Haematopoietic lineage cell-specific protein 1 (HS1) promotes actin-related protein (Arp) 2/3 complex-mediated actin polymerization

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HS1 (haematopoietic lineage cell-specific gene protein 1), a prominent substrate of intracellular protein tyrosine kinases in haematopoietic cells, is implicated in the immune response to extracellular stimuli and in cell differentiation induced by cytokines. Although HS1 contains a 37-amino acid tandem repeat motif and a C-terminal Src homology 3 domain and is closely related to the cortical-actin-associated protein cortactin, it lacks the fourth repeat that has been shown to be essential for cortactin binding to filamentous actin (F-actin). In this study, we examined the possible role of HS1 in the regulation of the actin cytoskeleton. Immunofluorescent staining demonstrated that HS1 co-localizes in the cytoplasm of cells with actin-related protein (Arp) 2/3 complex, the primary component of the cellular machinery

responsible for *de novo* actin assembly. Furthermore, recombinant HS1 binds directly to Arp2/3 complex with an equilibrium dissociation constant (K_d) of 880 nM. Although HS1 is a modest F-actin-binding protein with a K_d of 400 nM, it increases the rate of the actin assembly mediated by Arp2/3 complex, and promotes the formation of branched actin filaments induced by Arp2/3 complex and a constitutively activated peptide of N-WASP (neural Wiskott–Aldrich syndrome protein). Our data suggest that HS1, like cortactin, plays an important role in the modulation of actin assembly.

Key words: actin cytoskeleton haematopoietic cell, cortactin, tyrosine kinase, Wiskott–Aldrich syndrome protein (WASP).

INTRODUCTION

The actin cytoskeleton plays a fundamental role in the function of haematopoietic cells, including antigen-mediated activation of lymphatic receptors [1,2], platelet activation [3] and adhesion of leucocytes to vascular cells [4]. While signalling cascades from extracellular stimuli often lead to changes in the dynamics of the actin cytoskeleton, the molecular mechanism by which the actin cytoskeleton is regulated in haematopoietic cells had not been known until the recent discovery of Wiskott–Aldrich syndrome protein (WASP) and actin-related protein (Arp) 2/3 complex [5–8]. Arp2/3 complex contains two actin-related subunits, Arp2 and Arp3, and five other unique polypeptides [9]. Under physiological conditions, Arp2/3 complex binds to the side of an actin filament, serves as the nucleation site for the assembly of monomeric actin, and generates branched actin filaments, which compose a characteristic actin network, filling up the areas within cell leading edges including lamellipodia and membrane ruffles. However, the stimulation of the nucleation activity of Arp2/3 complex requires the function of WASP or its related proteins, which interact with and activate Arp2/3 complex upon binding to membrane-associated small GTPases such as Cdc42 [7,10,11]. Whereas WASP and small GTPases constitute an important pathway from extracellular signals to the actin network, other signalling molecules such as the non-receptor protein tyrosine kinase Src and its related proteins are also known to be implicated in the modulation of the actin cytoskeleton [12]. However, the key substrate(s) of the Src family kinases re-

sponsible for cytoskeletal reorganization in haematopoietic cells remain unknown.

HS1 (haematopoietic lineage cell-specific gene protein 1) was originally isolated as a putative transcription factor exclusively expressed in haematopoietic cells [13]. However, subsequent studies indicate that HS1 is mainly a cytoplasmic protein and is often phosphorylated at tyrosine residues upon activation of membrane receptors including B-cell antigen receptor [14,15], CD3 T-cell receptors [16] and Fc ϵ RI of mast cells [17]. Increased tyrosine phosphorylation of HS1 is also concomitant with the activation of one or several intracellular protein tyrosine kinases such as Lck, Syk, Lyn, Fgr and TPK-IIB [14,18–21]. Direct association of HS1 with Lyn and Lck has been described in T-cells and during the differentiation of erythroid cells mediated by erythropoietin [15,19,22,23]. The physiological function of HS1 is further illustrated by the study of HS1-knockout mice, which displayed a defect in antigen-induced proliferation and depletion of lymphocytes [24]. There is also evidence showing that HS1 is implicated in the apoptotic response to the activation of antigen receptors on lymphocytes, and lymphatic cells with low levels of HS1 expression have acquired a resistance to apoptosis [25]. Consistent with its role in apoptosis, HS1 is able to bind via its Src homology 3 (SH3) domain to HAX, a Bcl-2-like protein [26].

HS1 contains 3.5 37-amino acid tandem repeats and also an SH3 domain at the C-terminus. This structure is closely related to that of cortactin, an intracellular protein expressed primarily in adherent cells [27,28]. The amino acid sequences of the N-terminus, the repeat motif and the SH3 domain of HS1 share

Abbreviations used: Arp, actin-related protein; DTT, dithiothreitol; F-actin, filamentous actin; G-actin, globular actin; GFP, green fluorescent protein; GST, glutathione S-transferase; HS1, haematopoietic lineage cell-specific gene protein 1; N-WASP, neural WASP; SH3, Src homology 3; VCA, verprolin-cofilin-acidic motif; WASP, Wiskott–Aldrich syndrome protein.

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Figure 1 Structural comparison of cortactin and HS1

(A) Schematic presentation of cortactin and HS1. The N-terminal region, the 37-amino acid repeat and SH3 domain are indicated with different degrees of shading. (B) Alignment of the N-terminal sequence of human cortactin and human HS1. Identical amino acids are indicated by dots between them. The DDW motif, which is essential for cortactin binding to Arp2/3 complex, is boxed. (C) Alignment of the repeats of human cortactin and human HS1. Each repeat is denoted on the left by CortR or HS1R followed by a number corresponding to its position in the sequence. The consensus sequence, as defined using residues with an occurrence of more than 50%, is also shown.

identities of over 65% with those of cortactin (Figure 1), whereas the region between the repeat and the SH3 domain, which contains a putative α -helix, two or three phosphoryl residues and a proline-rich region, is less conserved. Like HS1, cortactin is also known to be implicated in growth-factor signal transduction and acts as a primary substrate of Src-related kinases [29,30]. Recent studies demonstrated further that cortactin is an Arp2/3-complex stimulator and modulates the dynamics of the actin cytoskeleton [31,32]. *In vivo*, cortactin is tightly associated with the cortical actin and co-localizes with the Arp2/3 complex within punctated structures and the cell leading edge. *In vitro*, cortactin binds directly to the Arp2/3 complex, enhances the actin nucleation activity of the Arp2/3 complex and promotes the formation of actin branches during actin polymerization [31,32].

The distinguishing structural feature of HS1 is that it contains only 3.5 repeat units, which is in contrast to the 5.5 or 6.5 repeat units frequently found in cortactin isoforms. Whereas the repeat domain of cortactin is known to be responsible for filamentous actin (F-actin) binding, it requires at least 4 repeat units for F-actin binding [29,31]. In particular, the cortactin fourth repeat has been implicated in a direct interaction with F-actin [33], which is apparently missing in HS1. Therefore, the role of HS1 in the modulation of the actin cytoskeleton has not yet been established. In fact, a previous study was unable to

determine a significant level of HS1 associated with F-actin [34], although its interaction with the actin cytoskeleton was assumed [35]. In the present study, we show that HS1 is able to bind to F-actin with modest affinity compared with cortactin. Furthermore, HS1 is able to interact directly with Arp2/3 complex and activate the Arp2/3-complex-mediated actin nucleation and branching in a manner similar to cortactin. Thus our study indicates that modulating actin assembly is a common function with both cortactin and HS1.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody against green fluorescent protein (GFP) was purchased from Molecular Probes. Polyclonal antibodies against a human Arp3 peptide and recombinant human HS1 tagged with glutathione S-transferase (GST) were prepared as described previously [31,34]. All polyclonal antibodies were purified by Protein A-Sepharose 4 Fast Flow (Amersham Biosciences) affinity chromatography as described previously [30].

Plasmid construction

Plasmid pGST-HS1 was constructed as described previously [34]. A His-tagged HS1 plasmid (pQEHS1) was constructed by

inserting a PCR-generated human HS1 DNA based on pGST-HS1 into the *Bam*HI and *Sal*I sites of pQE-30 (Qiagen). The pQEHS1 plasmid was selected in XL1-Blue bacterial cells (Stratagene). The nucleotide sequence of the coding region was confirmed by DNA sequencing. Plasmid pHS1-GFP was prepared with the aid of PCR using pGST-HS1 as the template and the primers 5'-AGCTCAGAATTCATGTGGAAGTCTGTAGTGGGC-3' and 5'-AGCTCAGTCGACCACTCCAGAAGCTTGACATA-3'. The resulting DNA fragment was inserted into *Eco*RI/*Sal*I sites of pEGFP1-N1 (Clontech).

Preparation of GST-HS1 and His-HS1 proteins

GST-HS1 was prepared as described previously [34]. To purify His-HS1 protein, XL1-Blue cells harbouring the pQEHS1 plasmid were grown at 37 °C to a density of $D_{600} = 0.6$, and expression was induced by adding 0.5 mM isopropyl β -D-thiogalactoside ('IPTG') followed by incubation at 34 °C with constant shaking at 250 rev./min for 4 h. The bacteria were harvested by centrifugation at 4000 *g* for 20 min and resuspended in lysis buffer (50 mM NaH_2PO_4 , pH 8.0, 300 mM NaCl and 10 mM imidazole) at a ratio of 4 ml/g (wet weight). Lysozyme was added to a final concentration of 1 mg/ml and the lysate was subsequently incubated on ice for 30 min. The lysate was further subjected to sonication with an ultrasonic microprobe using a setting of six 10 s bursts at 200–300 W with a 10 s cooling period between each burst. After sonication, Triton X-100 was added to a final concentration of 1% and the lysate was incubated on ice for 20 min. The lysate was then centrifuged at 10000 *g* for 20 min. The supernatant was mixed with 50% (v/v) slurry of Ni^{2+} -nitrilotriacetic acid ('Ni-NTA') resin and incubated for 1 h at 4 °C in a rotator at 20 rev./min. After washing with lysis buffer containing 20 mM imidazole, His-HS1 was eluted with the same buffer containing 250 mM imidazole. The eluate was loaded on to a Mono-Q column on a Waters 600 pump in 50 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol (DTT) and 1 mM EGTA, and eluted against a KCl gradient from 0 to 1 M in the same buffer at 1 ml/min. The major peak was collected and concentrated with Centricon-10 (Millipore). The concentration of purified protein was determined by the Bradford method using BSA as a standard. The purified His-HS1 protein was divided into small aliquots and stored at -80 °C.

Purification of Arp2/3 complex

Arp2/3 complex was purified from bovine brain extracts by a two-step procedure as described in [7] with a modification. Briefly, frozen bovine brain (100 g) was sliced with blades and homogenized with a dispersion device (Polytron PT 2100; Kinematica AG) on ice in 100 ml of buffer Q (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl_2 , 5 mM EGTA and 1 mM DTT) supplemented with 50 $\mu\text{g}/\text{ml}$ PMSF, 5 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ aprotinin. The homogenized sample was then clarified by centrifugation at 125000 *g* for 90 min at 4 °C, and the supernatant was subjected to chromatography in a 100 ml Q Sepharose column equilibrated with buffer Q. The flow-through containing Arp2/3 complex was collected, supplemented with 0.1 mM ATP and fractionated on a GST-verprolin-cofilin-acidic motif (VCA) glutathione-Sepharose column equilibrated with buffer B (50 mM Tris/HCl, pH 7.5, 25 mM KCl, 1 mM MgCl_2 , 0.5 mM EDTA, 1 mM DTT and 0.1 mM ATP). After washing with 0.2 M KCl in buffer B, the Arp2/3 complex was eluted with buffer B containing 0.2 M MgCl_2 . The protein was then dialysed against buffer Q and further subjected to additional chromatography using Q Sepharose to remove residues of GST-VCA. The protein in the flow-through fraction of the second Q

Sepharose fractionation was concentrated by a Centricon-30 (Millipore), and the buffer was changed to Ca^{2+} -free 1 $\times$ polymerization buffer (50 mM KCl, 2 mM MgCl_2 , 1 mM EGTA, 0.25 mM ATP, 10 mM Tris/HCl, pH 7.3, 3 mM NaN_3 and 0.5 mM DTT). Protein concentration was determined by Bradford method using BSA as the standard.

Analysis of the interaction between HS1 and Arp2/3 complex

Purified GST-HS1 (5 or 10 μg) immobilized on glutathione-Sepharose (Amersham Biosciences) was mixed with 10 pmol of Arp2/3 complex in 500 μl of buffer A (50 mM Tris/HCl, 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 $\times$ SDS loading buffer. The amount of Arp2/3 complex in the beads was quantified by immunoblotting using a polyclonal anti-Arp3 antibody after separation by SDS/PAGE.

To analyse the affinity of HS1 for Arp2/3 complex, purified Arp2/3 complex (4 nM) was incubated with immobilized GST-HS1 at different concentrations for 90 min at 4 °C in binding buffer (50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 0.1 mM CaCl_2 , 0.5 mM DTT, 3 mM NaN_3 , 50 mM KCl, 2 mM MgCl_2 , 0.2 mM ATP, 0.2 mg/ml BSA and 0.1% Tween-20). After incubation, samples were centrifuged at 800 *g* in a microcentrifuge for 10 s and the supernatants were then fractionated by SDS/PAGE (12%, v/v, gels) and immunoblotted with polyclonal anti-Arp3 antibody. The proteins that were reactive to Arp3 antibody were detected by enhanced chemiluminescence (ECL) with an optimal exposure time that yielded a near-linear detection range based on purified Arp2/3 complex at three known concentrations that were loaded on the same gel. The densities of Arp3 bands shown on the blot, which represented unbound Arp3, were scanned and quantified with Scion Image software. The amounts of Arp3 depletion by different concentrations of GST-HS1 were used to fit a single rectangular hyperbola using GraphPad Prism 3, and the K_d value was calculated on the basis of $V = V_{\text{max}}C/(K_d + C)$, where V is the depletion of Arp3 proteins in the supernatant and C is the concentration of unbound HS1. The same methodology was also used in the measurement of the affinity for GST-VCA and GST-cortactin.

Actin-polymerization assay

Polymerization of globular actin (G-actin; 10% pyrene-labelled) was performed as described in [31] with a modification. Briefly, Ca^{2+} ATP-G-actin in G-buffer (5 mM Tris/HCl, pH 8.0, 0.2 mM CaCl_2 , 0.5 mM DTT and 0.2 mM ATP) was converted into Mg^{2+} ATP-G-actin by mixing with 0.1 vol. of 10 $\times$ exchange buffer (2 mM EGTA/1 mM MgCl_2) at 20 °C for 2 min [36–38]. Actin polymerization was initiated by adding 60 μl of Mg^{2+} ATP-G-actin (10 μM) to 240 μl of 1.25 $\times$ polymerization buffer (62.5 mM KCl, 2.5 mM MgCl_2 , 12.5 mM imidazole, pH 7.3, 1.25 mM EGTA, 0.125 mM CaCl_2 , 0.625 mM DTT, 0.3125 mM ATP and 3.75 mM NaN_3) in the presence of Arp2/3 complex and His-HS1 at the concentrations indicated. Actin assembly was monitored by measuring the increase in pyrene fluorescence detected with an LS50B spectrophotometer (Perkin Elmer) with filters for excitation at 365 nm and emission at 407 nm.

F-actin branching assay

F-actin branching assay was based on a method that was described previously [39]. Briefly, polymerization of 2.0 μM monomeric actin was initiated by 30 nM His-HS1 in the presence

Figure 2 The interaction of HS1 and Arp2/3 complex

(A) Pull-down analysis of the interaction of HS1 with Arp2/3 complex. GST-VCA or GST-HS1 at various concentrations was mixed with 88 nM Arp2/3 complex as described in the Materials and methods section. After incubation for 90 min at 4 °C, GST-conjugated proteins were precipitated by glutathione beads. The presence of Arp2/3 complex in the pellet was analysed by immunoblotting with Arp3 antibody. Lane 1, 0.1 pmol of Arp2/3 complex; lane 2, 600 nM GST-VCA; lanes 3–5, GST-HS1 at 600, 300 and 150 nM, respectively; lane 6, GST, 300 nM. (B) Comparison of the affinity of GST-HS1, GST-cortactin and GST-VCA for Arp2/3 complex. Purified Arp2/3 complex (4.4 nM) was mixed with different amounts of immobilized GST-HS1, GST-cortactin or GST-VCA. After 90 min of incubation at 4 °C, samples were centrifuged at 800 *g* for 10 s. The presence of Arp3 in the supernatants was detected by immunoblot with anti-Arp3 antibody. Amounts of Arp3 were quantified by digital scanning and normalized to the percentage of depletion. The resulting data were used to fit a rectangular hyperbola, yielding apparent K_d values of 881, 647 and 115 nM for GST-HS1, GST-cortactin and GST-VCA, respectively. (C) Immunofluorescent staining of HS1-GFP and Arp2/3 complex within macrophage cells. Raw 264.7 macrophage cells were transiently transfected with pHS1-GFP. The transfected cells were fixed and double-stained with monoclonal anti-GFP antibody (panel a) and polyclonal anti-Arp3 antibody (panel b). The arrowheads indicate representative locations where HS1-GFP and Arp3 were apparently co-localized. A non-transfected cell, as shown in panels (a) and (b), is indicated by an arrow.

of 30 nM GST-VCA and 8 nM Arp2/3 complex. To visualize actin branches, a molar equivalent of rhodamine-phalloidin (Molecular Probes) was added to the reaction solution at given times after initiation. Samples were diluted 400-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 to coverslips precoated with 0.1% nitrocellulose in *n*-amyl acetate. Actin filaments were examined by an Olympus IX-70 inverted microscope using a 100×1.35 NA objective lens. Images were captured with a digital camera controlled by the SPOT advance software. The digital images were processed with Adobe Photoshop to generate monochromatic images. Actin branches were quantified by measuring the density of branches (number of branch points/filament length) from five randomly selected images using Scion Image software.

F-actin binding assay

F-actin was prepared by incubating G-actin in polymerization buffer at room temperature for 4 h. To compare the F-actin binding ability of HS1 with cortactin, F-actin at different concentrations was mixed with 30 nM His-HS1 or His-cortactin followed by incubation for 30 min at room temperature. The concentration of F-actin was calculated by the subtraction of

0.1 µM from the concentration of G-actin. The reaction solution was centrifuged at 200 000 *g* for 30 min to pellet the bound proteins. The unbound His-HS1 or His-cortactin proteins in the supernatant were fractionated by SDS/PAGE and detected by immunoblotting using antibodies against HS1 or cortactin.

Immunofluorescence analysis

Murine macrophage cell line Raw 264.7 was from the A.T.C.C. The cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. The cells at logarithmic phase were transfected with plasmids pHS1-GFP or pEGFP1-N1 with Superfect (Qiagen). After 24 h, the transfected cells were plated on fibronectin-coated glass coverslips in the growth medium. After an additional 12 h incubation, the cells were permeabilized by incubation for 20 s in 0.1 M Mes, pH 7.4, containing 0.2% Triton X-100, 1 mM MgCl₂, 1 mM EGTA and 4% poly(ethylene glycol) 6000. The permeabilized cells were subsequently fixed for 30 min in 3.7% paraformaldehyde in PBS followed by three washes with PBS. The cells were then incubated for 30 min in PBS containing 2% BSA followed by a 1 h incubation in the same buffer containing polyclonal anti-Arp3 antibody at a concentration of 50 µg/ml and monoclonal anti-GFP antibody at 10 µg/ml. After three washes with PBS, secondary antibodies (rhodamine-conjugated anti-rabbit IgG and fluorescein-conjugated anti-mouse IgG antibodies) were applied. After 1 h

incubation, cells were washed with PBS and mounted in Prolong antifade solution (Molecular Probes) on a glass slide. The samples were examined by confocal laser microscopy using a Bio-Rad Radiance 2100 laser scanning system equipped with a Nikon Eclipse E800 microscope.

RESULTS

HS1 interacts directly with Arp2/3 complex

The interaction between HS1 and Arp2/3 complex was first examined using a pull-down assay using GST-HS1 and purified

bovine Arp2/3 complex. As shown in Figure 2(A), GST-HS1 at concentrations ranging from 150 to 600 nM (Figure 2A, lanes 3–5) was able to pull down readily 88 nM Arp2/3 complex as indicated by the presence of Arp3, a subunit of Arp2/3 complex, in the pellets, whereas GST alone (Figure 2A, lane 6) precipitated no detectable Arp3 as analysed by immunoblot. Analysis of the Arp3 remaining in the supernatant after pull-down estimated a K_d of 881 nM for the binding of GST-HS1 to Arp2/3 complex. This K_d value is very similar to that of cortactin ($K_d = 647$ nM) but significantly greater than that of GST-VCA ($K_d = 115$ nM), a constitutively active peptide of human neural WASP (N-WASP; Figure 2B) [7]. The similar affinities of HS1 and cortactin

Figure 3 HS1 promotes actin polymerization nucleated by Arp2/3 complex

(A) Actin-polymerization analysis. Polymerization of 2 μ M 10% pyrene-labelled G-actin was initiated with 80 nM Arp2/3 complex and His-HS1 at the indicated concentrations. For comparison, 50 nM His-cortactin was also analysed in parallel. (B) His-HS1 alone does not have actin-assembly-promoting activity. The conditions were the same as in (A) except that His-HS1 was used at 80 nM and Arp2/3 complex at 14 nM. (C) Comparison of the activities of His-HS1 and His-cortactin for actin polymerization based on the time required for achieving 50% of a maximal actin polymerization ($t_{1/2}$). (D) Comparison of the affinities of HS1 and cortactin for F-actin: 30 nM His-HS1, His-cortactin or tag-free cortactin was co-precipitated with F-actin at concentrations ranging from 0.3 to 6 μ M. The K_d values were estimated as described in the Materials and methods section. The estimated K_d for His-HS1 was 395 nM and that for either His-cortactin or tag-free cortactin was less than 100 nM.

Figure 4 HS1 promotes the formation of stable F-actin branches

(A) Actin polymerization was initiated by 8 nM Arp2/3 complex and 30 nM GST-VCA in the absence (a, c) or presence (b, d) of 30 nM His-HS1. After 10 min (a, b) or 1 h (c, d), rhodamine-phalloidin at molar concentrations equivalent to monomer actin was added. The labelled actin filaments were examined under a fluorescent microscope. Scale bar (d), 5 μm . Panel (e), an enlarged image derived from (b) is shown on the left. On the right a sketch for the reconstruction of these branched actin complexes is presented. (B) Quantification of HS1-mediated actin branching. The branch density was calculated based on the ratio of the number of branching sites to the length of the filament in five randomly selected images from the same experiment as presented in (A). Each data point was calculated based on the measurement of actin filaments of more than 200 μm in length. (C) Comparison of the actin branching activity of His-HS1 with His-cortactin. Actin branching was initiated in the presence of 8 nM Arp2/3 complex, 30 nM GST-VCA, and His-HS1 or His-cortactin at the indicated concentrations. The density of actin branching at each concentration was calculated based on the average branching sites counted from 200 μm actin filaments.

for Arp2/3 complex is consistent with a conserved sequence of 63 % identity in their N-termini, which includes the DDW motif that has been demonstrated to be critical for cortactin binding to Arp2/3 complex (Figure 1B) [31].

The interaction of HS1 and Arp2/3 complex was further examined by analysing murine Raw 264.7 macrophage cells overexpressing a form of human HS1 tagged with GFP at its C-terminus (HS1-GFP). The cellular distributions of HS1-GFP and endogenous Arp2/3 complex were analysed by immunofluorescence staining with a monoclonal anti-GFP antibody and polyclonal anti-Arp3 antibody, respectively. As shown in Figure 2(C), both HS1-GFP and Arp3 are found in punctate-like structures enriched within the cell periphery and the cytoplasm. In many punctates, co-localization of HS1-GFP and Arp3 is evident. The specificity of the staining was verified by negative GFP staining of a non-transfected cell as indicated by an arrow

(Figure 2C, panels a and b) and cells expressing GFP only (results not shown).

HS1 promotes actin assembly nucleated by Arp2/3 complex

Arp2/3 complex is a primary cellular factor involved in actin nucleation, a rate-limiting step for *de novo* synthesis of actin filaments [9]. Therefore, we determined whether binding of HS1 to Arp2/3 complex had any influence on actin assembly. The effect of HS1 on the nucleation activity of Arp2/3 complex was evaluated by measuring the polymerization of pyrene-labelled G-actin. The rate of actin polymerization was apparently enhanced in the presence of both His-HS1 and Arp2/3 complex (Figure 3A). Half-maximal actin polymerization was reached within 600 s in the presence of 50 nM His-HS1 and 80 nM Arp2/3. However, the rate of His-HS1-stimulated actin growth appeared

to be lower than that of His-cortactin, which was able to induce half-maximal stimulation in approx. 300 s under the same conditions (Figure 3A). The apparent activity of His-HS1 in actin assembly was probably due to its interaction with Arp2/3 complex because no significant increase in actin polymerization was detected in the presence of either Arp2/3 (Figure 3A) or His-HS1 alone (Figure 3B). The activities of HS1 and cortactin were also compared by their dose-dependent stimulation of half-maximal actin assembly ($t_{1/2}$; Figure 3C). Whereas His-cortactin has an apparent higher potency than His-HS1, both proteins showed 50% stimulation at a concentration near 25 nM and maximal stimulation at 50 nM. Thus, like cortactin, HS1 has apparently a high functional efficacy for Arp2/3 complex during actin polymerization although its binding affinity for Arp2/3 is low.

HS1 is a modest F-actin-binding protein

Cortactin is known as a potent F-actin-binding protein through its repeat domain, and its affinity for F-actin appears to be crucial for its stimulation of Arp2/3-mediated actin assembly [31]. The apparent activity of HS1 to stimulate Arp2/3 complex suggests that HS1 may be an F-actin-binding protein as well. However, HS1 has a short repeat domain (3.5 37-amino acid tandem repeats) and its ability to bind to F-actin has not yet been established. We examined the interaction of HS1 with F-actin by co-precipitation of His-HS1 and F-actin and quantified the interaction by plotting co-precipitated HS1 as a function of F-actin concentration (Figure 3D). This analysis estimated the K_d of His-HS1 for F-actin binding to be approx. 395 nM. In a parallel experiment, we also measured the affinity of His-cortactin for F-actin and estimated the K_d of His-cortactin at less than 100 nM (Figure 3D). A precise prediction of the K_d value for His-cortactin has not been achieved because the critical concentration of actin, approx. 100 nM under typical conditions for F-actin binding, put a limit on the lowest amount of F-actin available for measuring the affinity of His-cortactin. The possible effect of the His tag was ruled out by comparing His-cortactin and tag-free cortactin, both of which showed identical affinity for F-actin (Figure 3D) and actin assembly (results not shown). Thus, in spite of the lack of a fourth repeat, HS1 is an F-actin-binding protein but with modest affinity compared with cortactin.

HS1 promotes the formation of a branched actin network

A hallmark of the actin polymerization initiated by Arp2/3 complex is the formation of characteristic branched actin network, an activity that can be significantly enhanced by cortactin [32]. To examine whether HS1 has a similar branching activity as cortactin, actin polymerization was initiated by adding His-HS1, Arp2/3 complex and GST-VCA. GST-VCA was included in this study to fully activate the Arp2/3 complex and facilitate the measurement of the activity of HS1 for actin branching. After either 10 min or 1 h of initiation, actin was labelled with rhodamine-phalloidin and examined by fluorescence microscopy. Although many branched actin filaments in the presence of Arp2/3 complex and GST-VCA were readily detected at an early time (10 min; Figure 4A, panel a), longer and less-branched actin filaments became dominant after 1 h incubation (Figure 4A, panel c), presumably due to a debranching process followed by an annealing reaction [39]. Quantitative analysis based on the branch density also indicated that about two-thirds of the actin filaments formed without His-HS1 had undergone the debranching process after 1 h (Figure 4B). In contrast, the presence of His-HS1 yielded short and highly branched actin filaments

with a density 1.8-fold higher than that formed in the absence of His-HS1 at 10 min and 4-fold higher at 1 h (Figure 4B).

The branching activity of His-HS1 was also compared with that of His-cortactin at different concentrations after 1 h of actin assembly. While the maximal actin branch density induced by His-HS1 appeared to be lower than that of His-cortactin by nearly 50%, both proteins were able to show a half-maximal stimulation near 25 nM and reach to a plateau at 100 nM (Figure 4C).

DISCUSSION

The data presented here demonstrate for the first time that HS1, a prominent substrate of intracellular protein tyrosine kinases in haematopoietic cells, may play a role in actin polymerization by its direct association with Arp2/3 complex, the primary component of the cellular machinery for actin assembly. Recombinant HS1 is able to bind to the Arp2/3 complex, and promote Arp2/3-mediated actin assembly and actin branching, the activity that is also observed with cortactin, the only protein in mammalian cells that shares significant sequence identity with HS1. However, the *in vivo* function of HS1 and cortactin may not be necessarily redundant because each of them shows a very distinct expression pattern. While HS1 is exclusively expressed in haematopoietic cells, cortactin is more frequently found in adherent types of cell. In addition, HS1 displays a relatively lower activity for the stimulation of actin nucleation and actin branching compared with cortactin. Such modest activity of HS1 may have a physiological function for haematopoietic cells, which have distinct morphology adapted to their circulation in blood vessels and interaction with vascular cells. Indeed, expression of cortactin in bone marrow is very restricted within limited types of cell, such as megakaryocytes and platelets, which have a characteristically large volume of cytoplasm and are ready to challenge stimuli for shape changes [28]. It is noteworthy that the WASP family, which currently includes WASP, N-WASP, WAVE1, WAVE2 and WAVE3, also shows a tissue-specific expression pattern for each of its members [9,40]. While WASP is exclusively expressed in haematopoietic cells, N-WASP is mostly found in a variety of adherent cells, particularly in the brain. In addition, each WASP member activates Arp2/3-dependent actin nucleation with differential efficiencies, with the strongest activity found with N-WASP [41,42]. Thus it seems that haematopoietic cells encompass a unique actin-polymerization system that involves HS1, WASP and the Arp2/3 complex. Characterization of the actin network assembled by these molecules may eventually illustrate a unique cytoskeleton more suitable for haematopoietic cells that circulate in the blood.

The apparent affinity of HS1 for Arp2/3 complex is significantly lower than that of GST-VCA, as analysed in the absence of actin. Interestingly, HS1 is able to induce half-maximal stimulation of Arp2/3-dependent actin polymerization and actin branching at a concentration near 25 nM (Figures 3C and 4C). However, the affinity of HS1 for Arp2/3, as determined by direct binding assay, was near 900 nM (Figure 2B). This apparent discrepancy between functional and binding affinity is also observed with cortactin (Figure 2B; see also [31]). One plausible explanation is that HS1 may acquire a higher affinity for Arp2/3 complex upon actin assembly. Indeed, a dramatic change in the configuration of Arp2/3 complex induced by WASP-related peptides has been postulated based on structural studies [43,44]. Such structural changes may influence the interaction between HS1 and Arp2/3 complex. Supporting this possibility is the observation that activated Arp2/3 complex is integrated with newly assembled F-actin at branched points, where cortactin and

WASP are apparently involved [31,32,45]. Alternatively, F-actin, especially newly assembled actin filaments, could also play a role in the regulation of the interaction between HS1 and Arp2/3 complex. Distinguishing these possibilities will require further delineation of the mechanism for actin polymerization and illustration of the structure of branched actin.

In addition to binding to Arp2/3 complex, HS1 shows a significant affinity for F-actin with an estimated K_d of 400 nM. Under the same conditions, we measured the K_d for cortactin to be less than 100 nM, which is slightly less than that estimated in previous studies [29,31]. This apparent discrepancy might be due to the improved methodology used in the current study, in which a low concentration (30 nM) of His-cortactin and freshly prepared F-actin were used. It has been reported that the repeat domain of cortactin is responsible for F-actin binding [29]. Particularly, the fourth repeat may participate in a direct interaction with F-actin because a series of truncated mutants lacking this repeat failed to show any F-actin binding activity [33]. However, HS1 contains only 3.5 repeats and lacks the fourth repeat found in cortactin. Thus binding of the cortactin family to F-actin seems not to be exclusively dependent on the existence of a particular repeat. It is more likely that F-actin binding requires a correct configuration of the repeat domain as a whole, which may be determined by both the number of repeats and their positions relative to F-actin at branching sites. Indeed, the sequence of the HS1/cortactin repeat unit is extremely conserved (Figure 1C). It is also possible that sequence elements other than the repeat domain of HS1 may also contribute to its F-actin binding activity. It is worth noting that the sequence that lies between the repeat and the SH3 domain of HS1 is much less conserved and shares only 25% identity with that of cortactin. This may reflect a different mechanism for the regulation of each protein *in vivo* and may impose different restrictions on their overall configurations as well.

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