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Expression, purification and structural characterization of functionally replete Thrombospondin-1 type 1 repeats in a bacterial expression system

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Abstract

The matrix glycoprotein Thrombospondin-1 (TSP-1) is a prominent regulator of endothelial cells and angiogenesis. The anti-angiogenic and anti-tumorigenic properties of TSP-1 are in part mediated by the TSP-1 type 1 repeat domains 2 and 3, TSR(2,3). Here, we describe the expression and purification of human TSR(2,3) in milligram quantities from an *Escherichia coli* expression system. Microvascular endothelial cell migration assays and binding assays with a canonical TSP-1 ligand, histidine-rich glycoprotein (HRGP), indicate that recombinant TSR(2,3) exhibits anti-chemotactic and ligand binding properties similar to full length TSP-1. Furthermore, we determined the structure of *E. coli* expressed TSR(2,3) by X-ray crystallography at 2.4 Å and found the structure to be identical to the existing TSR(2,3) crystal structure determined from a *Drosophila* expression system. The TSR(2,3) expression and purification protocol developed in this study allows for facile expression of TSR(2,3) for biochemical and biophysical studies, and will aid in the elucidation of the molecular mechanisms of TSP-1 anti-angiogenic and anti-tumorigenic activities.

Keywords

thrombospondin-1; angiogenesis; histidine-rich glycoprotein; GST; *Escherichia coli*; X-ray crystallography

Introduction

Progressive and unregulated angiogenesis is a hallmark of malignant tumors, rheumatoid arthritis, diabetic retinopathy and proliferative macular degeneration [1–3]. Thrombospondin-1 (TSP-1), a trimeric matrix glycoprotein, plays an important role in the

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regulation of vascular homeostasis and has been shown to inhibit tumor angiogenesis [4–7]. TSP-1 exerts anti-angiogenic activity by influencing microvascular endothelial cell function through interactions with endothelial cell surface proteins such as $\beta 1$ and $\beta 3$ integrins, CD47 and CD36 [8, 9]. *In vitro* and *in vivo* angiogenesis studies with synthetic TSP-1 peptides derived from the TSP-1 type 1 repeat domains 2 and 3 (TSR(2,3)) suggest that these domains are important for TSP-1 anti-angiogenic activity [10]. Additional studies have demonstrated that the individual TSP-1 TSR domains exhibit both anti-angiogenic and anti-tumorigenic activity [11, 12].

Previously, a TSR(2,3) construct was produced in a *Drosophila* expression system [13] which allowed for the structure of TSR(2,3) to be solved by X-ray crystallography. Protein expression in *Drosophila* cell culture can be time-consuming, requires specialized growth media and may be hampered by poor yields and inefficient transfection rates [14]. Further studies of the molecular mechanism of TSP-1 anti-angiogenic activity would be greatly aided by the ability to easily and inexpensively produce milligram quantities of TSR(2,3) in a more typically utilized expression system. Here, we describe the expression and purification of human TSR(2,3) using an *E. coli* expression system. We show that recombinant TSR(2,3) binds a canonical TSP-1 ligand, histidine-rich glycoprotein (HRGP), and exhibits anti-chemotactic properties similar to full length TSP-1. We solved the structure of recombinant TSR(2,3) by X-ray crystallography and found it to be identical to the existing TSR(2,3) structure determined using protein produced in a *Drosophila* expression system. The expression and purification protocol described here allows for production of high quantities of pure, correctly folded and functional TSR(2,3). This approach will promote further structural and functional investigation of the anti-angiogenic and anti-tumorigenic properties of TSP-1 TSR domains.

Material and Methods

Plasmid construction

The coding sequence for the second and third thrombospondin-1 type 1 repeats was generated via PCR using full length thrombospondin-1 (GenBank Accession No. [AAI36471](#)) as a template. The PCR primers introduced EcoRI and XhoI restriction sites at the 5' and 3' ends respectively. The amplified PCR product was digested with EcoRI and XhoI and ligated into a pGST-parallel-1 expression vector [15]. The resulting plasmid, pGST-parallel-1-TSR(2,3), codes for glutathione-S-transferase (GST), a tobacco etch virus (TEV) protease cleavage site and thrombospondin-1 residues 416 to 529. TEV protease digestion of GST~TSR(2,3) fusion protein leaves a 9 residue tag "GAMDPEFEL" at the amino terminus of the protein (Fig. 1) resulting in at 13.3 kDa construct. The pGST-parallel-1-TSR(2,3) plasmid was used as a template to prepare a second construct, pGST-parallel-1-TSR(2,3)~CD36tag. The vector encodes for GST, a TEV protease cleavage site, residues 416 to 529 of thrombospondin-1 and residues 90 to 117 of CD36. TEV protease digestion of GST~TSR(2,3)~CD36tag fusion protein yields a 9 residue tag "GAMDPEFEL" at the amino terminus of the protein (Fig. 1) resulting in at 16.8 kDa construct. The DNA for the CD36 fusion tag, residues 90–117, was generated via PCR from full length CD36 (GenBank Accession No. [AAH08406](#)), digested with XhoI and inserted into pGST-parallel-1-TSR(2,3) to produce pGST-parallel-1-TSR(2,3)~CD36tag.

Protein expression and purification

GST~TSR(2,3) and GST~TSR(2,3)~CD36tag were expressed and purified using identical procedures. Chemically competent *E. coli* RosettaGami cells (EMD Biosciences) were transformed with pGST-parallel-1-TSR(2,3) or pGST-parallel-1-TSR(2,3)~CD36tag and a single colony was used to inoculate a 2 ml LB starter culture with 100 μ g/ml ampicillin. The

starter culture was grown to saturation at 37°C, added to 100 ml LB with 100 µg/ml ampicillin and incubated overnight at 37°C with shaking at 250 rpm. The overnight culture was diluted 1:40 in 4 liters of LB with 100 µg/ml ampicillin and grown for 4 h at 37°C. Liter scale cultures were cooled on ice to 25°C and target protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 µM IPTG and grown at 25°C for 20 h.

Cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, pH 8.0, 1 mg/ml lysozyme and 20 µg/ml DNaseI. Resuspended cells were flash frozen in liquid nitrogen and stored at -80°C. Cells were thawed and lysed overnight with slow agitation at 4°C. Cell debris was removed by centrifugation at 21,000×g for 30 min and the supernatant was divided and loaded onto four 3 ml glutathione-Sepharose (GE Healthcare) columns pre-equilibrated with 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. Unbound proteins were washed from the column with 10 column volumes of the same buffer. GST fusion protein was eluted from the column with 3 column volumes of 20 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. Elution fractions were dialyzed twice in 1 L of 50 mM Tris-HCl, 50 mM NaCl, pH 8.0 to remove glutathione. Dialyzed fusion protein was incubated overnight at 4°C with TEV protease [16] at 1:10 ratio (TEV:fusion protein) to cleave target protein from the GST moiety. TEV and cleaved GST were removed by passing the sample over a 3 ml glutathione agarose column followed by a 0.5 ml Ni-NTA agarose column (5 Prime). The flowthrough was concentrated and loaded onto a Superdex-75 gel filtration column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl, 50 mM NaCl pH 8.0. Elution peaks from the gel filtration column were analyzed by SDS-PAGE and fractions containing pure target protein were combined and stored at -80°C.

Western blotting

Purified TSR(2,3), run on a 15% SDS polyacrylamide gel, was transferred to Immobilon-P PVDF transfer membrane (GE Healthcare) in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol, pH 8.3) for 90 min at 250 mA. The membrane was blocked with 5% nonfat dry milk (NFD) in Tris-buffered saline plus 0.1% tween 20 (TBST) overnight at 4°C. The blocked membrane was washed twice for 5 min in TBST and incubated with 1 µg/ml human thrombospondin-1 antibody (Abcam) diluted in 5% NFD in TBST for 1 h at RT. The antibody was removed and the blot washed once for 15 min followed by three washes for 5 min in TBST. After washing, enhanced chemiluminescence (ECL) anti-rabbit IgG, Horseradish Peroxidase-linked (Fab')₂ (GE Healthcare) antibody, diluted 1:5000 in 5% NFD in TBST, was added to the blot and incubated for 1 h at RT. The blot was washed once for 15 min and three times for 5 min in TBST to remove anti-rabbit IgG. The washed blot was exposed to ECL Plus detection reagent (GE Healthcare) and visualized by chemiluminescence.

HRGP binding assay

Human HRGP was purified from human plasma as previously described [17]. Purified human platelet TSP-1 was purchased from Sigma. In a 96-well polystyrene plate (Fisher Scientific), TSR(2,3), TSP-1 or 5% nonfat dry milk were added to each well at a concentration of 1 µg/well in 0.5 M NaHCO₃ pH 9.6 binding buffer. The plate was incubated overnight at 4°C to facilitate binding. Wells were washed twice with PBS and non-specific binding sites were blocked by addition of 1% BSA in PBS with 0.1% Tween 20 (PBST) for 1 h at room temperature (RT). Wells were washed twice with PBS followed by addition of HRGP, GST or PBS. Protein binding was carried out for 2 h at RT. Wells were washed twice with PBS followed by addition of HRGP monoclonal antibody (R&D Systems) or GST monoclonal antibody (Millipore) at 25 ng/well. Antibody binding was carried out for 1 h at RT. Wells were washed five times with PBST and horseradish

peroxidase-conjugated goat anti-mouse secondary antibody (diluted 1:2000) was immediately added and incubated for 1 h at RT. Wells were washed five times with PBST and 3,3',5,5'-tetramethyl benzidine (TMB) reagent (Pierce) was added. The colorimetric reaction was stopped after 10 min upon addition of 1M H₂SO₄. HRGP binding was determined by measuring absorbance at 450 nm.

Endothelial cell migration assay

Early passage human dermal microvascular endothelial cells (HDMVEC, passage 4–8, Lonza) grown in EBM-2 complete growth medium were used for all studies. 500 µl of minimal EBM-2 media (- control) or complete EBM-2 was added to the bottom of each well of a 24-well tissue culture plate. A suspension of 3.0×10^5 cells/ml in serum free media was prepared and 300 µl added to a millicell migration insert (8 µm pore size, Millipore) and placed into the migration plate. TSR(2,3) or TSP-1 was added to the cell suspension and the plate was incubated at 37°C with 5% CO₂ to allow the endothelial cells to migrate. After 4 h, the plate was removed from the incubator, the cell suspension was aspirated, and cells located on the inner side of the membrane were removed with a cotton swab. The membrane was cut away from the insert, placed onto a glass slide coated with DAPI, sealed, and visualized under fluorescence. Migrating cells were counted in sixteen random 10x fields per well and results were expressed as the number of migrating cells per field.

Protein crystallization

Crystallization conditions were identified from 96-condition commercial screens (Hampton Research and Qiagen) using a CrystalGryphon liquid handling robot (Art Robbins Instruments). Crystals of TSR(2,3) and TSR(2,3)~CD36tag grew in space groups *P*₃₂ and *C*222₁, respectively, appearing within 24 hours and growing to full size within one week. *P*₃₂ crystals grew to approximately 100 µm × 75 µm × 50 µm. *C*222₁ crystals grew to approximately 100 µm × 100 µm × 80 µm. TSR(2,3) crystals in space group *P*₃₂ were grown at 20°C by sitting drop vapor diffusion from equal volumes of 1 mM TSR(2,3) and reservoir solution consisting of 0.19 M ammonium sulfate, 0.1 M sodium acetate, pH 4.8, and 13% polyethylene glycol (PEG) 4000. TSR(2,3) crystals were cryoprotected in reservoir solution supplemented with 30% ethylene glycol and immediately flash frozen in liquid nitrogen. TSR(2,3)~CD36tag crystals in space group *C*222₁ were grown at 20°C by sitting drop vapor diffusion from equal volumes of 1 mM TSR(2,3)~CD36tag and reservoir solution consisting of 0.89 M sodium potassium phosphate, pH 4.9. TSR(2,3)~CD36tag crystals were cryoprotected in 0.89 M sodium potassium phosphate, pH 4.9 augmented with 25% ethylene glycol followed by flash freezing in liquid nitrogen. Native diffraction data for both crystals were collected at 1.54 Å wavelength with a Rigaku MicroMax-007HF generator and a Rigaku Saturn 944+ CCD detector.

X-ray crystallographic data collection and structure solution

Data reduction was performed with d*TREK [18]. Phases were calculated by molecular replacement utilizing the PHASER [19] component of PHENIX [20] with the TSR(2,3) structure [13] determined from a *Drosophila* expression system (PDB accession code [1LSL](#)). The molecular replacement solution was subjected to automated rebuilding in PHENIX with RESOLVE [21], followed by iterative model building in COOT [22] and refinement in PHENIX. The refinement protocol utilized isotropic atomic displacement parameters for all atoms and TLS motion analysis for protein chains [23]. Stereochemical analysis of the TSR(2,3)~CD36tag structure was completed with MolProbity [24]. All residues are within the allowed regions of the Ramachandran plot. No Cβ deviations or poor rotamers were found. The *MolProbity* score, a weighted measure of stereochemical statistics, was 1.78, which ranks the TSR(2,3)~CD36 structure in the 97th percentile of 8,058 structures of similar resolution deposited in the PDB. Atomic coordinates for

TSR(2,3)~CD36tag in spacegroup C222₁ were deposited with the Protein Data Bank with accession code **3R6B**.

Results

Expression and purification of TSR(2,3)

Plasmids for bacterial expression of GST~TSR(2,3) and GST~TSR(2,3)~CD36tag fusion proteins were based on the pGST parallel-1 vector [15]. The *E. coli* RosettaGami expression system was utilized to avoid negative effects on protein expression caused by rare codon usage in the coding sequence, and in order to maximize the potential for proper disulfide bonding in the TSP-1 type 1 repeats. Protein expression was induced by addition of IPTG and monitored by SDS-PAGE (Fig. 1). Cell lysates contained approximately 200 mg total soluble protein with 32 mg of GST fusion protein per liter of culture (Fig. 1, Table 1). GST~TSR(2,3) and GST~TSR(2,3)~CD36tag were purified from lysate using identical chromatographic procedures. The purification protocol yielded 5–7 mg target protein per liter of culture. The purity, estimated by SDS-PAGE, is greater than 99% (Fig. 1, Table 1). The identity of purified target protein was confirmed by western blot detection with human TSP-1 antibody (Fig. 1).

Recombinant TSR(2,3) binds HRGP – a TSP-1 ligand

HRGP is a plasma protein that binds to the type 1 repeat domains 2 and 3 of TSP-1 [25]. We examined the HRGP-binding capabilities of recombinant TSR(2,3) in an ELISA assay, using full length plasma-purified HRGP as the interacting protein (Fig. 2). Wells were coated with either full-length TSP-1, TSR(2,3) or 5% nonfat dry milk and assayed for HRGP binding by probing with an HRGP monoclonal antibody. The assay demonstrates that HRGP binds to both TSR(2,3) and full length TSP-1 to a similar extent (Fig. 2 “*TSR(2,3) + HRGP*” and “*TSP-1 + HRGP*”). In contrast, no signal was detected when the plates were probed with HRGP antibody alone (Fig. 2 “*Ab only*”), when the plate was incubated with GST and probed with a GST monoclonal antibody (Fig. 2 “*GST*”) or when wells blocked with 5% nonfat dry milk were incubated with HRGP and probed with HRGP antibody (Fig. 2 “*Blocked Well*”). The results establish that recombinant TSR(2,3) is capable of binding specifically to HRGP, a canonical TSP-1 ligand. The negative controls (Fig. 2 “*Ab only*”, “*GST*” and “*Blocked Well*”) demonstrate that the HRGP monoclonal antibody does not recognize TSR(2,3) and suggest that non-specific interactions, such as those between TSR(2,3) and GST, do not contribute to the observed binding signal. Furthermore, as our *E. coli*-expressed recombinant TSR(2,3) is not glycosylated, the results indicate that glycosylation of the TSP-1 type 1 repeat domains 2 and 3 is not required for interaction with HRGP.

TSR(2,3) exhibits anti-chemotactic properties similar to TSP-1

Within the microvasculature, the anti-chemotactic activity of TSP-1 is mediated by interactions between TSP-1 type 1 repeat domains 2 and 3 and the cell surface scavenger receptor CD36 [9, 26]. We investigated whether our recombinant TSR(2,3) exhibits similar anti-chemotactic activity using a migration assay. Human dermal microvascular endothelial cells (HDMVEC) in minimal media were allowed to migrate through a semi-permeable membrane toward complete media enriched with growth factors. In the presence of target media with a full complement of serum and growth factors HDMVECs migrated towards the complete media and accumulated with an average of 112 cells per 10x field (Fig. 3, “Complete Media”). In contrast, without the chemoattractant gradient, migration towards the target media was reduced by a factor of five (Fig. 3, “Minimal Media”). The addition of TSP-1 greatly reduced the migration of HDMVECs towards chemoattractant, resulting in migration levels similar to those observed in the absence of chemoattractant. Similarly,

addition of recombinant TSR(2,3) resulted in a nearly identical reduction in HDMVEC migration. These results demonstrate that recombinant TSR(2,3) robustly recapitulates anti-chemotactic properties of full-length TSP-1.

Structure of TSR(2,3) from a bacterial expression system

Our initial effort to determine the structure of recombinant TSR(2,3) yielded crystals in the $P3_2$ spacegroup that diffracted to 2.5 Å (Table 2). However, due to crystal packing, significant portions of TSR(2,3) loops were missing in the electron density map (data not shown). In an effort to crystallize TSR(2,3) in a space group that would allow for a more complete structural characterization, we explored alternative constructs. One such construct, a carboxy-terminal fusion chimera of TSR(2,3) and residues 90–117 of CD36 (TSR(2,3)~CD36tag), crystallized in the $C222_1$ spacegroup (Table 2). These crystals diffracted to 2.4 Å and electron density was observed for TSR(2,3) residues 416 through 529. The carboxy-terminal CD36 domain of the fusion chimera was disordered and did not give rise to electron density. The refined crystal structure of recombinant TSR(2,3)~CD36tag superimposes on the *Drosophila*-expressed TSR(2,3) crystal structure (PDB accession code [1LSL](#)) with less than 0.6 Å root mean square deviation for all heavy atoms (Fig. 4A). X-ray crystallographic data collection and refinement statistics for the recombinant TSR(2,3) structure solved in space group $C222_1$ are provided in Table 3. The structure confirms that recombinant TSR(2,3) expressed in *E. coli* is correctly folded and that the critical structural elements identified in *Drosophila*-expressed TSR(2,3), notably the Trp-Arg-Trp-Arg-Trp cation- π stacking motif and six disulfide bridges, are preserved (Fig. 4B). Although the *Drosophila* expressed TSR(2,3) crystal structure contains two core-glycosylated residues (threonine residues *O*-linked to fucose), the *E. coli* expressed TSR(2,3) has no post-translational modifications. The structural similarity between *E. coli* and *Drosophila* expressed TSR(2,3) indicates that glycosylation of TSP-1 does not fundamentally influence the structure of TSR(2,3). In addition, the relative orientation of the two type 1 repeats is identical in the *E. coli*- and *Drosophila*-expressed proteins and is thus an intrinsic property of the repeats, rather than a consequence of crystal packing or other extrinsic effects.

Discussion

It is well established that TSP-1 regulates pathophysiologic angiogenesis [4–7] as TSP-1 is required for physiological processes such as tissue remodeling and repair and the pathological processes of cancer growth and metastasis [27, 28]. TSP-1 is a multidomain matricellular protein important for cell-cell and cell-matrix contacts and each domain of TSP-1 has unique cell surface receptors. The amino-terminal domain binds integrin $\alpha_3\beta_1$, syndecan, and heparan sulfate proteoglycans, while the carboxy-terminal domain binds CD47. The type 1 (TSR) domains interact with β_1 and β_3 integrins, sulfatide and CD36 [8, 9]. Through these interactions, TSP-1 has been found to be important in cell attachment, cellular spreading, TGF β activation, nitric oxide signaling, and platelet aggregation. TSP-1 can bind multiple ligands simultaneously and the observed functions of TSP-1 appear to be dependent on both cell type and the specific ligands bound. Thus it is difficult to pinpoint the exact effect of each interaction within full length TSP-1 and the ability to produce individual domains of TSP-1 would aid in clarifying their roles within the intact molecule.

For example, TSP-1 activates TGF β through interactions mediated by an RFK sequence located between TSR1 and TSR2 [32]. While TGF β has been shown to suppress tumor growth of experimental human lung carcinomas [33], TGF β also stimulates the growth of melanoma, prostate cancer and colon carcinoma cells *in vitro* [34–36]. Interestingly, studies examining systemic injection of TSP-1 peptides have shown that TGF β activation is not essential for the anti-tumorigenic activity of TSP-1 [37]. Furthermore, the second and third

type 1 repeats, without the TGF β activating RFK sequence, are sufficient to recapitulate the anti-angiogenic and anti-chemotactic properties of TSP-1 within endothelial cell systems and the anti-tumorigenic properties of TSP-1 within subcutaneous tumor implantation models [11, 12]. The conflicting roles of TSP-1 mediated TGF β activation reported in the literature complicate efforts to deduce the mechanism by which the TSR domains convey the anti-angiogenic and anti-tumorigenic activities of TSP-1. The ability to delineate the effect of the TSR domains without TGF β activation could be addressed with a TSR(2,3) construct devoid of the RFK sequence that is found within full length TSP-1. Currently the only published protocol for producing TSR(2,3) requires the use of a *Drosophila* expression system [13]. We have developed a GST fusion expression construct that allows for large scale expression of TSR(2,3) in *E. coli*. Our expression and purification protocol allows for facile production of milligram quantities of >99% pure TSR(2,3) per liter of culture. We conducted endothelial cell migration assays and found that recombinant TSR(2,3), in comparison to full length TSP-1, causes a similar reduction in endothelial cell migration. These results indicate that our expression and purification protocol is capable of producing large quantities of protein which exhibits anti-chemotactic properties similar to full length TSP-1, yet lacks the TGF β activating RFK sequence. The recombinant TSR(2,3) construct described here provides a platform for examining the anti-angiogenic properties of TSP-1 TSR domains in a TGF β -independent manner.

In addition to examining the mechanism of TGF β -independent TSP-1 anti-angiogenic activity, the recombinant TSR(2,3) construct could prove valuable in probing the molecular basis of TSP-1 interactions with endothelial cell surface proteins such as CD36 and the β 1 and β 3 integrins. In addition to endothelial cell surface proteins, TSP-1 utilizes the TSR domains to interact with the human plasma protein HRGP [10], which potentially acts as a CD36 decoy [25, 38]. We have shown through ELISA assays that both recombinant TSR(2,3) and full length TSP-1 bind HRGP with similar affinities. Our results with recombinant TSR(2,3) confirm the findings of TSP-1 peptide interaction studies and demonstrate that the interaction between TSP-1 and HRGP can be fully replicated by the TSR(2,3) domains. The production of a construct capable of binding canonical TSP-1 ligands represents an important step towards characterizing the interaction between TSR(2,3) and various endothelial cell surface proteins. Our protocol, which produces significant quantities of TSR(2,3) makes a number of future experiments more feasible including *in vitro* binding assays to identify TSR(2,3) binding sites on endothelial cell surface proteins.

We have shown that our expression and purification protocol easily and rapidly produces milligram quantities of pure, functional protein. The ability to produce significant quantities of TSR(2,3) has allowed us to determine the structure of recombinant TSR(2,3). In light of the HRGP binding and anti-chemotactic activity of recombinant TSR(2,3), it is perhaps not surprising that the structure of recombinant TSR(2,3), determined by X-ray crystallography, is essentially identical to the structure of TSR(2,3) from a *Drosophila* expression system. Impressively, all six disulfide bonds (three per type-1 repeat) are properly maintained in the RosettaGami expression system. The primary difference between the TSR(2,3) structures is the *O*-linked glycosylation of two residues in the structure from the *Drosophila* expression system [13]. While TSP-1 is likely glycosylated *in vivo* [39], it was not known whether TSP-1 glycosylation is required for anti-chemotactic, anti-angiogenic, anti-tumorigenic or HRGP binding activities. Our functional assays comparing TSP-1 with TSR(2,3) indicate that TSR(2,3) in a non-glycosylated state faithfully reproduces both the HRGP binding and anti-chemotactic properties of TSP-1. In summary, the expression and purification protocol presented here is capable of producing high quantities of TSR(2,3) that is functionally replete and properly folded. This expression and purification protocol opens the door to a

number of future studies to probe the role of TSR(2,3) in pathophysiologic angiogenesis and to further characterize the interactions between TSP-1 and endothelial cell surface proteins.

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Abbreviations

GST	glutathione-S-transferase
HDMVEC	human dermal microvascular endothelial cell
HRGP	histidine-rich glycoprotein
IPTG	isopropyl- β -D-1-thiogalactopyranoside
NFDM	nonfat dry milk
TEV	tobacco etch virus
TMB	3,3',5,5'-tetramethyl benzidine
TSP-1	thrombospondin-1
TSR(2,3)	thrombospondin-1 type 1 repeat domains 2 and 3

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Highlights

- TSR(2,3) expression in *E. coli* as a GST fusion affords excellent yield and purity.
- Recombinant TSR(2,3) binds histidine-rich glycoprotein, a canonical TSP-1 ligand.
- Recombinant TSR(2,3) exhibits anti-chemotactic activity similar to full-length TSP-1.
- X-ray crystal structures of *E. coli* and *Drosophila* expressed TSR(2,3) are identical.

Fig. 1.

SDS-PAGE and western blot analysis of TSR(2,3) and amino acid sequences of TSR(2,3) and TSR(2,3)~CD36tag. (A) Whole cell fractions (“Cells”) were analyzed by SDS-PAGE for uninduced (*lane U*) or IPTG-induced (*lane I*) RosettaGami *E. coli* cells transformed with pGST-parallel-1-TSR(2,3). The supernatant of induced cells (*lane S*) was loaded onto a glutathione-sepharose column, eluted to yield GST~TSR(2,3) fusion protein (*lane 1*) and digested with TEV protease to yield GST and TSR(2,3) (*lane 2*). TSR(2,3) eluted from a gel filtration column (*lane 3*) was probed by western blot (*lane WB*) using an antibody to TSP-1. The expected size of each isolated construct is in agreement with the observed size as compared to the protein molecular weight marker (*lane M*, sizes shown on right). Amino acid sequence of (B) TSR(2,3) and (C) TSR(2,3)~CD36tag after digestion with TEV protease. Sequences are colored by domain; TSR-2 (cyan), TSR-3 (magenta) and CD36tag (black). Residues from the fusion tag are shown in italic (orange). Black lines indicate disulfide bonds between cysteine residues.

Fig. 2.

Purified TSR(2,3) binds the canonical TSP-1 ligand HRGP. An ELISA assay utilizing wells coated with TSP-1 or TSR(2,3) and probed with HRGP demonstrates that both TSP-1 and TSR(2,3) bind HRGP to a similar extent. All wells were probed with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody and visualized with TMB colorimetric reagent. Grey filled bars represent the average value over four replicates. Black bars represent the standard deviation. Statistically significant differences are denoted by asterisks (**) representing a two-tailed p value less than 0.01 calculated using an unpaired *t* test.

Fig. 3. Purified TSR(2,3) exhibits anti-chemotactic activity similar to TSP-1. (A) DAPI staining of cells collected on the membrane of an endothelial cell migration assays indicate that HDMVEC migration toward a chamber with minimal media is significantly reduced in comparison to migration toward a chamber with complete media. The addition of TSP-1 or TSR(2,3) significantly reduces HDMVEC migration toward a chamber with complete media. (B) HDMVEC migration expressed as number of migrated cells counted per 10x field. Grey filled bars represent the average value over sixteen fields. Black bars represent the standard deviation. Statistically significant differences are denoted by asterisks (**) representing a two-tailed p value less than 0.01 calculated using an unpaired t test.

Fig. 4. Structures of TSP-1 TSR domains 2 and 3 from *E. coli* and *Drosophila* expression systems. (A) Stereo overlay of structures for TSR(2,3) from an *E. coli* expression system (cyan) and from a *Drosophila* expression system (magenta, PDB ID 1LSL). Disulfide bonds are shown as sticks. (B) Overlay of the TSP-1 type 1 repeat domain 2 (TSR2) from both *E. coli* (cyan) and *Drosophila* (magenta) expression systems demonstrates that the core of the TSR domain, comprised of disulfide bonds and cation-pi stacking interactions between alternating tryptophan indole rings and arginine guanidino groups, is preserved in both structures. Side chains for residues in the TSR domain core are shown as sticks.

Table 1

Summary of TSR(2,3) protein purification.

Purification Step	TSR(2,3)	
	Total protein/gram cells (mg) ^a	Estimated purity (%)
Cleared lysate	60	10–15
GST-sepharose	6.3	95
TEV cleavage	4.1	50
Gel filtration	1.3	99

^aTSR(2,3) expression cultures typically generated 5 grams (wet weight) of *E. coli* cells. Protein content was determined by a Bradford assay against a bovine serum album standard.

Table 2

Crystallization of TSR(2,3) and TSR(2,3)~CD36tag.

Protein	TSR(2,3)~CD36tag	TSR(2,3)
Crystallization conditions	0.89 M sodium potassium phosphate (pH 4.9)	0.19 M ammonium sulfate, 0.1 M sodium acetate (pH 4.8), 13% PEG 4000
Crystal
Cryoprotection	Added ethylene glycol to 25%	Added ethylene glycol to 30%
Space group	$C222_1$	$P3_2$
Maximum resolution (Å)	2.4	2.5

Table 3

X-ray crystallographic data collection and refinement statistics

	TSR(2,3)~CD36tag
Data collection	
X-ray source	Rigaku MicroMax-007HF
Wavelength (Å)	1.5418
Space group	C222 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	66.4, 84.8, 60.7
α , β , γ (°)	90, 90, 90
Resolution (Å) ^a	30.35–2.40 (2.49–2.40)
R_{merge}^b	0.065 (0.227)
<i>I</i> / σ <i>I</i>	16.6 (4.7)
Completeness (%)	94.0 (87.6)
Redundancy	5.8 (3.8)
No. unique reflections	6,564
Refinement	
Resolution (Å)	30.35–2.40
No. unique reflections	6519
R_{work}/R_{free}	0.235/0.273
No. atoms	
Protein	853
Water	100
<i>B</i> -factors	
Protein	47.9
Water	50.3
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.286
Ramachandran	
Favored (%)	99.12
Allowed (%)	100.00
Disallowed (%)	0.00
MolProbity quality scores	
C β deviations (>0.25 Å)	0
Poor rotamers (%)	0
MolProbity score ^c	1.78 (97 th percentile, N=8058, 2.400 Å $\pm$ 0.25 Å)
Residue with bad bonds (%)	0
Residues with bad angles (%)	0
PDB ID	3R6B

^aValues in parentheses are for the highest-resolution shell.

$$R_{merge} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \overline{I(hkl)}|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

^bThe merging R factor is defined as

^cThe MolProbity score is weighted sum of stereochemical statistics [24]. 100th percentile is the best among N structures of comparable resolution.