of flowthrough and wash was loaded onto on a Q-Sepharose column (50 mL). The column was equilibrated with buffer A (without protease inhibitors), and the Arp2/3 complex was eluted with a salt gradient from 30 to 300 mM KCl in the same buffer. A pyrene−actin polymerization assay was used to monitor the elution of the Arp2/3 complex. Active fractions were pooled, diluted five times, and loaded onto an affinity GST-WA-His Ni-NTA agarose column. GST-WA-His was overexpressed in *E. coli*, and the crude extract was purified on glutathione−Sepharose (Amesham) and loaded onto a Ni-NTA column (QIAGEN). After loading, enriched Arp2/3 complex pooled fractions from Q-Sepharose on an affinity column, and the column was washed with 5 volumes of buffer A (without protease inhibitors). The Arp2/3 complex was eluted with 250 mM KCl in buffer A. Protein was diluted seven times and stored in glycerol 30% v/v at −80 °C.

Pyrene−Actin Polymerization in Vitro. Actin polymerization was monitored as previously described, with slight modifications (*33*). Actin polymerization reactions were carried out in 50 mM KCl, 0.05 mM EGTA, and 0.8 mM $MgCl_2$ in buffer G (2 mM Tris-HCl at pH 8.0, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM $MgCl_2$, and 0.02% w/v NaN_3). Final actin concentration in the reactions was 3.5 μM, including

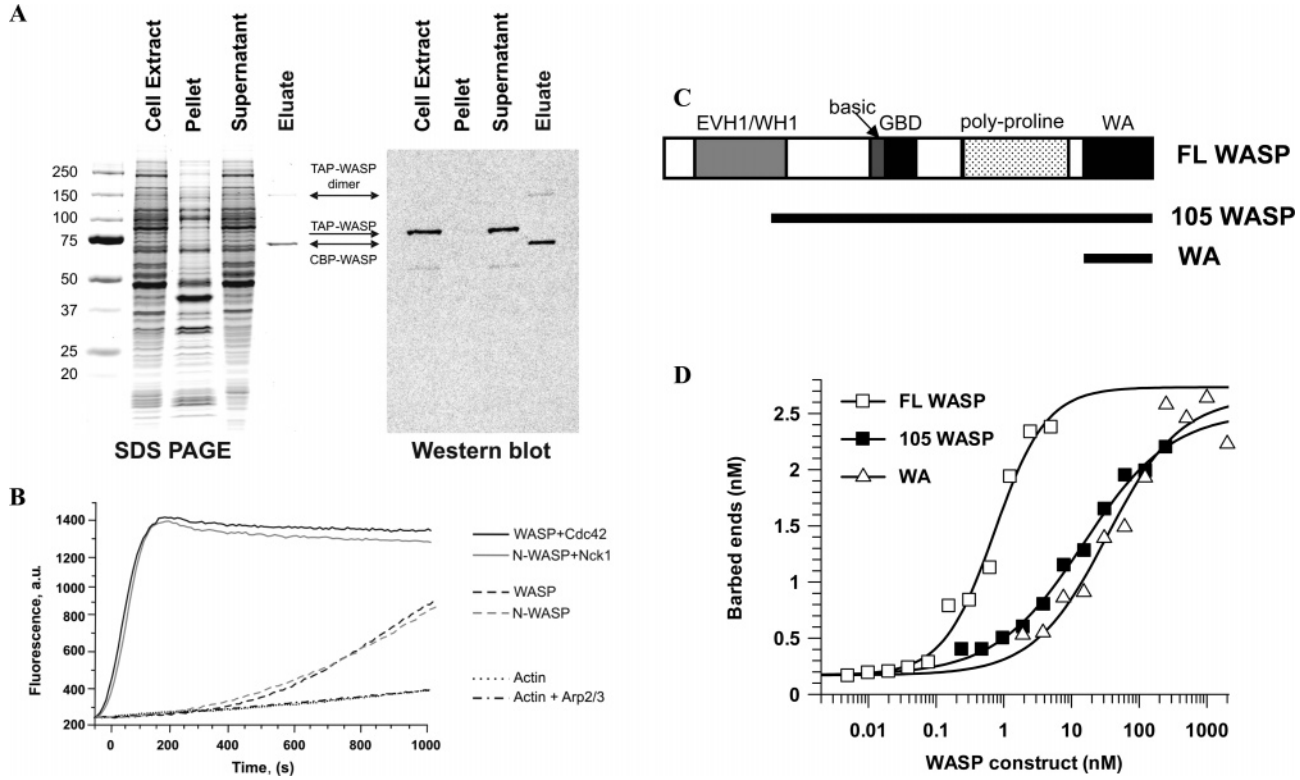

FIGURE 1: Purification and test of activity of recombinant full-length WASP and N-WASP from 293 cells. (A) Cells (293) were transiently transfected with the expression vector encoding full-length WASP that was tagged at the C-terminus with the TAP tag and with the Myc tag at the N-terminus. The protein fractions were analyzed on 4−20% SDS−PAGE, and the separated proteins were either stained with Coomassie Blue or Western blotted with Myc antibodies. TAP-tagged WASPs before (TAP-WASP) and after digestion (CBP-WASP) are depicted. (B) The regulation of full-length WASP and N-WASP was tested in a pyrene−actin polymerization assay by using 3.5 μM chicken muscle actin that was 15% labeled (black), 6 nM Arp2/3 complex from human platelets (black dotted), 1 nM full length WASP with (black) or without (black dotted) 300 nM GTP-Cdc42 (G12V mutant), and 1 nM N-WASP with (gray) or without (gray dotted) 60 nM Nck1. (C) Diagram representing WASP's domains and constructs used in this study. (D) Different concentrations of full-length WASP, 105WASP, and WA were tested in a pyrene−actin polymerization assay. The observed concentrations of WASP, 105WASP, and WA required for half-maximal activation were 0.7 ± 0.2 nM, 15 ± 4 nM, and 34 ± 11 nM, respectively. In addition to WASP or its fragments, we have used 500 nM Cdc42, 2.5 nM Arp2/3 complex, and 3.5 μM actin (15% labeled). Each data point represents an average of two measurements. The averaged standard deviation in this experiment was 6.7%.

15% pyrene−actin. Cdc42 and Rac1 were charged with GTP by incubating the small GTPases with 2 mM GTP in buffer G for 10 min at room temperature. PIP_2 vesicles containing egg phosphatidylcholine (PC), brain phosphatidylserine (PS), and brain PIP2 (48:48:4) were prepared as described (*12*). Fluorescence data were collected on a 96 well plate reader, Spectramax Gemini XS (Molecular Devices). The concentration of barbed ends was determined at 80% polymerization and calculated by using the following formula

$$[\text{barbed ends}] = \text{rate of polymerization}/(k_+[\text{actin monomers}])$$

Where $k_+ = 11.6\ \mu M^{-1}\ s^{-1}$ at pH 7.0 (*33*).

The bell-shaped dependence of barbed-end formation as a function of concentration of WASP and N-WASP activators was analyzed using a simplified activation−inhibition model (reaction scheme 1)

$$\begin{array}{c} A + W \leftrightarrow AW \rightarrow \text{nucleation} \qquad (1) \\ K_a \quad + \\ \quad A \\ \quad \updownarrow \quad K_i \\ \quad A_2W \end{array}$$

In this model, the activator (**A** = Cdc42, Rac1, Nck1, or Nck2) can bind to WASP (**W** = WASP or N-WASP) with a dissociation constant K_a. The formation of this complex leads to Arp2/3 complex activation and eventual nucleation of a new actin filament. At high activator concentrations, a second molecule of **A** can bind to **W** (with a higher dissociation constant K_i), leading to a nonproductive complex that is unable to stimulate actin nucleation. Experimental data were fitted to this model using the nonlinear regression software Grafit 5 (Erithacus software, Inc.).

RESULTS

Purification and Characterization of Recombinant Full-Length Human WASP and N-WASP. In order to express and purify human FL WASP and N-WASP, we transfected 293 adherent and nonadherent cells with FL WASP-TAP and FL N-WASP-TAP constructs. After 48 h of transfection, $2 \cdot 10^8$ cells expressed about 40 μg of WASP according to a quantitative Western blot analysis (data not shown). The vast majority of the overexpressed protein was soluble, and only traces of WASP and N-WASP were observed in the 163,000*g* pellet by a Western blot analysis (Figure 1A). We used tandem-affinity purification (TAP) to purify WASP and N-WASP from the crude cell extracts. As shown in Figure

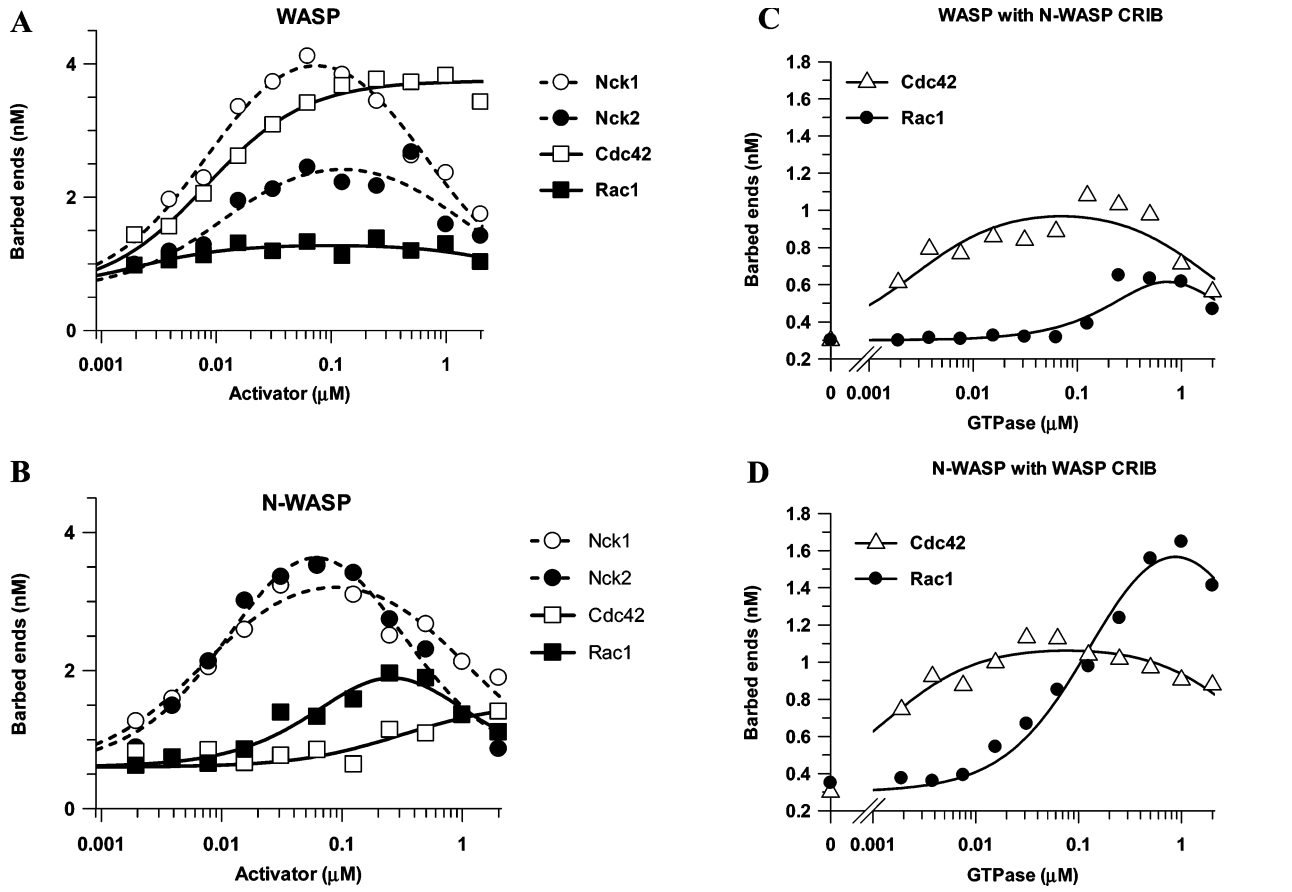

FIGURE 2: Activation of WASP and N-WASP by small GTPases and Nck proteins. (A) The pyrene−actin polymerization assay was used to monitor the polymerization of 3.5 μM chicken muscle G-actin (15% labeled) by 4 nM Arp2/3 complex, 2 nM full-length WASP, and indicated concentrations of Cdc42, Rac1, Nck1, and Nck2. The concentration of barbed ends was determined at 80% polymerization and calculated by using the formula [barbed ends] = rate of polymerization/(rate constant[actin monomers]) (*33*). The lines represent nonlinear fits to the data to the activation−inhibition model as described in Materials and Methods. (B) Activation of full-length N-WASP was measured with the same assay, with the analysis methods and conditions as described for Figure 2A. See Table 1 for numerical results. (C) WASP and (D) N-WASP mutants with swapped-CRIB domains (2 nM) were tested in a pyrene−actin polymerization assay with small GTPases. In the reaction mix, the Arp2/3 complex was present at 6 nM and actin at 3.5 μM (15% labeled). Each data point represents an average of two measurements. The averaged standard deviation in this experiment was 3.5%. Data were fitted to the model used in A and B with the following results: (C) Cdc42 $K_a = 1.4 \pm 0.4$ nM, $K_i = 4.2 \pm 1.2$ μM, Rac1 $K_a = 150 \pm 40$ nM, $K_i = 5.2 \pm 2.9$ μM; (D) Cdc42 $K_a = 2.8 \pm 1.2$ nM, $K_i = 1.8 \pm 0.8$ μM, Rac1 $K_a = 940 \pm 17$ nM, $K_i = 570 \pm 11$ nM.

Table 1: Regulation of WASP and N-WASP by Different Activators[a]

	WASP			N-WASP		
activator	maximum barbed ends (nM)[b]	K_a (nM)	K_i (nM)	maximum barbed ends (nM)[b]	K_a (nM)	K_i (nM)
Cdc42	3.1 ± 0.07	8 ± 1	ND	0.9 ± 0.2	310 ± 180	ND
Rac1	ND[c]	ND	ND	2.5 ± 0.8	120 ± 60	550 ± 330
Nck1	4.2 ± 0.2	8.8 ± 1.4	620 ± 100	3.0 ± 0.2	7.4 ± 2.1	1000 ± 300
Nck2	2.1 ± 0.3	12 ± 5	1300 ± 600	4.6 ± 0.4	16 ± 3	240 ± 50

[a] The total concentration of the Arp2/3 complex in the assay was 4.2 nM. [b] The value after subtracting baseline. [c] Not determined.

1A (Eluate), besides the dominant band for FL WASP, only one other weak band (150 kD) was observed, representing 8% of the total protein content. LC-MS/MS analysis of the peptides generated by trypsin digestion of the 150 kDa band confirmed it to be WASP (data not shown). Similar levels of expression, solubility, and purity were observed for N-WASP production (data not shown).

We next measured the ability of purified WASP and N-WASP to activate the Arp2/3 complex and nucleate actin polymerization using a pyrene−actin assay. WASP and N-WASP alone had a weak effect on Arp2/3 complex-stimulated actin polymerization (Figure 1B). The addition of WASP and N-WASP activators, Cdc42 and Nck1, dramatically accelerated actin polymerization with an elongation rate 13-fold higher than that of the polymerization without activators (Figure 1B). We used Nck1 to test the regulation of N-WASP because of its ability to fully activate N-WASP as shown in Table 1 and Figure 2B. This significant regulation of both WASP and N-WASP suggested that the recombinant proteins were properly folded. The highly pure and active Arp2/3 complex and the completely monomeric actin have enabled us to measure the activity of

WASP and N-WASP using the pyrene−actin polymerization assay in an accurate way and with a low level of spontaneous actin polymerization (example in Figure 1B).

The majority of the *in vitro* characterization of WASP reported to date has been performed with truncated constructs. Therefore, we decided to compare their properties with full-length WASP in the pyrene−actin polymerization assay. All constructs were capable of producing a maximal concentration of barbed ends equivalent to the concentration of the Arp2/3 complex (Figure 1C and D). There were, however, significant differences in affinities as reported by the half-maximal effect. The effect of GST-WA was maximal at a concentration of 250 nM, and the half-effect was observed at 34 nM. A version of WASP lacking the WH1 domain (GST-105WASP) was approximately 2 times more active than WA exhibiting a half-effect at 15 nM (Figure 1D) in the presence of 300 nM Cdc42. Full-length WASP (WASP-CBP, calmodulin binding protein tag) was markedly more active than either of the truncated versions of WASP, and a half-effect was achieved at 0.7 nM (Figure 1D).

To address the effect of tags on WASP and N-WASP activity, we have expressed and tested the activity of a WASP derivative lacking the WH1 domain (105-WASP) with and without the N-terminal GST tag as well as with the C-terminal CBP tag. No significant differences were observed among the constructs in the pyrene−actin polymerization assay, indicating that the CBP tag does not alter the characteristics of the recombinant WASP family member proteins we have used in the study (data not shown).

Differential Regulation of WASP and N-WASP with Small GTPases. We next examined the ability of small GTPases to activate WASP and N-WASP and to stimulate actin nucleation by the Arp2/3 complex in the pyrene−actin polymerization assay. Saturating concentrations of WASP and N-WASP were used for the experiments (Figure 1B). We tested constitutively active (GTP type) Cdc42 and Rac1. In the absence of PIP2 vesicles, Cdc42 preferentially activated FL WASP over N-WASP. As shown in Figure 2A, at 200 nM, Cdc42 fully activated WASP, generating 3.8 nM filaments. At the same concentration, Cdc42 had a modest effect on FL N-WASP-stimulated actin nucleation. The opposite result was observed with Rac1. Rac1 robustly activated N-WASP, producing half of the theoretical maximum of barbed ends. In the WASP assay, Rac1 was inactive.

Similar data were observed in the experiments where actin polymerization was monitored in an *in vitro* bead assay. As shown in Figure S1 in Supporting Information, Cdc42 was a more potent activator of WASP, whereas Rac1 preferentially activated N-WASP.

To examine the possibility that the differential activity of Cdc42 and Rac1 on WASP family members is due to the variations in their GTPase-binding CRIB domains, we expressed and characterized WASP and N-WASP mutants with swapped CRIB domains. According to pyrene−actin polymerization data, both chimeric proteins were highly autoinhibited (data not shown). CRIB-chimeras were equally responsive to Cdc42 activation (Figure 2C and D), unlike wild type WASP (Figure 2A) and N-WASP (Figure 2B) that were, respectively, fully and weakly activated. Interestingly, the activity of both chimeric constructs was reduced by higher GTPase concentrations. At peak activity, Cdc42 stimulated the generation of approximately 17% of the theoretical maximal number of barbed ends with both WASP and N-WASP CRIB mutants. In contrast to the wild type WASP that was unresponsive to Rac1, the WASP mutant with the N-WASP CRIB domain produced 10% of the theoretical maximal number of actin filaments upon activation with Rac1 (Figure 2D). Finally, Rac1 activation of the N-WASP mutant with the swapped CRIB domain was moderately impaired compared to that of wild type N-WASP (Figure 2D) but with much attenuated inhibition by higher Rac1 concentrations. In conclusion, CRIB replacement altered the small GTPase specificity and response profiles but was unable to fully reproduce the specificity spectrum of corresponding native WASP and N-WASP proteins. This indicates that the CRIB domain is an important but not autonomous determinant of the specificity of WASP family regulation by Cdc42 and Rac1.

Nck Adaptor Proteins Are Very Potent WASP and N-WASP Activators. In addition to the Rho family GTPases, we tested WASP- and N-WASP-activating potency of Nck1 and Nck2 adaptor proteins. We found that Nck1 is a powerful activator of both WASP and N-WASP in the absence of PIP2 vesicles. There is a bell-shaped dependence between the concentration of Nck1 and the concentration of barbed ends (Figure 2A and B). Maximal stimulation of actin polymerization was observed by using 60 nM Nck1 in both FL WASP and N-WASP assays. To achieve the full activation of WASP and N-WASP, Nck1 had to be present in a ratio of 10:1 compared to WASPs in the assay. At the highest concentration used (2 μM), Nck1 had significantly less effect on either WASP or N-WASP activation (Figure 2A and B). Similar to Nck1, 60 nM Nck2 fully activated N-WASP, producing a maximal concentration of barbed ends and exhibiting the same bell-shaped dose dependence (Figure 2B). In contrast to the N-WASP assay, Nck2 partially stimulated actin polymerization in the WASP assay, producing approximately half of the maximal concentration of barbed ends (Figure 2A). Both Nck1 and Nck2 had no effect on actin polymerization if GST-WA (VCA) was used instead of FL WASPs (data not shown). This eliminates the possibility that the adaptor proteins affect actin polymerization independent of WASP. In conclusion, among the activators tested in this study, Nck1 and 2 are the most potent activators of both WASP and N-WASP, and they do not require PIP2 to fully activate WASP and N-WASP.

PIP2 Vesicles Preferentially Activate N-WASP but Not WASP. Next, we tested the effect of PIP2 vesicles on WASP and N-WASP activation alone or in the presence of small GTPases. Our data from the pyrene−actin polymerization assay indicated that PIP2 alone had no effect on FL WASP (Figure 3A). In a parallel assay using N-WASP, PIP2 vesicles alone partially stimulated actin polymerization (Figure 3B). Surprisingly, when used with Cdc42, PIP2 vesicles had a strong inhibitory effect on WASP-stimulated actin polymerization but no effect on Rac1 activation. PIP2 had a marked synergistic effect on N-WASP activation by Rac1 and Cdc42 (Figure 3B). We found that PIP2 increased the elongation rate 3-fold compared to that of polymerization with GTPases only, causing an almost full activation of N-WASP (Figure 3B). To rule out the concentration dependence of WASP activators on PIP2, we have tested the effect of PIP2 vesicles on WASP using small GTPases at both peak and half-maximal effect concentrations. Very similar results were observed in

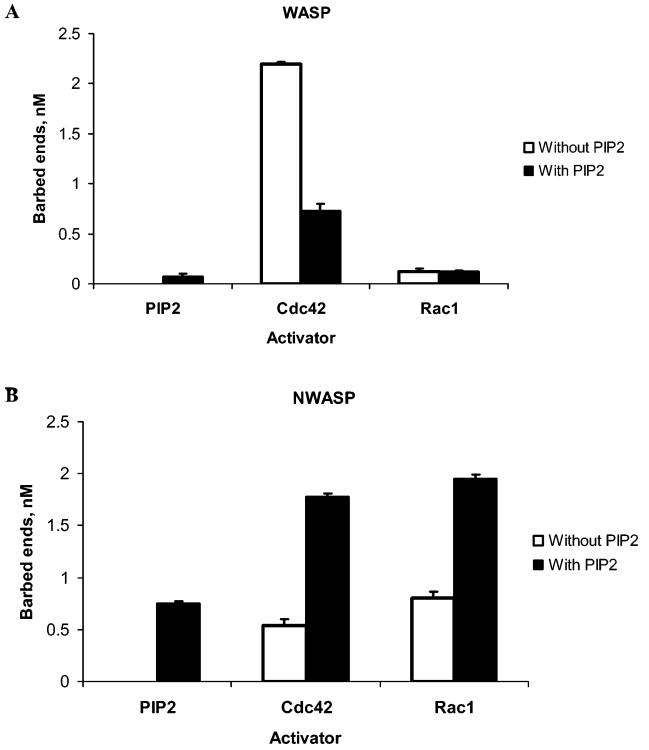

FIGURE 3: Effects of PIP2 on full-length WASP and N-WASP activation. (A) The regulation of full-length WASP by PIP2 vesicles was tested in a pyrene−actin polymerization assay by using 3.5 μM chicken muscle actin that was 15% labeled, 6 nM Arp2/3 complex, 1 nM full-length WASP with (black bars) or without (white bars) 20 μM lipid vesicles. As indicated, the effects of PIP2 on WASP were analyzed in the presence or absence of 300 nM Cdc42 and 300 nM Rac1. (B) Dependence of N-WASP activation on PIP2 was performed as described in A, by using 1 nM full-length N-WASP.

both experiments (data not shown). In addition, PIP2 exhibited dose-dependent activation of N-WASP in the presence of Cdc42, but PIP2 inhibited WASP-stimulated actin polymerization in the presence of Cdc42 in a dose-dependent manner (data not shown). From this data, we concluded that PIP2 has a differential effect on WASP and N-WASP.

DISCUSSION

Numerous studies have shown that the regulation of WASP and N-WASP is a complex process involving proteins from different pathways (*2*). Although intensively studied, the regulation of WASP family proteins is still not well understood. Problems associated with the expression of autoinhibited full-length WASP are the major reason for the lack of *in vitro* data that would describe the molecular mechanism of WASP regulation. Also, most reports assume that activation of WASP should be analogous to that of N-WASP, whose regulation is better characterized. This study represents the first characterization of human, recombinant full-length WASP and N-WASP expressed in human cells. We have observed that diverse WASP and N-WASP activators have qualitatively and quantitatively distinct effects on WASP and N-WASP regulation. Our *in vitro* data demonstrate that the differential regulation of WASP and N-WASP by cellular activators reflects fundamental differences of response at the protein−protein level.

Recombinant Full-Length WASP and N-WASP Are Highly Active and Autoinhibited. It was shown that native WASP from human leukocytes is autoinhibited and can be regulated with Cdc42 or PIP2 vesicles (*9*). Recombinant full-length WASP and N-WASP are either insoluble (*9*, *28*, *34*) or not very well regulated (*35*, *8*, *14*). We have purified human, recombinant full-length WASP and N-WASP expressed in 293 human cells that are completely soluble, highly active, and tightly regulated. The elongation rate of actin polymerization stimulated by WASP in the presence of an activator is 13 times higher than that without the activator. This well-controlled behavior suggests either the proper folding of the proteins or an effect of post-translational modifications of the proteins upon expression in human cells.

When activated with Cdc42, full-length WASP was 20 times more efficient in activating the Arp2/3 complex than the 105WASP derivative of WASP lacking the WH1 domain. We expressed 105WASP in *E. coli* and found that this protein is regulated with Cdc42. The higher potency of full-length WASP versus that of 105WASP may suggest that the WH1 domain has an important effect on actin polymerization, although expression in different systems should also be taken into consideration. It was reported that the WH1 domain of N-WASP interacts with F-actin (*8*). However, Suetsugu et al. showed that the WH1 domain binds the Arp2/3 complex and complements its activation by the WA portion (*36*). Both interactions of WH1 with actin or the Arp2/3 complex may as a consequence enhance the ability of N-WASP to stimulate actin polymerization. We have observed that the level of autoinhibition of full-length WASP is significantly higher than that of the 105WASP (data not shown). This suggests that the WH1 domain is crucial for the formation of a properly folded, autoinhibited WASP molecule.

Rac1 Is a More Potent N-WASP Activator than Cdc42. Our analysis of the activation of full-length WASP and N-WASP by Rho family small GTPases has yielded several novel results. We found that Rac1 is a more potent activator of N-WASP than Cdc42. In general, Cdc42 and Rac1 showed a differential profile of WASP and N-WASP activation. Rac1 preferentially activated N-WASP over WASP. Cdc42 is much more potent in activating WASP over N-WASP (Figure 2). Our *in vitro* data suggest a possibility of a novel signaling pathway that triggers an Arp2/3 complex-mediated actin nucleation through N-WASP and Rac1.

It is known that Rac1 has a significant effect on actin dynamics in cells. In general, it is believed that Rac1 functions through Scar/WAVE proteins, although it does not interact with them (*20*, *37*−*40*). This is the first study to show a direct *in vitro* effect of Rac1 on N-WASP activation.

One of the possible explanations for the differential regulation of WASP and N-WASP by Rac1 and Cdc42 can be based on the differences in the CRIB domains of the WASP family members. To address this, we expressed and characterized full-length WASP and N-WASP mutants with swapped CRIB domains (Figure 2C and D). We found that these mutants, to some extent, resembled the small GTPase specificity profile of the corresponding wild type proteins. Our data indicates that the CRIB domain is important, but it does not exclusively determine the specificity of WASP family regulation by Cdc42 and Rac1. It is likely that in addition to CRIB, other WASP and N-WASP regions modulate the interaction with GTPases. It was reported that the structure of a WASP CRIB domain attached to the WA domain of WASP is very different from the one when CRIB

was bound to Cdc42, suggesting dramatic structural changes upon the binding of Cdc42 (*13*). These significant structural changes could reflect or be dependent on other WASP regions. We speculate that in addition to CRIB-GTPase binding, the other WASP structural elements in the vicinity of CRIB influence the interaction with GTPases, providing an extra element that controls the specificity of interaction. This additional binding site may have an important role in determining the binding affinities of CRIB-interacting proteins and even lead to the inhibition at high activator concentrations, which will be discussed later. It is clear that additional work on the structural determinants of WASP/N-WASP activation will be required.

Our data differ from previously reported work on small GTPase stimulation of the Arp2/3 complex in *Xenopus* egg extracts (*41*). In our assay, we use the human Arp2/3 complex and human N-WASP expressed in human cells, whereas Ma et al. used *Xenopus* proteins (*41*). In addition, we run *in vitro* assays using purified proteins instead of crude extracts. *Xenopus* N-WASP and/or Rac1 may behave differently from human homologues, or there may be an inhibitory activity in the crude *Xenopus* egg extract preventing Rac1 from activating N-WASP. The influence of other N-WASP-interacting proteins present in crude extracts such as WIP and Toca-1 cannot be ruled out as well (*42*).

Nck1 and Nck2 Are the Most Powerful WASP and N-WASP Activators. Nck1 and Nck2 are SH2/SH3-domain-containing adaptor proteins. Here, for the first time we report *in vitro* evidence of WASP activation by Nck adaptor proteins. We have observed that the adaptor protein is a more potent activator of WASP than Cdc42. At 60 nM, Nck1 fully activated WASP (Figure 2A). In addition, we have shown that Nck1 fully activates N-WASP, producing concentrations of barbed ends close to the theoretical maximum. Interestingly, Nck1 does not require PIP2 vesicles to fully activate human N-WASP. This is inconsistent with reported data showing that Nck1 alone has a very modest effect on rat N-WASP activation (*14*, *26*). In addition to species differences, the activities of the two respective N-WASP recombinant proteins differ significantly. To produce a maximal concentration of barbed ends in the pyrene−actin polymerization assay, we used a concentration of N-WASP that was 50 times lower than that reported in the previous study by Rohatgi et al. (*14*, *26*). Unexpectedly, our data indicate that in addition to integrin signaling (*27*), Nck2 may play a direct role in actin cytoskeleton rearrangements by preferential activation of N-WASP. Unlike Nck1, which fully activated both WASP family member proteins, Nck2 manifested the differential regulation of WASP and N-WASP.

According to our dose−response data, it is clear that both Nck1 and Nck2 are more powerful N-WASP activators than the Rho family small GTPases (Figure 2B; Table 1). This suggests that the alternative mechanism of N-WASP activation through the poly proline domain is more efficient than activation through the CRIB domain. Our data correlate with reports showing that *in vitro* Nck-stimulated actin nucleation by the N-WASP−Arp2/3 complex is not further stimulated by Cdc42 (*26*).

Interestingly, most of the analyzed WASP and N-WASP activators have exhibited bell-shaped dose dependence, becoming very weak activators at high (μM) concentrations. This may explain why our observations were missed in previously reported studies. A trivial explanation for the bell-shaped response curves could be that higher concentrations of WASP and N-WASP activators sequester free actin, thereby impairing actin polymerization. That possibility was ruled out by the observation that high concentrations of the activators have no effect on actin polymerization mediated by the Arp2/3 complex and the WA domain of WASP (data not shown). This observation indicates that the activators of WASP/N-WASP have no effect on the Arp2/3 complex and the C-terminal domain of WASP. It is feasible that when present at markedly high concentrations (WASP/activator ratio is 1:1000 and higher), poly proline domain-binding activators (Nck) attach to more than one binding site per WASP molecule, impairing its ability to form a complex with Arp2/3 and actin. Similarly, excess amounts of small GTPases may cause substrate-type inhibition by interacting with a putative secondary low-affinity binding site on WASP or N-WASP. The observed dose−response data with Nck1 and Nck2 and Rac1 fit well to this simple model (Figure 2A and B; Table 1).

Cdc42 and Rac are prenylated *in vivo*. It has been reported that the prenylation of Cdc42 is essential for its activity (*9*). In this study, we used non-prenylated small GTPases that were expressed in *E. coli*. We performed a full dose-dependence test of the purified proteins as an appropriate biochemical characterization of the competency to stimulate actin polymerization. Our data show that non-prenylated Cdc42 is capable of fully activating WASP because the maximum amount of generated barbed ends is comparable to the concentration of the Arp2/3 complex present in the polymerization mix.

Lipid Vesicles Containing PIP2 Have a Differential Effect on WASP and N-WASP. Several studies have shown that PIP2 vesicles have a significant impact on WASP- and N-WASP-stimulated actin polymerization (*9*, *12*, *14*). In this study, we have demonstrated that PIP2 vesicles have differential effects on WASP and N-WASP. We found that PIP2 vesicles alone stimulate actin polymerization by N-WASP but not by WASP. Unexpectedly, PIP2 vesicles alone had no effect on WASP; moreover, PIP2 had and inhibitory effect on Cdc42-stimulated activation of full-length WASP. This experiment was done with two Cdc42 concentrations that would cause full and partial WASP activation. At both conditions, inhibition upon the addition of PIP2 was observed. Differential regulation of WASP and N-WASP by PIP2 vesicles might correlate with the distinctive affinities of WASP and N-WASP for the lipids. Previously, it was suggested that both WASP and N-WASP interact with PIP2 by their pleckstrin homology domains (PH) that partially include WH1 domains (*10*). A more recent model proposes that PIP2 binds to the basic region of N-WASP that has a net charge of +9 and is located N-terminal to the CRIB (*11*, *12*). Several lines of evidence have demonstrated that the basic region is essential for the regulation of N-WASP by PIP2 (*11*, *12*). In contrast to N-WASP, WASP's basic domain weakly interacts with PIP2 and has a net charge of +5 (*9*). It is still not clear whether the basic domain of WASP is the primary PIP2-interacting site. Although different affinities of WASP and N-WASP for PIP2 vesicles may have an effect on the differential regulation of WASP and N-WASP by PIP2, additional work is required to further clarify this complex cellular process.

Our data differ from a previously reported study showing that prenylated Cdc42 synergized with PIP2 in stimulating actin nucleation by native WASP and the Arp2/3 complex (*9*). At this moment, we do not have a reliable explanation for the discrepancies between the data. The most obvious difference is that Higgs and Pollard were using native WASP, whereas we used recombinant WASP. We have confirmed the consistency of our observations by using lipids from several sources, including the one used in Higgs' and Pollard's work.

This report shows that WASP and N-WASP can be regulated by a range of proteins and lipids implicated in essential cellular pathways, including the previously unknown activation of N-WASP by Rac1. We have demonstrated that the ability of full-length WASP and N-WASP to stimulate actin polymerization is distinct and generally higher from their truncated forms commonly used in previous studies. Data gathered with the full-length proteins have shown that WASP and N-WASP are differentially regulated by several cellular activators. Our observations indicate that despite the superficially similar architecture, the distinct cellular roles of WASP and N-WASP may reflect intrinsic differences in potency and specificity of regulation in addition to the known external factors such as their cellular localizations or expression levels.

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SUPPORTING INFORMATION AVAILABLE

Activation of WASP and N-WASP by small GTPases on beads and the cloning of expression constructs. This material is available free of charge via the Internet at http://pubs.acs.org.

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