Recombinant hepatocyte growth factor activator inhibitor 1: expression in *Drosophila* S2 cells, purification and crystallization

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Hepatocyte growth factor activator inhibitor 1 (HAI-1) is a multi-domain membrane-associated protease inhibitor that potently inhibits a variety of serine proteases such as hepatocyte growth factor activator and matriptase. Different truncates of HAI-1 show varying potencies for inhibition of target proteases, suggesting that the domain organization of HAI-1 plays a critical role in its function. Here, the soluble full-length extracellular part of HAI-1 (sHAI-1) was expressed using the *Drosophila* S2 insect-cell expression system. Diffraction-quality crystals of sHAI-1 were produced using ammonium sulfate as precipitant. The crystal diffracted to 3.8 Å resolution and belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 95.42$, $c = 124.50$ Å. The asymmetric unit contains one sHAI-1 molecule.

1. Introduction

Hepatocyte growth factor activator inhibitor 1 (HAI-1) is a Kunitz-type serine protease inhibitor that was originally identified as a potent inhibitor of hepatocyte growth factor activator (HGFA; Shimomura *et al.*, 1997). *In utero*, HAI-1-deficient mice die owing to undifferentiated chorionic trophoblasts (Fan *et al.*, 2007; Hong *et al.*, 2016), indicating that HAI-1 is essential for placental development. After birth, HAI-1-deficient mice were found to have enhanced epithelial cell apoptosis and increased mucosal permeability (Kawaguchi *et al.*, 2011; Itoh *et al.*, 2000), showing that HAI-1 is essential to maintain epithelium integrity. In addition, HAI-1 is important to maintain keratinization of the epidermis *via* maintaining the assembly of keratin into desmosomes in keratinocytes (Kawaguchi *et al.*, 2015). HAI-1 also affects the proliferation and cell fate of neural progenitor cells (Koivuniemi *et al.*, 2013). Abnormal HAI-1 expression levels have also been observed in various types of human cancer (Parr & Jiang, 2001; Zheng *et al.*, 2014). Breast, pancreatic, prostate and oral carcinoma cells exhibited enhanced invasive properties *in vitro* in response to HAI-1 knockdown (Parr & Jiang, 2006; Sanders *et al.*, 2007; Baba *et al.*, 2012; Cheng *et al.*, 2009). Forced expression of HAI-1 significantly inhibited the invasion and migration of cervical, endometrial and uterine cancer cells *in vitro* (Nakamura *et al.*, 2010, 2011). HAI-1 suppresses tumorigenesis and progression *via* regulation of the activity of a range of serine proteases in tumour microenvironments (Zheng *et al.*, 2014), including matriptase (Oberst *et al.*, 2002),

HGFA (Kataoka *et al.*, 2000), hepsin (Kirchhofer *et al.*, 2005), prostaticin (Fan *et al.*, 2005), plasmin (Kirchhofer *et al.*, 2003), TMPRSS13 (Hashimoto *et al.*, 2010) and human airway trypsin-like protease (HAT; Kato *et al.*, 2012).

HAI-1 is produced by epithelial cells as a membrane-associated form containing a MANEC PAN/apple-like domain (MANEC or N), an internal domain (I) with two Kunitz domains (K1 and K2) separated by a low-density lipoprotein receptor (LDLR)-like domain (L), a transmembrane region and a short cytoplasmic region (Shimomura *et al.*, 1997; Fig. 1*a*). The membrane-bound form of full-length HAI-1 can be liberated from the cell surface *via* proteolytic cleavage in the C-terminal transmembrane domain (Shimomura *et al.*, 1999). Several soluble forms of truncated HAI-1 have been identified in the conditioned media of cells (Shimomura *et al.*, 1997; Tsuzuki *et al.*, 2005) and human milk (Lin *et al.*, 1999), with molecular weights of 58, 40/39 and 25 kDa, respectively (Shimomura *et al.*, 1999; Lin *et al.*, 1999). *In vitro*, soluble forms of recombinant HAI-1 variants made up of various combinations of domains have different inhibitory activities, indicating different roles of these domains in protease inhibition (Kojima *et al.*, 2008).

Structural studies of HAI-1 are limited and are restricted to HAI-1 fragments. Crystal structures of the HAI-1 IK1 fragment (Hong *et al.*, 2016), the HAI-1 K1–matrilysin complex (Zhao *et al.*, 2013), the HAI-1 K1–HGFA complex (Shia *et al.*, 2005) and an NMR structure of the MANEC domain (Hong *et al.*, 2015) have been reported. However, how HAI-1 arranges its domains in space and regulates its inhibitory activity remains unclear and needs to be illustrated in detail. Here, we

report the purification, crystallization and diffraction of the full-length extracellular domain of HAI-1 (sHAI-1; NIK1LK2; Fig. 1*a*) in order to study the molecular mechanism of its regulation of inhibitory activity.

2. Materials and methods

2.1. Macromolecule production

The cDNA for sHAI-1 (residues Gly36–Phe441) was generated by PCR using the full-length cDNA of HAI-1 isoform 2 (Fig. 1*a*), and was digested with the MluI restriction enzyme (Zhao *et al.*, 2013). The digested fragments were cloned into the expression vector pMT/Bip/V5-His-A (Invitrogen), which was digested with the SmaI and MluI restriction enzymes (Table 1). The sequence of the final expression vector pMT/Bip/sHAI-1 was confirmed by DNA sequencing. A total of 2 µg expression vector (pMT/Bip/sHAI-1) and selection vector pCoBlast (Invitrogen) were mixed in a ratio of 19:1 and the mixture was co-transfected into exponentially growing *Drosophila* S2 cells (Invitrogen) using Cellfectin II reagent (Invitrogen) according to the manufacturer's instructions. Blasticidin (Invitrogen) was added to the medium to a final concentration of 25 µg ml⁻¹ 2 d after transfection to select stable cell lines. The selection medium was replaced every 3 d, and stably transformed polyclonal cell populations were isolated after 9 d. For large-scale production, stably transfected S2 cells were cultured in EX-CELL-420 serum-free medium (Sigma–Aldrich) at 25°C and 120 rev min⁻¹ in a shaker incubator. At a cell density of 4 ×

Figure 1 Purification of recombinant sHAI-1. (*a*) Schematic domain construction of HAI-1. Domains are shown as ovals and labelled as follows: N (MANEC domain), I (internal domain), K1 (Kunitz domain 1), L (LDLR-like domain) and K2 (Kunitz domain 2). TM represents the transmembrane region and C the cytoplasmic region. Linker regions are shown as black lines. The previously characterized regions of the MANEC domain and the IK1 fragment are highlighted in rectangles (dashed lines) coloured olive and magenta, respectively. sHAI-1 is highlighted by a blue rectangle. Residues of the N-terminal and C-terminal region of each domain are labelled on the left. (*b*) SDS–PAGE analysis of sHAI-1 elution fractions from Ni Sepharose Excel resin (Ni-Excel). Lane M, molecular-weight markers (labelled in kDa). Lanes 1 and 2, supernatant from medium; lanes 3, 4 and 5, washes from Ni-Excel; lane 6, elution fractions from Ni-Excel. (*c*) SDS–PAGE analysis of elution fractions (lanes 1–8) from Resource Q anion-exchange chromatography. (*d*) Size-exclusion chromatography of sHAI-1 on a Superdex 75 10/300 GL column. The protein elutes with a retention volume of 10.5 ml, which corresponds to a molecular weight of approximately 54 kDa, corresponding to a monomer. The inset shows SDS–PAGE analysis of the sHAI-1 protein eluted at 10.5 ml.

Table 1
Construction and expression of sHAI-1.

Source organism	<i>Homo sapiens</i>
DNA source	cDNA
Forward primer	GGGCCACCGCCGCGC
Reverse primer†	CGACGCGTGGGATTCCCGCTCAGG
Cloning vector	pMT/Bip/V5-His-A
Expression vector	pMT/Bip/V5-His-A
Expression host	<i>Drosophila</i> S2 cells
Complete amino-acid sequence of the construct produced‡	RSPWPGPPAPPGLPAGADCLNSFTAGVPGFVLD-TNASVNGATFLESPTVRRGDCVRACCTTQN-CNLALVELQPDREGDAIAACFLINCLYEQNFV-CKFAPREGFINYLTRVYRSYRQLRTQGFGGS-GIPKAWAGIDLKVQPQELVLKDVENTDWRLL-RGDTDVRVERKDPNQVELWGLKEGYLFLTLV-TSSDHPEDTANVTVTVLSTKQTEDYCLASNKV-GRCRGSFPRWYDPTQICKSFVYGGCLGNKN-NYLREEECILACRGVQGPSMERRHPVCSGTCQ-PTQFRCSNGCCIDSFLECDTPNCPDASDEAA-CEKYTSGFDELQRIHFPSDKGHCVDLPDTGLC-KESIPRWYYPFSEHCARFTYGGCYGNKNFE-EEQQCLESCRGISKDVFGLRREIPTRTGHHH-HHH

† The MluI restriction site is underlined. ‡ The His₆ tag and vector residues are underlined.

10⁶ cells per millilitre, a final concentration of 500 µM CuSO₄ was added for induction of expression. The conditioned medium was harvested after one week of incubation. The cultured supernatant was clarified by centrifugation at 1000 rev min⁻¹ for 10 min at 4°C, and the supernatant was centrifuged again at 10 000 rev min⁻¹ for 30 min at 4°C. Before purification, the supernatant was filtered through a 0.45 µm filter membrane. The recombinant protein was captured by Ni Sepharose Excel resin (GE Healthcare Life Sciences). After washing with buffer containing 10 mM imidazole, the target protein was finally eluted with elution buffer consisting of 200 mM imidazole, 50 mM Tris-HCl pH 8.0 (Fig. 1b). Fractions containing sHAI-1 protein were pooled and dialyzed into buffer consisting of 50 mM Tris-HCl pH 8.0 for further purification. Using Resource Q anion-exchange chromatography (GE Healthcare Life Sciences), the eluted protein was further purified with a linear gradient of 50–300 mM NaCl at pH 8.0 (Fig. 1c). The peak fractions were concentrated and further purified using size-exclusion chromatography (Superdex 75 10/300 GL) with elution buffer consisting of 50 mM Tris-HCl pH 8.0 (Fig. 1d). The sHAI-1 protein was concentrated to 40 mg ml⁻¹ for crystal-growth experiments. The cDNA of the HAI-1 K1 domain (residues Ser245–Val303; Table 2) was also generated by PCR using the full-length cDNA of HAI-1 isoform 2 as a template. PCR products were digested with EcoRI and SalI restriction enzymes and were then cloned into the pPICZαA expression vector (Invitrogen; Table 2). The recombinant plasmid encoding HAI-1 K1 was linearized with the MssI restriction enzyme and transformed into *Pichia pastoris* X-33 cells (Invitrogen) by electroporation (1500 V, 25 µF, 200 Ω). The positive transformants were selected on a YPDS plate (1% yeast extract, 2% tryptone, 2% glucose, 1 M sorbitol, 1.2% agarose) containing 100 µg ml⁻¹ Zeocin at 28°C for 2 d. For protein expression, the expression clones were inoculated into 100 ml BMGY medium (1% yeast extract, 2% tryptone, 1%

Table 2
Construction and expression of HAI-1 K1.

Source organism	<i>Homo sapiens</i>
DNA source	cDNA
Forward primer†	CGGAATTCAGACAGAAGACTACTGCCTCG
Reverse primer†	ACGCGTCGACCACACCCGACAGGCTAG
Cloning vector	pPICZαA
Expression vector	pPICZαA
Expression host	<i>Pichia pastoris</i> X-33 cells
Complete amino-acid sequence of the construct produced‡	EAEAEFTQEDYCLASNKVGRGRGSFPRWYDPTQ-ICKSFVYGGCLGNKNYLREEECILACRGVV-DHHHHHH

† The EcoRI and SalI restriction sites are underlined. ‡ The His₆ tag and vector residues are underlined.

glycerol, 100 mM potassium phosphate pH 6.0) until the culture reached an A₆₀₀ of 6. The cells were harvested by centrifugation at 1500 rev min⁻¹ for 5 min at room temperature and resuspended in 500 ml BMMY medium (1% yeast extract, 2% tryptone, 0.5% methanol, 100 mM potassium phosphate pH 6.0). The cultures were then induced every 24 h with 1% (v/v) methanol for 4 d. Target protein was captured using Ni Sepharose Excel resin and was eluted with 200 mM imidazole in 50 mM Tris-HCl pH 8.0. Fractions containing HAI-1 K1 protein were pooled and concentrated to 0.5 mg ml⁻¹ for further use. All fractions were analysed on 12% polyacrylamide gels, and proteins were stained with Coomassie Brilliant Blue R-250.

The inhibitory activities of sHAI-1 and its fragment (HAI-1 K1) against the serine protease domain of matriptase (matriptase-SPD) were determined by a chromogenic assay in a reaction buffer consisting of 150 mM NaCl, 0.5% Tween 20,

Figure 2
Inhibition arrays of sHAI-1 (a) and HAI-1-K1 (b) against matriptase-SPD measured using the chromogenic assay.

Table 3
Crystallization of recombinant sHAI-1.

Method	Sitting-drop vapour diffusion
Plate type	48-well plates
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	40
Buffer composition of protein solution	50 M Tris-HCl pH 8.0
Composition of reservoir solution	1.6 M ammonium sulfate, 20%(v/v) glycerol, 0.08 M sodium acetate pH 4.6
Volume and ratio of drop	1.6 µl, 1:1
Volume of reservoir (µl)	100

20 mM Tris-HCl pH 7.4. sHAI-1 with an initial concentration of 6 mg ml⁻¹ was serially diluted in a ratio of 1:3 using the reaction buffer, and 10 µl of each diluted sample was then incubated with matriptase-SPD at room temperature for 10 min in a 96-well polystyrene plate. The proteolytic reactions were initiated by adding 40 µM of the chromogenic substrate Pefachrome tPA to the system to a final volume of 100 µl. The kinetics of *p*-nitroanilide release were monitored spectrophotometrically at 405 nm for 10 min (Fig. 2).

2.2. Crystallization

We screened more than 1000 crystallization conditions using a Phoenix crystallization robot (Art Robbins Instruments) with commercial screening kits (from Qiagen, XtalQuest and Hampton Research) by the sitting-drop vapour-diffusion method, and obtained preliminary crystals of sHAI-1 with a tetrahedral morphology. Reproducible crystals were finally obtained in 1.6 M ammonium sulfate, 20%(v/v) glycerol, 0.08 M sodium acetate pH 4.6 after 7 d at 25°C (Fig. 3a). Crystals were picked up from the drop, washed with reservoir solution, dissolved in SDS-PAGE loading buffer and subsequently checked by SDS-PAGE (Fig. 3b). After optimization, the final diffraction-quality crystals of sHAI-1 were

Table 4
Data collection and processing for recombinant sHAI-1 crystals.

Values in parentheses are for the outer shell.	
Diffraction source	BL18U1
Wavelength (Å)	1.0308
Temperature (K)	100
Detector	PILATUS 6M
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	0.5
Total rotation range (°)	360
Exposure time per image (s)	2
Space group	<i>P</i> 4 ₁ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	95.43, 95.43, 124.50
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.76
Resolution range (Å)	38.09–3.82 (3.96–3.82)
Total No. of reflections	150678 (15675)
No. of unique reflections	5987 (567)
Completeness (%)	97.20 (85.37)
Multiplicity	25.2 (27.6)
$\langle I/\sigma(I) \rangle$	17.47 (4.45)
<i>R</i> _{r.i.m.}	0.157 (0.977)
<i>R</i> _{merge}	0.1537 (0.922)

soaked briefly in a cryoprotectant solution consisting of 1.8 M ammonium sulfate, 0.08 M sodium acetate pH 4.6 with 25%(v/v) glycerol prior to data collection (Table 3). These crystals were cooled in liquid nitrogen for X-ray data collection.

2.3. Data collection and processing

Data collection was performed under cryogenic conditions at 100 K on beamline BL18U1 at Shanghai Synchrotron Radiation Facility (SSRF). A total of 720 images were collected at a crystal-to-detector distance of 300 mm with 2 s exposure time for every 0.5° oscillation frame. The data were processed using the *HKL-3000* package (Minor *et al.*, 2006). Data-collection and processing statistics are summarized in Table 4.

Figure 3
Crystallization and X-ray data collection of sHAI-1. (a) Crystals of sHAI-1 obtained using 1.6 M ammonium sulfate, 20%(v/v) glycerol, 0.08 M sodium acetate pH 4.6. (b) SDS-PAGE analysis of sHAI-1 crystals reveals no degradation during crystallization. Lane 1 contains sHAI-1 used in the crystallization experiment and lane 2 contains dissolved sHAI-1 crystals. (c) The X-ray diffraction pattern of the sHAI-1 crystal to 3.8 Å resolution.

3. Results and discussion

We constructed the expression vector for sHAI-1 with a hexa-His tag, and transfected it into *Drosophila* S2 cells. The secretory expression level of sHAI-1 was up to 80 mg per litre of culture. The target protein was initially captured and purified using Ni Sepharose Excel resin and fractions containing sHAI-1 protein were confirmed by SDS-PAGE (purity of ~85%; Fig. 1*b*). A subsequent anion-exchange chromatographic purification (Resource Q) further increased the purity of the sHAI-1 protein (to ~90%; Fig. 1*c*). Fractions containing sHAI-1 were pooled, concentrated and applied onto a gel-filtration column (Superdex 75 10/300), and the target protein was collected and concentrated to 40 mg ml⁻¹. SDS-PAGE analysis revealed that sHAI-1 was homogenous with high purity (up to 95%; Fig. 1*d*). The target protein was shown to exist in a monomeric form based on its elution volume (10.5 ml) on Superdex 75 (Fig. 1*d*). The IC₅₀ value of sHAI-1 towards matriptase-SPD was 160-fold weaker than that of the isolated HAI-1 K1 fragment (Fig. 2), demonstrating that the full-length extracellular domain of HAI-1 was less potent in inhibition of matriptase, which was consistent with a previous study with rat HAI-1 and matriptase (Kojima *et al.*, 2008).

To investigate the role of the domains in the regulation of the inhibitory activity of sHAI-1, sHAI-1 was crystallized in 1.6 M ammonium sulfate, 20% (v/v) glycerol, 0.08 M sodium acetate pH 4.6 using the sitting-drop vapour-diffusion method (Table 3 and Fig. 3*a*). SDS-PAGE analysis of the crystals showed only a single band near 54 kDa (Fig. 3*b*); thus, no degradation took place during crystallization. This result was encouraging because degradation was a large problem in our previous work on this protein (Zhao *et al.*, 2013).

The initial crystals of sHAI-1 were too small and diffracted to only 7–8 Å resolution. Intensive efforts were made to improve the resolution, including trying different additives (e.g. calcium) or pH, decreasing the concentration of sHAI-1 protein to 20 mg ml⁻¹, changing the concentration of glycerol to 4% and screening different cryoprotectants. Finally, a data set to moderate resolution (3.8 Å) was obtained on beamline BL18U1 at Shanghai Synchrotron Radiation Facility (SSRF; Fig. 3*c*) using the best cryoprotection, which consisted of the reservoir solution containing 25% (v/v) glycerol (Table 3). The crystal belonged to space group *P*₄₁₂₁₂, with unit-cell parameters *a* = *b* = 95.42, *c* = 124.50 Å. The asymmetric unit contained one sHAI-1 molecule (54 kDa) with approximately 53% solvent content and a Matthews coefficient (*V*_M) of 2.62 Å³ Da⁻¹ (Matthews, 1968). We are working towards phase solution by molecular replacement. The relatively low resolution of the crystal is likely to be owing to the multi-domain nature of this protein. Fortunately, we are able to obtain molecular-replacement solutions for most domains. Further structural analysis is currently under way.

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