Calponin h1-S175T Point Mutation Enhances Resistance to Actin Cytoskeleton Perturbation in Human Cancer Cells

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Abstract. *To investigate Calponin h1 (CNh1) regions responsible for suppressing cancer cell motility, the interaction between CNh1 and actin in HeLa cells was examined. First, it was observed that the actin binding of CNh1 depends on the calponin repeat 1 (CNR 1), region more than the actin binding site region. Next, point mutantions were generated at S175 and/or T184 in CNR1, substrates of protein kinase C, and it was observed by cellular immunostaining that the actin binding of CNh1 depends on 175th amino acid. Furthermore, the point mutation S175T exhibited more resistance to actin rearrangement by cytochalasin D and PDBu than intact CNh1, suppressing cell motility induced by PDBu. This result indicates that S175T may be an effective target for new cancer treatments.*

Smooth muscle calponin (CNh1) is a 34 kDa actin binding protein originally isolated from vascular smooth muscle (1). The functions of CNh1 are well known, and include inhibition of myosin ATPase in smooth muscle contraction (2-4). CNh1 expression has also been demonstrated to be a critical determinant in the prediction of tumour metastasis and patient outcome. Down-regulation of CNh1 in tumour cells has been observed in human leiomyosarcoma (5), and suppression of CNh1 in tumour blood vessels in human melanoma (6), hepatocellular carcinoma (7), renal cell carcinoma (8) and colon cancer (9) has been associated with tumour progression. Ramaswamy *et al*. (10) highlighted CNh1 as one of the nine down-regulated genes associated with adenocarcinoma metastasis by assessment with micro cDNA arrays. In knock-out experiments, CNh1-deficient mice exhibited morphological fragility in tissues, including blood vessels and peritoneal membranes, which is an important factor influencing cancer metastasis (11). Furthermore, peritoneal dissemination occurring in CNh1-deleted mice was suppressed by treatment with CNh1 (12), indicating an ability of CNh1 to protect against cancer dissemination *via* the stabilization of peritoneal membranes (13). Transfection of CNh1 into cancer cell lines has demonstrated that CNh1 exhibits anti-tumourigenic functions (14-17). Taken together, these observations indicate a strong possibility that CNh1 can simultaneously suppress cancer phenotypes and protect normal cells from the attack of cancer cells (13, 18).

Through binding with actin, CNh1 stabilises actin filament systems (19) and regulates cytoskeletal organization in non-muscle cells (20). Several CHh1 actin-binding site candidates have been investigated in normal cells and tissues, and include the actin-binding site (ABS) (21), calponin repeats (CNRs) (22), and 175th and 184th residues in calponin repeat 1 (CNR1) that are substrates of protein kinase C (PKC) (23) and Rho kinase (24). Based on these studies, it is hypothesized that the anti-tumorigenic effects of CNh1 are imparted through stabilization of the actin cytoskeleton *via* one of these regions. Therefore in this study, several candidate CNh1 ABS in HeLa cells were screened.

Materials and Methods

Expression plasmids and mutations. Human full-length CNh1 cDNA was cloned into a *HindIII-BamHI* site of a pEGFP-C2 vector (GFP-V: Clontech, Palo Alto, CA, USA) and named GFP-CNh1. Deletion mutants of CNh1 were produced by polymerase chain reaction (PCR) using GFP-CNh1 as a template. Point mutations were introduced into GFP-CNh1 by PCR using primer sets that included the mutations. The expand High-Fidelity system (Roche Diagnostics, Mannheim, Germany) was used for PCR and a Takara BKL kit (Takara Biotechnology, Tokyo, Japan) was used for the cloning of PCR products into blunt end vectors. All constructs were sequenced using an ABI PRISM 310 genetic Analyzer (Applied Biosystems, CA, USA).

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Cell culture and DNA transfection. Cells from the human cervical adenocarcinoma cell line (HeLa cells: American Type Culture Collection (ATCC) of Global Bioresource Center, Manassas, VA, USA) were cultured in Dulbecco's modified medium supplemented with 10% fetal bovine serum and 1% antibiotic solution at 37˚C in 5% CO_2 air. GFP-V, GFP-CNh1, and GFP-CNh1 deletion or point mutants were then transfected transiently into HeLa cells to analyze the stability of actin filaments. Transfection was performed using FuGENE HD Transfection Reagents (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Immunofluorostaining and Western blotting. For immunofluorostaining, cells were fixed with 10% formaldehyde/PBS for 30 minutes, permeabilized with 0.1% Triton X-100 in PBS for 2 minutes, and then stained for actin filaments with rhodamine-phalloidin (RP: Eugene, OR, USA) for 1 hour. Association of CNh1 or its mutants with actin filaments was examined with a fluorescence microscope (Axiovert S100; Zeiss, Thornwood, NY, USA).

For Western blotting of CNh1 deletion mutants, transfected HeLa cells were subjected to 10% SDS-PAGE and immunoreacted with anti-human calponin antibodies (DAKO Japan, Kyoto, Japan).

Motility assay using gold colloid methods. Random cell motility was estimated by the phagokinetic track method on gold colloid coated glass plates as previously described (25) The phagokinetic track made by Hela cells was assessed after they were seeded onto coated glass coverslips and cultured for 18 hours. The phagokinetic area was analyzed using an ARGUS Image Processor System (Hamamatsu Photonics Co., Hamamatsu, Japan).

Actin stability assay by cytochalasin D (CytoD) or phorbol 12,13-dibutyrate (PDBu). GFP-V, GFP-CNh1, and a GFP-CNh1 point mutant were first transfected into HeLa cells. Two days later, cells were treated for different time periods with 2 or 3 μM cytoD or 1 μM PDBu. Resistance to actin cytoskeleton depolymerization by these reagents was observed with a fluorescence microscope after RP staining of actin filaments.

Statistical analysis. Student's *t*-test or ANOVA followed by Fisher's post-hoc test was performed for statistical analysis using Stat View 5.0 software (SUS Institute, Inc., Berkley, CA, USA). Differences were considered to be statistically significant at $p<0.05$. All values were expressed as mean±standard error (SE) of the mean.

Results

Exogenously expressed CNh1 co-localized with actin filaments and suppressed cell motility in HeLa cells. Transferred CNh1 (GFP-CNh1) was seen to co-localize with and strengthen the actin cytoskeleton in HeLa cells (Figure1A). Cell motility, as measured by the gold colloid method (25), was significantly suppressed in GFP-CNh1 transfected cells compared with GFP-V transfectant controls (**$p<0.01$, Figure 1B). Thus, these results confirm that CNh1 strengthened the structure of actin cytoskeleton and suppressed cell motility in HeLa cells.

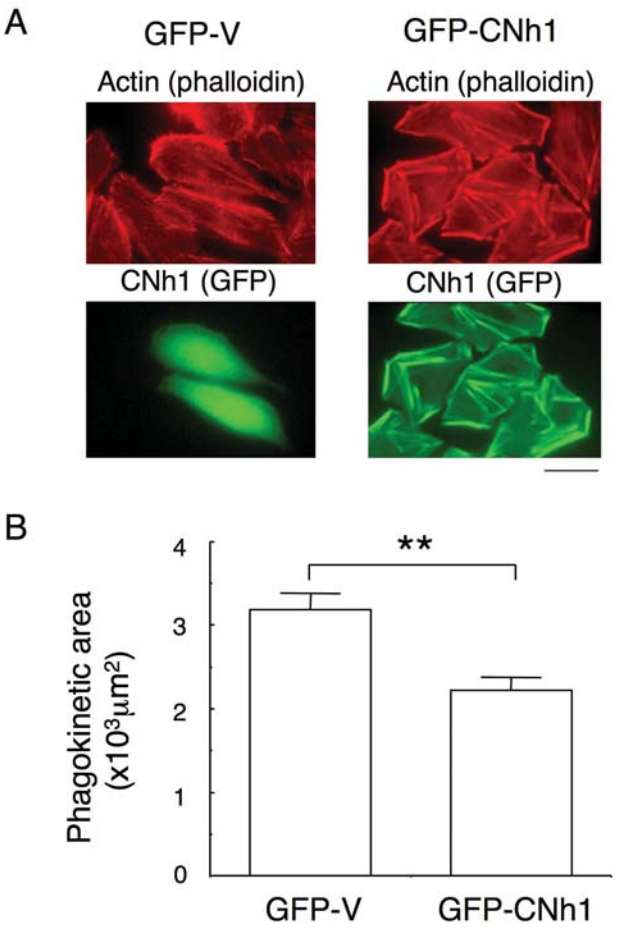

Figure 1. *Effects of CNh1 on the stabilization of actin filaments in HeLa cells. A: GFP-V (pEGFP-C2 vector) and GFP-CNh1 (full-length human CNh1 in a pEGFP-C2 vector) were transiently transfected into HeLa cells. Actin filaments were stained with rhodamine phalloidin. CNh1 is shown by GFP. Scale bar: 10 μm. B: Cell motility was measured by the gold colloid method 18 hours after seeding and expressed as the area of phagokinetic tracks. N=50 for GFP-V or GFP-CNh1, **p<0.01.*

Both ABS and CNR1 of CNh1 are required for actin binding and stabilization of the actin cytoskeleton. To elucidate the CNh1 domain responsible for actin binding, deletion mutants of CNh1 lacking ABS (GFP-ΔABS: Δ142nd to 163rd amino acid: aa) or CNR1 (GFP-ΔCNR1: Δ164th to 203rd aa) were constructed from GFP-CNh1 (Figure 2A, left) and transfected into HeLa cells (Figure 2A, right). Western blotting results are shown in Figure 2B. Neither GFP-ΔABS nor G-ΔCNR1 was able to maintain the binding ability of CNh1 with actin filaments; however, the binding ability of GFP-ΔCNR1 was more strongly suppressed than that of GFP-ΔABS. These results suggest that both ABS, and to a greater degree CNR1, of CNh1 are necessary for binding with actin filaments.

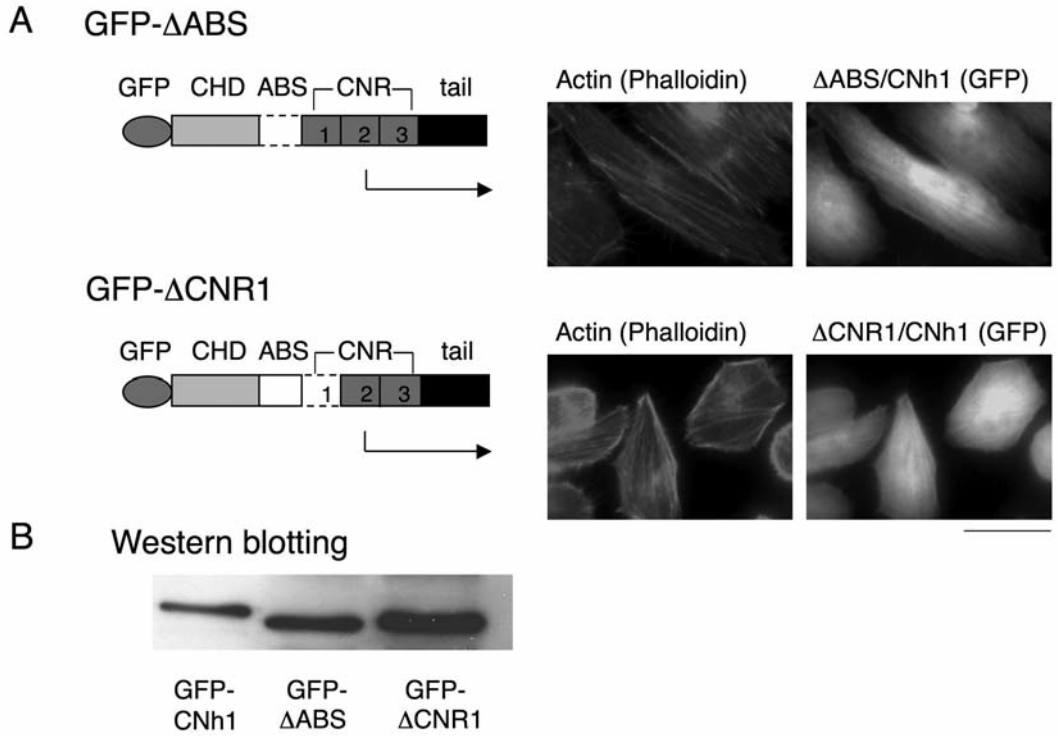

Figure 2. *Significance of ABS (actin binding site) and CNR1 (calponin repeats 1) of CNh1 for actin binding. A: Actin binding ability was compared between ABS-deleted CNh1 mutants (GFP-ΔABS) and CNR1-deleted CNh1 mutants (GFP-ΔCNR1: left) in transiently transfected HeLa cells. ΔABS and ΔCNR1 are shown by GFP. Actin filaments were stained with rhodamine phalloidin (right). CHD: calponin homology domain. Scale bar:10 μm. B: Western blotting of GFP-CNh1, GFP-ΔABS, and GFP-ΔCNR1.*

The 175th residue of CNh1 is more important for actin binding than the 184th residue. After establishing the importance of ABS, and especially CNR1, for actin binding, serine 175 (S175) and threonine 184 (T184) of CNh1 were investigated next. These are both known phosphorylation sites of PKC and Rho-kinase in the CNR1 domain. CNh1 point mutants were constructed of these amino acids, either separately or together. It was observed that alanine-substituted S175A&T184A or S175A, but not T184A, exhibited a decrease in actin binding, indicating that binding regulation of CNh1 to actin was largely dependent on the 175th aa (Figure 3A). Glutamate-substituted S175E and/or T184E point mutants exhibited consistent results with the alanine mutants (Figure 3B), but a decrease in actin binding ability by S175E appeared noticeably greater. Since this 175th residue was already suspected to play an important role in actin binding, S175 was then substituted with threonine (GFP-S175T). Surprisingly, GFP-S175T demonstrated the strongest actin binding and actin cytoskeleton stabilization compared to the other point mutants (Figure 3C).

Threonine substitution of the 175th serine (GFP-S175T) stabilized actin filaments against CytoD. To further investigate the stabilization of actin filaments through association with CNh1, resistance to actin degradation caused by CytoD was examined in GFP-V, GFP-CNh1, and GFP-S175T transfected cells. Thirty minutes after treatment with 3μM CytoD, most of the actin filaments in GFP-V-transfected cells were depolymerized, while those in GFP-CNh1-transfected cells showed some resistance to depolymerization. Interestingly, GFP-S175T cells resisted actin depolymerization caused by CytoD by considerably retaining their original actin cytoskeletons (Figure 4).

Threonine substitution of the 175th serine (GFP-S175T) imparted actin filament resistance to reorganization caused by PDBu. The motility of HeLa cells transfected with S175T mutants was investigated in order to confirm the correlation between cytoskeletal stabilization and motility in cancer cells. Without any stimulation, the motility of HeLa cells transfected with GFP-CNh1 or GFP-S175T was significantly lower compared with GFP-V transfectant controls (n=20 for each, $p<0.05$, Figure 5A). There were no discernible differences observed in the degree of motility suppression between GFP-CNh1 and GFP-S175T transfectants.

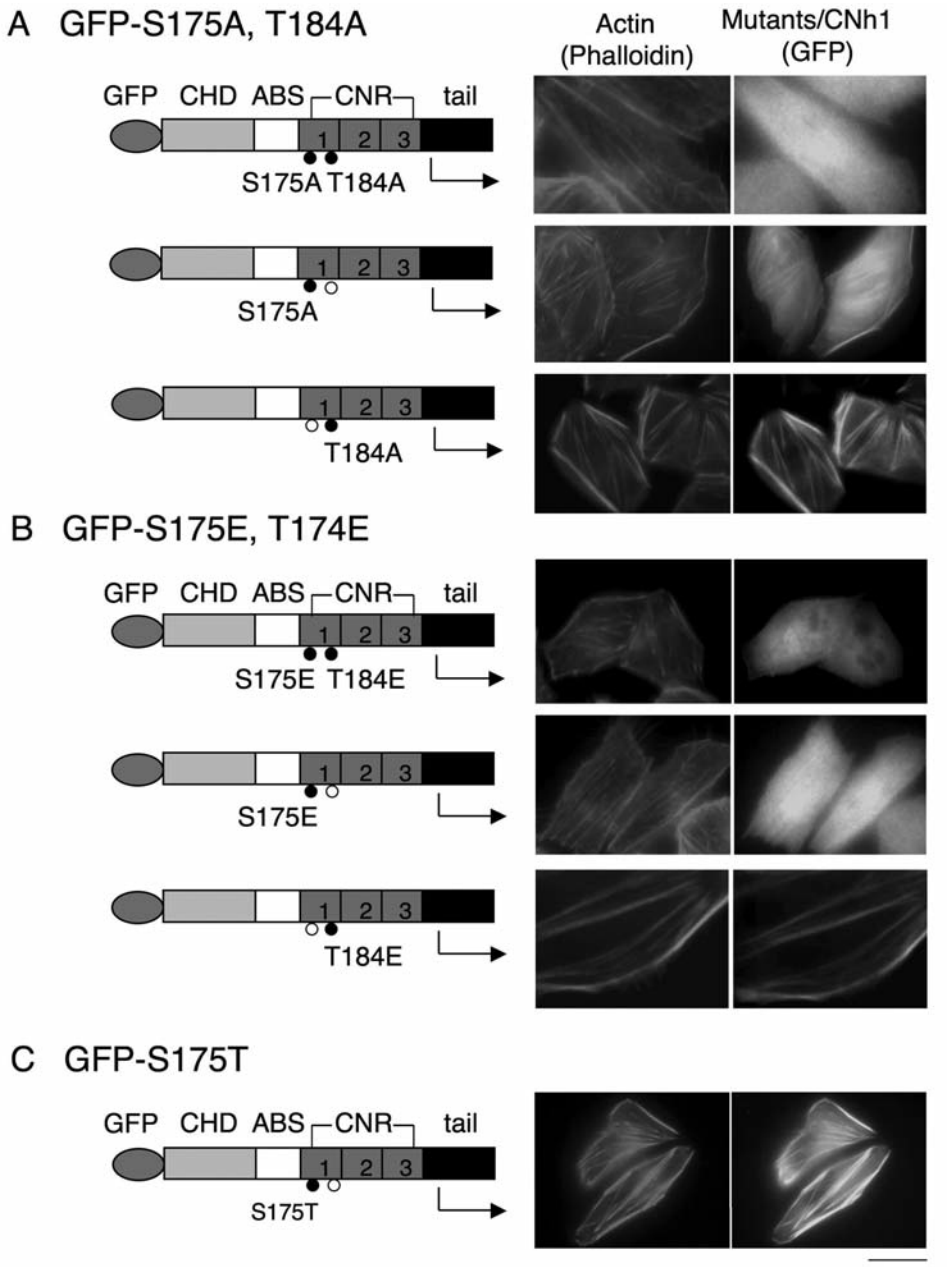

Figure 3. *Significance of alanine, glutamate, and threonine substitution of the 175th and/or 184th residues of CNh1 for actin binding. A: S175A and/or T184A point mutated CNh1 introduced into pEGFP-C2 vectors (GFP-S175A and/or GFP-T184A, respectively) were transiently transfected into HeLa cells. Actin binding ability was evaluated by fluorescence microscopy. B: S175E and/or T184E point mutants were examined in the same way as in A). C: The S175T point mutant was examined in the same way as in A). Scale bar: 10 μm.*

As it has been hypothesised that CNh1 interactions with actin are regulated by PKC (26), transfectants were treated with PDBu (1μM), a phorbol ester that activates PKC, for 1 hour. GFP-S175T transfectants showed a stronger inhibitory effect on motility than transfectants of wild-type GFP-CNh1 (n=20 for each, $p<0.05$, Figure 5B). Furthermore, GFP-S175T transfectants also exhibited stronger resistance to rearrangement of actin cytoskeletons than did wild-type transfectants under PDBu stimulation (Figure 5C, left). The association rate of CNh1-S175T to actin cytoskeletons (24.1%) was higher than that of wild-type CNh1 (16.1%, Figure 5C, right). These findings confirmed the stabilization of actin filaments by CNh1-S175T transfectants in HeLa cells.

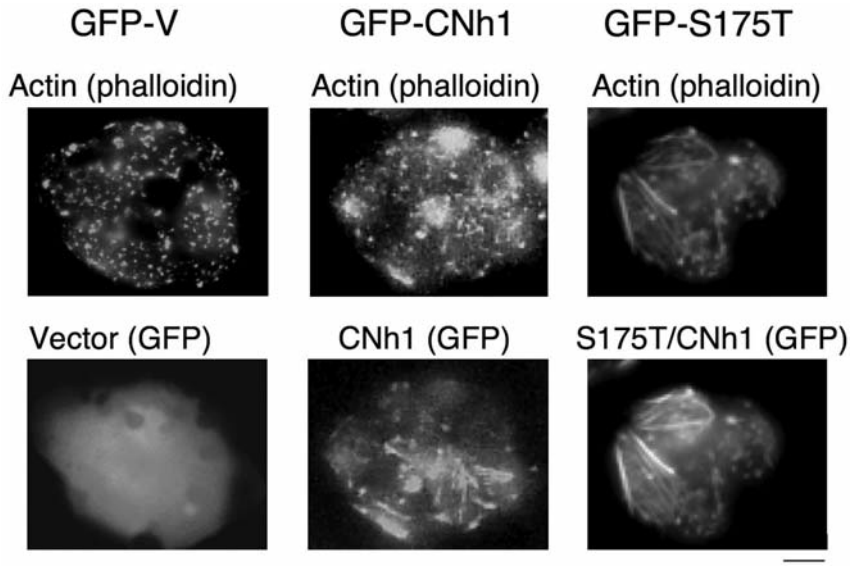

Figure 4. *Comparison of stability against cytochalasin D (CytoD) treatment among GFP-V, GFP-CNh1, and GFP-S175T transfectants. HeLa cells transfected with GFP-V, GFP-CNh1, or GFP-S175T were stimulated by 3 μM CytoD for 30 minutes and degradation of actin filaments was investigated. Scale bar: 10 μm.*

Discussion

CNh1 has been reported to cause rapid polymerization of actin filaments, with virtually no depolymerization occurring in its presence (19). Normal cells made to overexpress CNh1 revealed that CNh1 reduced myosin-based actin motility, and stress fibers in these cells were resistant to actin depolymerization under CytoD and latrunculin B stimulation (20, 27) which demonstrated the effect of CNh1 on cytoskeletal stabilization in NIH3T3, REF52, and A7r5 cell lines. The present study using tumour cells supported these results; CNh1 transfected into HeLa cells suppressed cell motility and resisted degradation of the cytoskeleton caused by CytoD or PDBu (data not shown) stimulation.

Since suppression of tumour motility and cytoskeleton degradation by CNh1 is seemingly related to its ability to bind and stabilise actin filaments, the study began by investigating the responsible sites on CNh1 for actin binding with the HeLa human cancer cell line. Mezgueldi *et al*. originally suggested the 145th to 182nd aa of CNh1 as the actin filament binding site (21), and later narrowed this region to the 142nd to 163rd aa of CNh1 (28) after co-sedimentation assays. As a second independent actin binding site, the 172nd to 187th aa has been proposed since it bound to actin filaments without inhibition by the actin-activated ATPase activity of myosin (29). Gimona *et al*. (22) proposed CNR to be as an important site for actin binding. According to them, although calponin h2 lacked a consensus ABS at 142nd to 163rd aa, recombinant full-length calponin h2 co-sedimented with actin filaments, suggesting the presence of another binding site in the molecule. Gimona *et al*. (22) demonstrated that the isolated CNR (164th to 173rdth aa), but not the calponin homology domain (CHD), associated with actin filaments in REF 52 fibroblasts. Furthermore, it has been shown that ectopic expression of multiple 23 amino acid calponin-like repeats (similar to CNR) from *C. elegans* effectively inhibited cell motility and suppressed soft agar colony formation of melanoma cells (17). Therefore, in this study, CNR1 was identified as a more effective binding site for actin filaments, even though both ABS and CNR1 are necessary for efficient actin binding in HeLa cells.

It has been well established that PKC associates with CNh1 (30-33), and that CNR1 contains the substrates of PKC, such as Ca^{2+} calmodulin-dependent kinase II2 and Rho kinase (24). According to Kaneko *et al*. (24), unphosphorylated CNh1 binds to actin filaments, but this property is lost upon phosphorylation. Therefore, in order to enhance actin stabilization through binding of CNh1 to actin filaments, this study sought to determine the most effective site on CNh1 for phosphorylation by blocking such sites. Whereas Winder *et al*. (23, 34) reported that both serine and threonine residues in CNh1 are phosphorylated, however, the major site of phosphorylation is S175 in CNR1 and S175 is important for binding to actin. In contrast, Nakamura *et al*. (26) demonstrated that T184 in CNR1 is the preferred site of phosphorylation and is functionally the most important site phosphorylated by PKC. In the present study, substitutes of alanine or glutamate for S175 and/or T184 indicated that S175 had a greater actin binding ability than T184 in HeLa cells. S175E was a substitution to an acidic amino acid to

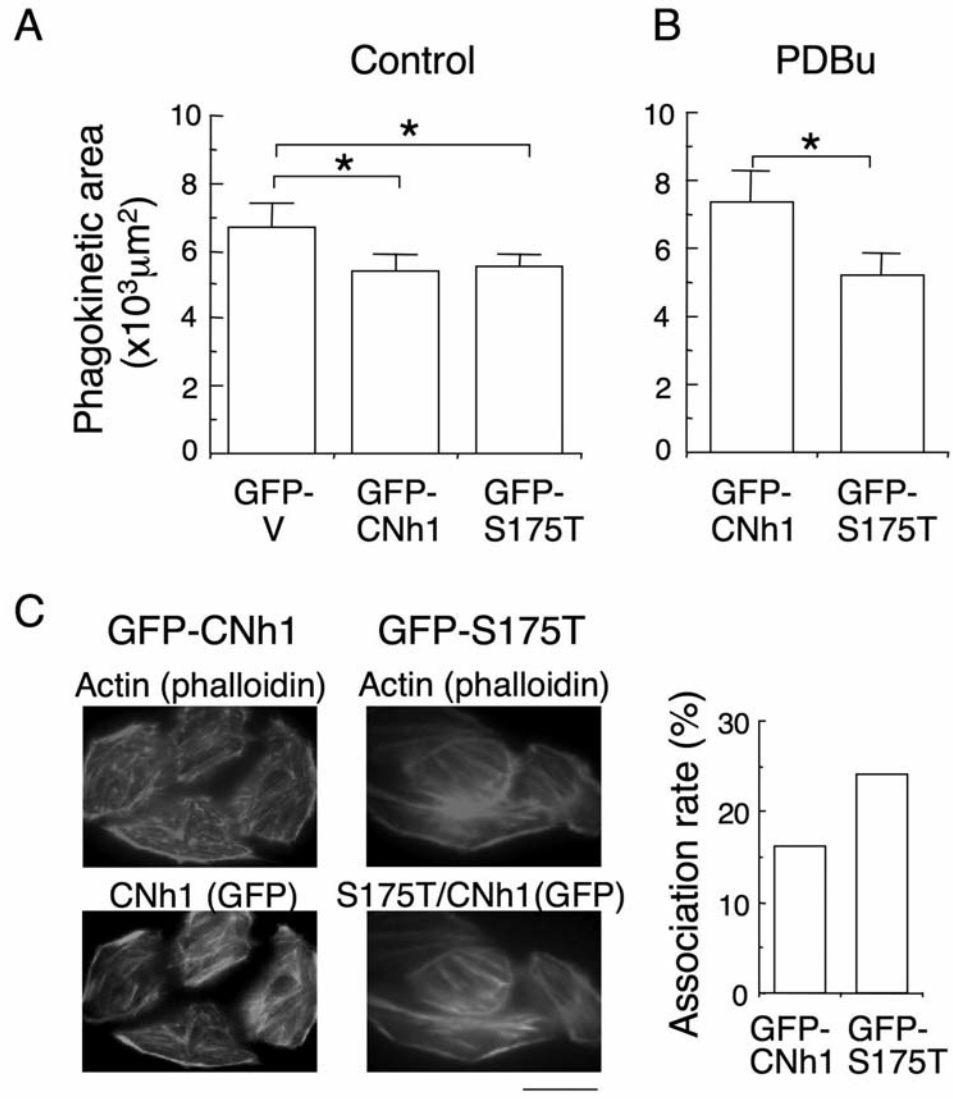

Figure 5. *Comparison of stability against PDBu treatment between GFP-CNh1 and GFP-S175T. A: Motility of HeLa cells transfected with GFP-V, GFP-CNh1, or GFP-S175T was measured by the gold colloid method. N=20 for each, *p<0.05. B: Motility affected by PDBu stimulation (1 μM for 1 hour) was evaluated by the same method as in A) in GFP-CNh1 and GFP-S175T transfected HeLa cells. N=20 for each, *p<0.05. C: HeLa cells transfected with GFP-CNh1 or GFP-S175T were stimulated by 1 μM PDBu for 1 hour, and the association rate of CNh1 or CNh1-S175T with actin filaments was assessed by manual counting of random fields. Scale bar: 10 μm.*

mimic phosphorylation, while S175A was a substitution to a neutral amino acid that nonetheless influenced actin binding like that seen in glutamate substitution.

It is noteworthy that replacement of the 175th serine with threonine strengthened binding to actin filaments, that is, S175T made the actin cytoskeleton resistant to PDBu and CytoD more greatly than wild-type CNh1. These results are in agreement with the perturbation of CNh1 binding to actin filaments in S175T being less affected by PKC treatment than wild CNh1, which led to a decrease in cell motility. These results are also consistent with a previous report that S175T bound to actin filaments with higher affinity and inhibited the actin-activated myosin MgATPase more than wild type CNh1 (35). Taken together, the 175th amino acid seems to be involved more sensitively in the regulation of actin filaments, and phosphorylation at this residue greatly affects the function of CNh1 to bind and stabilize actin filaments.

Conclusion

In this study, it was confirmed that CNh1 inhibits cell motility through its binding and stabilization of actin filaments. Furthermore, it was shown that the 175th amino

acid of CNh1 strongly affects the dynamics of actin filaments and that a S175T mutant of CNh1 could more effectively inhibit cell motility and cytoskeletal perturbation induced by CytoD or PDBu than wild type CNh1 in HeLa cells. Further studies on cancer therapy using CNh1 with increased attention to this mutant will be designed.

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