

# Cortactin regulates cell migration through activation of N-WASP

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## Summary

Cortactin is an actin-associated scaffolding protein that regulates cell migration. Amplification of the human gene, *EMSI*, has been detected in breast, head and neck tumors, where it correlates with increased invasiveness. Cortactin can regulate actin dynamics directly via its N-terminal half, which can bind and activate the Arp2/3 complex. The C-terminal portion of cortactin, however, is thought to have limited function in its regulation of the actin polymerization machinery. In this report, we identify a role for the cortactin C-terminus in regulating cell migration and, more specifically, actin dynamics. Overexpression of either full-length cortactin or cortactin C-terminus is

sufficient to enhance migration of mammary epithelial cells. In vitro, cortactin binds to and activates, via its SH3 domain, a regulator of the Arp2/3 complex, neural Wiskott Aldrich Syndrome protein (N-WASP). This in vitro activation of N-WASP is likely to be important in vivo, as cortactin-enhanced migration is dependent upon N-WASP. Thus, our results suggest that cortactin has multiple mechanisms by which it can recruit and modulate the actin machinery and ultimately regulate cell migration.

Key words: Cortactin, N-WASP, SH3 domain, Cell migration, Actin polymerization

## Introduction

Cell migration is critical for many physiological processes such as gastrulation and wound healing, and misregulation of migration can contribute to tumor metastasis and cardiovascular disease. Cortactin is a cytoskeletal protein that regulates cell migration in response to a number of extracellular stimuli (Weed and Parsons, 2001). It was initially identified as a target of the oncogenic tyrosine kinase, *v-src*, and the human gene, *EMSI*, is amplified in 13% of breast cancers and 29% of head and neck tumors (Kanner et al., 1990; Wu et al., 1991; Schuurin et al., 1992). Overexpression of cortactin both in tumorigenic and non-tumorigenic cell lines leads to increased cellular motility and invasiveness (Okamura and Resh, 1995; Huang et al., 1998; Patel et al., 1998; Bowden et al., 1999). These phenotypes may be related to the localization of cortactin at sites of actin rearrangement where it regulates actin dynamics (Wu and Parsons, 1993; Kaksonen et al., 2000; Weed et al., 2000; Uruno et al., 2001; Weaver et al., 2001).

Cortactin is composed of a series of modular domains that mediate interactions with a variety of binding partners (Fig. 1A) (Wu and Parsons, 1993; Weed and Parsons, 2001). In vitro, the N-terminal half of cortactin (amino acids 1-326) binds to F-actin and induces actin filament assembly through activation of the Arp2/3 complex, a major cellular regulator of de novo actin polymerization (Welch et al., 1998; Uruno et al., 2001; Weaver et al., 2001). Isolated Arp2/3 complex is inactive unless activated by nucleation promoting factors (Pollard and Borisy, 2003). Once activated, Arp2/3 complex binds the side

of a pre-existing actin filament and generates a new filament as a 70° branch (Amann and Pollard, 2001).

WASP family proteins, including WASP, N-WASP and Scar/WAVE, are potent activators of the Arp2/3 complex. The WH2-Acidic (WA) domain of WASP proteins binds the Arp2/3 complex through an acidic motif and G-actin via a WH2 domain (Machesky and Insall, 1998; Egile et al., 1999; Higgs et al., 1999). Formation of a ternary complex between the WA domain, an actin monomer and the Arp2/3 complex promotes a conformational change that leads to its activation (Higgs and Pollard, 2001).

Cortactin, which binds Arp2/3 complex through the N-terminal acidic region (NTA) and F-actin through the repeat region, is thought to activate the Arp2/3 complex by enhancing its interaction with the sides of actin filaments (Weed et al., 2000; Uruno et al., 2001; Weaver et al., 2001). As the cortactin N-terminus is sufficient for both its ability to activate Arp2/3 and targeting cortactin to sites of actin assembly at the leading edge, the C-terminal half of cortactin (amino acids 327-546) was thought to be of little importance in the regulation of actin reorganization. However, recent studies show that the cortactin SH3 domain interacts with several regulators of the actin machinery, including dynamin, WASP-interacting protein (WIP) and N-WASP (McNiven et al., 2000; Mitzutani et al., 2002; Kinley et al., 2003).

Isolated N-WASP exists in an autoinhibited conformation, in which the WA domain is rendered inaccessible. This autoinhibition can be relieved by binding of phosphatidylinositol 4,5-bisphosphate (PIP2) and Cdc42-GTP to the basic region and CRIB domain of N-WASP, respectively

(Higgs and Pollard, 2000; Prehoda et al., 2000; Rohatgi et al., 2000). SH3 domain-containing adaptor proteins, such as Nck and Grb2, can also activate N-WASP through binding to the proline-rich region (Carlier et al., 2000; Rohatgi et al., 2001). Activation of N-WASP thus unmasks the WA domain, which binds and activates the Arp2/3 complex (Egile et al., 1999; Kim et al., 2000; Rohatgi et al., 2000; Higgs and Pollard, 2001).

Given that (1) cortactin and N-WASP colocalize in podosomes; (2) the cortactin SH3 domain binds N-WASP; and (3) other SH3 domain-containing proteins can activate N-WASP, we tested whether the cortactin C-terminus can regulate cellular actin-based processes, specifically cell migration, through activation of N-WASP (Carlier et al., 2000; Rohatgi et al., 2001; Mituzani et al., 2002). Our results demonstrate that expression of cortactin C-terminus enhances the migration of mouse mammary epithelial cells in an SH3-dependent manner; and, in vitro, the cortactin SH3 domain stimulates Arp2/3-mediated actin polymerization via activation of N-WASP. This interaction is important for actin-based processes in cells, as cortactin-enhanced migration requires N-WASP.

## Materials and Methods

### Cell culture

SCp2 cells were grown in DMEM/F12 supplemented with 2% fetal bovine serum (FBS), 5  $\mu$ g/ml insulin and penicillin/streptomycin (Desprez et al., 1993). N-WASP null (NW<sup>-/-</sup>) and rescued mouse embryonic fibroblasts (MEFs) were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (Snapper et al., 2001).

For generation of cortactin-expressing lines, SCp2 cells, NW<sup>-/-</sup> or rescued MEFs were infected twice with pBABE retrovirus encoding hemagglutinin (HA)-tagged full-length (FL), HA-Ct, HA-CtW525L (C-terminus with W525L mutation) cortactin or vector only (V) as a control (Morgenstern and Land, 1990). Infected cells were selected in puromycin and the polyclonal puromycin-resistant population assayed as described below. Expression of the HA-tagged proteins and endogenous cortactin were analyzed by western blotting with anti-HA.11 mAb (1:1000 dilution; Covance, Princeton) and anti-cortactin 4F.11 mAb (1:1000 dilution; Upstate Biotechnologies, Lake Placid).

### Migration assays

Cell migration was assayed as described previously (Hagel et al., 2002). Briefly, a confluent monolayer of cells was scratched with a pipette tip to make two or three independent wounds for each sample and placed in growth media containing 2% serum. Phase images of each wound (10 $\times$  magnification) were taken at 0 hours and at various times after wounding. Care was taken to photograph the wound in the same region at each time point. Wound area at each time point was measured using NIH Image software. The change in wound area (area at  $x$  hours – area at 0 hours) for each independent wound was determined and an average change in area calculated for the sample. Fold change in area = average change in area of sample cells / average change in area of control cells. The statistical significance of the data was determined by performing one-tailed paired  $t$ -tests using the Prism software program. Differences in migration were considered significant if  $P < 0.05$ .

### Protein purification

Actin was purified from rabbit muscle and isolated as Ca<sup>2+</sup>-ATP-G-actin in G buffer (5 mM Tris-Cl, pH 7.8, containing 0.1 mM CaCl<sub>2</sub>, 0.2 mM ATP and 1 mM DTT) as described (Pardee and Spudich, 1982) and pyrenyl or rhodamine labeled. Bovine Arp2/3 complex was purified from brain extracts as described (Egile et al., 1999). His<sub>6</sub>-

GST-N-WASP (GST-N-WASP) and His<sub>6</sub>-N-WASP were expressed in insect cells and purified on glutathione-sepharose and/or Ni<sup>2+</sup>-NTA agarose as described (Egile et al., 1999; Rohatgi et al., 2000). GST-cortactin proteins and GST-Nck were expressed in *Escherichia coli* and affinity purified on glutathione-sepharose beads using standard protocols. Cleavage of the GST tags from the cortactin C-terminus and SH3 proteins where described was performed using bovine thrombin protease (Sigma).

### GST pull-down assays

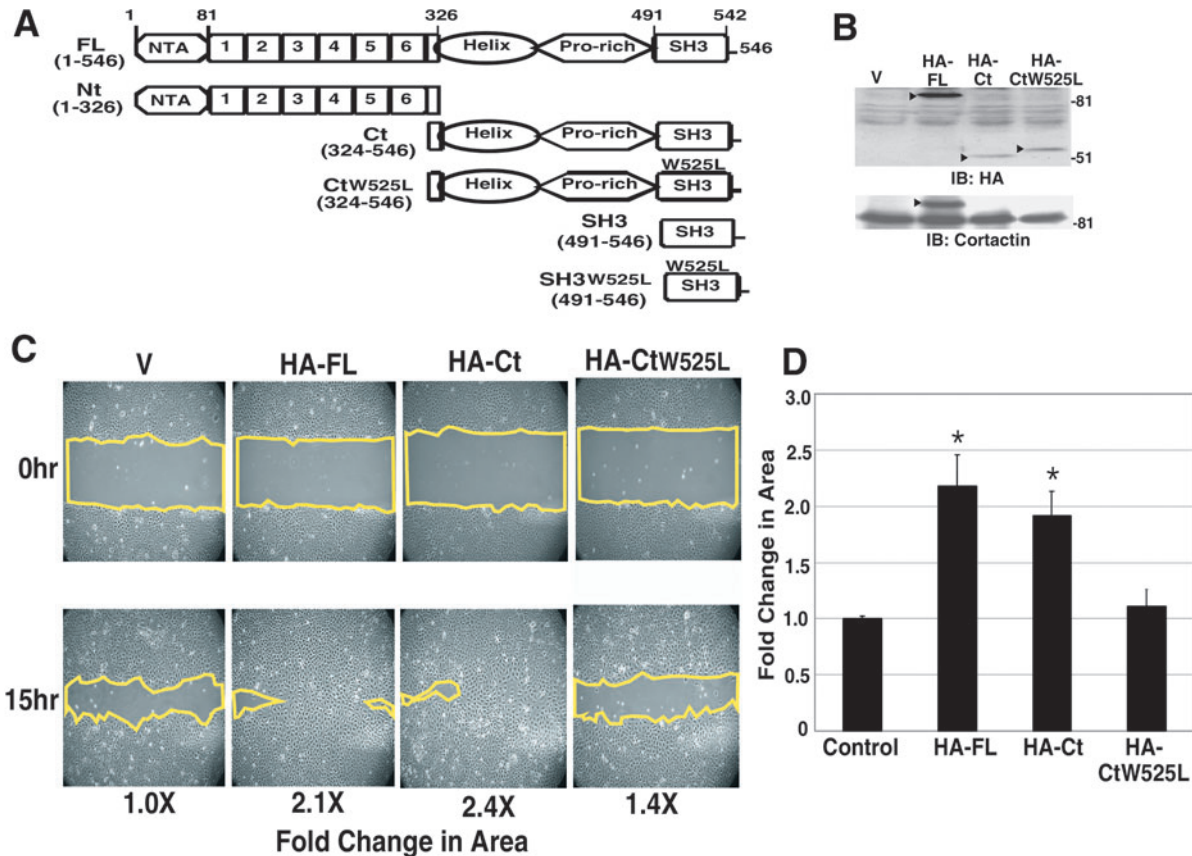
GST fusions of cortactin variants were immobilized on glutathione beads. Equivalent amounts of each fusion protein (225 pmol) were incubated with 800  $\mu$ g NIH3T3 cell lysate in lysis buffer (20 mM Tris, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol) for 1 hour at 4°C, then washed five times with lysis buffer. Bound proteins were eluted in sample buffer, resolved by SDS-PAGE and probed with an anti-N-WASP antibody (gift of H. Ho and M. Kirschner, Harvard Medical School). For peptide competition assays, ~700 pmol GST-SH3 protein bound to glutathione beads was pre-incubated with the indicated concentrations of 'cortoptimal' or 'control' peptide (see below) for 10 minutes at 25°C prior to incubation with 200  $\mu$ g NIH3T3 cell lysate.

### Pyrene actin polymerization assays

Pyrene actin assays were performed essentially as described (Amann and Pollard, 2001). Briefly, GST-cortactin fusion proteins and GST-N-WASP or His<sub>6</sub>-N-WASP (25 nM) were added to purified bovine Arp2/3 complex (10 nM) in KMET buffer (50 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM Tris, pH 7.0, 0.5 mM ATP and 1 mM DTT) at 25°C. Actin polymerization was initiated by the addition 1.5  $\mu$ M G-actin (5-10% pyrene labeled) and monitored by continuous pyrene fluorescence measurements ( $\lambda_{ex}$ =386 nm,  $\lambda_{em}$ =407 nm) (Cooper et al., 1983; Mullins et al., 1998). Fold activation = maximum polymerization rate (GST-protein+N-WASP) / maximum polymerization rate (N-WASP). Fold activation curves represent a logarithmic 'best-fit' of the data points. Maximum polymerization rates were taken as the slope of the linear phase of each polymerization curve and represent the average of two to three experiments. For peptide inhibition experiments, a 16 amino acid peptide ('cortoptimal': LGFEFSKPPIPQKPTWM) or a random 14 amino acid peptide ('control': GGNFAPQLSYGYDE) was pre-incubated with 500 nM GST-SH3 for 5 minutes at 25°C prior to use with GST-N-WASP and bovine Arp2/3 complex in the pyrene-actin assay.

## Results

Overexpression of cortactin enhances the migration and invasiveness of both normal and malignant cells (Okamura and Resh, 1995; Huang et al., 1998; Patel et al., 1998; Bowden et al., 1999). As the N-terminal half of the molecule can bind and activate the Arp 2/3 complex, it is generally presumed that cortactin regulates migration via this pathway (Weed et al., 2000; Uruno et al., 2001; Weaver et al., 2001). The C-terminal half of cortactin, however, also interacts with several proteins that are linked to the actin polymerization machinery (McNiven et al., 2000; Mitutani et al., 2002; Kinley et al., 2003). To investigate the importance of the cortactin C-terminus in cell motility, we expressed full-length cortactin or cortactin C-terminus (amino acids 324-546) in the SCp2 mouse mammary epithelial cell line and tested the ability of these cells to migrate in wound healing assays (Fig. 1). After 15 hours, the wound area closed in cells expressing either full-length cortactin or cortactin C-terminus was twofold greater than that



**Fig. 1.** Overexpression of full-length or C-terminal cortactin enhances migration of mouse SCp2 mammary epithelial cells. (A) Domain structure of cortactin variants used in this study. FL, full-length cortactin; Nt, N-terminal (1-326 amino acids) cortactin; Ct, C-terminal (324-546 amino acids) cortactin; CtW525L, C-terminus of cortactin with W525L mutation; SH3, SH3 domain; SH3W525L, SH3 domain with W525L mutation. (B) Expression of cortactin variants in SCp2 cells. Western blot showing expression of the HA-FL, HA-Ct and HA-CtW525L cortactin (upper blot) or endogenous cortactin (lower blot). Constructs were expressed at or below the level of endogenous cortactin. Arrowheads denote HA-FL, HA-Ct and HA-CtW525L bands. V, control vector expression. (C) Migration of SCp2 cells in a representative wounding experiment is shown for control vector only infected (V) or cortactin variant infected (HA-FL, HA-Ct, HA-CTW525L) cells with images at 0 and 15 hours. (D) Quantification of wound closure data from three to seven experiments. Fold change in wound area was calculated as described in Materials and Methods. Error bars indicate s.e.m. and asterisks indicate statistically significant differences from change in area calculated for control cells ( $P < 0.05$ ).

observed in control cells (Fig. 1C,D compare cortactin variant infected cells to control infected cells). Similar results were also observed with expression of the N-terminal half alone (amino acids 1-326; data not shown). In addition, when SCp2 cells expressing these different cortactin variants were cultured in a 3-dimensional matrix, differentiation was disrupted (S.G. and S.M.T., unpublished). The migration results do not reflect any differences in the number of cell divisions, as no significant differences in the proliferative rates of the cells were observed and differences in wound closure were detected as early as 6-8 hours. Interestingly, the ability of cortactin C-terminus to enhance cell migration requires an intact SH3 domain, as mutation of a critical residue in the SH3 binding pocket strongly inhibited this effect (Fig. 1C,D).

Although the ability of the N-terminal half to bind and activate Arp2/3 complex may contribute to the cortactin-induced migration, the mechanism by which cortactin C-terminus enhances cell migration was unclear. Given that the cortactin SH3 domain interacts with N-WASP, an activator of the Arp2/3 complex, we hypothesized that the cortactin C-

terminus-enhanced migration could be mediated through its ability to activate N-WASP.

As previously reported, cortactin full-length, C-terminus and SH3 proteins interacted with N-WASP in GST pull-down assays (Fig. 2A) (Mizutani et al., 2002). Conversely, no N-WASP bound to a cortactin SH3W525L mutant protein (inset in Fig. 3D). Neither cortactin N-terminus nor GST alone interacted with N-WASP, as expected (Fig. 2A) (Mizutani et al., 2002). To determine whether cortactin C-terminus binding can activate N-WASP, *in vitro* pyrene-actin assembly assays were performed with purified proteins (Fig. 2A-E) (Cooper et al., 1983; Mullins et al., 1998).

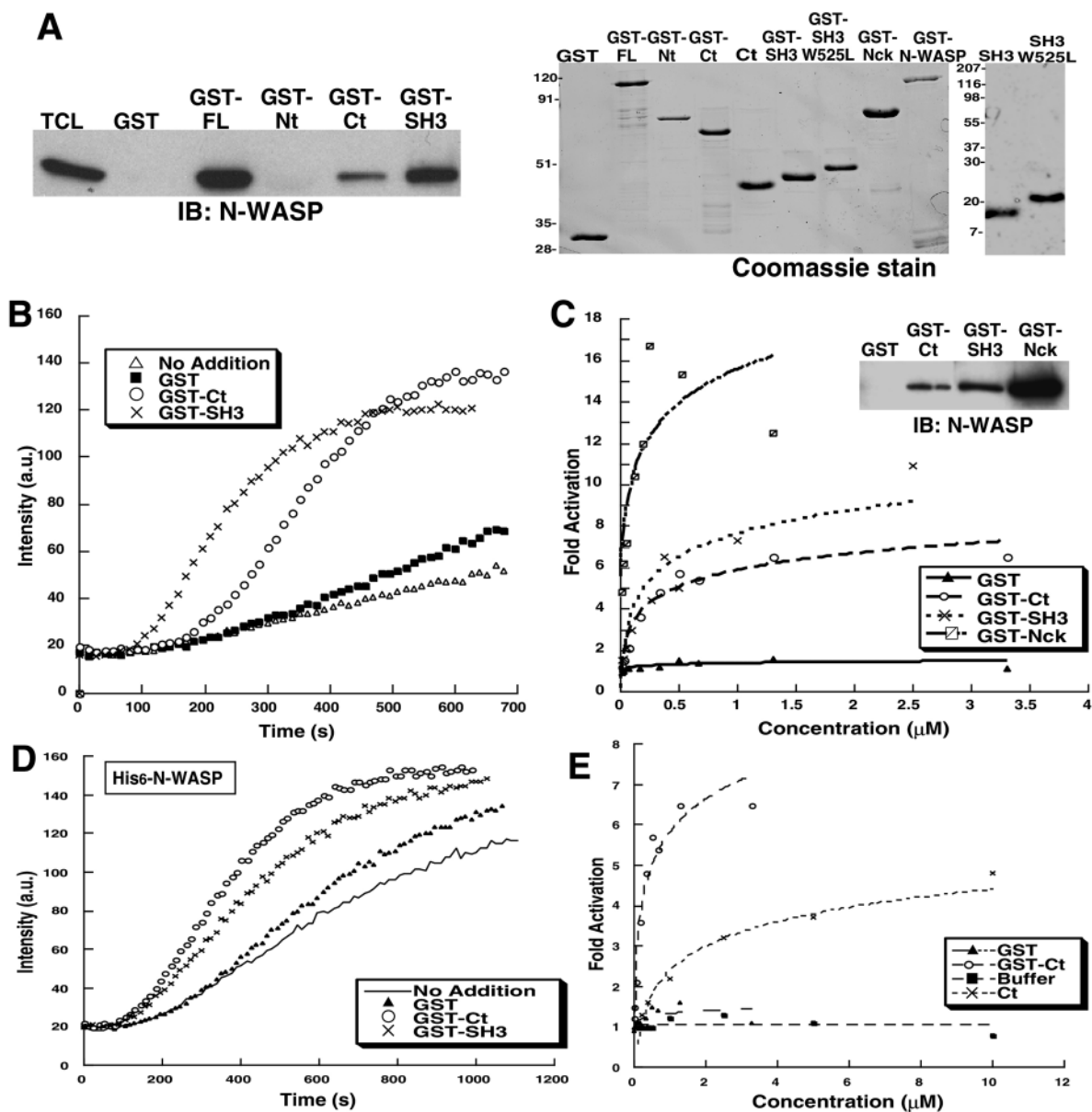
In contrast to GST alone, GST-cortactin C-terminus (GST-Ct) and GST-cortactin SH3 (GST-SH3) fusion proteins induced up to six- and tenfold activation of GST-N-WASP, respectively (Fig. 2B,C). These differing capacities for N-WASP activation correlate with the abilities of each cortactin variant to bind N-WASP, with GST-SH3 showing a roughly twofold higher relative affinity for N-WASP compared to GST-Ct (data not shown). At the concentrations of Arp2/3 complex used in these



experiments, none of the cortactin variants directly activated Arp2/3 complex, thus all actin polymerization represents effects on N-WASP (data not shown). Although Nck, an SH3-containing adaptor, was a more robust activator of N-WASP (17-fold), GST-Nck also precipitated an average of twofold more N-WASP than did the cortactin fragments over a range of concentrations (Fig. 2C, data not shown). Although it is known that not all three of the Nck SH3 domains contribute

equally to N-WASP activation, the presence of all three domains is required for both optimal binding to and activation of N-WASP (Rohatgi et al., 2001). However, the single cortactin SH3 domain can still activate N-WASP up to tenfold.

To rule out the possibility that activation of N-WASP by cortactin was solely due to the GST tags present on both the cortactin and N-WASP proteins, two complementary experiments were performed. First, we used a His<sub>6</sub>-N-WASP

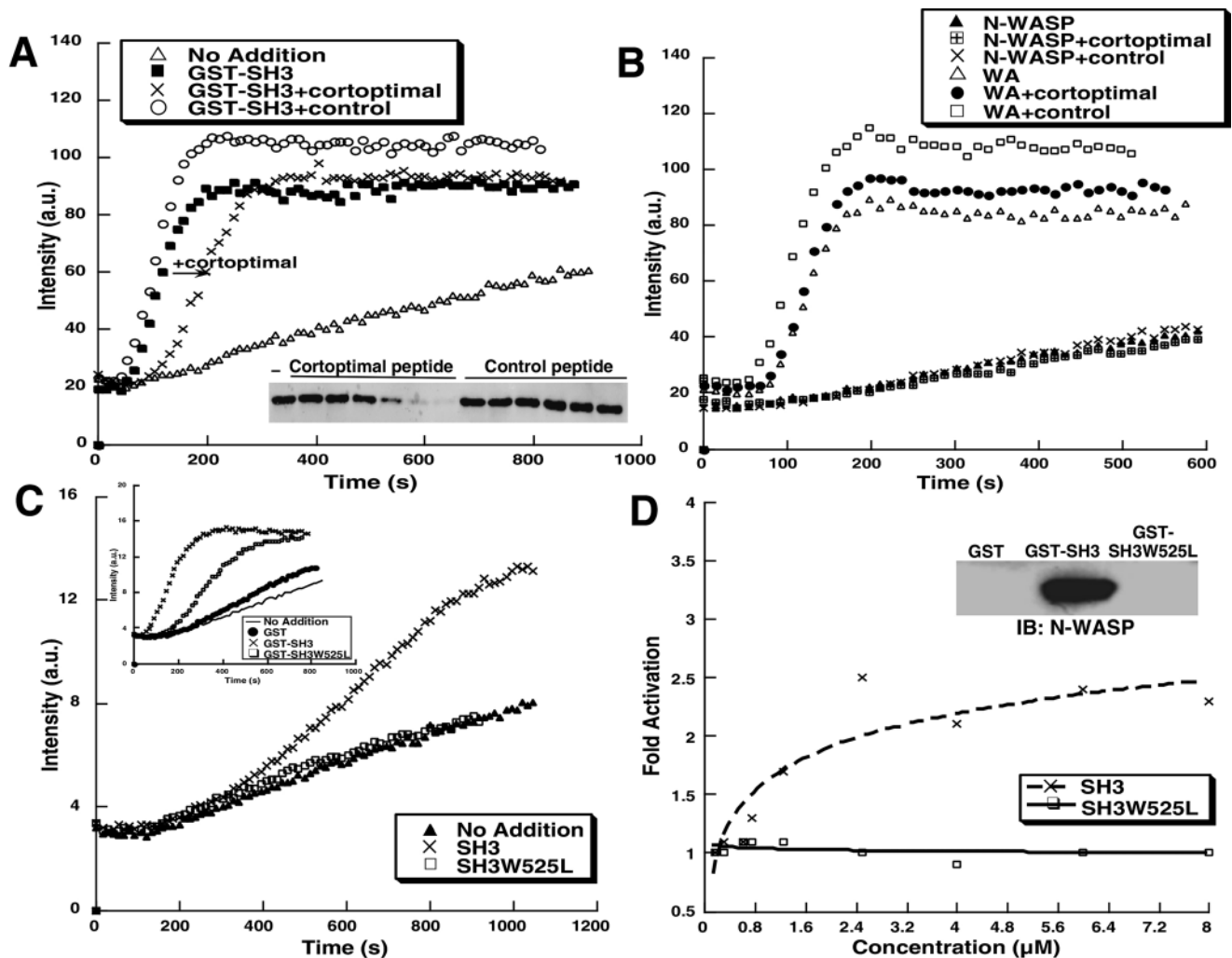


**Fig. 2.** The SH3 domain of cortactin is sufficient to activate N-WASP. (A) Western blot (left) of GST pull-down assays showing association of GST-cortactin fusions with endogenous N-WASP. TCL, total cell lysate; 1/40th of the amount of lysate used in the pull down was used for TCL. Right panel shows comparative size of GST fusion proteins used in this study on Coomassie-stained gels. Values down the side of the gel show the positions of protein standards in kDa. (B-E) Pyrene Assays. 10 nM Arp2/3 complex, 1.5 μM pyrene-labeled G-actin and 25 nM GST-N-WASP or His<sub>6</sub>-N-WASP were incubated together with GST-tagged or untagged cortactin proteins at the specified concentrations. (B) Optimal activation of GST-N-WASP by GST-cortactin proteins (GST, 1.3 μM; GST-Ct, 1.3 μM; GST-SH3, 1 μM). (C) Fold activation of GST-N-WASP by GST, GST-Ct or GST-SH3 cortactin, or GST-Nck. All fold activation curves are a logarithmic 'best fit' of the data points shown. Inset shows N-WASP binding to GST-Ct, GST-SH3 and GST-Nck beads. (D) GST tag on N-WASP is not required for N-WASP activation by cortactin. Sample polymerization curves are shown for each GST-cortactin protein at the concentration giving optimal N-WASP activation in the pyrene assay (GST, 250 nM; GST-Ct, 1 μM; GST-SH3, 250 nM). (E) GST tag is not required for N-WASP activation by cortactin C-terminus. Plots show fold activation of GST-N-WASP by GST, GST-Ct, untagged cortactin C-terminus (Ct) or buffer control (Buffer).

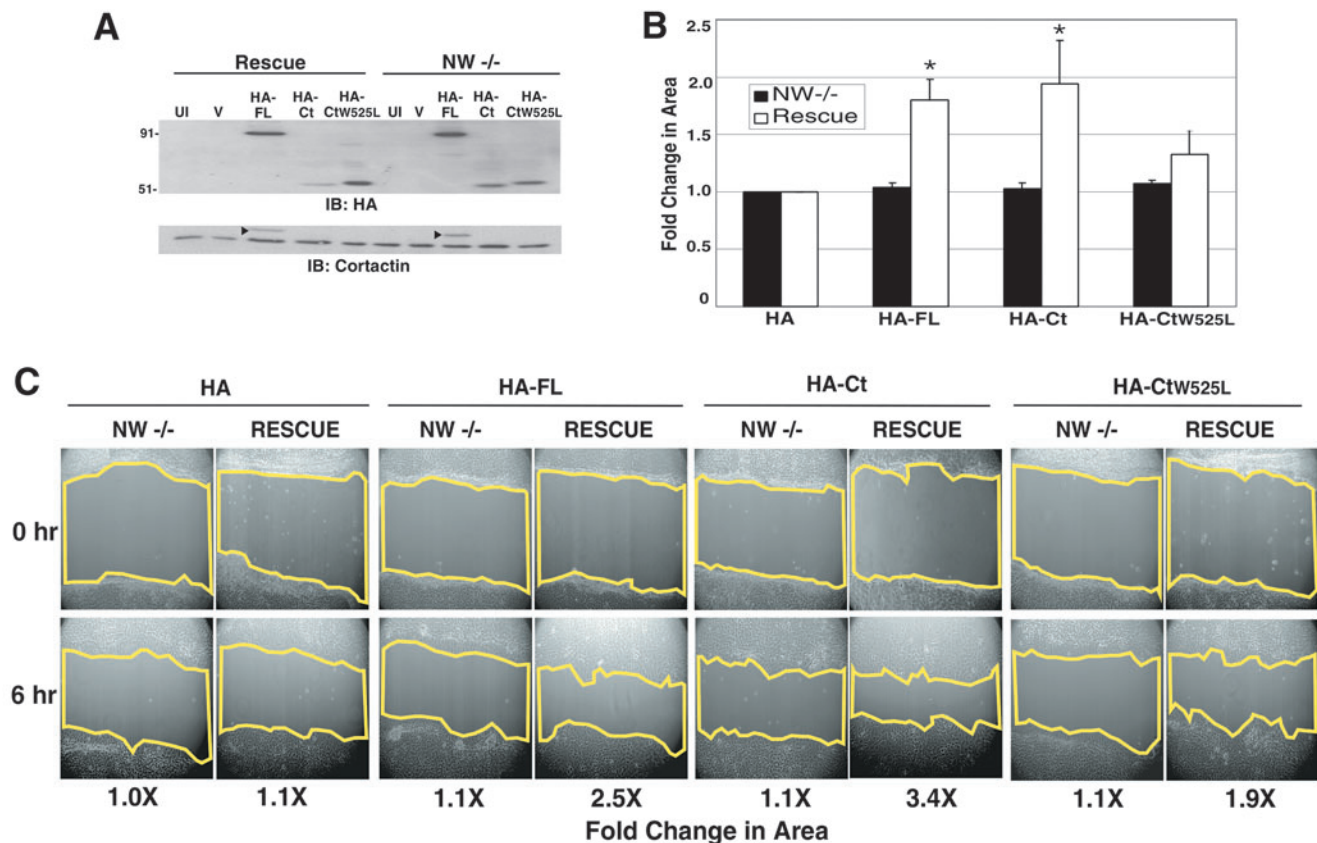
protein with GST-cortactin in the pyrene-actin polymerization assays (Fig. 2D). This His<sub>6</sub>-N-WASP protein has a high basal activity, which is probably because of a partial unfolding and loss of autoinhibition (data not shown) (Egile et al., 1999). In fact, in our hands, the His<sub>6</sub>-N-WASP protein gives 5.5-fold basal activation of the Arp2/3 complex, whereas basal activation by GST-N-WASP is only 1.7-fold (data not shown). Despite the elevated basal activity, GST-Ct and GST-SH3 cortactin still induced 1.6- to 1.8-fold activation of His<sub>6</sub>-N-WASP, whereas GST alone did not activate His<sub>6</sub>-N-WASP (Fig. 2D and data not shown). In addition, untagged cortactin C-terminus promoted up to fivefold activation of GST-N-WASP (Fig. 2E). Finally, untagged cortactin SH3 domain also

activated N-WASP up to 2.5-fold (Fig. 3C,D). Thus, although the GST tag can enhance the ability of cortactin C-terminus and cortactin SH3 to activate N-WASP, probably through stabilization of protein folding or by promoting cortactin multimerization, it is not required.

To examine directly the role of the SH3 domain in N-WASP activation, we used blocking peptides and an SH3 domain mutant in the pyrene actin polymerization assays (Fig. 3). The optimal proline-rich sequence for binding the cortactin SH3 domain, which was identified by phage display (+PP $\Psi$ PXKPXWL), was used to design a 16 amino acid blocking peptide designated 'cortoptimal' (Sparks et al., 1996). This peptide and a random control peptide were first tested for



**Fig. 3.** N-WASP activation requires an intact, accessible SH3 domain. (A,B) Peptide inhibition. Pyrene assays were performed with 10 nM Arp2/3 complex, 1.5  $\mu$ M pyrene-labeled G-actin, 25 nM GST-N-WASP and 0.5  $\mu$ M GST-SH3 in the presence of peptide inhibitors at the specified concentrations. (A) Activation of N-WASP by cortactin SH3 protein in the presence of 100  $\mu$ M cortoptimal or 100  $\mu$ M control peptides. The inset shows a western blot of competitive GST pull-down assays showing association of GST-SH3 cortactin with endogenous N-WASP in the presence of increasing concentrations (0, 25, 50, 100, 200, 500, 1000  $\mu$ M, left to right) of cortoptimal or control peptide. (B) 100  $\mu$ M cortoptimal and control peptides have no effect on N-WASP alone or on WA-mediated actin polymerization. (C,D) Mutation of the SH3 domain. Pyrene assays were performed with 10 nM Arp2/3 complex, 1.5  $\mu$ M pyrene-labeled G-actin and 25 nM GST-N-WASP, in the presence of either GST-tagged or untagged cortactin SH3 domain. (C) Sample polymerization curves showing maximal GST-N-WASP activation by 2.5  $\mu$ M SH3 or SH3W525L. Inset shows sample polymerization curves of maximal GST-N-WASP activation by 0.75  $\mu$ M GST, GST-SH3 or GST-SH3W525L proteins. (D) Fold activation of GST-N-WASP in the presence of untagged cortactin SH3 and SH3W525L proteins over a range of concentrations. Curves are a logarithmic 'best fit' of the data points shown. Inset shows GST pull-down assays for N-WASP binding performed in the presence of GST, GST-SH3 or GST-SH3W525L beads.



**Fig. 4.** Maximal cortactin-enhanced migration requires N-WASP. (A) Western blot showing expression of the HA-FL, HA-Ct and HA-CtW525L cortactin (upper blot) or endogenous cortactin (lower blot) in NW<sup>-/-</sup> or rescued MEFs. Arrowheads indicate HA-FL cortactin. Expression of all HA-tagged constructs was equivalent to or less than endogenous cortactin levels. UI, uninfected cells. (B) HA-tagged, or in some assays GFP-tagged, cortactin-expressing cells were assayed for wound closure at 0, 2, 4 and 6 hours after wounding. Data are the average of four to five experiments at the 6-hour time point  $\pm$  s.e.m. Asterisks indicate statistically significant differences from wound closure results in control cells ( $P < 0.05$ ). (C) Representative results from wounding assay experiments are shown with images taken at 0 and 6 hours after wounding. The fold change in wound area is indicated underneath each figure.

their ability to block the cortactin-N-WASP interaction, then each was added along with the cortactin SH3 protein in the pyrene-actin polymerization assays (Fig. 3A). Neither peptide had any effect on actin polymerization mediated by N-WASP alone or the active WA fragment of N-WASP (Fig. 3B). However, addition of 100  $\mu$ M cortoptimal peptide inhibited up to 40% of the cortactin SH3 domain-induced N-WASP activation and as much as 60% inhibition was observed at higher concentrations of the peptide (Fig. 3A and data not shown). The cortoptimal peptide also inhibited N-WASP binding to cortactin in competitive pull-down assays (half-maximal inhibition  $\sim$ 100  $\mu$ M peptide) (Fig. 3A, inset). In contrast, there was no inhibition by the control peptide of N-WASP binding or activation. Furthermore, a cortactin SH3W525L mutant deficient in N-WASP binding was impaired in its ability to activate N-WASP (Fig. 3C,D). Taken together, these data clearly indicate that an intact SH3 domain is required for maximal N-WASP binding and activation by cortactin.

To examine the role of cortactin and N-WASP binding in actin filament formation further, we performed bead assays in a reconstituted actin-based motility system composed of purified proteins (Loisel et al., 1999; Wiesner et al., 2003). In the presence, but not the absence, of N-WASP, actin assembly followed by the appearance of actin comet tails was observed

at the surface of carboxylated beads coated with GST-SH3 or GST-Ct (data not shown). In contrast, GST-SH3W525L-coated beads were significantly impaired in generating actin tails (data not shown). This ability of the SH3 domain to form actin comets in an N-WASP-dependent manner demonstrates that this domain is sufficient to promote actin tail formation through N-WASP and supports a role for cortactin in N-WASP recruitment and activation.

As shown in Fig. 1, expression of the cortactin C-terminus induces migration and this correlates with the ability of this region to bind to and activate N-WASP (Fig. 1C,D; Figs 2, 4). Although loss of N-WASP has no significant effect on wound closure (Snapper et al., 2001) (data not shown), it is still possible that N-WASP may be important for cortactin-induced migration. Significantly, we found that the ability of full-length and C-terminal cortactin to enhance migration was largely dependent upon N-WASP (Fig. 4). Whereas reconstituted N-WASP-deficient mouse embryonic fibroblasts (MEFs) ('rescue' cells) expressing full-length cortactin or cortactin C-terminus enhanced wound closure (1.8- and 1.9-fold, respectively) compared to control vector-infected MEFs, expression of full-length cortactin or cortactin C-terminus in N-WASP-deficient MEFs (NW<sup>-/-</sup> cells) did not enhance wound closure compared to control or CtW525L-expressing N-WASP<sup>-/-</sup> cells (Fig. 4).



These effects are unlikely to be due to expression differences, as the full-length protein was expressed at equivalent levels in both the N-WASP null and rescued MEFs, and cortactin C-terminus was always expressed at higher levels in the N-WASP null cells (Fig. 4A). Taken together, these data demonstrate that N-WASP is necessary for maximal migration induced by either full-length cortactin or cortactin C-terminus.

## Discussion

It was previously thought that cortactin functions to recruit N-WASP to sites of Arp2/3 localization (Mitzutani et al., 2002). In this study, however, we show that the cortactin C-terminus, via the SH3 domain, can directly stimulate the activity of N-WASP. Moreover, our results demonstrate that the C-terminal half of cortactin can regulate cell motility independently of the presence of the N-terminal portion, and this correlates with its ability to stimulate actin polymerization *in vitro*. This SH3-mediated activity is clearly relevant to the function of the full-length molecule, as full-length cortactin both enhances migration in an N-WASP-dependent manner and induces N-WASP activation in pyrene-actin nucleation assays (Fig. 4 and data not shown). Thus, cortactin, via its SH3 domain, may be important for both localization and activation of N-WASP.

Clustering of N-WASP can enhance its activation and may be necessary for its biological effects, and the data presented here support this hypothesis (Castellano et al., 1999; Hufner et al., 2001). Specifically, GST-Ct and GST-SH3 proteins were more potent activators of N-WASP than the untagged proteins. Although there is no direct evidence for cortactin multimerization, cortactin is a scaffold protein with many binding partners and it is likely that clustering of cortactin does occur in cells because of these interactions. Clustering of other N-WASP activators, such as activated Cdc42 and Nck, as well as clustering of the VCA fragment enhances activation (Castellano et al., 1999; Higgs and Pollard, 2000; Hufner et al., 2001; Rivera et al., 2004). Thus, although N-WASP can be activated by several different molecules, *in vivo* these activators are likely to do so by both relieving autoinhibition and by clustering N-WASP.

Previous studies defined a role for cortactin NTA in activation of the Arp2/3 complex, and here we show a role for the SH3 domain in activation of N-WASP. In addition, while this work was under review, activation of N-WASP via the cortactin SH3 domain was also reported (Martinez-Quiles et al., 2004). Given that cortactin and N-WASP binding to Arp2/3 complex have been shown to be mutually exclusive events, our data suggest a potential model: cortactin bound to the side of a filament may first bind and activate N-WASP to initiate daughter filament formation, then subsequently bind activated Arp2/3 at the branch point to stabilize the newly branched network (Urano et al., 2003). These functions may be important at the leading edge but could be more significant in other subcellular locations. For example, although actin turnover at the leading edge is generally thought to be the driving force in cell migration, vesicle trafficking can also contribute to this process (Nabi, 1999). N-WASP is not prominently localized to the leading edge, where cortactin and Arp2/3 complex can be found (Wu and Parsons, 1993; Weed and Parsons, 1998; Kaksonen et al., 2000; Weed et al., 2000). Both N-WASP and cortactin, however, are colocalized in podosomes of *v-Src* transformed cells, and in filopodia and

the perinuclear region in bradykinin-stimulated 3T3 cells (Mitzutani et al., 2002; Martinez-Quiles et al., 2004). In addition, both proteins are found in cytoplasmic puncta associated with the actin comet tails of trafficking vesicles (Kaksonen et al., 2000; Taunton et al., 2000; Cao et al., 2003). Furthermore, the cortactin C-terminus, which promotes cell migration in an N-WASP-dependent manner, does not localize to the leading edge but is found in cytoplasmic puncta (data not shown). Accordingly, cortactin may be important for regulating N-WASP in such structures, whereas its direct activation of the Arp2/3 complex may be more important in modulating actin dynamics at the leading edge. At either subcellular location, the SH3 domain also may serve to enhance the function of the NTA domain through SH3 binding partners such as dynamin and WIP. Both of these proteins have been shown to modulate the ability of the NTA domain of cortactin to bind and directly activate the Arp2/3 complex (Schafer et al., 2002; Kinley et al., 2003).

Although we believe that the ability of cortactin to activate N-WASP is of importance for basic cellular functions like migration and vesicle trafficking, it remains unclear whether this cortactin-N-WASP pathway plays a primary or an accessory role in these events. This is particularly true given the existence of other cortactin SH3 domain binding partners that might also enhance actin polymerization at either the leading edge or on vesicles to stimulate migration. For example, the interaction of dynamin with the cortactin SH3 domain is important for the formation of dorsal membrane ruffles in PDGF-stimulated cells, which probably contributes to a motile phenotype (McNiven et al., 2000; Krueger et al., 2003). Furthermore, cortactin and dynamin are colocalized on PIP5 kinase-induced vesicles and have been implicated in trafficking (Orth et al., 2002; Cao et al., 2003). Given that protein and membrane trafficking to and from the leading edge is important for migration, such interactions might also be important for cortactin-induced motility (Nabi, 1999).

Regardless of the potential functions of these other binding partners, our results clearly indicate a role for the cortactin-N-WASP interaction, as we have shown that: (1) cortactin binds and activates N-WASP; (2) activation of N-WASP by cortactin is more robust than its ability to directly activate the Arp2/3 complex; and (3) cortactin-induced migration is dependent upon N-WASP. As N-WASP is a potent activator of Arp2/3 complex, activation of N-WASP via cortactin would serve to amplify the signals transduced by this complex. It is plausible, therefore, that the cortactin-N-WASP pathway is important in normal actin-based processes. Moreover, this regulation may be of even greater significance in tumorigenesis. Cortactin is overexpressed in head, neck and mammary tumors where its expression correlates with increased tumor invasiveness (Schuurung et al., 1992). Thus, given that the enhanced cell migration observed with overexpression of full-length cortactin is dependent upon N-WASP, this cortactin-N-WASP pathway may be of great importance in the invasive phenotypes of cortactin-overexpressing tumors.

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