A Neural Wiskott-Aldrich Syndrome Protein-mediated Pathway for Localized Activation of Actin Polymerization That Is Regulated by Cortactin*[S]

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Activation of the epidermal growth factor (EGF) receptor can stimulate actin polymerization via the Arp2/3 complex using a number of signaling pathways, and specific stimulation conditions may control which pathways are activated. We have previously shown that localized stimulation of EGF receptor with EGF bound to beads results in localized actin polymerization and protrusion. Here we show that the actin polymerization is dependent upon activation of the Arp2/3 complex by neural Wiskott-Aldrich Syndrome protein (N-WASP) via Grb2 and Nck2. Suppression of Grb2 or Nck2 results in loss of localization of N-WASP at the activation site and reduced actin polymerization. Although cortactin has been found to synergize with N-WASP for Arp2/3-dependent actin polymerization *in vitro*, we find that cortactin can restrict N-WASP localization around EGF-bead-induced protrusions. In addition, cortactin-deficient cells have increased lamellipod dynamics but show reduced net translocation, suggesting that cortactin can contribute to cell polarity by controlling the extent of Arp2/3 activation by WASP family members and the stability of the F-actin network.

Binding of chemoattractants such as EGF[1] to cell surface receptors induces actin polymerization and membrane protrusion (1–3). In cancer, the ability of cells to sense spatial gradients of chemoattractants can contribute to invasion and metastasis (4–6). EGF-induced actin polymerization depends on the severing activity of cofilin to generate barbed ends and the *de novo* branching of actin filaments by the Arp2/3 complex (7–9). Cofilin severing activity is controlled by phosphorylation, pH, and phosphatidylinositol 4,5-biphosphate binding (10, 11). The Arp2/3 complex is activated by a conformational change induced by protein binding (12). Biochemical studies have shown

that WASP and WAVE family proteins as well as cortactin can induce Arp2/3-dependent actin polymerization (13).

N-WASP and WAVE family proteins use their C-terminal verprolin, cofilin, acidic (VCA) homology sequence domains to bind and activate the Arp2/3 complex (13). N-WASP was initially shown to be activated by GTP-Cdc42 binding (14). Subsequently, Grb2 (15) and Nck (16) were demonstrated to activate N-WASP-induced actin polymerization by binding to the proline-rich region. Rac1 or Nck are thought to activate WAVE family members by disruption of a five-protein complex (17).

Cortactin induces actin polymerization by binding to the Arp2/3 complex via its N-terminal acidic region (18, 19). Unlike N-WASP and WAVE proteins, cortactin requires binding to F-actin filaments to induce Arp2/3-dependent actin polymerization (18, 19). Binding assays have shown that cortactin cooperatively binds the Arp2/3 complex with N-WASP (19). Recent findings have suggested that cortactin can also displace N-WASP from the Arp2/3 complex, stabilizing filament branches and maintaining actin polymerization (20). Therefore, the role of cortactin in actin polymerization may vary depending on cell type and stimulation condition.

To define the spatial patterns of pathways that can link the EGF receptor to Arp2/3 complex activation, we have developed a method of stimulating cells with EGF-coated beads to allow us to evaluate local stimulation of actin polymerization (21). Stimulation with EGF-coated beads induced localized actin polymerization within a 2 μm radius, and in 25% of the cases the actin polymerization was accompanied by a protrusion extending the bead several microns off the surface of the cell. The response was inhibited by blocking EGF receptor activity or actin polymerization. This type of stimulation did not induce focal adhesion clustering (21), which may integrate other signals influencing actin dynamics. We found that both cofilin and the Arp2/3 complex were required to stimulate protrusions and localized actin polymerization by EGF immobilized on beads but that these responses were independent of phosphatidylinositol 3-kinase (21), whereas responses to soluble EGF are sensitive to this kinase (22). EGF beads may mimic ligands bound to extracellular matrix *in vivo*, which could elicit a different response compared with the addition of soluble growth factor.

In this report, we analyze the signaling pathways contributing to regulation of the Arp2/3 complex in the EGF-bead response. We find N-WASP to be the critical factor in Arp2/3 activation by EGF beads. Furthermore, both Grb2 and Nck2 are required for activation of N-WASP. Removal of cortactin enhanced the EGF-bead protrusion response together with an increase in N-WASP around the bead. We conclude that under our stimulation condi-

tions, cortactin restricts N-WASP activation of actin polymerization and stabilizes the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Cell Culture and Stimulation—MTLn3 cells were maintained in α minimal essential media with 5% fetal calf serum and antibiotics (23). For experiments, cells were plated on MatTek dishes in complete media overnight. Typically, cells were then starved for 3 h in L15 media (Invitrogen) supplemented with 0.35% bovine serum albumin before stimulation with EGF-bound beads for 3 min or with 10 nM soluble EGF (Invitrogen) for 1 min. Biotin EGF (Molecular Probes, Eugene, OR) was bound to streptavidin-bound magnetic beads (Pierce, or Dynal, Brown Deer, WI) brought down onto cells within 20 s using a magnet; this was followed by fixation and staining as described (21, 24). Observation in the F-actin (rhodamine) channel was used to characterize the response as either: 1) a protrusion (a three-dimensional structure where the bead has been "pushed" away from the cell and is not in the same plane of focus as neighboring cell components), 2) local positive responses (increased F-actin staining near the bead but in the same plane of focus as neighboring cell components with no three-dimensional structure clearly visible), or 3) no responses (no protrusion or increased F-actin staining near the bead). Data were then plotted as fraction of cells showing protrusions only or total responses (the sum of protrusions and local positive responses).

For analysis of net path length and net flow, 60-min-long time lapse movies were analyzed with DIAS at 5-min intervals (Solltech, Oakdale, IA). Net path length was provided by DIAS as the straight line distance from the path starting point to the ending point during the entire 60-min interval. Net flow represents the total fraction of cell area that changes position between successive time points and is the sum of the positive and negative flow parameters calculated by DIAS. Net path length represents the net movement of the cell centroid, whereas net flow is a measure of all changes in cell shape and protrusion independent of their contribution to cell translocation.

Small Interfering RNAs and Rescue—All siRNAs were purchased from Qiagen. Control, cofilin, and p34 siRNAs have been described (21). N-WASP (AAGACGAGATGCTCCAAATGG), WAVE1 (AATGCCTC-CGTCCCCACCTTC), WAVE2 (AAACCTATAACAGCTGTGACG), cortactin (CAAGCTTCGAGAGAATGTCTT), Grb2 (AACATCCGTGTC-CAGGAACCA), Nck1 (GATGATAGCTTTGTTGATCCA), and Nck2 (CAAGCACTGATGCGGAATACC) siRNA sequences were designed for the rat sequence. Cells were transfected with 100 nM siRNA using Oligofectamine (Invitrogen) 36 or 48 h before use. Rescue cDNA constructs were: *Bos taurus* GFP-N-WASP (Dr. Tadaomi Takenawa, University of Tokyo), human GFP-Grb2 and GFP-Nck2 (25), human cortactin (Dr. Roger Daly, Garvan Institute, Sydney, Australia), Myc-tagged N-terminal deleted and full-length mouse cortactin (Dr. Xi Zhan, American Red Cross, Rockville, Maryland). In siRNA rescue, cells were first treated with siRNA and allowed to rest until 80–90% confluent. Cells were then transfected with cDNA constructs using Lipofectamine 2000 (Invitrogen). Rescued cells were identified by GFP or antibody staining using anti-N-WASP (Santa Cruz Biotechnology) and anti-cortactin (Upstate Biotechnology) antibodies.

Triton Permeabilization Assay—Cells were either fixed in 4% formaldehyde for 10 min and then permeabilized with 0.1% Triton X-100 for an additional 10 min (total filaments) or fixed with 4% formaldehyde and 0.1% Triton X-100 for 20 min (stable filaments). Both groups were then washed in 1× Tris-buffered saline five times, stained with rhodamine-phalloidin in 1× Tris-buffered saline containing 1% bovine serum albumin and 1% fetal calf serum, and then washed with 1× Tris-buffered saline five times.

Staining Intensity Measurements—The F-actin or N-WASP staining ratio of the bead site compared with the edge of cell was then determined as described (21, 24). In brief, using an image processing program such as NIH Image or ImageJ, the area of increased fluorescence intensity in the cell membrane next to the bead is outlined and the average intensity determined. The average intensity of the membrane staining in an equivalent area far from the bead is also determined. The ratio of the two is then calculated for each cell followed by averaging of the ratios from all the cells for each condition.

Western Blot—Western blots were performed as described (21). Antibodies used were for p34 and cortactin (Upstate Biotechnology), N-WASP, WAVE1 and WAVE2 (Dr. Tadaomi Takenawa, University of Tokyo, Japan) and Nck (Dr. Tony Pawson, University of Toronto, Canada).

RESULTS

The EGF-Bead Response Requires N-WASP—To determine the pathway upstream of Arp2/3 required for the response to EGF beads, siRNAs were designed to suppress expression of the major activators of the Arp 2/3 complex: N-WASP, WAVE1, WAVE2, and cortactin (26). WAVE3 and WASP were not tested because they are not expressed in MTLn3 cells (data not shown). Measurements were scored by identifying beads in contact with cells and then using rhodamine-phalloidin staining to determine whether the bead induced no response or localized actin polymerization or a protrusion (Fig. 1*A*). WAVE1 and WAVE2 suppression resulted in little or no reduction of responses (Fig. 1*B*). N-WASP suppression resulted in a decrease in protrusions and total responses observed by 50% (Fig. 1*B*). A second siRNA to a different region of N-WASP yielded a similar level of inhibition (data not shown). Expression of *Bos taurus* N-WASP (which contains two base pair mismatches in sequence compared with the siRNA used) restored the EGF-bead response in N-WASP siRNA-treated cells (Fig. 1*C*). Thus, N-WASP is important for activation of the EGF-bead induced response, whereas neither WAVE1 nor WAVE2 play a substantial role.

Simultaneous suppression of both Arp2/3 complex and cofilin function is required for near complete inhibition of the EGF-bead-induced response, suggesting two parallel pathways to actin polymerization (21). Simultaneous suppression of N-WASP and cofilin also dramatically decreased the EGF-bead-induced response (Fig. 1*D*). The combination of N-WASP and p34 (a component of the Arp2/3 complex) siRNA did not reduce responses to EGF-coated beads more than N-WASP siRNA alone (Fig. 1*D*), indicating that N-WASP is the major upstream activator of the Arp2/3 complex in actin polymerization induced by localized EGF.

Grb2 and Nck2 Are Required in the EGF-Bead Response to Recruit and Activate N-WASP—We have shown previously that phosphatidylinositol 3-kinase and cdc42 are not required for the responses induced by EGF beads (21). We therefore tested Grb2, Nck1, and Nck2, which can also activate N-WASP (15, 16). Suppression of Grb2 decreased responses by 40% (Fig. 2*A*, *diagonal lines bars*) and was confirmed using a second siRNA (data not shown). A GFP-tagged human Grb2 sequence, which deviates from the rat Grb2 siRNA by three base pairs, restored responses in cells treated with rat Grb2 siRNA (Fig. 2*B*, *gray bars*). To monitor Nck protein expression, we used an antibody that recognizes both Nck1 and Nck2 SH3 domains (27). Either Nck1 or Nck2 suppression alone resulted in a partial decrease in protein expression; however, when both siRNAs were added simultaneously little or no protein remained (Fig. 2*E*). This result is consistent with Nck knock-out experiments utilizing mouse embryonic fibroblast lysates in which much of the antibody staining was caused by Nck1 (27). Interestingly, suppression of Nck1 had no effect on the EGF-bead response (Fig. 2*A*, *dotted bars*). Nck2 suppression (Fig. 2*A*, *gray bars*) inhibited the bead response to an extent similar to that seen in Grb2 suppression or simultaneous suppression of both Nck1 and Nck2 (Fig. 2*A*, *horizontal striped bars*). Thus Nck2, like Grb2, mediates EGF-bead-induced responses. This was confirmed by a second Nck2 siRNA (data not shown) and rescue of the siRNA treatment using GFP-tagged human Nck2, which had four mismatched base pairs to the rat Nck2 siRNA used (Fig. 2*C*). Simultaneous reduction of the levels of Grb2 and Nck2 did not inhibit the bead response more than reduction of either protein alone (Fig. 2*A*, *black bars*).

Suppressing N-WASP in combination with either Grb2 or Nck2 (Fig. 2*D*) did not significantly differ from suppression of N-WASP, Grb2, or Nck2 alone. However, suppressing cofilin in
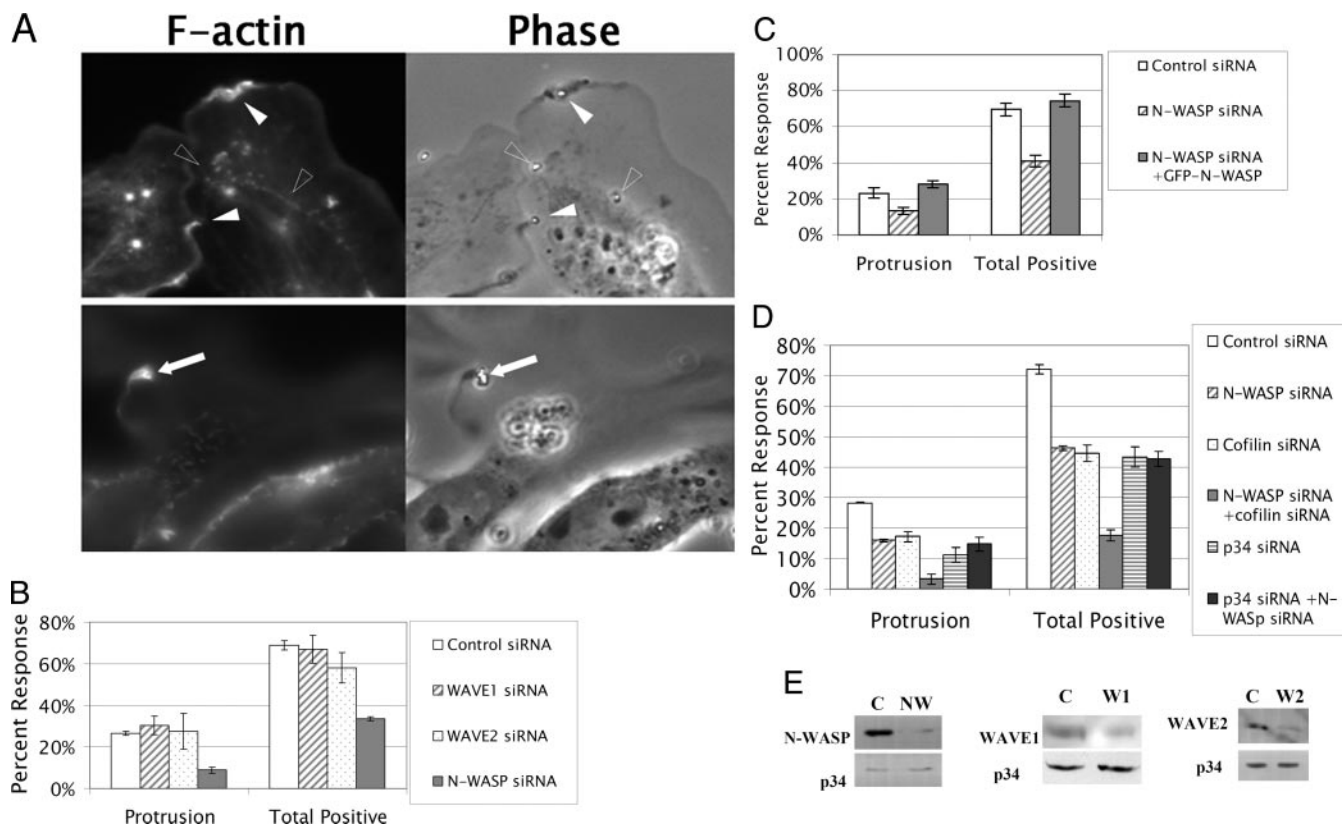
FIG. 1. **N-WASP, not WAVE1 or WAVE2, is required for EGF bead-induced actin polymerization.** *A*, examples of responses induced by EGF beads. No responses (*top*, *outlined arrowheads*), localized responses (*top*, *white arrowheads*), and protrusion responses (*bottom*, *white arrows*) are detected by observing rhodamine-stained F-actin (*left*) and phase images (*right*). Beads are detected as phase bright objects in the phase images and then increased F-actin next to the bead marks an actin polymerization response. Responses are categorized as localized responses if the neighboring cell components are in the same plane of focus as the bead, whereas for protrusion responses the bead and F-actin are above the plane of focus of surrounding cell components. *B*, the response of cells treated with control (*open bars*), WAVE1 (*diagonal lines bars*), WAVE2 (*dotted bars*) and N-WASP (*gray bars*) siRNAs to EGF beads. *C*, cells treated with control (*open bars*) or N-WASP siRNAs (*diagonal lines* and *gray bars*) were transfected with *Bos taurus* GFP-N-WASP and stimulated with EGF beads. GFP-negative cells (*diagonal lines bars*) and GFP-positive cells (*i.e.* expressing GFP-N-WASP, *gray bars*) on the same coverslip were analyzed. *D*, cells were treated with control (*open bars*), N-WASP (*diagonal lines bars*), cofilin (*dotted bars*), cofilin and N-WASP (*gray bars*), p34 (*horizontal striped bars*) or p34 and N-WASP (*black bars*) siRNAs followed by stimulation with EGF beads. Data represent the mean ± S.E. of 3–4 experiments where $n > 70$ for each experiment for *B–D*. *E*, Western blot analysis of siRNA suppression with p34 as loading control. *C*, control siRNA; *NW*, N-WASP siRNA; *W1*, WAVE1 siRNA; *W2*, WAVE2 siRNA.

combination with either Grb2 or Nck2 showed a decrease in responses similar to suppression of both N-WASP and cofilin (Fig. 1*C*) or both p34 and cofilin (21). These data suggest that Grb2 and Nck2 might act in concert to activate N-WASP in response to EGF-beads.

To test this hypothesis we evaluated N-WASP recruitment to the bead site in the presence or absence of Grb2 or Nck2. In a normal response, N-WASP staining is increased next to the bead (Fig. 3, *A*, *open bars*, and *B*, *top panels*). In the absence of either Grb2 or Nck2, N-WASP staining at the bead site decreased to levels seen at the edge of cells that were not touched by a bead (Fig. 3, *A*, *striped* and *solid bars*, and *B*, *bottom panels*). Furthermore, the beads that showed positive responses under these conditions had a decrease in F-actin staining at the bead site compared with control siRNA-treated cells (Fig. 3*A*), indicating that the loss of Grb2 and Nck2 along with a decrease in N-WASP recruitment impeded actin polymerization at the bead site. Grb2 and Nck2 siRNA had no effect on N-WASP, p34, or cofilin expression (data not shown). We conclude that Grb2 and Nck2 are necessary for recruitment of N-WASP to the EGF receptor to activate Arp2/3-induced actin polymerization.

Cortactin Is Antagonistic to Actin Polymerization Induction but Is Required for Integration of Freshly Polymerized Actin into the Cell Cytoarchitecture—Suppression of cortactin expression unexpectedly resulted in the enhancement of protrusion responses by 50% (Fig. 4*A*, *diagonal lines bars*) and was confirmed by another siRNA against a different region of the cortactin gene (data not shown). Next we rescued the rat cortactin siRNA response by overexpressing a human cortactin sequence, which had three mismatches to the siRNA used. Overexpression of human cortactin in rat cortactin siRNA-treated cells decreased responses (Fig. 4*A*, *gray bars*), whereas expression of an N-terminal deletion of cortactin, which does not bind Arp2/3 but does have its F-actin binding region (18), also enhanced protrusion responses (Fig. 4*B*, *gray bars*). This overexpression analysis suggested that the antagonistic function of cortactin was occurring at its Arp2/3 binding region and may be regulating N-WASP function. Because it has been shown that cortactin can displace N-WASP from the Arp2/3 complex in the presence of F-actin (20), we compared the localization of N-WASP in control and cortactin siRNA-treated cells. In control siRNA-treated cells, N-WASP staining was tightly localized around the bead site, whereas cortactin localized with F-actin staining just outside the site of N-WASP localization (Fig. 4*E*) similar to actin comet tails formed by vaccinia virus (28) and *Shigella* (20). Cells treated with cortactin siRNA had increased N-WASP protein staining at EGF beads and more uniform colocalization of N-WASP with F-actin throughout protrusions (Fig. 4, *C* and *E*). Thus, suppression of cortactin increases N-WASP recruitment and the number of protrusions found at the EGF-bead site.
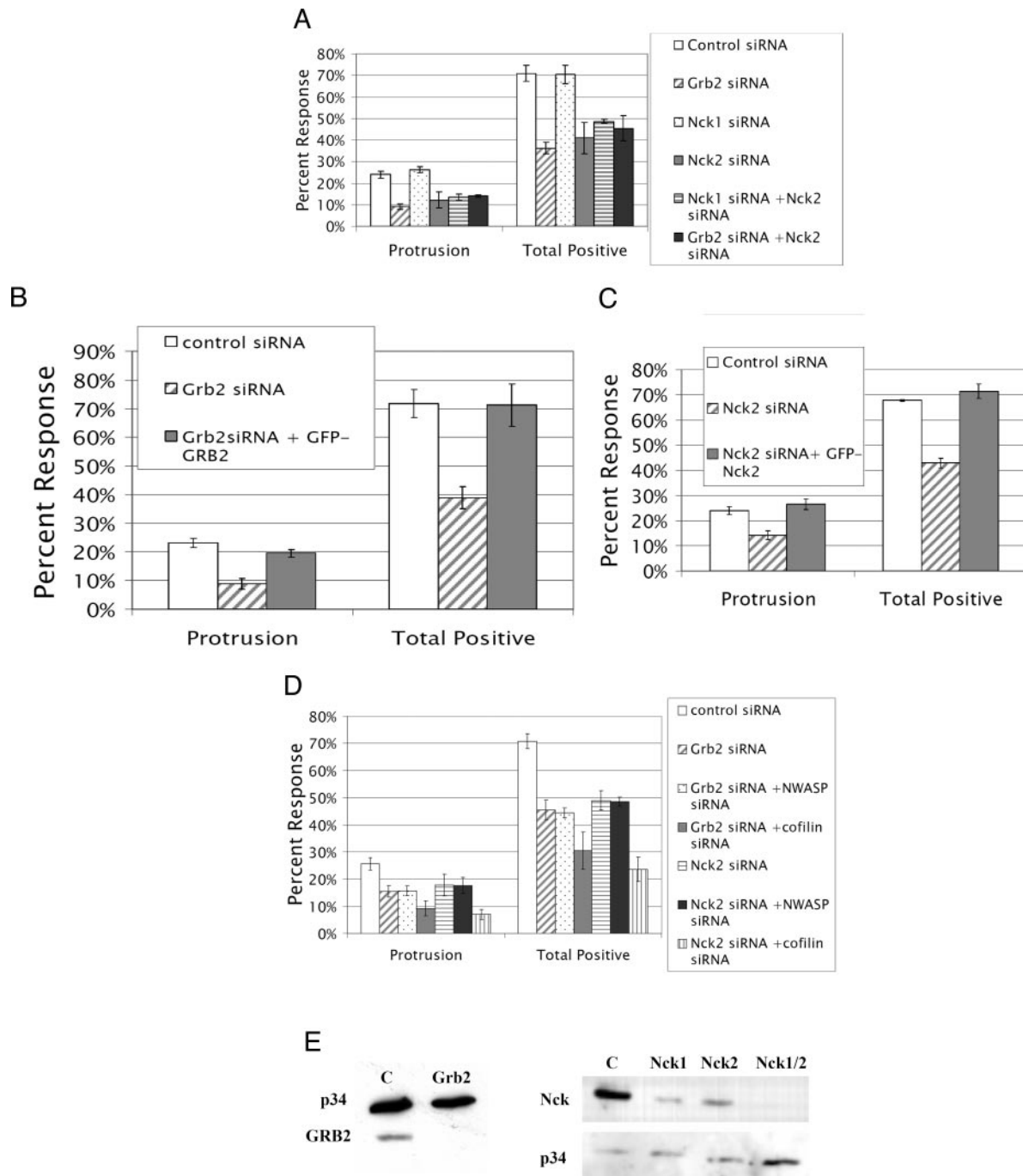
FIG. 2. **Grb2 and Nck2 are required for activation of N-WASP.** *A*, cells were treated with control (*open bars*), Grb2 (*diagonal lines bars*), Nck1 (*dotted bars*), Nck2 (*gray bars*), Nck1 and Nck2 (*horizontal striped bars*), or Grb2 and Nck2 (*black bars*) siRNA followed by stimulation with EGF beads. *B*, cells treated with control (*open bars*) or Grb2 (*diagonal lines and gray bars*) siRNAs were then transfected with GFP-tagged human Grb2 and stimulated with EGF beads. GFP-negative cells (*diagonal lines bars*) and GFP-positive cells (*gray bars*) in the Grb2 siRNA samples on the same coverslip were analyzed. *C*, cells transfected with control (*open bars*) or Nck2 (*diagonal lines and gray bars*) were then transfected with GFP-tagged human Nck2 and stimulated with EGF beads. GFP-negative cells (*diagonal lines bars*) and GFP-positive cells (*gray bars*) in the Nck2 siRNA samples on the same coverslip were analyzed. *D*, cells treated with control (*open bars*), Grb2 (*diagonal lines bars*), Grb2 and N-WASP (*dotted bars*), Grb2 and cofilin (*gray bars*), Nck2 (*horizontal striped bars*), Nck2 and N-WASP (*black bars*) or Nck2 and cofilin (*vertical lines bars*) siRNA were analyzed for their response to EGF-coated beads. Data represent the mean ± S.E. of three experiments where $n > 70$ for each experiment. *E*, Western blot analysis of siRNA suppression (*C*, control siRNA; Grb2, Grb2 siRNA; Nck1, Nck1 siRNA; Nck2, Nck2 siRNA, Nck1/2, Nck1 and Nck2 siRNA) with p34 as loading control.

Cells with reduced cortactin also displayed increased F-actin staining at the lamellipod edge compared with control siRNA cells (Fig. 5*A*) suggesting that increased actin polymerization was occurring there. Live cell imaging was performed to compare the cortactin to control siRNA-treated cells. There was an increase in multiple actively protruding cell edges in cortactin siRNA-treated cells compared with control siRNA-treated cells,

which tended to have one dominant lamellipod (supplemental Movies 1 and 2 and net flow in Fig. 5*B*). Net flow (Fig. 5*B*), which is a measure of retraction and extension of cell protrusions, was significantly higher in the cortactin siRNA-treated cells, consistent with the presence of multiply protruding cell edges. However, cells with reduced cortactin showed decreased total translocation compared with control treated cells (net path length in Fig. 5*B*) in
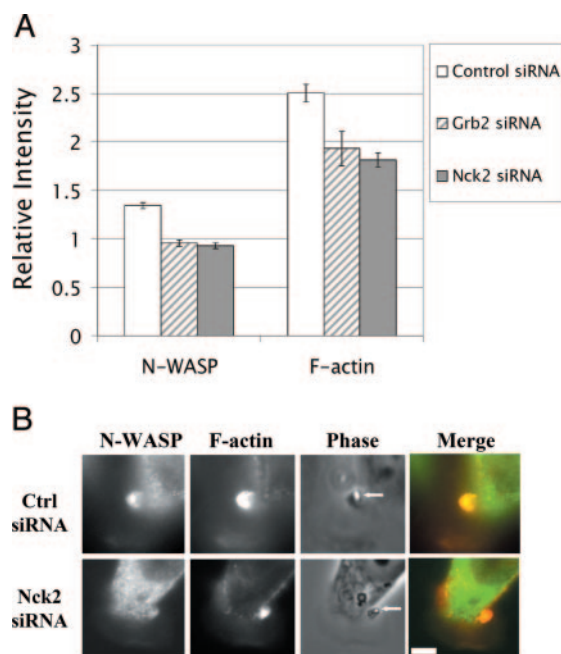
FIG. 3. **Grb2 and Nck2 enhance (whereas cortactin suppresses) N-WASP localization to EGF beads.** *A*, cells were treated with control (*open bars*), Grb2 (*diagonal lines bars*) or Nck2 (*gray bars*) siRNAs stimulated with EGF-coated beads and stained for N-WASP and F-actin. The relative intensity of each molecule at the bead site compared with a region of the same cell not binding a bead was evaluated. Data represent the mean ± S.E. of five experiments where $n > 25$ for each experiment. *B*, cells treated with control (*top row*) or Nck2 (*bottom row*) siRNAs were stimulated with EGF-coated beads and then stained for F-actin (*red*) or N-WASP (*green*). The EGF beads are marked by *open arrows* in the phase image. *Scale bar*, 5 μm.

line with previous reports of cortactin contributing to effective cell movement (29, 30). These data suggest that cortactin is required for cell movement but not for the induction of actin polymerization in spontaneous cell motility.

If cortactin is involved in binding F-actin filaments in conjunction with the Arp2/3 complex (19), then loss of cortactin might result in reduced stability. The stability of the actin filaments in control and cortactin siRNA-treated cells after EGF-bead stimulation was determined using a Triton permeabilization assay. Cells were fixed in the presence of Triton X-100, which can enhance the loss of labile structures within the cell. Although suppression of cortactin produced increased actin polymerization around the beads in the absence of Triton (Fig. 5*C*, total F-actin), the amount of F-actin was significantly decreased after Triton treatment in cortactin siRNA-treated cells compared with control siRNA-treated cells (Fig. 5*C*, stable F-actin). Therefore, cortactin contributes to the stability of actin filaments induced by EGF beads.

DISCUSSION

Previously, we found that actin polymerization induced by EGF beads is dependent on cofilin and the Arp2/3 complex but independent of phosphatidylinositol 3-kinase and Cdc42/Rac (21) suggesting that Arp2/3 activation in this response was independent of Rho family proteins. In the current manuscript we examined the mechanism of Arp2/3 activation. Of Arp2/3 complex activators, only N-WASP siRNA treatment significantly inhibited the response. Next we inhibited the expression of N-WASP activators, Grb2 and Nck1 and Nck2 (15, 16) and found that both Grb2 and Nck2 were required in the EGF-bead response. When used in combination with cofilin siRNA, the N-WASP, Grb2, or Nck2 siRNAs were able to inhibit the protrusion response almost completely, similar to suppression of
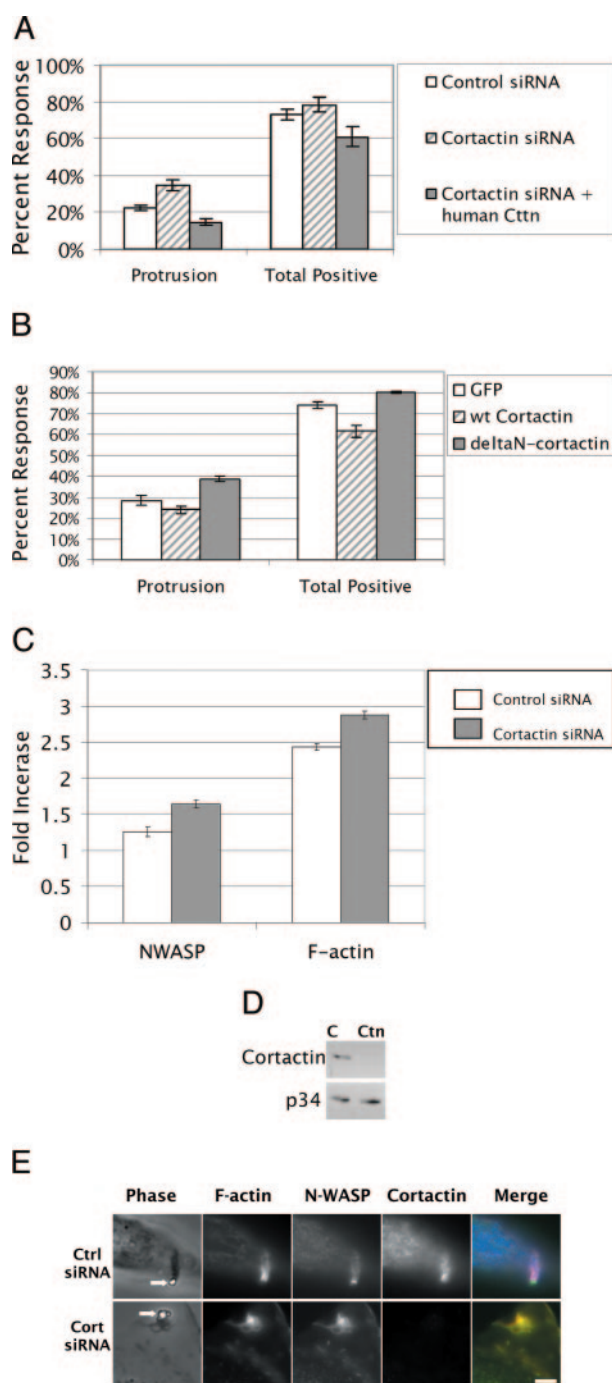

FIG. 4. **Cortactin regulates EGF-bead responses.** *A*, cells transfected with control (*open bars*) or cortactin (*diagonal lines* and *gray bars*) siRNAs were then transfected with human cortactin (+ *human Cttn*), stimulated with EGF beads, and stained for cortactin. Cortactin-negative cells (*diagonal lines bars*) and cortactin-positive cells (*gray bars*) on the cortactin siRNA-treated sample were analyzed separately. *B*, cells were transfected with GFP (*open bars*), full-length mouse cortactin (*diagonal lines bars*), or deltaN mouse cortactin and stimulated with EGF beads. For *A* and *B*, data represent the mean ± S.E. of three experiments where $n > 70$ for each experiment. *C*, cells treated with control (*open bars*) or cortactin (*gray bars*) siRNA and starved for 3 h were then stimulated with EGF-coated beads and were stained for N-WASP and F-actin. The relative intensity of staining at the bead site compared with a region of the same cell not binding a bead was evaluated. Data represent the mean ± S.E. of five experiments where $n > 25$ for each experiment. *D*, immunoblots for cells treated with control (*C*) or cortactin (*Ctn*) siRNAs. *E*, cells treated with control (*top row*) or cortactin (*bottom row*) siRNAs were stimulated with EGF-coated beads and then stained for F-actin (*red*), N-WASP (*green*), and cortactin (*blue*). The EGF beads are marked by *open arrows* in the phase image. *Scale bar*, 5 μm.
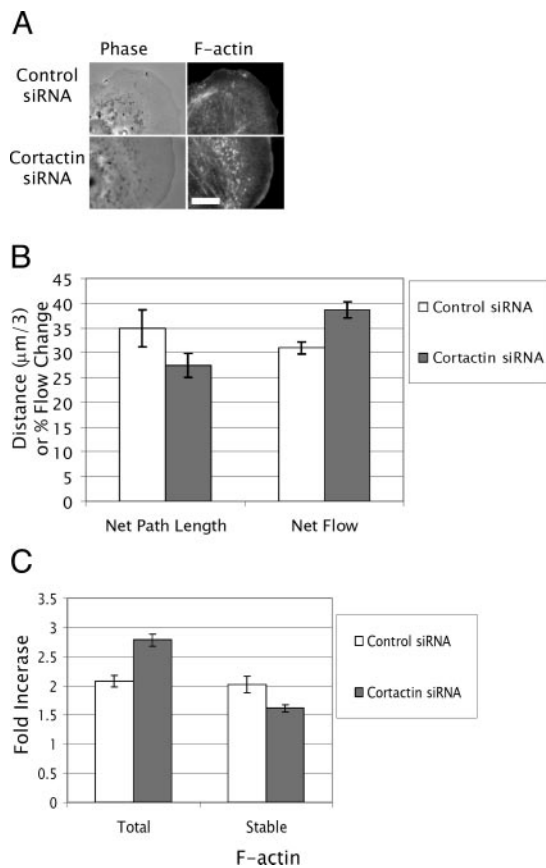
FIG. 5. **Cortactin is required for cell translocation and the stability of the actin cytoarchitecture.** *A*, cells treated with control or cortactin siRNA were stained for F-actin. Images are of leading edges of cells. *Scale bar*, 10 μm. *B*, analysis of net path length and net flow of control siRNA and cortactin siRNA-treated cells. Net flow represents the percent area in a cell that changes position between each frame observed. Data represent the mean $\pm$ S.E. of three experiments where $n > 50$. *C*, cells were treated with either control or cortactin siRNA for 48 h. Cells were starved for 3 h and then stimulated with EGF-coated beads for 3 min, and total (fixed in the absence of Triton X-100) and stable (fixed in the presence of Triton X-100) F-actin was measured. Data are the mean $\pm$ S.E. for three experiments, $n > 30$.

cofilin and p34 (21). However, the combination of p34 with N-WASP siRNA or N-WASP with Grb2 or Nck siRNA had no additional effect over any one siRNA treatment alone, indicating that Grb2, Nck2, N-WASP, and p34 are all on the same pathway leading to activation of the Arp2/3 complex. Remarkably, we found that suppression of cortactin, another Arp2/3 activator, increased the number of EGF bead-induced protrusions. This increase in responses was also seen with overexpression of a cortactin fragment that lacked the N-terminal acidic domain, which mediates binding to the Arp2/3 complex (18, 19). This suggested that the antagonistic function of cortactin was caused by its interaction with the Arp2/3 complex. Because cortactin can displace the VCA homology sequence domain of N-WASP from Arp2/3 complex in the presence of F-actin (20), we tested if cortactin was regulating N-WASP localization at the EGF-bead site. After cortactin loss, there was increased N-WASP found at the EGF-bead site as well as increased localization of N-WASP throughout protrusions. This increased binding of N-WASP was concurrent with an increase in F-actin staining at the bead site as well as other areas of actin polymerization, such as the leading edge, demonstrating that cortactin is regulating the function as well as location of N-WASP.

Both Grb2 and Nck1 and -2 have been shown to bind the activated EGF receptor, either directly at phosphotyrosine res-

idues in the case of Grb2, or via other proteins, such as Dok in the case of Nck (31). In addition, Grb2 and Nck have been shown to bind and stimulate N-WASP activation of the Arp2/3 complex (15, 16, 32). Localized accumulation of the Nck SH3 domains induces actin polymerization dependent on N-WASP (33). Furthermore, both Ncks and Grb2 were shown to contribute to the recruitment of N-WASP in vaccinia-induced and phosphatidylinositol 4,5-biphosphate-induced motility (25, 34, 35). We find that N-WASP recruitment to the bead site is decreased after removal of either Grb2 or Nck2, indicating that both Grb2 and Nck2 are required for the proper localization of N-WASP in the EGF-bead response. Both Grb2 and Nck2 bind the polyproline region of N-WASP (26), possibly at different sites; however, *in vitro* studies of Grb2 and Nck1 used in combination did not show synergistic activation of N-WASP-induced Arp2/3 dependent actin polymerization (14). In our studies, Nck1 did not mediate activation of N-WASP, raising the possibility that the mechanism of Nck2 activation of N-WASP may differ from that of Nck1. Because Grb2 and Nck2 bind to the activated EGF receptor at different locations (31), the receptor may orient these molecules so they synergize to localize and activate N-WASP *in vivo*. Alternatively, Grb2 and/or Nck2 may interact with additional proteins, such as Toca-1 (<u>t</u>ransducer <u>o</u>f <u>C</u>dc42-dependent <u>a</u>ctin assembly) (36), or WIP (34, 36, 37).

As part of our study of Arp2/3 activators, we also examined the role of cortactin in the EGF-bead response. It was surprising to see an increase in the number of protrusion responses when cortactin was suppressed. However, cortactin can displace N-WASP from the Arp2/3 complex after N-WASP has initiated actin polymerization (20). Our immunostaining demonstrated that N-WASP is at the site of EGF receptor activation and cortactin is juxtaposed to N-WASP, similar to what has been reported for vaccinia virus (28) and *Shigella*-stimulated (20) actin tails. Furthermore, the loss of cortactin resulted in enhanced actin polymerization as shown by increased F-actin staining at the EGF-bead site as well as at lamellipods. This information shows that cortactin can regulate actin polymerization by maintaining N-WASP at sites of actin polymerization and limiting its localization. Cortactin may have similar effects on WAVEs, which have a VCA homology sequence terminal sequence similar to N-WASP (26) and have been shown to be required for lamellipod protrusions (38). Cortactin has also been implicated in stabilizing actin branches (19). The Triton permeabilization assay showed reduced F-actin staining in cortactin siRNA-treated cells, demonstrating that cortactin can contribute to the stability of F-actin induced in the EGF-bead response. Our data are consistent with a model in which cortactin can displace N-WASP from the Arp2/3 complex (20, 39) and stabilize newly formed actin branches (19). Under other conditions, such as during contact-stimulated spreading and adhesion (40, 41), cortactin may also stimulate (or regulate) actin polymerization.

In summary, we have mapped out an actin polymerization pathway from the receptor to output by observing the EGF-bead response. Grb2 and Nck2 can bind the activated receptor and then recruit/activate N-WASP to the EGF-bead site. This simple pathway differs from the main pathway of N-WASP activation triggered by soluble EGF and may reflect the effect of restraining EGF receptors by binding them to immobilized ligands. Such responses may occur *in vivo* in the presence of ligands bound to extracellular matrix elements.

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