Interaction of N-WASP with hnRNPK and Its Role in Filopodia Formation and Cell Spreading*

Received for publication, November 2, 2005, and in revised form, March 28, 2006. Published, JBC Papers in Press, March 30, 2006. DOI 10.1074/jbc.M511825200

Youngdong Yoo‡, Xiaoyang Wu‡, Coumaran Egile‡, Rong Li‡, and Jun-Lin Guan‡

From the ‡Department of Molecular Medicine, Cornell University, Ithaca, New York 14853 and the §Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

N-WASP is a member of the WASP family of proteins, which play essential roles in actin dynamics during cell adhesion and migration. hnRNPK is a member of the heterogeneous nuclear ribonucleoprotein complex, which has also been implicated in the regulation of cell spreading. Here, we identify a direct interaction between N-WASP and hnRNPK. We show that this interaction is mediated by the N-terminal WH1 domain of N-WASP and the segment of hnRNPK containing its K interaction (KI) domain. Furthermore, these two proteins are co-localized at the cell periphery in the spreading initiation center during the early stage of cell spreading. We found that co-expression of hnRNPK with N-WASP reverses the stimulation of cell spreading by N-WASP, and this effect is correlated with hnRNPK binding to N-WASP. Expression of hnRNPK does not affect subcellular localization of N-WASP protein. However, co-expression of hnRNPK with N-WASP reduced filopodia formation stimulated by N-WASP in spreading cells. Together, these results identify hnRNPK as a new negative regulator of N-WASP and suggest that hnRNPK may regulate the initial stage of cell spreading by direct association with N-WASP in the spreading initiation center.

Cell migration is an important biological process in embryonic development as well as wound repair, angiogenesis, and tumor metastasis (1, 2). Cell migration is a multistep process involving protrusion of the cell membrane and formation of a new attachment in the leading edge, myosin-actin-mediated cell contraction and release of attachment at the rear part of the cell. When they first contact the substrate, cells spread to maximize the contact area by protruding the membrane, which is considered to be similar to the forward extension of filopodia and lamellipodia in the initial step of cell migration (3). Cell spreading is driven by actin polymerization, which is regulated by actin nucleation machinery involving Arp2/3 complex (4). Arp2/3 complex is activated by the Wiskott-Aldrich syndrome protein (WASP)5 protein family through direct binding to the conserved verproline homology, cofilin homology, acidic (VCA) domain of WASP (5). N-WASP is a member of the WASP family and an effector in Cdc42-mediated regulation of actin cytoskeleton and filopodia formation (6–11). N-WASP is inactive in quiescent cells, and it is activated by Cdc42 and Toca-1 in a two-step activation mechanism (12, 50, 54–57, 61). GTP-Cdc42 first binds to Toca-1, and this complex binds to the G protein-binding domain and the polyproline domains of N-WASP. Activation of N-WASP exposes the VCA domain, which interacts with Arp2/3 complex and induces actin polymerization (5, 12).

hnRNPK is a well conserved RNA binding protein, originally identified as a component of the heterogeneous nuclear ribonucleoprotein complex (13–15). It is a modular protein with three conserved KH (K homology) domains for RNA/DNA binding and the KI region between KH2 and KH3 involved in interaction with a variety of other proteins (16–25). hnRNPK has been found not only in the nucleus but also in the cytoplasm and is proposed to be involved in a variety of cellular functions such as regulation of transcription and translation, RNA splicing, mRNA stability, chromatin remodeling, and signal transduction (19, 25–40). Recently, de Hoog et al. (41), using a proteomic approach, discovered that hnRNPK is also involved in cell adhesion. They showed that hnRNPK is present in a novel structure called the spreading initiation center (SIC), which is similar, but distinct, from the more mature focal adhesions. A functional role of hnRNPK in cell adhesion is supported by the observation that inhibition of hnRNPK by antibodies led to an increased spreading of the cell. This study suggests a potential role of hnRNPK in cell spreading; nevertheless, little is known about the mechanism by which hnRNPK regulates cell spreading.

In this study, we identify a direct interaction between N-WASP and hnRNPK, which is mediated by the N-terminal WH1 domain of N-WASP and the segment of hnRNPK containing its KI domain. We found that N-WASP and hnRNPK are co-localized at the cell periphery, which resembles the SIC, in the early spreading stage. Furthermore, co-expression of hnRNPK reverses N-WASP-induced filopodia formation and cell spreading. These results suggest that hnRNPK regulates cell spreading through its inhibition of N-WASP.

EXPERIMENTAL PROCEDURES

Antibodies—The rabbit polyclonal α-HA (Y11) antibody, the mouse monoclonal α-c-Myc-tag (9E10) antibody, the rabbit polyclonal α-hnRNPK antibody, and the rabbit polyclonal α-ParP antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Affinity-purified antibody against GST was prepared from anti-GST serum using GST immobilized on glutathione-Sepharose as an affinity matrix. The mouse monoclonal α-vinculin antibody was obtained from Sigma. The rabbit antibody against N-WASP was a generous gift of Dr. H. Miki (University of Tokyo).

Cell Culture and Transfection—293 cells were cultured in DMEM with 10% FBS. mouse embryonic fibroblast (MEF) cells derived from mouse embryo were maintained in DMEM supplemented with 10% FBS. CHO cells were cultured in Ham’s F-12 with 10% FBS, and NIH3T3 cells were maintained in DMEM with 10% calf serum. Transient transfections were performed using Lipofectamine (Invitrogen) according to

* This work was supported by National Institutes of Health Grants GM48050 and HL73394 (to J.-L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel: 607-253-3586; Fax: 607-253-3708; E-mail: jg19@cornell.edu.

‡ The abbreviations used are: WASP, Wiskott-Aldrich syndrome protein; hnRNPK, heterogeneous nuclear ribonucleoprotein K; VCA, verproline homology, cofilin homology, acidic; KH, K homology; KI, K interaction; SIC, spreading initiation center; HA, hemagglutinin; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; GFP, green fluorescent protein; FN, fibronectin; MEF, mouse embryonic fibroblast; FAK, focal adhesion kinase; WCL, whole cell lysates.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
hnRNPK Inhibition of N-WASP in Cell Spreading

A) Myc–N-WASP

\[ \text{IP : HA, IB : Myc, WCL : GST-hnRNPK} \]

B) GST pulldown

\[ \text{IB : N-WASP} \]

C) Cell staining

\[ \text{Ponceau S Staining} \]

Figure 1. Association of N-WASP with hnRNPK. A, 293 cells were co-transfected with plasmids encoding Myc-tagged N-WASP and HA-tagged hnRNPK or vector control (−) as indicated. Lysates were prepared and immunoprecipitated with anti-HA antibody, followed by Western blotting with anti-Myc (top panel) or anti-HA (bottom panel). Aliquots of the lysates (whole cell lysates (WCL)) were also analyzed directly by blotting with anti-Myc to verify equal amounts of N-WASP in samples (middle panel). B, lysates were prepared from NIH3T3 cells and immunoprecipitated with anti-N-WASP antibody or control IgG. The immunoprecipitates (IP) and aliquots of the lysates (WCL) were analyzed by Western blotting with anti-hnRNPK. C, GST fusion protein containing hnRNPK and recombinant His-tagged N-WASP protein were purified from bacteria and insect Sf21 cells, respectively. GST pulldown assays were performed using a mixture of His-N-WASP and GST-hnRNPK or GST alone as control, followed by Western blotting with anti-N-WASP antibody. The membrane used for Western blotting was stained with Ponceau S to show equal amounts of proteins.

Nuclear and Cytoplasmic Fractionation—Fractionation was performed essentially as described (45). Briefly, cells were lifted by trypsinization, washed with phosphate-buffered saline, then lysed in a lysis buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 2 mM MgCl2, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 20 mg/ml leupeptin) for 10 min. The lysates were centrifuged at 1,500 \( \times g \) for 5 min to sediment nuclei. The supernatant was then centrifuged at 15,000 \( \times g \) for 10 min, and the supernatant formed the cytoplasmic fraction. The nuclear pellet was washed three times with lysis buffer and then resuspended in the same lysis buffer supplemented with 0.5 mM NaCl to extract nuclear proteins. The extracted nuclear proteins were sedimented at 15,000 \( \times g \) for 10 min, and the resulting supernatant was harvested as the nuclear fraction.

Fluorescent Microscopy—Cells were processed for immunofluorescent staining as described previously (46). Cells were lifted by trypsinization, pelleted, resuspended in serum-free media, and incubated at 37 °C for 1 h with gentle agitation. The cells were pelleted, resuspended in media with 10% FBS, and then allowed to spread on the fibronectin (FN)-

the manufacturer’s guidelines. Transfection efficiency was about 90% in 293T cells, 10–20% in NIH3T3 cells and 40–50% in CHO cells as detected by fluorescent microscopy using GFP as a marker. In case of co-transfection, each plasmid encoding protein was used in a 1:1 ratio. Co-transfection efficiency was determined to be nearly 100% by indirect immunofluorescent staining using anti-Myc and anti-HA. In some experiments, GFP-encoding plasmid was used as transfection marker. The ratio between GFP, HA-tagged and Myc-tagged protein-coding plasmid was 1:3:3. After transfection, GFP-positive cells were considered as co-transfected cells.

Plasmid Construction—pKH3-N-WASP, pHAN-N-WASP, pDHGST-N-WASP, pDH-N-WASP, pDH-N-WASP1, pDH-N-WASP2, pDH-N-WASP3, pDH-N-WASP4 and pEGFP-N-WASP have been described previously (42). The hnRNPK cDNA, a generous gift from Dr. David Levens (National Institutes of Health), was used as a template for PCR amplification. The PCR product with the primers 5‘-tcagatgattcatgaaactcgaagccgcaagacctctc-3’ and 5‘-taaaccgaattctaaacctttcacagccgctca-3’ was digested with EcoRI and then ligated to a linearized pKH3 vector with EcoRI sites on the ends to generate pKH3-hnRNPK. The EcoRI fragment digested from pKH3-hnRNPK was inserted into linearized pHAN and pGEX2T-hnRNPK, respectively. hnRNPK truncation mutant encoding residues 1–337(K1), 1–209(K2), 1–104(K3) and 171–337(K4) were made by PCR using primers: 5‘-tcagatccggaattctacaccctatc-3’ and 5‘-tcagatgattcatgaaactcgaagccgcaagacctctc-3’ (hnRNPK-5) and 5‘-taaaccgaattcattacagccgactcttgcacttc-3’ (337-3), hnRNPK-5 and 5‘-taaaccgaattcattacagccgactcttgcacttc-3’ (209-3), hnRNPK-5 and 5‘-taaaccgaattcattacagccgactcttgcacttc-3’ (171-5) and 337-3, respectively. The PCR products were digested with Smal and EcoRI and then inserted into pKH3 at the corresponding sites to generate pKH3-K1, pKH3-K2, pKH3-K3, and pKH3-K4, respectively.

Immunoprecipitation and Western Blotting—Subconfluent cells were washed with ice-cold phosphate-buffered saline twice and lysed with 1% Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 20 mg/ml leupeptin). Lysates were cleared by centrifugation for 20 min at 4 °C, and protein concentration was determined by Bio-Rad protein assay. Immunoprecipitations were performed by incubating cell lysates with appropriate antibodies, as indicated, for more than 2 h at 4 °C. For the experiment to detect interaction between endogenous proteins, Immunoprecipitations were followed by incubation with protein A-Sepharose for another 2 h. After three times washing, the immune complex was resolved by SDS-PAGE. Western blotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody and ECL system for detection.

Preparation of GST Fusion Proteins and in Vitro Binding Assay—GST fusion proteins were produced and purified as described previously (44). GST fusion proteins were immobilized on glutathione-agarose beads and then incubated with recombinant His-tagged N-WASP for more than 2 h at 4 °C in 300 μl of Nonidet P-40 buffer. After washing, the bound proteins were analyzed by Western blotting with antibody for N-WASP. For in vitro binding assays to measure the binding constant, a serially increasing amount of His-tagged N-WASP (0.003–0.2 μM) and GST-hnRNPK or GST-K4 fragment were used. The intensity of the bands was quantified with Image J software. To calculate the binding constant \( K_d \), the resulting data were fit to a single rectangular hyperbola equation with Prism 3.0 (Graph Pad Software, San Diego, CA): \( B = B_{max}C/(K_d + C) \), where \( B \) is the relative value of bound protein, and \( C \) is the concentration of samples tested.

JUNE 2, 2006•VOLUME 281•NUMBER 22 JOURNAL OF BIOLOGICAL CHEMISTRY 15353
FIGURE 2. Analysis of interaction between N-WASP and hnRNPK. A, schematic diagram of N-WASP segments used in the binding assay in B, B. 293 cells were co-transfected with plasmids encoding Myc-hnRNPK and expression vector pDHGST encoding different N-WASP fragments or vector alone (GST), as indicated. Lysates were prepared, and N-WASP fragments were pulled down by adding glutathione-coupled agarose beads. The samples were resolved by SDS-PAGE and blotted with anti-Myc (top panel) or anti-GST (bottom panel). Aliquots of the lysates (WCL) were also analyzed directly by blotting with anti-Myc to verify equal amounts of hnRNPK in samples. C, schematic diagram of hnRNPK and its truncated mutants used in the binding assay in D, D. 293 cells were co-transfected with plasmids encoding Myc-tagged N-WASP and HA-tagged hnRNPK or its truncated mutants or vector control (293/H11022) as indicated. Lysates were prepared and immunoprecipitated using anti-HA. The samples were resolved in SDS-PAGE and blotted with anti-Myc (top panel) or anti-HA (bottom panel). Aliquots of the lysate (WCL) were analyzed directly by blotting with anti-Myc to show equal levels of N-WASP in samples. E, equal amounts of immobilized GST-hnRNPK or GST-hnRNPK-K4 fragment were incubated with an increasing amount of recombinant His-tagged N-WASP. The bound proteins were resolved on SDS-PAGE, followed by Western blotting, and quantified by digital scanning and densitometry. The values were converted into relative binding to the value of maximal binding. The resulting data were fit to a single rectangular hyperbola equation, yielding $K_d$ values of 0.06 and 0.057 M for GST-hnRNPK and GST-K4 fragment, respectively. The results show mean $\pm$ S.E. for three independent experiments.
coated coverslip. After 25 min, cells were fixed with 3.7% formaldehyde and subjected to fluorescent immunostaining. The primary antibodies were rabbit anti-HA antibody and mouse monoclonal anti-Myc antibody. For secondary antibodies, Texas Red-conjugated goat anti-rabbit antibody (1:200, Jackson ImmunoResearch Laboratory, West Grove, PA) and fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody were used. For confocal microscopy, coverslips were imaged using Leica TCS SP2 (sequential scan). For phalloidin staining to detect filament formation, cells in suspension were pelleted, resuspended in media with 0.2% calf serum, and then allowed to spread on the FN-coated coverslip. 30 min after plating, cells were fixed and subject to immunofluorescent staining. GFP was observed directly by fluorescent microscopy.

Cell Spreading Assay—Cell spreading assays were performed as described previously (47). Briefly, cells were lifted by trypsinization, pelleted, resuspended in serum-free media and incubated at 37 °C for 1 h with gentle agitation. The cells were pelleted, resuspended in media with 0.2% FBS, and then allowed to spread on the FN-coated plate. After 1.5 h, cells were fixed with 3.7% formaldehyde and photographed. Spread cells were defined as cells with irregular morphology and lacking phase brightness; non-spread cells were rounded and phase-bright under the microscope. Multiple fields were imaged and ~200 transfected cells were monitored and counted blindly for each experiment. Three independent experiments were performed, and the Student’s t test was used to determine the statistical significance.

Pyrenyl Actin Polymerization Assays—Bovine Arp2/3 complex 10 nM and activator were added to 1.5 μM Mg2+/ATP-G-actin (10% pyrene labeled) in KMET buffer (50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 10 mM Tris, pH 7.0) supplemented with 0.5 mM ATP. Actin polymerization was monitored by continuous pyrene fluorescence measurements (λex = 386 nm, λem = 407 nm) in a Cary Eclipse fluorescence spectrophotometer (Varian). Actin was purified from rabbit muscle and isolated as Ca2+-ATP-G-actin in G buffer (5 mM Tris-Cl, pH 7.8, 0.1 mM CaCl2, 0.2 mM ATP, and 1 mM dithiothreitol) according to Pardee and Spudich (48) and pyrenyl labeled. Bovine GST-N-WASP WA and bovine Arp2/3 complex were purified as described previously (49).

RESULTS

Identification of N-WASP Interaction with hnRNPK—To further investigate the potential mechanisms of regulation of N-WASP, we searched for additional proteins that interact with N-WASP via tandem tag affinity purification followed by mass spectrometry for protein identification. One of the novel N-WASP-associated proteins was identified as an RNA-binding protein, hnRNPK. Although hnRNPK was not known to be involved in actin-related cellular function, de Hoog et al. (41) recently reported that hnRNPK is localized in the SIC and the inhibition of hnRNPK by antibodies led to an increased cell spreading, suggesting a potential role for hnRNPK in the down-regulation of cell adhesion. To validate association of N-WASP with hnRNPK, 293 cells were co-transfected with plasmids encoding Myc-tagged N-WASP and HA-tagged hnRNPK or HA alone as a control. The cell lysates were then immunoprecipitated with antibody against HA and blotted with anti-Myc. Fig. 1A shows that N-WASP was associated with hnRNPK but not present in the immunoprecipitates from control cells. To examine the interaction between endogenous N-WASP and hnRNPK, co-immunoprecipitation experiments were performed using cell lysates prepared from NIH3T3 cells. The lysates were immunoprecipitated by antibodies against N-WASP, and the immune complexes were subjected to Western blotting with anti-hnRNPK to detect associated hnRNPK. Fig. 1B shows the co-precipitation of hnRNPK with N-WASP but not with the control antibody. To determine whether the interaction between N-WASP and hnRNPK is direct or not, we prepared a GST fusion protein containing hnRNPK and a recombinant His-tagged N-WASP protein and used these for an in vitro binding assay. Fig. 1C shows the association of recombinant His-tagged N-WASP with GST-hnRNPK but not with control GST alone. Together, these results identify a specific and direct interaction between N-WASP and hnRNPK.

To determine which domain(s) of N-WASP is responsible for binding to hnRNPK, 293 cells were co-transfected with plasmids encoding Myc-tagged hnRNPK and vectors encoding GST fusion proteins containing N-WASP fragments (see Fig. 2A). Lysates were prepared from the transfected cells, and GST fusion proteins were pulled down with glutathione-agarose beads followed by Western blotting with anti-Myc antibody to detect associated Myc-hnRNPK. Fig. 2B shows that hnRNPK was associated with the N-terminal fragment of N-WASP containing EVH1/WH1 domain (residues 1–148) but not with other fragments corresponding to G protein-binding domain, proline-rich, and VCA domains. A similar strategy was used to define the N-WASP binding site on hnRNPK by co-transfection of 293 cells with plasmids encoding Myc-tagged N-WASP and vectors encoding HA-tagged hnRNPK or its fragments (see Fig. 2C). Fig. 2D shows that the full-length hnRNPK, the K1 (residues 1–337) and K4 (residues 171–337), which contains KI domain) fragments were associated with N-WASP, but K2 (residues 1–209) and K3 (residues 1–104) fragments were not. Together, these results suggest that the interaction between N-WASP and hnRNPK is mediated by the EVH1/WH1 domain of N-WASP and the region of hnRNPK containing its KI domain.

To obtain the binding constant for the interaction between N-WASP and hnRNPK, an in vitro binding assay using a serially increasing amount of recombinant His-tagged N-WASP and GST-hnRNPK was performed as described under “Experimental Procedures.” The GST pulldown assay was followed by Western blotting with anti-N-WASP antibody, and the intensity of each band was measured by densitometry. We estimated that the binding constant (Kd) is 60 nM. (Fig. 2E). Similar assays showed an approximate Kd of 57 nM for association of N-WASP and hnRNPK K4 fragment (Fig. 2E). These results suggested that the K4 fragment of hnRNPK is primarily responsible for hnRNPK interaction with N-WASP.
Co-localization of N-WASP and hnRNPK in the SIC during Early Stage of Cell Spreading—Consistent with its role in the regulation of actin polymerization, N-WASP is localized in the cell periphery (42). Interestingly, recent studies suggested that hnRNPK is localized in the SIC, a distinctive patch-like structure associated with the cell periphery, in the early stage of cell spreading (41). Thus, we examined potential co-localization of N-WASP and hnRNPK in cells during their early spreading stage. Primary MEFs were co-transfected with plasmids encoding HA-tagged hnRNPK and Myc-tagged N-WASP or a control irrelevant protein, GST. Transfected cells were suspended, and replated on FN-coated coverslips. They were fixed in the early spreading stage and subjected to double-label immunofluorescence using rabbit anti-HA and mouse anti-Myc antibody. Fig. 3 shows that hnRNPK is localized in a distinct circular patch in the cell periphery resembling the SIC described previously (41). N-WASP is co-localized with hnRNPK in a punctuated pattern in the cell periphery, whereas GST is uniformly distributed in the cytoplasm. These results suggest specific interaction between N-WASP and hnRNPK in the SIC in the early spreading stage of the cell.

Inhibition of N-WASP Promoted Cell Spreading by hnRNPK—N-WASP is a well established critical regulator of actin polymerization, which is important for filopodia formation and cell spreading. Interestingly,
hnRNPK Inhibition of N-WASP in Cell Spreading

To explore potential mechanisms by which hnRNPK interacts with N-WASP, CHO cells were co-transfected with plasmids encoding Myc-tagged N-WASP or HA-tagged hnRNPK, or both plasmids, along with a vector encoding GFP as a transfection marker. Filopodia formation was evaluated by staining of polymerized actin with phalloidin for cells re-plated on FN. At this early time after re-plating, the majority of cells were attached to FN but still appeared rounded with phalloidin encompassing the entire cell (Fig. 6A). In contrast, cells transfected with N-WASP exhibited filopodia structures, indicating a role for N-WASP in the stimulation of filopodia formation as described previously (42). Expression of hnRNPK did not affect filopodia formation under these conditions. However, co-expression of hnRNPK with N-WASP reversed the stimulatory effect of N-WASP on filopodia formation. Furthermore, the hnRNPK K1 fragment that binds to N-WASP but not the K2 fragment, which does not associate with N-WASP, also reduced stimulation of filopodia formation by N-WASP. Together, these results suggest that the inhibition of cell spreading by hnRNPK is mediated by its ability to interact with N-WASP and to negatively regulate filopodia formation by N-WASP.

We next investigated whether hnRNPK can influence filopodia formation induced by N-WASP, which is a critical step in the early stage of cell spreading. NIH3T3 cells were co-transfected with plasmids encoding Myc-tagged N-WASP or HA-tagged hnRNPK, or both plasmids, along with a vector encoding GFP as a transfection marker. Filopodia formation was evaluated by staining of polymerized actin with phalloidin for cells re-plated on FN. At this early time after re-plating, the majority of cells were attached to FN but still appeared rounded with phalloidin encompassing the entire cell (Fig. 6A). In contrast, cells transfected with N-WASP exhibited filopodia structures, indicating a role for N-WASP in the stimulation of filopodia formation as described previously (42). Expression of hnRNPK did not affect filopodia formation under these conditions. However, co-expression of hnRNPK with N-WASP reversed the stimulatory effect of N-WASP on filopodia formation. Furthermore, the hnRNPK K1 fragment that binds to N-WASP but not the K2 fragment, which does not associate with N-WASP, also reduced stimulation of filopodia formation by N-WASP. Together, these results suggest that the inhibition of cell spreading by hnRNPK is mediated by its ability to interact with N-WASP and to negatively regulate filopodia formation by N-WASP.

We next investigated whether hnRNPK can influence filopodia formation induced by N-WASP, which is a critical step in the early stage of cell spreading. NIH3T3 cells were co-transfected with plasmids encoding Myc-tagged N-WASP or HA-tagged hnRNPK, or both plasmids, along with a vector encoding GFP as a transfection marker. Filopodia formation was evaluated by staining of polymerized actin with phalloidin for cells re-plated on FN. At this early time after re-plating, the majority of cells were attached to FN but still appeared rounded with phalloidin encompassing the entire cell (Fig. 6A). In contrast, cells transfected with N-WASP exhibited filopodia structures, indicating a role for N-WASP in the stimulation of filopodia formation as described previously (42). Expression of hnRNPK did not affect filopodia formation under these conditions. However, co-expression of hnRNPK with N-WASP reversed the stimulatory effect of N-WASP on filopodia formation. Furthermore, the hnRNPK K1 fragment that binds to N-WASP but not the K2 fragment, which does not associate with N-WASP, also reduced stimulation of filopodia formation by N-WASP. Together, these results suggest that the inhibition of cell spreading by hnRNPK is mediated by its ability to interact with N-WASP and to negatively regulate filopodia formation by N-WASP.

To further explore the mechanisms by which hnRNPK interacts with N-WASP, we examined whether hnRNPK could affect the activity of N-WASP to stimulate Arp2/3 complex-mediated actin polymerization in vitro. Surprisingly, GST-hnRNPK relieves the autoinhibitory conformation of N-WASP and appears to increase Arp2/3-dependent actin polymerization in vitro (Fig. 7). These results suggest that hnRNPK inhibition of cell spreading is not due to the direct inhibition of N-WASP conformational change but rather may occur through other mechanisms in the cell.

DISCUSSION

N-WASP is a well established regulator of actin polymerization through its interaction with Arp2/3 complex, a major actin nucleation
**FIGURE 6. Regulation of N-WASP-dependent filopodia formation by hnRNPK.** A, NIH3T3 cells were co-transfected with plasmids encoding Myc-tagged N-WASP, HA-tagged hnRNPK or truncated mutants, along with a vector encoding GFP as a transfection marker, as indicated. Transfected cells were replated onto FN-coated coverslips, and 30 min after replating, cells were fixed and subjected to immunofluorescent staining with Texas-red conjugated phalloidin to view actin filaments. GFPs were observed directly by fluorescence microscope. B, filopodia formation assays were performed as in panel A. The mean ± S.E. of percentage of cells with filopodia (among positively transfected cells as identified by GFP) from three independent experiments are shown as a relative index of filopodia-positive cells after normalization to that in mock-transfected cells. *, p < 0.05; **, p = 0.21 in comparison with value from mock cells. ***, p < 0.05; ****, p = 0.7 in comparison with value from Myc-N-WASP-transfected cells.

**FIGURE 7. Effect of hnRNPK on N-WASP-stimulated Arp2/3 complex-mediated actin polymerization.** A, purified GST-hnRNPK or GST alone was added to the N-WASP and Arp2/3 complex in pyrene actin assays as described under "Experimental Procedures." B, differential amounts of GST-hnRNPK were used in the pyrene actin assays as described for A. Three independent experiments were performed, and one representative experiment is shown.
apparatus in cells. It is activated by GTP-bound Cdc42 and Toca-1 (12, 50, 54–57, 61) and plays an essential role in mediating Cdc42 regulation of actin cytoskeleton and cell motility (9–11, 57). In addition to Cdc42 and Toca-1, recent studies have identified a number of other regulatory mechanisms of N-WASP. Src family kinases have been shown to associate with N-WASP through their SH2 and SH3 domains and phosphorylate N-WASP, which lead to activation of N-WASP (58, 59). More recently, Abi has been shown to activate N-WASP through a similar mechanism (60). Our recent studies suggested that FAK also binds to and phosphorylates N-WASP (42). However, FAK appears to affect N-WASP through a different mechanism. Phosphorylation of N-WASP by FAK did not stimulate N-WASP activity directly but rather prevented nuclear translocation of the activated N-WASP, resulting in increased ability of N-WASP to promote actin polymerization in the cytoplasm and cell migration (42). The differential effect of FAK and Src family kinases on N-WASP activity is likely due to FAK not having either an SH2 or SH3 domain, whose binding to N-WASP (rather than phosphorylation of N-WASP per se) is largely responsible for the stimulation of N-WASP activity (42). In the present study, we report a direct interaction between N-WASP and hnRNPK, which functions to inhibit N-WASP activity in filopodia formation and cell spreading. These results add to our knowledge of regulation mechanisms of N-WASP by providing an inhibitory regulation mechanism of N-WASP in mammalian cells. Given the central role of N-WASP in the regulation of actin polymerization, it is not surprising that N-WASP itself is subjected to multiple regulatory mechanisms.

Although both N-WASP and hnRNPK are present in the nucleus of growing cells, the inhibitory effect of hnRNPK on N-WASP likely occurs in the cell periphery during the initial stage of cell spreading. These two proteins are co-localized in the SIC (see Fig. 3), a cellular structure that has been proposed to play an important role in cell spreading (41). We also found that hnRNPK did not alter the cytoplasmic versus nuclear localization of N-WASP. Therefore, it is unlikely that hnRNPK affected N-WASP-promoted cell spreading via facilitating N-WASP translocation into the nucleus to reduce its presence in the actin polymerization site as proposed previously for FAK regulation of N-WASP (42). It is more likely that association of hnRNPK with N-WASP inhibits cell spreading through inhibition of N-WASP-induced filopodia formation, a process that occurs in the early stage of cell spreading.

A well established role of N-WASP is its ability to stimulate Arp2/3 complex-mediated actin polymerization, which drives filopodia formation. Surprisingly, however, hnRNPK did not inhibit, but rather enhanced, the function of N-WASP in Arp2/3 complex-mediated actin polymerization in in vitro pyrene actin assays (see Fig. 7). Such differential results between in vitro actin polymerization assay and in vivo activity has been described recently for WIP protein, another regulator of N-WASP. WIP association with N-WASP increases cellular F-actin contents and filopodia formation in vivo, but it inhibited N-WASP induced Arp2/3 complex-mediated actin polymerization in pyrene actin assays in vitro (62, 63). In any case, these data suggested that hnRNPK inhibition of N-WASP does not occur through a direct effect on its conformational change but may be via other mechanisms in vivo. It is possible that hnRNPK binding to N-WASP may displace a positive regulator of N-WASP in vivo, which may not be present in the purified in vitro pyrene actin assay systems, to reduce its function in filopodia formation and cell spreading. It is also possible that hnRNPK association with N-WASP could sequester it in a cellular localization to reduce its ability to promote actin polymerization in filopodia formation.

We found that overexpression of hnRNPK alone showed little effect on either cell spreading or filopodia formation. Interestingly, previous studies demonstrated that inhibition of endogenous hnRNPK by antibody injection increases cell spreading (41). This is consistent with an inhibitory function of hnRNPK in filopodia formation and cell spreading as proposed here. It is possible that endogenous hnRNPK is sufficient to inhibit endogenous N-WASP functions under normal conditions so that further increase of hnRNPK level by overexpression may not result in any additional inhibitory effects except when N-WASP is also overexpressed as observed in the current study.

Together with the previous paper by de Hoog et al. (41), our results suggest a novel function for hnRNPK in the regulation of cell spreading. It is interesting that hnRNPK and a number of other RNA binding proteins as well as RNA are localized in the SIC, raising the possibility that localized translation of selective messenger RNAs may play a role in cell spreading. However, previous studies showed that actin and FAK mRNA were not present in the SIC, and it is still unclear which mRNAs may be present whose translation may help to promote cell spreading. Data from our studies provide an alternative mechanism for a role of hnRNPK in the regulation of cell spreading by protein-protein interactions. Indeed, besides RNA and DNA binding activities in the KH domains, the region containing KI domain of hnRNPK, which has been shown to interact with proteins (39), is responsible for association with N-WASP and inhibition of cell spreading. Nevertheless, it will be interesting to determine in future studies whether the other RNA binding proteins as well as RNAs present in the SIC could modulate hnRNPK interaction with N-WASP and affect cell spreading.

Acknowledgments—We are grateful to Dr. H. Miki of University of Tokyo for the antibody against N-WASP, Dr. D. Levens of NIH for hnRNPK cDNA, and Dr. H. Sondermann for software program and help in data analysis of Fig. 2E. We thank our colleagues Zara Melkoumian, Xu Peng, Boyi Gan, Dan Rhoads, Richard Liang, and Huei Jin Ho for their critical reading of the manuscript and helpful comments.

REFERENCES
1. Christopher, R. A., and Guan, J. L. (2000) Int. J. Med. 5, 575–581
2. Laufenburger, D. A., and Horwitz, A. F. (1996) Cell 84, 359–369
3. Nakamura, M., and Dreyfuss, G. (1994) Trends Cell Biol. 4, 242–247
4. Welch, M. D. (1999) Trends Cell Biol. 9, 423–427
5. Rokutag, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999) Cell 97, 221–231
6. Worthylake, R. A., and Barridge, R. K. (2001) Curr. Opin. Cell Biol. 13, 569–577
7. Benesch, S., Lommel, S., Steffen, A., Stradal, T. E., Scapelhorn, N., Way, M., Weihland, J., and Rottner, K. (2002) J. Biol. Chem. 277, 37771–37776
8. Banzai, Y., Miki, H., Yamaguchi, H., and Takenawa, T. (2000) J. Biol. Chem. 275, 11987–11992
9. Takenawa, T., and Miki, H. (2001) J. Cell Sci. 114, 1801–1809
10. Miki, S., Sasaki, T., Takai, Y., and Takenawa, T. (1998) Nature 391, 93–96
11. Snapper, S. B., Takeshima, F., Anton, I., Liu, C. H., Thomas, S. M., Nguyen, D., Dudley, D., Fraser, H., Purich, D., Lopez-Ilasaca, M., Klein, C., Davidson, L., Bronson, R., Mulligan, R. C., Southwick, F., Gehr, G., Goldberg, M. B., Rosen, F. S., Hartwig, J. H., and Alt, F. W. (2001) Nat. Cell Biol. 3, 897–904
12. Prehoda, K. E., Scott, J. A., Mullins, R. D., and Lim, W. A. (2000) Science 290, 801–806
13. Swanson, M. S., and Dreyfuss, G. (1988) Mol. Cell. Biol. 8, 2237–2241
14. Matunis, M. J., Michael, W. M., and Dreyfuss, G. (1992) Mol. Cell. Biol. 12, 164–171
15. Nacket, K., Arnould, N., and Chesneau, J. (1994) Nucleic Acids Res. 22, 1509–1510
16. Grishin, N. V. (2001) Nucleic Acids Res. 29, 638–643
17. Siomi, M., Matunis, M. J., Michael, W. M., and Dreyfuss, G. (1993) Nucleic Acids Res. 21, 1193–1198
18. Siomi, H., Choi, M., Siomi, M. C., Nussbaum, R. L., and Dreyfuss, G. (1994) Cell 77, 33–40
19. Shreve, M., Schullery, D. S., Suzuki, H., Higaki, Y., and Bomsztyk, K. (2000) J. Biol. Chem. 275, 15498–15503
20. Denisenko, O. N., O'Neill, B., Ostrowski, J., Van Seuningen, I., and Bomsztyk, K. (1996) J. Biol. Chem. 271, 27701–27706
HnRNPK Inhibition of N-WASP in Cell Spreading

21. Collier, B., Goobar-Larsson, L., Sokolowski, M., and Schwartz, S. (1998) J. Biol. Chem. 273, 22648–22656
22. Van Seuningen, I., Ostrowski, J., Bustelo, X. R., Sleath, P. R., and Bomsztyk, K. (1995) J. Biol. Chem. 270, 26976–26985
23. Ostrowski, J., Sims, J. E., Sibley, C. H., Valentine, M. A., Dower, S. K., Meier, K. E., and Bomsztyk, K. (1991) J. Biol. Chem. 266, 12722–12733
24. Weng, Z., Thomas, S. M., Bickles, R. J., Taylor, J. A., Brauer, A. W., Seidel-Dugan, C., Michael, W. M., Dreyfuss, G., and Brugge, J. S. (1994) Mol. Cell. Biol. 14, 4509–4521
25. Michelot, E. F., Michelot, G. A., Arousson, A. I., and Levison, D. (1996) Mol. Cell. Biol. 16, 2350–2360
26. Denisenko, O. N., and Bomsztyk, K. (1997) Mol. Cell. Biol. 17, 4707–4717
27. Denisenko, O., and Bomsztyk, K. (2002) Mol. Cell. Biol. 22, 286–297
28. Ostareck-Lederer, A., Ostareck, D. H., Cans, C., Neubauer, G., Bomsztyk, K., Superti-Furga, G., and Hentze, M. W. (2002) Mol. Cell. Biol. 22, 4535–4543
29. Cao, R., Wang, L., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002) Science 298, 1039–1043
30. Du, W., Thanos, D., and Maniatis, T. (1993) Cell 74, 887–898
31. Haieh, T. Y., Matsumoto, M., Chou, H. C., Schneider, R., Hwang, S. B., Lee, A. S., and Lai, M. M. (1998) J. Biol. Chem. 273, 17651–17659
32. Expert-Bezancon, A., Le Caer, J. P., and Marie, J. (2002) J. Biol. Chem. 277, 16614–16623
33. Evdokimova, V., Ruzanov, P., Imataka, H., Raught, B., Svitkin, Y., Ovchinnikov, L. P., and Sonenberg, N. (2001) EMBO J. 20, 5491–5502
34. Ostareck-Lederer, A., Ostareck, D. H., Standart, N., and Thiele, B. J. (1994) EMBO J. 13, 1476–1481
35. Chen, C. Y., Gherzi, R., Andersen, J. S., Gaietta, G., Jurchott, K., Roey, H. D., Mann, M., and Karin, M. (2000) Genes Dev. 14, 1236–1248
36. Kim, J. H., Hahm, B., Kim, Y. K., Choi, M., and Jang, S. K. (2000) J. Mol. Biol. 298, 395–405
37. Ostareck-Lederer, A., Shatsky, I. N., and Hentze, M. W. (2001) Cell 104, 281–290
38. Habelhah, H., Shah, K., Huang, L., Ostareck-Lederer, A., Burlingame, A. L., Shokat, K. M., Hentze, M. W., and Boruki, Z. (2001) Nat. Cell Biol. 3, 325–330
39. Bomsztyk, K., Denisenko, O., and Ostrowski, J. (2004) BioEssays 26, 629–638
40. Bomsztyk, K., Van Seuningen, I., Suzuki, H., Denisenko, O., and Ostrowski, J. (1997) FEBS Lett. 403, 113–115
41. de Hoog, C. L., Foster, L. J., and Mann, M. (2004) Cell 117, 649–662
42. Wu, X., Suetsugu, S., Cooper, L. A., Takenawa, T., and Guan, J. L. (2004) J. Biol. Chem. 279, 9565–9576
43. Han, D. C., Shen, T. L., Miao, H., Wang, B., and Guan, J. L. (2002) J. Biol. Chem. 277, 45665–45661
44. Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F., and Chen, H. C. (1999) J. Biol. Chem. 274, 12361–12366
45. Lin, S. Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K. Y., Bourguignon, L., and Hung, M. C. (2001) Nat. Cell Biol. 3, 802–808
46. Zhao, J. H., Reiske, H., and Guan, J. L. (1998) J. Cell Biol. 143, 1997–2008
47. Han, D. C., Rodriguez, L. G., and Guan, J. L. (2001) Oncogene 20, 346–357
48. Padree, J. D., and Spudich, J. A. (1982) Methods Cell Biol. 24, 271–289
49. Egle, C., Loisel, T. P., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P. J., and Carlier, M. F. (1999) J. Cell Biol. 146, 1319–1332
50. Hikari, M., Mura, K., and Takenawa, T. (1996) EMBO J. 15, 5326–5335
51. Suetsugu, S., Miki, H., and Takenawa, T. (1998) EMBO J. 17, 6516–6526
52. Yamaguchi, H., Miki, H., Suetsugu, S., Ma, L., Kirschner, M. W., and Takenawa, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12631–12636
53. Fukuoka, M., Suetsugu, S., Miki, H., Fukami, K., Endo, T., and Takenawa, T. (2001) J. Cell Biol. 152, 471–482
54. Ma, L., Rohatgi, R., and Kirschner, M. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15362–15367
55. Rohatgi, R., Ho, H. Y., and Kirschner, M. W. (2000) J. Cell Biol. 150, 1299–1310
56. Kim, A. S., Kakalis, L. T., Abdul-Manan, N., Liu, G. A., and Rosen, M. K. (2000) Nature 404, 151–158
57. Ma, L., Cantley, L. C., Janney, P. A., and Kirschner, M. W. (1998) J. Cell Biol. 140, 1125–1136
58. Suetsugu, S., Hattori, M., Miki, H., Tezuka, T., Yamamoto, T., Mikoshiba, K., and Takenawa, T. (2002) Dev. Cell 3, 645–658
59. Torres, E., and Rosen, M. K. (2003) Mol. Cell 11, 1215–1227
60. Burton, E. A., Oliver, T. N., and Pendergast, A. M. (2005) Mol. Cell. Biol. 25, 8834–8843
61. Ho, H. Y., Rohatgi, R., Lebensohn, A. M., Le, M., Li, J., Gygi, S. P., and Kirschner, M. W. (2004) Cell 118, 203–216
62. Ramesh, N., Antoon, I. M., Hartwig, J. H., and Geha, R. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14671–14676
63. Martinez-Quiles, N., Rohatgi, R., Antoon, I. M., Medina, M., Saville, S. P., Miki, H., Yamaguchi, H., Takenawa, T., Hartwig, J. H., Geha, R. S., and Ramesh, N. (2001) Nat. Cell Biol. 3, 484–491