RESEARCH ARTICLE

REVISED Structure of the transmembrane protein 2 (TMEM2) ectodomain and its apparent lack of hyaluronidase activity [version 2; peer review: 2 approved]

Previous title: Structure of the transmembrane protein 2 (TMEM2) ectodomain and its lack of hyaluronidase activity

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Abstract

Background: Hyaluronic acid (HA) is a major polysaccharide component of the extracellular matrix. HA has essential functions in tissue architecture and the regulation of cell behaviour. HA turnover needs to be finely balanced. Increased HA degradation is associated with cancer, inflammation, and other pathological situations. Transmembrane protein 2 (TMEM2) is a cell surface protein that has been reported to degrade HA into ~5 kDa fragments and play an essential role in systemic HA turnover.
Methods: We produced the soluble TMEM2 ectodomain (residues 106-1383; sTMEM2) in human embryonic kidney cells (HEK293) and determined its structure using X-ray crystallography. We tested sTMEM2 hyaluronidase activity using fluorescently labelled HA and size fractionation of reaction products. We tested HA binding in solution and using a glycan microarray.
Results: Our crystal structure of sTMEM2 confirms a remarkably accurate prediction by AlphaFold. sTMEM2 contains a parallel β-helix typical of other polysaccharide-degrading enzymes, but an active site cannot be assigned with confidence. A lectin-like domain is inserted into the β-helix and predicted to be functional in carbohydrate binding. A second lectin-like domain at the C-terminus is unlikely to bind carbohydrates. We did not observe HA binding in two assay formats, suggesting a modest affinity at best. Unexpectedly, we were unable to observe any HA degradation by sTMEM2. Our negative results set an upper limit for k_{cat} of approximately 10^{-5} min^{-1}.

Open Peer Review

Approval Status ✓ ✓

	1	2
version 2 (revision) 02 May 2023	✓ view	✓ view
version 1 14 Feb 2023	? view	? view

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Conclusions: Although sTMEM2 contains domain types consistent with its suggested role in TMEM2 degradation, its hyaluronidase activity was undetectable. HA degradation by TMEM2 may require additional proteins and/or localisation at the cell surface.

Keywords
Glycosaminoglycan, hyaluronidase, X-ray crystallography, parallel β-helix, lectin

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Author roles: Niu M: Formal Analysis, Investigation, Writing – Original Draft Preparation; **McGrath M**: Formal Analysis, Investigation, Writing – Original Draft Preparation; **Sammon D**: Formal Analysis, Investigation, Writing – Original Draft Preparation; **Gardner S**: Formal Analysis, Investigation, Writing – Original Draft Preparation; **Morgan RM**: Formal Analysis, Investigation, Writing – Original Draft Preparation; **Di Maio A**: Formal Analysis, Investigation, Writing – Review & Editing; **Liu Y**: Formal Analysis, Funding Acquisition, Investigation, Writing – Review & Editing; **Bubeck D**: Formal Analysis, Project Administration, Supervision, Writing – Original Draft Preparation; **Hohenester E**: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Project Administration, Supervision, Validation, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED **Amendments from Version 1**

The revised version differs from version 1 in the following respects:

1. The title has been changed to emphasise that the lack of hyaluronidase activity is not absolute but only "apparent".

2. Two authors have been added: Antonio Di Maio and Yan Liu. They carried out the new glycan microarray analysis.

3. Two sentences on HA binding have been added to the abstract.

4. More experimental detail has been added (HMW-HA, sperm hyaluronidase, SEC column).

5. A figure showing the quality of the electron density map has been added to Figure 1.

6. The comparison with other β-helix enzymes has been expanded (revised text and revised Figure 2 and Figure 3).

7. It is now stated that the hyaluronidase assay with fluorescently labelled HMW-HA has been used to detect CEMIP activity in two independent studies (references 24 and 25 of the revised manuscript).

8. CD spectroscopy has been used to demonstrate thermal stability of sTMEM2 (new Figure 4C).

9. The HA interaction data have been moved to a new Figure 5. sTMEM2 was tested in a glycosaminoglycan-focused microarray. No binding to any of the probes was observed. The negative data for HA probes have been added to the revised manuscript (new Figure 5B).

10. Two new data sets were added to Figshare (CD spectra, microarray data).

11. Grant support for the glycan microarray facility is acknowledged.

Any further responses from the reviewers can be found at the end of the article

Introduction

Hyaluronic acid (HA), also called hyaluronan, is a polysaccharide consisting of alternating glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) sugars, linked $[GlcA\text{-}\beta1,3\text{-}GlcNAc\text{-}\beta1,4]_n$[1]. It is the only glycosaminoglycan that exists as a free polysaccharide; all other glycosaminoglycans, such as heparan sulphate or chondroitin sulphate, are attached to core proteins to form proteoglycans. HA is synthesised by a plasma membrane-embedded processive enzyme, which produces chains of molecular mass >1 MDa (high-molecular-weight HA, HMW-HA). HMW-HA retains a large amount of water and forms gel-like networks with high viscoelasticity, which makes it essential for tissue hydration and biomechanics. HMW-HA is anti-inflammatory, anti-proliferative, and anti-angiogenic[1,2]. In contrast, HA fragments derived from HMW-HA degradation often have opposite biological activities, contributing to a range of pathological conditions[1,2]. Therefore, the enzymes involved in HA turnover are of considerable interest.

The human genome encodes six members of the HYAL (hyaluronidase) family. HYALs are generally associated with acidic intracellular compartments such as the lysosome and sperm acrosome, but some members also exist as glycsoylphosphatidylinositol-anchored proteins at the cell surface where they may contribute to extracellular HA degradation[3]. HYAL1 is considered to be the major lysosomal hyaluronidase. Recently, two homologous proteins that are not members of the HYAL family have been implicated in extracellular HA degradation: CEMIP (cell migration-inducing and hyaluronan-binding protein, also called KIAA1199 or HYBID) and TMEM2 (transmembrane protein 2). The two proteins share the same domain structure (see below) but differ in one important respect: CEMIP is a secreted protein, whereas TMEM2 is a type II transmembrane protein in the plasma membrane[3]. CEMIP was found to be required for HA degradation in normal skin fibroblasts, and transfection with CEMIP endowed other cell types with HA-degrading capability[4].

Interestingly, active CEMIP appeared to be associated with clathrin-coated pits, and purified soluble CEMIP had no hyaluronidase activity. TMEM2 was shown to degrade HMW-HA into 5 kDa fragments in a cell contact and Ca^{2+}-dependent manner[5]. Unlike in the case of CEMIP, a soluble version of TMEM2 had hyaluronidase activity, allowing a pH optimum of 6-8 to be determined. Subsequent studies showed that TMEM2 is essential for systemic HA turnover[6] and that it regulates cell adhesion and migration via HA degradation at focal adhesion sites[7]. Interest in TMEM2 was further raised by a screen in *C. elegans* that identified TMEM2 as a promotor of endoplasmic reticulum homeostasis and longevity[8].

We set out to characterise TMEM2 structurally and enzymologically. Prior to the structure prediction of the human proteome by AlphaFold[9], the domain structure of TMEM2 was assigned only incompletely. Prediction by Phyre2[10] identified the two lectin-like domains and a central β-helix. A G8 domain had been assigned at the N-terminus by a previous bioinformatic study[11], but was not picked up by Phyre2 because of the lack of structural information. Here, we describe the crystal structure of the entire TMEM2 ectodomain and our unsuccessful efforts to demonstrate its hyaluronidase activity. Together with other recent findings, our study should prompt a fresh look into the mechanism of TMEM2-mediated HA degradation.

Methods

Expression vector

DNA encoding the human TMEM2 ectodomain (soluble TMEM2, sTMEM2) was assembled from two partial cDNA clones (IMAGE clones 9021641 and 9053037; Horizon Discovery) using strand overlap extension polymerase chain reaction (PCR). The PCR product was cloned into the N-His-TEV-pCEP vector[12] using *Nhe*I and *Not*I. The correct sequence was verified by DNA sequencing. The vector encodes a protein consisting of the basement membrane protein 40 signal peptide, a hexahistidine tag, a tobacco etch virus protease cleavage site, and TMEM2 residues 106-1383 (KYAPDE...QASKAH). After cleavage of the signal peptide, the following vector-derived sequence remains at the N-terminus of secreted sTMEM2: APLVHHHHHHALDENLYFQGALA.

Protein production

sTMEM2 was produced in Expi293 cells maintained in FreeStyle 293 medium (Thermo Fisher Scientific, catalogue number

A14635) at 37°C and 8% CO_2 in shaking flasks (125 rpm). The cells were transfected at a density of 10^6 cells/ml using polyethylenimine (PEI MAX 40000; Polysciences) and a DNA: PEI ratio of 1:3 (1 µg of DNA/ml of cell culture). Five days after transfection the medium was harvested and the cells were pelleted by centrifugation at 500 g. The supernatant was passed through a 0.45 µm cellulose acetate filter (Sartorius) and loaded onto a 1-ml HisTrap column (Cytiva) using an ÄKTA pure chromatography system. The column was washed with 50 mM HEPES, 150 mM NaCl, pH 7.5 and the bound protein eluted in wash buffer containing 500 mM imidazole. Fractions containing protein were concentrated to 0.5 ml using a Vivaspin 30,000 MWCO centrifugal filter (Sartorius). The concentrated sample was loaded onto a Superdex 200 increase 10/300 column (Cytiva) and eluted in 50 mM HEPES, 125 mM NaCl, 2 mM $CaCl_2$, pH 7.5. sTMEM2 eluted in a symmetric peak at 11.4 ml. The peak fractions were pooled, concentrated to 3 mg/ml, and snap-frozen in liquid nitrogen in 50 µl aliquots. The final yield was 8 mg of sTMEM2 protein from 1 litre of cell culture medium.

Crystal structure determination

Snap-frozen purified sTMEM2 was thawed and rerun on a Superdex 200 increase 10/300 column (Cytiva). Peak fractions were concentrated to 9 mg/ml and a range of commercial crystallisation screens were set up using a Mosquito nanolitre liquid handler (STP Labtech). Large single crystals were obtained at room temperature using 0.1 M sodium acetate, pH 4.5, 30% polyethylene glycol 3000 as precipitant. The crystals were frozen in liquid nitrogen using precipitant solution supplemented with 20% ethylene glycol as cryoprotectant. Diffraction data were collected on beamline I04 at the Diamond Light Source (λ = 0.9795 Å) and processed using the XIA2 DIALS pipeline (version 3.dev.661-g1a4ae04e6)[13]. The structure was solved by molecular replacement using PHENIX (version 1.18rc1_3769)[14] and AlphaFold 2.0 model Q9UHN6 as search model[9]. Manual rebuilding and refinement were done using COOT (version 0.8.9.2)[15] and PHENIX. A conservative refinement protocol using a single B-factor per residue gave the same R_{free} as models with more parameters. Crystallographic data are summarised in Table 1. Structural comparisons were done using the DALI server[16]. All software used in the study are freely available to academic users.

Enzyme activity assays

Fluorescein-labelled high-molecular-weight HA (HMW-HA; average molecular mass, 800 kDa; 4-6 mol-% substitution; Carbosynth) was incubated for 18 h at 37°C with sTMEM2 or hyaluronidase from bovine testes (750-3,000 U/mg; Sigma-Aldrich, H3884) in 100 mM MES, pH 6.0, 1 mM $CaCl_2$ (total reaction volume, 1 ml). The HMW-HA concentrations ranged from 10 to 250 µg/ml, and protein concentrations ranged from 10 to 100 µg/ml. The reaction products were size-fractionated on a HiPrep 16/60 Sephacryl S200 HR column (Cytiva) with phosphate-buffered saline as running buffer. The useful separation range of this column for dextran standards is quoted as 1-80 kDa by the manufacturer. A fluorescein-labelled HA 22-mer (5 kDa, Iduron) was used as a reference sample. Aliquots of each fraction were transferred into black 96-well plates (Greiner Bio-One) and fluorescence was measured using a Tecan Spark microplate reader (excitation, 485 nm; emission, 535 nm). In a separate set of experiments, 0.1 µg/ml HMW-HA was incubated with 100 µg/ml of sTMEM2 or sperm hyaluronidase. The reaction products were passed through a Vivaspin 50,000 MWCO centrifugal filter (Sartorius) and the fluorescence in the filtrate measured as described above. The mock experiment contained 100 µg/ml of an irrelevant protein that is not known to bind or cleave HA (Endo180 D1-4[17]).

Circular dichroism spectroscopy

sTMEM2 at a concentration of 0.12 mg/ml in 50 mM phosphate buffer, pH 6.0 was incubated overnight at 4°C and 37°C. Spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics) in a 1-mm path length quartz cuvette at 20°C, and corrected for buffer signals.

HA interaction assay

sTMEM2 (1 mg/ml) was incubated with unlabelled HMW-HA (200 µg/ml; average molecular mass, >950 kDa; R&D Systems) for 30 min at room temperature in 50 mM HEPES, 125 mM NaCl, 2 mM $CaCl_2$, pH 7.5 (total volume, 200 µl). The mixture was loaded onto a Superdex 200 increase 10/300 column (Cytiva)

Table 1. Crystallographic statistics.

Data collection	
Resolution range (Å)	92.38–3.50 (3.56–3.50)
Space group	$P4_22_12$
Unit cell: *a*, *b*, *c* (Å)	184.76, 184.76, 105.40
Unique reflections	23570 (1159)
Multiplicity	26.8 (28.3)
Completeness (%)	100.0 (100.0)
Mean $I/\sigma(I)$	7.1 (0.5)
$CC_{1/2}$	0.997 (0.424)
R_{pim}	0.073 (0.871)
Refinement	
Non-hydrogen atoms	
Protein	9992
Glycan	333
Ion	1 Ni^{2+}, 1 Cl^-
R_{work}/R_{free}	0.265/0.319
Root-mean-square deviations	
Bonds (Å)	0.004
Angles (°)	0.638
Ramachandran plot	
Favoured (%)	90.1
Allowed (%)	9.2
Outliers (%)	0.7

and eluted in the same buffer. The control experiment was done in the same way, but without HA.

Glycan microarray

Microarray analyses were carried out using a glycosaminoglycan (GAG)-focused array based the neoglycolipid (NGL) technology[18,19]. The preparation of the GAG NGL probes, their formulation as liposomes, and printing using a noncontact robotic arrayer Nano-Plotter 2.1 (GeSim, Germany) onto 16 pad nitrocellulose-coated glass slides (Sartorius, Germany) at 2 and 5 fmol per spot in duplicate were as described previously[18]. After blocking the slides for 1 h with 2% (w/v) bovine serum albumin (BSA; Merck A7030) in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) containing 5 mM $CaCl_2$, the microarrays were overlaid with sTMEM2 at 300 µg/mL for 90 minutes, followed by 1 h incubation with the detection antibody solution composed of mouse monoclonal anti-polyhistidine antibody (Merck SAB4200620) and the biotinylated goat anti-mouse IgG (Merck B7264) precomplexed at a 1:1 ratio and diluted in at 10 µg/mL, diluent: 1% (w/v) BSA in HBS containing 5 mM $CaCl_2$. Binding was detected with Alexa Fluor-647-labelled streptavidin (Molecular Probes) at 1 µg/mL for 30 minutes. The control HA-binding protein was a native protein isolated from bovine nasal cartilage (complex of aggrecan G1 and link protein; Merck 385911), which was overlaid at 50 µg/mL followed by single step detection with Alexa Fluor-647-labelled streptavidin as mentioned above. The microarray images were recorded using a GenePix 4300A scanner (Molecular Devices).

Results

Crystal structure of sTMEM2

We expressed the soluble ectodomain of human TMEM2 (residues 106-1383; sTMEM2) with a N-terminal hexahistidine tag in Expi293 cells. We obtained a monodisperse glycoprotein that formed single crystals readily. We collected a high-redundancy X-ray diffraction data set (deposited in the PDB, see *Underlying data*) to 3.5 Å resolution and solved the structure by molecular replacement using the AlphaFold model of TMEM2[9]. After rebuilding residues 812-824 and adding N-linked glycans, we refined the structure conservatively to R_{free} = 0.319. Nearly the entire ectodomain (residues 112-1381) is resolved in the crystal structure, which matches the AlphaFold model with a root-mean-square (r.m.s.) deviation of only 1.1 Å (1270 Cα atoms). Thus, we have experimentally confirmed the remarkably accurate prediction of this multidomain protein.

The sTMEM2 structure is built around a 70 Å-long right-handed parallel β-helix that starts at the N terminus (Figure 1). The predicted G8 domain[11] is part of this continuous β-helix.

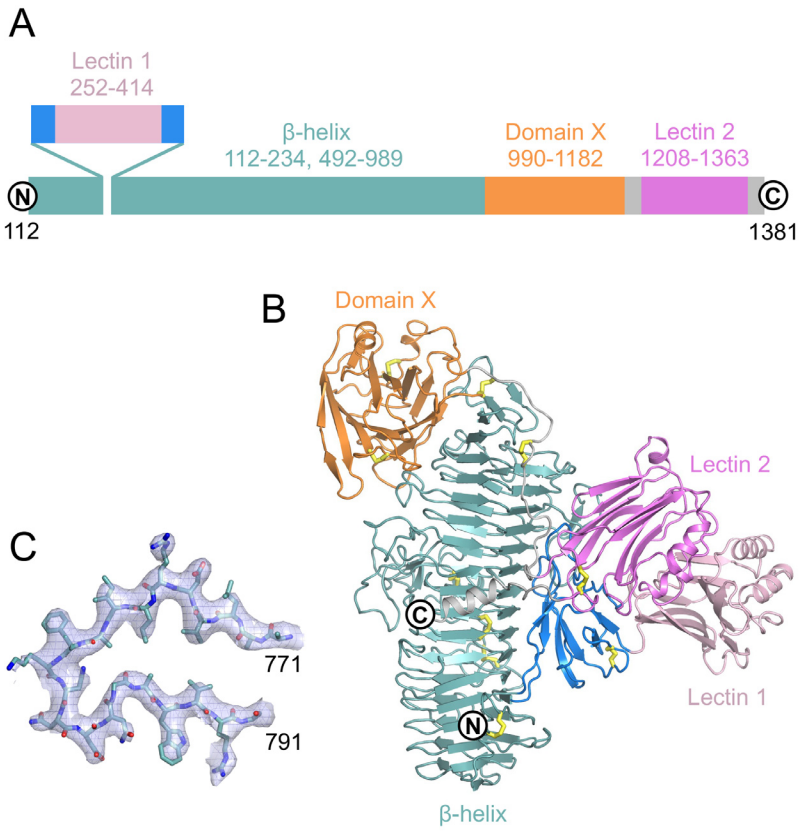

Figure 1. Crystal structure of soluble Transmembrane protein 2 (sTMEM2). (**A**) Schematic representation of the sTMEM2 domain structure. Sequence numbers refer to full-length TMEM2. (**B**) Cartoon drawing of the sTMEM2 structure using the same colour code as in A. Disulfide bonds are indicated by yellow sticks. *N*-linked glycans have been omitted for clarity. (**C**) Electron density map for residues 771-791. Shown is the final $2F_{obs}$-F_{calc} map contoured at 1 σ level. This figure was produced using PyMOL version 2.5.2. UCSF Chimera is a free alternative to PYMOL.

A small β-sandwich and a larger lectin-like domain (26% sequence identity to the N-terminal domain of protein O-linked-mannose β-1,2-N-acetylglucosaminyltransferase 1 (POMGNT1)[20]) are inserted into the β-helix after three complete turns without disrupting its regular structure. The β-helix is followed by a large β-sandwich that is not closely related to any other known structure (termed domain X). The polypeptide chain then runs halfway down the β-helix before forming a second lectin-like domain (20% identity to POMGNT1) and, finally, a C terminal helix. The two lectin-like domains interact through a modest interface, and together form a bulky addition to the central β-helix. The longest dimension of sTMEM2, from the tip of lectin-like domain 1 to domain X, is over 110 Å.

sTMEM2 contains 24 cysteine residues, 20 of which are involved in disulphide bonds. Cys361, Cys554, Cys750, and Cys940 are unpaired and too distant from each other to plausibly form disulphide bonds. sTMEM2 contains 15 sequons for N-linked glycosylation, nine of which showed extra electron density for glycans in our structure. The electrostatic surface potential of sTMEM2 (Figure 2A) shows a relatively even distribution of positive and negative potential, and no extended basic (positive) surface regions as may have been expected for a protein interacting with polyanionic HA. Sequence conservation is highest in the β-helix, with another notable region of high sequence conservation at the tip of lectin-like domain 1 (Figure 2B).

Comparison to other structures

The β-helix is a common structural motif of enzymes involved in polysaccharide degradation, encompassing both hydrolases (using water to break the glycosidic bond) and lyases (using a β-elimination mechanism). The latter family is restricted to enzymes acting on uronic polysaccharide substrates, such as HA and chondroitin sulphate[21]. We used the DALI server[16] to identify structurally similar β-helix enzymes and mapped all active sites for which experimental confirmation was available (bound substrate and/or mutagenesis). Active sites are invariably located on the concave face of the β-helix (Figure 2). The majority of active sites are located roughly in the middle of the β-helix, which in sTMEM2 is obstructed by two long loops, made up of residues 627-662 and 733-756 (Figure 3A). A smaller number of β-helix enzymes have their active sites closer to the C-terminus of the β-helix (i.e. nearer the top in the view of Figure 2). The most relevant example is the *Pedobacter heparinus* chondroitin B lyase structure, which can be superimposed onto sTMEM2 with a r.m.s. deviation of 3.3 Å for 325 Cα atoms (8% sequence identity). The active site of chondroitin B lyase features a catalytic Ca^{2+} ion bound by two acidic residues, Glu243 and Glu254

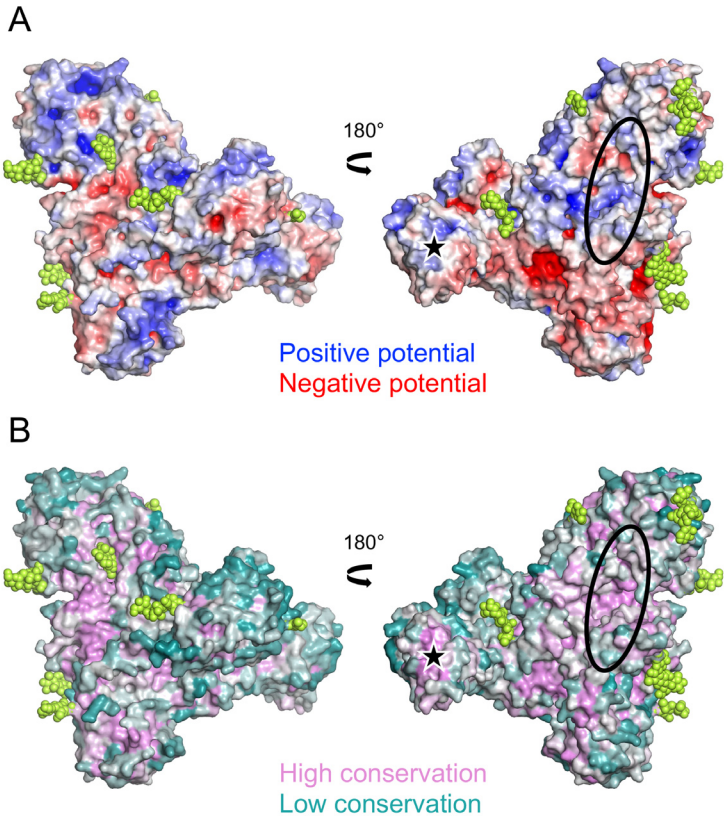

Figure 2. Surface properties of soluble Transmembrane protein 2 (sTMEM2). (**A**) Electrostatic surface potential calculated using the APBS function of PyMOL (version 2.4.1). UCSF Chimera is a free alternative to PYMOL. Positive and negative potential is shown in blue and red, respectively. Glycans are shown in lime green. (**B**) Sequence conservation calculated using the Consurf server[22]. High and low sequence conservation is shown in magenta and teal, respectively. Glycans are shown in lime green. In **A** and **B**, the views on the left are the same as in Figure 1B. The black oval indicates the general location of active sites in related β-helix enzymes (see text). The black star indicates the tip of lectin-like domain 1.

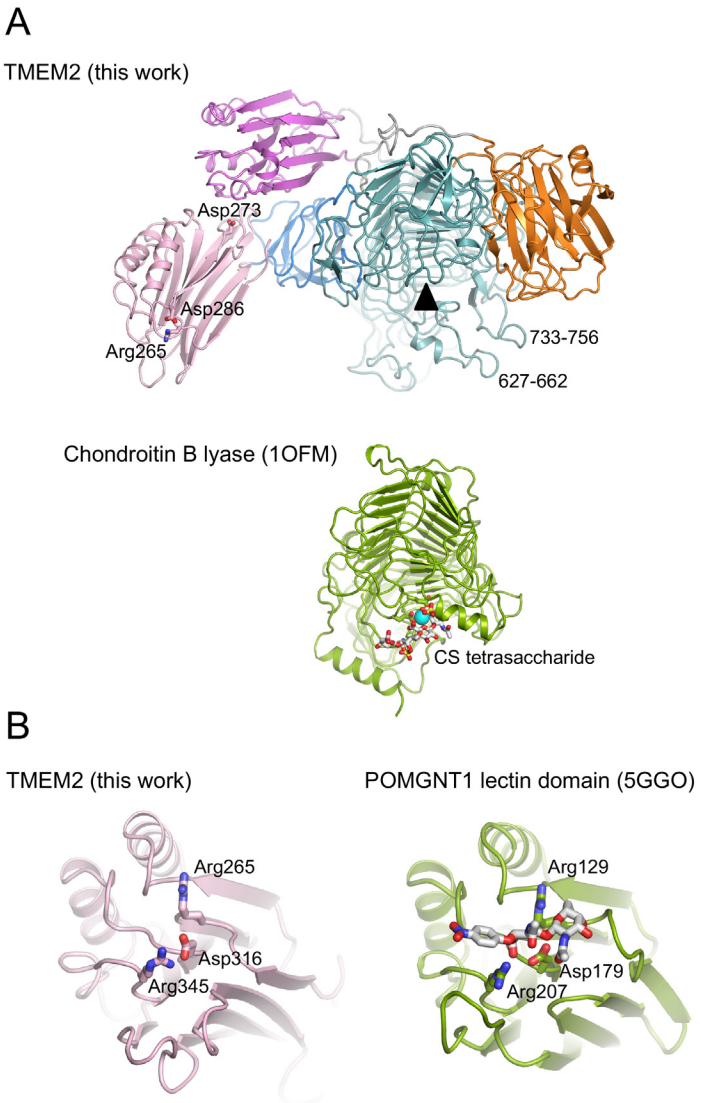

Figure 3. Comparison of soluble Transmembrane protein 2 (sTMEM2) to other proteins. (**A**) Chondroitin lyase (PDB 1OFM) was superimposed onto the β-helix of sTMEM2 with a r.m.s. deviation of 3.3 Å for 325 Cα atoms. Chondroitin lyase contains a Ca^{2+} ion in the active site; the equivalent position in sTMEM2 is indicated by the black triangle. Three residues in the lectin-like domain 1 whose mutation has been reported to reduce TMEM2 activity[5] are shown in atomic detail. (**B**) The N-terminal lectin domain of POMGNT1 (PDB 5GGO) was superimposed onto the lectin-like domain 1 of sTMEM2 with a r.m.s. deviation of 2.3 Å for 153 Cα atoms. The disaccharide bound to POMGNT1 is shown in atomic detail, as are selected residues that are conserved between sTMEM2 and POMGNT1. This figure was produced using PyMOL version 2.5.2. UCSF Chimera is a free alternative to PYMOL.

(Figure 3A)[23]. sTMEM2 contains a similarly sized cleft, but only one of the acidic residues is conserved, and there are no other suitable Ca^{2+} ligands in the vicinity. Given that our biochemical experiments failed to detect any hyaluronidase activity of sTMEM2 (see below), the location of the putative active site on TMEM2 remains an open question.

As already mentioned, sTMEM2 contains two lectin-like domains, one inserted into the N-terminal region of the β-helix and another one at the very C-terminus. The closest homologue of known structure is the N-terminal domain of the glycosyltransferase POMGNT1, which binds β-linked GlcNAc[20]. The three key sugar-binding residues of POMGNT1 are conserved in TMEM2, suggesting that lectin-like domain 1 may bind carbohydrates (Figure 3B). It is also interesting that all of the substitutions that have been reported to abrogate TMEM2 activity[5] map to lectin-like domain 1: R265C, D273N, D286N. None of the three sugar-binding residues is conserved in lectin-like domain 2 of sTMEM2, suggesting that this domain does not bind carbohydrates.

Hyaluronidase activity of sTMEM2

As a first step towards identifying catalytic residues in sTMEM2, we established the enzymatic activity assay used by Yamamoto *et al.*[5] for TMEM2 and by two independent groups for CEMIP[24,25]. Fluorescently labelled high-molecular-weight

(HMW) HA was incubated with sTMEM2 or hyaluronidase from bovine testes under the reported optimum conditions for sTMEM2 (pH 6.0, 1 mM $CaCl_2$)[5]. Size-fractionation of the reaction products revealed complete degradation of HMW-HA by the sperm hyaluronidase, but to our surprise we could detect no degradation of HMW-HA by sTMEM2 (Figure 4A). We also tested other sTMEM2:HA ratios, but failed to detect any hyaluronidase activity of TMEM2 in this assay (the column we used would have separated fragments <80 kDa from intact HMW-HA). We then carried out experiments at the very low HMW-HA concentration of 0.1 µg/ml, using ultrafiltration to separate intact and degraded HA, but again failed to observe HMW-HA degradation by sTMEM2 (Figure 4B). To exclude the possibility that sTMEM2 may not be stable at 37°C, we recorded circular dichroism spectra of protein samples incubated overnight at 4°C and 37°C (Figure 4C). We observed no differences and concluded that unfolding or aggregation of sTMEM2 during the overnight incubation cannot be the reason for the failure to detect hyaluronidase activity. The data associated with Figure 4 are available in *Underlying data*.

We estimated an upper limit of k_{cat} as follows. In our experimental setup, an enzyme that degrades 0.1 µg/ml HMW-HA (molecular mass, 1 MDa) into 5 kDa fragments[5] would have to cleave 20 nM glycosidic bonds over the course of 18 hours, or 0.019 nM bonds per minute. The sTMEM2 concentration of 100 µg/ml corresponds to 700 nM putative enzyme. Thus, a k_{cat} of 0.019/700 = 2.6 x 10^{-5} min^{-1} would be sufficient to observe complete degradation of the HMW-HA into 5 kDa fragments. For comparison, the k_{cat} value of human HYAL1 is 350 min^{-1} (calculated from the v_{max} value reported in ref 26). Therefore, if sTMEM2 indeed has hyaluronidase activity, it is >10^7-fold less active than a conventional HYAL enzyme.

Next, we tested whether sTMEM2 interacts with HMW-HA in solution. sTMEM2 eluted in a sharp symmetrical peak at 11.4 ml from a Superdex S200 increase 10/300 column regardless of whether it had been pre-incubated with HMW-HA or not (Figure 5A). Any sTMEM2 associated with HMW-HA would have eluted either in the void volume of the column (stable complex) or between the void volume and the elution volume of free sTMEM2 (complex dissociating during chromatography). We also tested HA binding using a glycosaminoglycan-focused microarray[18]. sTMEM2 did not bind to any of the HA probes on this array, whereas a known HA-binding protein (aggrecan G1-link protein complex) showed robust binding to oligosaccharides longer than 10 sugar residues (Figure 5B). We conclude that if sTMEM2 interacts with HA, it does so only weakly. We note that the K_M value of sperm hyaluronidase for a HA octasaccharide is 130 µM[27]. A similarly low affinity of sTMEM2 for HA could explain

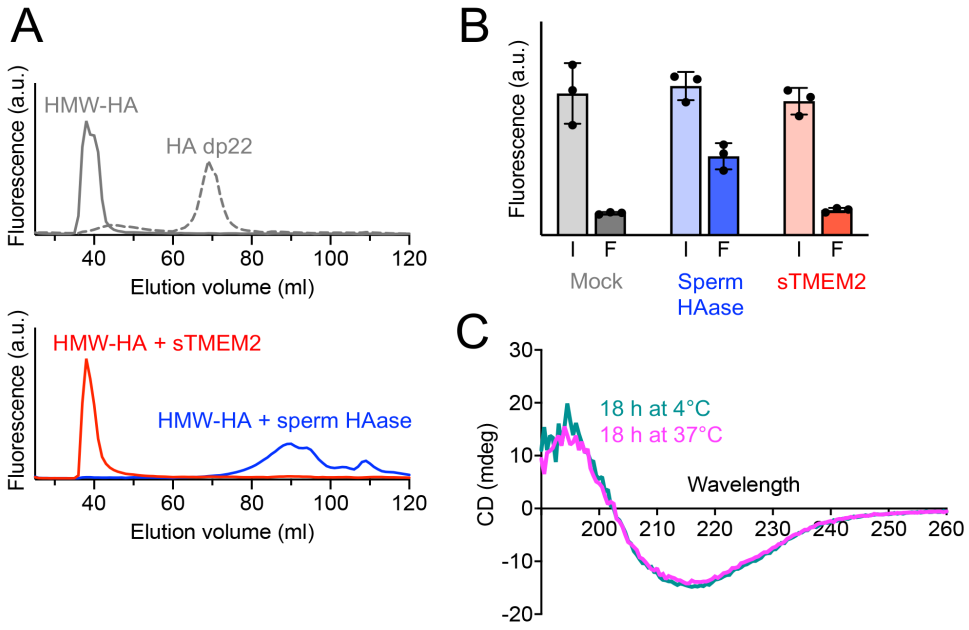

Figure 4. Hyaluronidase activity of soluble Transmembrane protein 2 (sTMEM2). (**A**) Top, size exclusion chromatography of 100 µg/ml fluorescein-labelled high-molecular-weight Hyaluronic acid (HMW-HA) or HA 22-mer (dp22, ~5 kDa). Bottom; size exclusion chromatography of 100 µg/ml fluorescein-labelled HMW-HA incubated overnight with 100 µg/ml sTMEM2 or sperm hyaluronidase (pH 6.0, 1 mM $CaCl_2$). Only the sperm hyaluronidase was able to degrade HMW-HA. Shown is a representative of n = 3 experiments. (**B**) Ultrafiltration of 0.1 µg/ml fluorescein-labelled HMW-HA incubated overnight with 100 µg/ml sTMEM2 and sperm hylauronidase (pH 6.0, 1 mM $CaCl_2$). The fluorescence was measured in the solution before and after filtration (I, input; F, filtrate). Only the sperm hyaluronidase was able to degrade HMW-HA. Data are presented as mean values ± s.e.m. for n = 3 independent experiments. (**C**) Circular dichroism spectra of sTMEM2 incubated overnight at 4°C (teal) and 37°C (magenta). The minimum at 217 nm is characteristic of β-sheet structure. There is no evidence of denaturation at the higher temperature. The raw measurements are available in *Underlying data*.

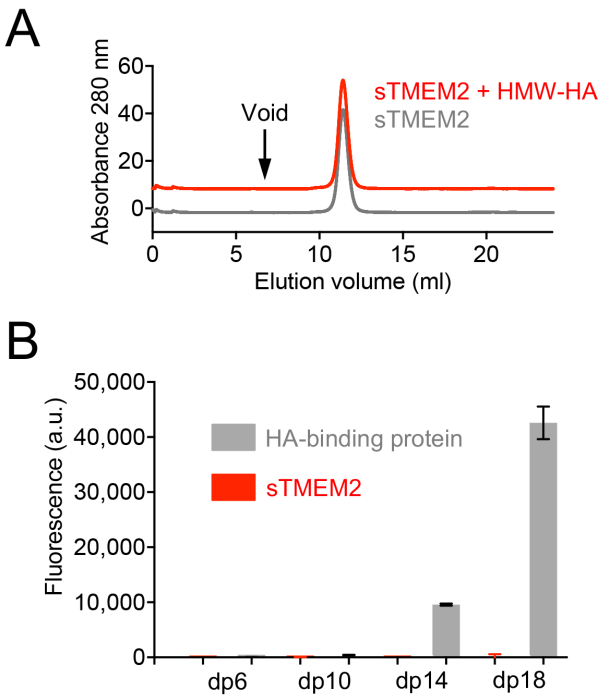

Figure 5. Hyaluronan binding by soluble Transmembrane protein 2 (sTMEM2). (**A**) Size exclusion chromatography of sTMEM2 in the absence or presence of unlabelled HMW-HA. To avoid overlap of the two traces, the red trace has been shifted by +10 mAU. No sTMEM2 protein is associated with HMW-HA eluting in the void volume of the column (arrow). (**B**) Binding of sTMEM2 and the aggrecan G1-link protein complex (HA-binding protein) to HA oligomers of different length (dp, degree of polymerisation). The experiment was done using a neoglycolipid-based microarray[18,19]. Data (fluorescence intensities of HA probes arrayed at 5 fmol/spot level) are presented as mean values ± range for n = 2 duplicate spots on the array. The raw measurements are available in *Underlying data*.

why we did not observe an interaction in two different assay formats. The data associated with Figure 5 are available in *Underlying data*.

Discussion

We found that pure, crystallisable, soluble TMEM2 has no detectable hyaluronidase activity. Two possibilities need to be considered: (1) TMEM2 is not a hyaluronidase, contrary to what has been reported[5]; or (2) TMEM2 is a hyaluronidase, but activity is lost when the ectodomain is severed from the plasma cell membrane. With regards to the former possibility, we are not the first ones to observe negligible HA degradation by TMEM2. Knock-down experiments in human skin fibroblasts[24] and chondrocytes from osteoartritic knee cartilage[25] demonstrated involvement of CEMIP but not of TMEM2 in HA degradation. Moreover, hyaluronidase activity in skin homogenates was found to be robust at the acidic pH optimum of HYALs, but negligible at the neutral pH optimum reported for TMEM2, even though TMEM2 is highly expressed in skin[28]. How these negative results may be reconciled with reports that TMEM2 is an extracellular hyaluronidase essential for systemic HA turnover[5,6] is a matter for future studies. Other functions ascribed to TMEM2, such as its roles in development[29–31] and endoplasmic reticulum homeostasis[8], do not require TMEM2 itself to be a hyaluronidase. For instance, TMEM2 may be a component of the machinery that translocates HA into the cell for eventual lysosomal degradation. The second possibility is that TMEM2 hyaluronidase activity requires localisation of the protein at the plasma membrane, either because of some steric reason or because of the presence of an essential protein partner or cofactor. This situation would be akin to what has been reported for CEMIP, which appears to be active only in clathrin-coated pits[4]. Clustering of TMEM2 at the cell surface, which would greatly amplify its avidity for HA, may be required for HA degradation. Whatever the reason, our finding that pure sTMEM2 is at least 10^7-fold less active than conventional HYALs complicates further enzymological studies.

What can be learned from the sTMEM2 structure? First of all, it dramatically highlights the power of AlphaFold in predicting complex multi-domain structures[9]. Second, it allows a better interpretation of previous mutagenesis experiments. Mutation of Arg187 in CEMIP and its equivalent Arg265 in TMEM2 reduced HA degradation[4,5]. Arg265 is located at the tip of lectin-like domain 1, in an equivalent position as a critical sugar-binding residue in a related domain (Figure 3). It is unlikely that Arg265 participates in catalysis, but it may bind HA weakly and present it to an active site elsewhere on TMEM2 or an associated protein. Such a "molecular ruler" effect could explain why TMEM2 products are ~5 kDa fragments and not ~400 Da disaccharides as in the case of HYALs[5]. Finally, the finding that the G8 domain is actually part of the β-helix explains why previous attempts to functionally dissect the TMEM2 ectodomain were unsuccessful[30].

Data availability

Underlying data

Protein Data Bank: Structure factors and coordinates of the sTMEM2 structure. Accession number 8C6I; https://www.rcsb.org/structure/8C6I.

Figshare: Hyaluronidase activity of sTMEM2 (SEC). https://doi.org/10.6084/m9.figshare.21975914.v2.

This project contains the following underlying data:

- Hyaluronidase activity of sTMEM2 (SEC).

Figshare: Hyaluronidase activity of sTMEM2 (Filtration). https://doi.org/10.6084/m9.figshare.21975920.v2.

This project contains the following underlying data:

- Hyaluronidase activity of sTMEM2 (Filtration).

Figshare: Circular dichroism spectra of sTMEM2. https://doi.org/10.6084/m9.figshare.22548601.

This project contains the following underlying data:

- Circular dichroism spectra of sTMEM2.

Figshare: Hyaluronan binding of sTMEM2 (SEC). https://doi.org/10.6084/m9.figshare.21975917.v2

This project contains the following underlying data:

- Hyaluronan binding of sTMEM2 (SEC).

Figshare: Hyaluronan binding of sTMEM2 (Microarray). https://doi.org/10.6084/m9.figshare.22434085

This project contains the following underlying data:

- Hyaluronan binding of sTMEM2 (Microarray).

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 23 May 2023

https://doi.org/10.21956/wellcomeopenres.21506.r56861

✔ **Kristina Nesporova**
R&D Department, Contipro a.s, Dolni Dobrouc, Czech Republic

The revised version (v2) of the article sufficiently addresses previous shortcomings.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: hyaluronan structure and hyaluronan/protein interactions, hyaluronidase activity assessment

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 15 May 2023

https://doi.org/10.21956/wellcomeopenres.21506.r56862

✔ **Rebekka Wild**
Institut de Biologie Structurale, UMR 5075, CNRS, CEA, University Grenoble Alpes, Grenoble, France

The revised version (v2) of the article sufficiently addresses previous shortcomings.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Glycosaminoglycan biosynthesis, structural biology, glycobiology, cryo-EM

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 01 March 2023

https://doi.org/10.21956/wellcomeopenres.20995.r55041

?

Kristina Nesporova
R&D Department, Contipro a.s, Dolni Dobrouc, Czech Republic

The manuscript by Niu et al. deals with the interesting topic of structural and biochemical analysis of relatively novel hyaluronidase TMEM2. Authors are trying to broaden the knowledge about this protein which seems to play an important role in physiological and pathological processes in various tissues.
The first (and possibly the main) part of this work is focused on the structure of the soluble ectodomain of TMEM2 which should be responsible for hyaluronan degradation. The second part of this study then deals with the biochemical/enzymological measurements. The overall study is rather brief and straightforward but it doesn't necessarily lessen its impact.
I don't find any obvious problems with the structural study and it seems that the very high correlation of AlphaFold prediction with the actual crystal structure is a good indicator of the reliability of these results. It is also important to see this high level of correlations which might promote using this in silico method. As my area of expertise is more in hyaluronidase activity detection, I will focus more on the second part of the study.

The method used for hyaluronidase activity assessment has been already used by Yamamoto et al. and for well-characterized samples of hyaluronidases I don't doubt its usefulness. For a partial protein whose optimum for enzymatic activity is not well known, I would recommend alternative methods which don't rely on the labeled HA as the chemical modification can affect the degradation process. Such a method can be agarose electrophoresis with subsequent HA staining or more quantitative analytical methods such as SECMALS or even LC-MS for small fragment identification and quantification. When using the fluorescently labeled substrate, at least the degree of substation should be included in the substrate description in the method section. And I would recommend the analyzed the content of free fluorophore molecules, as those are often present. Those can be eliminated by an ultracentrifugation filter with a relatively low cut-off (only low molecular fluorescein and other possible residues from the chemical reaction will be filtered off). As you used the mock protein with no hyaluronidase activity the possible contamination with free fluorescein can be evaluated, but using pure labeled HMW HA is always preferable.

I am also missing information about the enzymatic activity of sperm hyaluronidase used as a

positive control. As the term sperm hyaluronidase is used broadly for various preparation of PH20 (various animal origin, various purity of preparations) the activity is more important information than the concentration (add the supplier or manufacturer of BTH used and its activity). I am also rather surprised that the sperm hyaluronidase at the 100 ug/ml concentration during the 18h degradation protocol hasn't degraded the HA fully (Fig 4B). But without the information about BTH activity, I cannot judge this. Even without the activity info, I would like to ask the authors to explain why the increase of LWM HA fragment in the sperm hyaluronidase sample (Fig 4B) is not accompanied by a decrease in HMW HA.

I would be also very careful with the conclusion from very preliminary results using the SEC for the detection of the sTMEM2/HA complexes. The composition of the mobile phase can drive the formation and disruption of noncovalent complexes of enzymes and substrates. Would the same method enable us to see the hyaluronidase/HA complexes formation with the control sperm hyaluronidase?

The section of the article dealing with enzymatic activity provides mostly preliminary data. I appreciate that the authors are not making any major conclusions from them but the final version of this manuscript would benefit from the addition of some optimization steps or better discussion of obtained results from activity assays.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: hyaluronan structure and hyaluronan/protein interactions, hyaluronidase activity assessment

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Apr 2023

Erhard Hohenester

1. "*The method used for hyaluronidase activity assessment has been already used by Yamamoto et al. and for well-characterized samples of hyaluronidases I don't doubt its usefulness. For a partial protein whose optimum for enzymatic activity is not well known, I would recommend alternative methods which don't rely on the labeled HA as the chemical modification can affect the degradation process. Such a method can be agarose electrophoresis with subsequent HA staining or more quantitative analytical methods such as SECMALS or even LC-MS for small fragment identification and quantification.*"

   We refer to our response to reviewer 1 (point 4). The assay has been used not only by Yamamoto et al., but also by two other groups to show hyaluronidase activity of cell-associated CEMIP (references 23 and 24 of the revised manuscript). We therefore feel that this is a validated assay for this class of proteins.

2. *"When using the fluorescently labeled substrate, at least the degree of substation should be included in the substrate description in the method section."*

   The degree of substitution is ~5%. This information has been added to the revised manuscript.

3. *"And I would recommend the analyzed the content of free fluorophore molecules, as those are often present. Those can be eliminated by an ultracentrifugation filter with a relatively low cut-off (only low molecular fluorescein and other possible residues from the chemical reaction will be filtered off). As you used the mock protein with no hyaluronidase activity the possible contamination with free fluorescein can be evaluated, but using pure labeled HMW HA is always preferable."*

   We would have detected any free fluorophore during size exclusion experiments. There is no fluorescence signal between 50 and 120 ml (the column volume).

4. *"I am also missing information about the enzymatic activity of sperm hyaluronidase used as a positive control. As the term sperm hyaluronidase is used broadly for various preparation of PH20 (various animal origin, various purity of preparations) the activity is more important information than the concentration (add the supplier or manufacturer of BTH used and its activity)"*

   The supplier and catalogue number of the sperm hyaluronidase was given in the original manuscript (Methods). We have added information on the specific activity, as requested.

5. *"I am also rather surprised that the sperm hyaluronidase at the 100 ug/ml concentration during the 18h degradation protocol hasn't degraded the HA fully (Fig 4B). But without the information about BTH activity, I cannot judge this. Even without the activity info, I would*

like to ask the authors to explain why the increase of LWM HA fragment in the sperm hyaluronidase sample (Fig 4B) is not accompanied by a decrease in HMW HA."

There seems to be a misunderstanding here. Figure 4A shows that the sperm hyaluronidase is able to fully degrade 100 ug/ml HMW-HA (complete disappearance of the void peak). The experiment in Figure 4B used a 1000-fold lower HMW-HA concentration. The bars labelled "I" do not show the HMW-HA remaining *after* incubation with protein/enzyme. Rather, they show fluorescence *before* incubation, to demonstrate equal input in all experiments. Why not all input fluorescence is recovered in the filtrate is unclear. The device has a dead volume and some of the fluorescent molecules may have stuck to the ultrafiltration membrane.

6. *"I would be also very careful with the conclusion from very preliminary results using the SEC for the detection of the sTMEM2/HA complexes. The composition of the mobile phase can drive the formation and disruption of noncovalent complexes of enzymes and substrates. Would the same method enable us to see the hyaluronidase/HA complexes formation with the control sperm hyaluronidase?"*

   The K_M value of sperm hyaluronidase for HA appears to be quite high (>100 µM), so it is not clear whether binding would be detected. A sentence on this point has been added to the revised manuscript. In response to a comment by reviewer 1, we tested glycan binding by sTMEM2 in a glycosaminoglycan-focused microarray. We observed no binding to any of the probes and have added the negative data for HA probes to the revised manuscript (new Figure 5B).

7. *"The section of the article dealing with enzymatic activity provides mostly preliminary data. I appreciate that the authors are not making any major conclusions from them but the final version of this manuscript would benefit from the addition of some optimization steps or better discussion of obtained results from activity assays."*

   We refer to our response to reviewer 1 (point 4). We are confident that our sTMEM2 protein is not detectably active under the optimum conditions reported by Yamamoto et al. (2017). We feel that this is a valuable observation that should be in the public domain. Of course, we cannot categorically exclude that sTMEM2 has hyaluronidase activity under certain, untested, conditions. We believe we have been careful in the discussion of our negative finding. We concede that the original title did not convey our uncertainty well enough, and we have therefore changed it to *apparent* lack of hyaluronidase activity. We would like to add that, as a matter of courtesy, we informed Dr Yamaguchi of our negative finding a year before we submitted the study for publication.

Competing Interests: No competing interests were disclosed.

Reviewer Report 01 March 2023

https://doi.org/10.21956/wellcomeopenres.20995.r55040

?

Rebekka Wild
Institut de Biologie Structurale, UMR 5075, CNRS, CEA, University Grenoble Alpes, Grenoble, France

In their article, Niu et al. describe the crystal structure of TMEM2, a cell surface protein previously reported to have hyaluronidase activity. Hyaluronic acid (HA) is a long, linear polysaccharide with diverse biological functions and its regulated degradation helps to fine-tune its activity. The described structure at 3.5 Å resolution reveals that TMEM2 folds into a large β-helix and three globular domain. Structural similarities of the β-helix with the chondroitin B lyase from Pedobacter heparinus indicates a potential catalytic site in the concave face of the β-helix. Similarities between the lectin 1 domain and the N-terminal domain of POMGNT1, involved in N-acetylglucosamine binding, suggest a potential involvement in glycan recognition. Despite the structural similarities of TMEM2 with other enzymes involved in glycan metabolism, the purified TMEM2 does not show any detectable *in vitro* hyaluronidase activity and no binding of high-molecular weight hyaluronic acid to TMEM2 was observed in size-exclusion chromatography.

- The TMEM2 structure at 3.5 Å resolution was solved by molecular replacement using the AlphaFold predicted model. A panel illustrating the quality of the map (map around the model) would be helpful to assess the structure.

- A soluble, secreted form of TMEM2 was previously reported to have hyaluronidase activity (Yamamoto et al., JBC 2017). Using similar reaction conditions, Niu et al. don't observe any hydrolysis of high-molecular weight HA (HMW-HA). This article raises the big question if TMEM2 has hyaluronidase activity or not. Although some possible explanations addressing the question why no activity is observed are provided by the authors, a more extensive analysis of this is needed (see experiments suggested below).

- A more comprehensive analysis of the structure with other glucosidases could help to pin-point conserved and distinct residues in a potential active site in more detail.

- The activity assay is carried out at 37°C for 18h, however, the thermal stability of purified TMEM2 is unknown. A thermal stability assay (nano differential scanning fluorimetry or others) would help to confirm that the chosen reaction conditions (T) are appropriate.

- A more extensive screening of different reaction conditions (pH range, metal ions,...) should be considered.

- A 800 kDa HMW-HA is used for enzymatic assays. Partial cleavage of this large polysaccharide would lead to products that could still elute in the void volume of the Sephacryl S200 HR column. It is not discussed in the article, what is the maximum length of

generated fragments that is distinguishable from the uncleaved substrate.

- Only one substrate (800 kDa) HMW-HA was used to study potential TMEM2 activity. Additional activity assays using HA of different/shorter lengths could help to detect hyaluronidase activity.

- Binding of HMW-HA to TMEM2 was studied by size-exclusion chromatography. However, size exclusion chromatography is more useful to study high-affinity interactions rather than low affinity interactions. A second approach to measure TMEM2 interaction with HA or even another glycosaminoglycan could help to reveal lower affinity interactions.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Glycosaminoglycan biosynthesis, structural biology, glycobiology, cryo-EM

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Apr 2023

Erhard Hohenester

1. *"The TMEM2 structure at 3.5 Å resolution was solved by molecular replacement using the AlphaFold predicted model. A panel illustrating the quality of the map (map around the model) would be helpful to assess the structure."*

   A figure of a representative portion of the map has been added to Figure 1.

2. *"A more comprehensive analysis of the structure with other glucosidases could help to pin-point conserved and distinct residues in a potential active site in more detail."*

   Our analysis is now described in more detail. The majority of β-helix enzymes have their active sites in a region that is obstructed by two long loops in TMEM2 (now labelled in Figure 3A). Among the few enzymes that have their active sites in a more C-terminal location, chondroitinase B is the most relevant example in terms of the reaction catalysed and the dependence on calcium ions. Neither electrostatic potential nor sequence conservation are of help in locating a putative active site (revised Figure 2).

3. *"The activity assay is carried out at 37°C for 18h, however, the thermal stability of purified TMEM2 is unknown. A thermal stability assay (nano differential scanning fluorimetry or others) would help to confirm that the chosen reaction conditions (T) are appropriate."*

   We have measured circular dichroism spectra before and after incubation at 37°C for 18 hours and found no evidence of thermal instability. These data are now presented in a revised Figure 4.

4. "*A more extensive screening of different reaction conditions (pH range, metal ions,...) should be considered."*

   We measured activity under the reported optimum conditions for sTMEM2 (Yamamoto et al., 2017). It is important to note that the assay with fluorescently labelled HMW-HA has been used not only by Yamamoto et al., but also by two other groups who showed hyaluronidase activity of cell-associated CEMIP (references 23 and 24 of the revised manuscript). Our experiments include a positive control and we now have experimental verification that our sTMEM2 protein is thermally stable. Therefore, we are confident that our sTMEM2 protein is not detectably active under the reported optimum conditions. We feel that this is a valuable observation that should be in the public domain. Of course, we cannot categorically exclude that sTMEM2 has hyaluronidase activity under certain, untested, conditions. We believe we have been careful in the discussion of our negative finding. We concede that the original title did not convey our uncertainty well enough, and we have therefore changed it to *apparent* lack of hyaluronidase activity. We would like to add that, as a matter of courtesy, we informed Dr Yamaguchi of our negative finding a year before we submitted the study for publication.

5. *"A 800 kDa HMW-HA is used for enzymatic assays. Partial cleavage of this large polysaccharide would lead to products that could still elute in the void volume of the Sephacryl S200 HR column. It is not discussed in the article, what is the maximum length of*

generated fragments that is distinguishable from the uncleaved substrate."

The S200 column would have separated fragments as large as 80 kDa from the void peak. This information has been added to the text.

6. *"Only one substrate (800 kDa) HMW-HA was used to study potential TMEM2 activity. Additional activity assays using HA of different/shorter lengths could help to detect hyaluronidase activity."*

   See our reponse to point 3. We used a substrate that was degraded by cell-associated CEMIP in two independent studies using the same assay.

7. *"Binding of HMW-HA to TMEM2 was studied by size-exclusion chromatography. However, size exclusion chromatography is more useful to study high-affinity interactions rather than low affinity interactions. A second approach to measure TMEM2 interaction with HA or even another glycosaminoglycan could help to reveal lower affinity interactions."*

   In response to this comment, we tested binding of sTMEM2 to a glycosaminoglycan-focused microarray. We observed no binding to any of the probes and have added the negative data for HA probes to the revised manuscript (new Figure 5B). We have also added a line to the discussion that the high avidity at cell-matrix contact sites may allow HA binding and degradation.

Competing Interests: No competing interests were disclosed.