The Coiled-coil Domain Is Required for HS1 to Bind to F-actin and Activate Arp2/3 Complex*

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HS1 (hematopoietic lineage cell-specific protein 1), a substrate of protein tyrosine kinases in lymphocytes, binds to F-actin, and promotes Arp2/3 complex-mediated actin polymerization. However, the mechanism for the interaction between HS1 and F-actin has not yet been fully characterized. HS1 contains 3.5 tandem repeats, a coiled-coil region, and an SH3 domain at the C terminus. Unlike cortactin, which is closely related to HS1 and requires absolutely the repeat domain for F-actin binding, an HS1 mutant with deletion of the repeat domain maintains a significant F-actin binding activity. On the other hand, deletion of the coiled-coil region abolished the ability of HS1 to bind to actin filaments and to activate the Arp2/3 complex for actin nucleation and actin branching. Furthermore, a peptide containing the coiled-coil sequence only was sufficient for F-actin binding. Within cells overexpressing green fluorescent protein-tagged HS1 proteins, wild type HS1 co-localizes with cortical F-actin at the cell leading edge, whereas mutants with deletion of either the coiled-coil region or the repeat domain diffuse in the cytoplasm. Immunoprecipitation analysis reveals that the coiled-coil deletion mutant binds poorly to F-actin, whereas the mutant without the repeat domain fails to bind to both Arp2/3 complex and F-actin. These data suggest that the HS1 coiled-coil region acts synergistically with the repeat domain in the modulation of the Arp2/3 complex-mediated actin polymerization.

Actin dynamics plays a fundamental role in immune system such as immune response, phagocytosis, and adhesion to cells or extracellular substratum. Assembly of cortical actin filaments that lie underneath the plasma membrane is the function of actin-related protein (Arp) 2/3 complex and its associated proteins. At least two types of proteins that bind to and subsequently activate the Arp2/3 complex in a sequential manner have been described in mammalian cells, WASP and cortactin. WASP, a protein family that includes WASP, N-WASP, and three WAVE/Scar-related proteins (1), initiates actin assembly by recruiting G-actin to the proximity of the Arp2/3 complex, resulting in a heterogeneous nucleation site for efficient actin extension (2). The initial product of actin assembly, where the Arp2/3 complex is still linking to a nascent filamentous actin (F-actin), acquires a high affinity for cortactin, binding of which provokes further actin nucleation and extension (3). Unlike WASP/WAVE proteins, which interact with the Arp2/3 complex through an acidic rich sequence at their C termini, cortactin binds to the complex through a DDW-containing N terminus (4). In addition, cortactin contains a unique F-actin binding motif that consists of 6.5 tandem repeats, with each having exactly 37 amino acids (5). The binding of F-actin appears to be an important property of cortactin and absolutely required for the cortactin/Arp2/3-mediated actin assembly (4).

In immune cells, the function of cortactin is likely carried out by HS1 (6), which contains 3.5 cortactin characteristic repeated units, a coiled-coil (CC)5 region, an SH3 domain at the C terminus, and an Arp2/3 binding domain at the N terminus (see Fig. 1). Like cortactin, HS1 shows a significant F-actin binding activity and markedly promotes actin assembly mediated by Arp2/3 complex when analyzed with purified proteins (7, 8). Despite this similarity, the mechanism for HS1 to associate with F-actin is unclear because it has notably less repeat units than cortactin. In fact, it has been described that cortactin mutants with 3.5 repeats bind poorly to F-actin and are unable to activate Arp2/3 complex (4, 9, 10), indicating that the minimal number of the repeat units for F-actin binding is 4.5. One possibility is that HS1 could have an additional F-actin binding motif. In the exploration of the detailed mechanism for HS1 to bind to F-actin, we found that HS1 mutant with deletion of the repeat domain maintains an F-actin binding activity but is impaired in Arp2/3 binding. We also found that the CC region is necessary for F-actin binding and activation of the Arp2/3 complex-mediated actin assembly. These data suggest a new function of the repeat domain and indicate for the first time that the CC region plays an important role in the regulation of the activity of HS1, the property that may be adapted to the actin dynamics in hematopoietic cells.

MATERIALS AND METHODS

Cell Lines and Antibodies—Human vein umbilical endothelial cells were purchased from Clonetics and cultured as previously described (11). Immature mouse B cell line WEHI-231 was obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and penicillin-streptomycin (Invitrogen). Antibody specific to β-actin monoclonal antibody is from Sigma. Polyclonal antibody specific to mouse HS1 was raised in rabbits against a peptide corresponding to amino acids 306–320. Rabbit polyclonal antibody to Arp2/3 was prepared against a human p41 peptide (QSSQRLTARERFQNLDKKC).

DNA Constructs—HS1 constructs based on MGIN viral vector were prepared as previously described (12). To prepare glutathione S-transferase (GST)-tagged HS1 constructs, including GST-HS1, GST-HS1-

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CC, GST-HS1-R, and GST-HS1ΔRPS, DNA fragments were produced by polymerase chain reaction with primers encoding the corresponding mutations and then subcloned into BamHI and SalI sites of pGEX-4T-2 (Pharmacia Biotech). The deletion mutants GST-HS1ΔCC and GSTHS1ΔR were generated using QuickChange site-directed mutagenesis kit (Stratagene). All mutations were verified by DNA sequencing. The constructs were transformed into strain BL21(DE3) Escherichia coli cells as described previously (12).

Protein Preparation and Purification—Plasmids encoding GST-HS1 constructs were transformed into Escherichia coli strain BL21(DE3) pLysS. The transformed bacteria were grown at 37 °C to the density of A600 = 0.6, and expression of GST-HS1 proteins was induced by adding isopropyl-1-thio-β-D-galactoside to a final concentration of 0.5 mM and by incubation for additional 3 h. The bacteria were harvested by centrifugation at 6,000 × g for 10 min, and the pellet was resuspended in 1/10 volume of starting culture with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and incubated at 4 °C on a rotating wheel for 90 min. After brief centrifugation, the beads were washed twice with F buffer and in 20 μl of 2× SDS sample buffer. Actin present in the beads and the supernatants were revealed by Coomassie Blue staining after SDS-PAGE.

F-actin binding was also assessed by co-sedimentation. Briefly, the purified GST proteins (1 μl) were mixed with F-actin at different concentrations as indicated and incubated on ice. After incubation for 30 min the mixture was centrifuged at 200,000 × g for 45 min at 4 °C. The supernatant was transferred to a new tube, and the pellet was resuspended in 2× SDS loading buffer at equal volume. Aliquots of the supernatant and pellets were resolved by SDS-PAGE, and the gel was stained with Coomassie Blue.

Analysis of the Interaction with Arp2/3 Complex—Im mobilized GST-tagged proteins (0.25 μl) were mixed with 10 pmol of purified Arp2/3 complex in 500 μl of buffer A (50 mM Tris/Cl, pH 8.0, 150 mM NaCl, and 1% Triton X-100) and incubated at 4 °C on a rotating wheel for 90 min. After brief centrifugation, the beads were washed twice with buffer A and dissolved in 20 μl of 2× SDS loading buffer. The amount of Arp2/3 complex in the beads was detected by immunoblotting using a monoclonal anti-Arp3 antibody after separation by SDS-PAGE.

Actin-polymerization Assay—Polymerization of globular actin (G-actin; 10% pyrene-labeled) was performed as described (4) with a modification. Briefly, Ca2+ ATP-G-actin in G-buffer (5 mM Tris/Cl, pH 8.0, 0.2 mM CaCl2, 0.5 mM DTT, and 0.2 mM ATP) was converted into Mg2+ ATP-G-actin by mixing with 0.1 volume of 10× exchange buffer (2 mM EGTA and 1 mM MgCl2) at 20 °C for 2 min. Actin polymerization was initiated by adding 60 μl of Mg2+ ATP-G-actin (10 μM) to 240 μl of 1.25× polymerization buffer (62.5 mM KCl, 2.5 mM MgCl2, 12.5 mM imidazole, pH 7.3, 1.25 mM EGTA, 0.125 mM CaCl2, 0.625 mM DTT, 0.3125 mM ATP, and 3.75 mM NaN3) in the presence of Arp2/3 complex and GST-HS1 at the concentrations as indicated. Actin assembly...
bly was monitored by measuring the increase in pyrene fluorescence detected by LS50B spectrophotometer (PerkinElmer Life Sciences) with filters for excitation at 365 nm and emission at 407 nm.

**F-actin Branching Assay**—The F-actin branching assay was based on a method that was described previously (3). Briefly, actin polymerization was initiated by adding 2.0 μM G-actin to the mixture of 25 nM GST-HS1 proteins, 100 nM VCA, and 50 nM Arp2/3 complex. To visualize actin branches, rhodamine-phalloidin (2 μM) was added to the reaction solution at 15 min after initiation. Samples were diluted 200-fold in fresh fluorescence buffer (100 mM KCl, 1 mM MgCl₂, 100 mM DTT, 10 mM imidazole, pH 7.3, 0.5% methylcellulose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, and 3 mg/ml glucose). Diluted samples (2.0 μl) were applied on coverslips precoated with poly-D-lysine. Actin filaments were examined by a Nikon inverted microscope TE2000-U using a 100X 1.5 NA objective lens. Images were captured with a digital camera equipped on the microscope and controlled by the ACT software. The digital images were processed with Adobe Photoshop to generate monochromatic images. Actin branches were quantified by counting the number of branched filaments based on five randomly selected digital images.

**Immunofluorescence Microscopy**—Human umbilical vein endothelial cells expressing GFP-HS1 variants were seeded on fibronectin-coated glass coverslips in a 6-well plate at a density of 1 × 10⁵ cells/well. After 24 h, the cells were fixed in 4% formaldehyde, permeabilized with 0.2% Triton X-100 in PBS, and stained with rhodamine phalloidin. The stained samples were inspected under Nikon TE2000-U microscope equipped with a digital camera. The digital images were captured and processed by Adobe Photoshop.

**Immunoprecipitation and Immunoblot**—1 × 10⁷ of WEHI-231 cells expressing HS1-GFP or mutants were washed once with PBS and lysed in 500 μl of TNE buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM NaN₃VO₄, and 5 mM EDTA) containing protease inhibitor mixture (Roche Applied Science). The cell lysates were centrifuged briefly to remove insoluble debris. The supernatants were immunoprecipitated with protein G-Sepharose coupled with GFP monoclonal antibody. The immunoprecipitates were resolved by 10% (v/v) SDS-PAGE, transferred to a PVDF membrane, and immunoblotted using appropriate primary antibodies followed by secondary horse radish peroxidase-conjugated antibodies (Bio-Rad). The membrane was stripped using Restore Western blot stripping buffer (Pierce) and reprobed with appro-
The CC Motif Is Necessary and Sufficient for F-actin Binding—To explore the structural motifs necessary for F-actin binding, a series of HS1 mutants were prepared as GST fusion proteins (Fig. 1). Purified recombinant proteins at either 1 or 5 μM were incubated with spontaneously assembled F-actin. The resulting complex of F-actin and HS1 was pulled down by glutathione beads. 7% of the supernatant (s) and 40% of the pellet (p) were resolved in SDS-PAGE and the gel was subsequently stained by Coomassie Blue (Fig. 2). Although GST itself at either 1 or 5 μM failed to pull down any detectable F-actin, which displayed a gel motility of 42 kDa, GST-HS1 precipitated readily F-actin at both concentrations (Fig. 2A, right). Likewise, the mutant GST-HS1ΔPS, which lacks the proline and SH3 domains, also precipitated a significant amount of F-actin, indicating that the C-terminal region is dispensable for F-actin binding. Interestingly, the repeat deletion mutant GST-HS1ΔR was still able to pull down F-actin, although it did so less efficiently at 1 μM. On the other hand, the mutant GST-HS1ΔCC with the deletion of the amino acids from 215 to 280 failed to do so at both concentrations. To confirm whether the CC and the repeat domains are sufficient for F-actin binding, peptides GST-HS1-R, which contains the repeat domain only (Fig. 1), and GST-HS1-CC, which contains the sequence from 215 to 280, were prepared and used in the pull-down assay. As shown in Fig. 2A, actin was readily detected in the pellet of GST-HS1-CC, whereas it was absent in that of GST-HS1-R, indicative of a poor interaction between the repeat domain and F-actin.

To verify that the pull-down assay reflects a true F-actin binding, the mixture of GST fusion proteins and F-actin were co-sedimented at a high centrifugation force, the assay that has been most commonly used for F-actin binding. As shown in Fig. 2B, the CC domain peptide itself bound readily to F-actin, and deletion of it nearly abolished the binding. The results with other mutants by co-sedimentation were also similar to those based on pull-down assay. A quantitative co-sedimentation was performed at different concentrations of F-actin, from which we estimated that GST-HS1 and GST-HS1ΔPS have equilibrium dissociation constant (Kd) values of 0.52 and 0.46 μM, respectively, whereas GST-HS1-CC peptide shows a Kd = 1.63 μM. It is apparent that the CC motif does not account for the full F-actin binding activity, suggesting that other sequence of HS1 may likely play a role in the optimal F-actin binding as well. One possible situation is that the CC domain acts synergistically with the repeat domain for F-actin binding. Indeed, the repeat deletion mutant GST-HS1ΔR has a slightly lower affinity for F-actin than the wild type HS1 with a Kd = 0.68 μM, indicating that the repeat domain also plays a role in F-actin binding, though to a less degree. It should be also noticed that the only mutant that was able to be nearly 100% depleted by F-actin in the co-sedimentation assay is GST-HS1ΔPS (Fig. 2C), whereas trace amounts of the other HS1 forms, including the wild type, were apparently maintained in the supernatant even in the presence of 5 μM F-actin. This suggests that HS1 may possess a configuration that is resistant to F-actin binding and negatively regulated by the C-terminal region.

The CC Motif Is Required for HS1 to Promote the Arp2/3 Complex-mediated Actin Polymerization—HS1 is known to bind to and activate Arp2/3 complex (8). To determine whether the CC domain plays a role in the interaction with Arp2/3 complex, the recombinant GST-HS1 mutants were used to pull down purified Arp2/3 complex, which was further detected by immunoblot using antibody against Arp3, a subunit of Arp2/3 complex. As shown in Fig. 3A, GST-HS1 and GST-HS1ΔPS bound effectively to Arp2/3 complex. Deletion of the CC motif or the repeat domain as shown by GST-HS1ΔCC and GST-HS1ΔR had little effect on Arp2/3 binding under the same condition. Likewise, GST-HS1-CC or GST-HS1-R itself was unable to bind to Arp2/3 binding activity. Thus, the CC motif and repeat domain are dispensable for Arp2/3 binding under the condition without actin polymerization.

Our previous study has shown that the functional affinity of cortactin for Arp2/3 complex is influenced by actin polymerization as a result of binding to branched actin (3). Therefore, it is possible that the CC and repeat motifs could regulate the affinity for the Arp2/3 complex only when actin polymerization takes place. As a result, we analyzed the effect of these GST-HS1 fusion proteins on the Arp2/3 complex-medi-
ated actin assembly, providing an indirect measurement of the interaction with the complex. Actin polymerization was initiated by adding ATP-actin to the mixture of HS1 proteins and Arp2/3 complex. As reported previously (8), GST-HS1 enhanced markedly the kinetic growth of actin filaments in the presence of Arp2/3 complex (Fig. 3B, curve c compared with curve a). The mutant of GST-HS1^H9004PS also promoted actin polymerization at an efficiency that is slightly higher than GST-HS1 (Fig. 3B, curve f), which is consistent with a possible inhibitory activity of the C-terminal domain, a phenomenon that was also found with cortactin (14). In contrast, both GST-HS1^H9004R and GST-HS1^H9004CC failed to induce significant increase in actin polymerization under the same condition (Fig. 3B, curves d and e), indicating that both the CC motif and the repeat domain are essential for the activation of the Arp2/3 complex.

The HS1 mutants were also evaluated for the effect on Arp2/3 complex and N-WASP mediated actin branching, the characteristic feature for the Arp2/3 complex-mediated actin assembly that can be enhanced by wild type HS1 (8). To visualize actin filamentous morphology, actin filaments were assembled and stained immediately by rhodamine-conjugated phalloidin. The labeled actin filaments were subsequently inspected by fluorescent microscopy. Under this condition, spontaneously assembled actin proteins were shown as long and non-branched filaments (Fig. 4A, a). In contrast, actin was assembled into short and branched filaments in the presence of Arp2/3 complex and GST-VCA (Fig. 4A, b), which is a constitutively activated form of N-WASP (15). As previously reported (8), the branched actin filaments were significantly enhanced in the presence of either GST-HS1 or GST-HS1^H9004PS as shown by the apparently increased density of branched filaments (Fig. 4A, a and d). On the other hand, both GST-HS1^H9004R and GST-HS1^H9004CC failed to increase actin branching (Fig. 4A, c and f). A quantitative analysis further indicates that the mutants GST-HS1^H9004R and GST-HS1^H9004CC at 25
nm actually slightly inhibited branching as compared with that without HS1 (Fig. 4B). At a higher dose (75 nM), the inhibition was more apparent, suggesting a possible competition between HS1 mutants and GST-VCA, presumably for Arp2/3 complex binding.

Analysis of the Role of the CC and Repeat Motifs in the Interaction between HS1 and F-actin in Cells—To facilitate examination of the distribution of HS1 mutants in cells, MGIN retroviruses carrying HS1-GFP fusions were used to infect human umbilical vein endothelial cells. The infected cells were stained with rhodamine phalloidin (Fig. 5, red). As shown in Fig. 5, HS1-GFP, which was shown as green staining, localized at the cell leading edges and the perinucleus. In the cell leading edge, it was primarily found in lamellipodia and membrane ruffles where F-actin is evidently enriched. This pattern is quite similar to that of cortactin (16), indicating that HS1-GFP functions properly. In contrast, HS1ΔCC-GFP randomly distributed in the cytoplasm and showed little association with F-actin within the membrane protrusive structures. Similarly, HS1ΔR-GFP diffused and did not exhibit a clear association with any distinct cellular structures in the leading edge. Because endothelial cells express few endogenous HS1 proteins (data not shown), we also evaluated the interaction between HS1 mutants and F-actin in B cells where HS1 is abundant (17). The murine WEHI-231 B cells expressing GFP-tagged HS1 mutants were established similarly by retroviral infection (12), and the cell lysates were immunoprecipitated by GFP antibody. The pellets were blotted with antibody either against β-actin or HS1. All HS1 proteins were expressed at similar levels (Fig. 6A). Two lower bands of HS1ΔR-GFP likely present degraded proteins. It is apparent that HS1ΔCC-GFP diffused and did not exhibit a clear association with any distinct cellular structures in the leading edge. Because endothelial cells express few endogenous HS1 proteins (data not shown), we also evaluated the interaction between HS1 mutants and F-actin in B cells where HS1 is abundant (17). The murine WEHI-231 B cells expressing GFP-tagged HS1 mutants were established similarly by retroviral infection (12), and the cell lysates were immunoprecipitated by GFP antibody. The pellets were blotted with antibody either against β-actin or HS1. All HS1 proteins were expressed at similar levels (Fig. 6A). Two lower bands of HS1ΔR-GFP likely present degraded proteins. It is apparent that HS1ΔCC-GFP diffused poorly with F-actin as compared with wild type HS1-GFP, the result that is consistent with the cell staining and in vitro data. Interestingly, the F-actin binding activity of HS1ΔR-GFP was nearly abolished. Although this result agrees with the cell staining (Fig. 5), it was paradoxical because the same mutant still maintains a significant F-actin binding activity when analyzed with purified actin alone (Fig. 2). One possibility is that HS1 binds only to cortical F-actin that is nucleated by the Arp2/3 complex in cells as cortactin does (17). Therefore, we analyzed the interaction of the

DISCUSSION

Our major finding in this report is that the HS1 repeat domain is not the only one contributing to F-actin binding, which is very different from cortactin. Although deletion of the repeat domain abolished acti-
New Function of F-actin Binding Domain

vation of Arp2/3 complex, it has only a minor effect on direct F-actin binding. Instead, in vitro F-actin binding activity was nearly diminished by deletion of the CC domain. The finding of the CC motif as a main F-actin binding domain strongly implies a new function for the HS1 repeat domain. Because both the CC motif and the repeat domain are necessary for activation of the Arp2/3 complex and co-localization with F-actin in cells, these two structural domains likely act synergistically to regulate the Arp2/3 complex-mediated actin assembly. One possible function of the repeat domain is that it aligns along the side of the nascent actin filament at a branching site (Fig. 7), other than simply binding to F-actin through the fourth repeat unit as previously suggested (10). Although the repeat domain itself does not bind to the Arp2/3 complex, it follows immediately the Arp2/3 binding domain, which may facilitate a strong association of the Arp2/3 complex at the pointed end of the nascent actin filament, resulting in further increase in actin nucleation and branching stabilization. Indeed, deletion of the repeat domain has nearly abolished binding to the Arp2/3 complex in cells as well as F-actin, which was likely generated from the Arp2/3 complex (Fig. 6). On the other hand, the CC domain may bind directly to F-actin and enhance the association of HS1 with the complex of Arp2/3 and branched actin. Additional evidence supporting this model is that the sequence of the repeat domain is highly conservative (over 70% identity), which may be necessary to accommodate a conserved but repetitive structural profile on the side of actin filaments. In contrast, the sequence of the CC domain, which mainly consists of charged amino acid residues, shows little homology to that of cortactin. Although this may indicate that the F-actin binding mediated by the CC motif is less specific, its affinity for F-actin, when it is analyzed alone, is less than that of the wild type HS1 (1.63 vs. 0.52 μM). Thus, interaction of HS1 with F-actin in cells may be determined by the synergistic interactions among the repeat, the CC, and the Arp2/3 complex binding domains; lacking any of these components would cause its dislocation in relation to cortical F-actin (18).

Cortactin also contains a coiled-coil-like region. Although the functional role of the region is unknown, it is not indispensable for its binding to F-actin and activation of the Arp2/3 complex as analyzed with purified proteins (data not shown). This suggests that the repeat domain with more than four repeat units as in the case of cortactin is sufficient to achieve a significant F-actin binding, resulting in a less demanding for the function of the CC motif. Although it appears that the function of the CC domain is unique to HS1, the HS1-mediated actin cytoskeleton reorganization is likely to adapt to a special property of hematopoietic cells that is distinguished from adherent cells by assuming a rounding shape necessary for their circulation in the blood. The characterization of the difference in details between HS1 and cortactin may eventually explore the molecular mechanisms that determines specific cellular morphogenesis.

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