Chapter 7

Identification of HMGB1-Binding Components Using Affinity Column Chromatography

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1. Introduction

High Mobility Group B1 (HMGB1) is a 30 kDa protein widely expressed in mammalian cells. HMGB1 has a high content of charged amino acids and has a bipolar structure consisting of two highly positive amino terminal HMG-box domains and an acidic carboxy terminal tail. HMGB1 has nuclear functions regulating chromatin structure and gene expression and extracellular functions regulating immune response and cell motility. Biochemical and cell biological studies have revealed that HMGB1 binds to various kinds of biomolecules and these interactions are crucial for determining the *in vivo* functions of HMGB1. Albeit several different biochemical methods have been used to detect HMGB1-binding components, HMGB1-affinity column chromatography has rarely been applied in such studies. Here, we describe an affinity chromatography method that we have applied to isolation and identification of HMGB1-binding molecules from different cell types. Biomolecules recovered with HMGB1-affinity chromatography include proinflammatory bacterial DNA and glioblastoma cell histones H1 and H3 which all have previously been reported as HMGB1-binding molecules by other methods. Furthermore, an entirely new HMGB1-binding protein, Multimerin-1 containing complex, was identified from platelet lysates by HMGB1-affinity chromatography. Endogenous Multimerin-1 and HMGB1 were shown to associate on the surface of endothelial cells and activated platelets, and endogenous Multimerin-1 also regulated the release of HMGB1 from activated platelets. In conclusion, HMGB1-affinity chromatography can be used to isolate and characterize novel HMGB1-binding partners from a variety of cellular sources. Such new interactions reveal further complexity in the multi-faceted biology of the HMGB1.

2. HMGB1

2.1. HMGB1 (Amphoterin) as a heparin-binding protein

Extracellular form of HMGB1 was originally identified from the developing rat brain as an adhesive neurite outgrowth promoting molecule. This finding was done in studies where brain lysates were fractionated with heparin affinity chromatography and the activity of brain tissue fractions to induce neurite outgrowth was monitored [1]. Since the isolated neurite outgrowth promoting protein had a bi-polar structure it was named as "Amphoterin" [2]. Its structure turned out to be identical with a previously characterised nuclear DNA-binding protein "High Mobility Group -1 [3]. In current nomenclature this protein is called HMGB1 [4].

In addition to heparin-Sepharose affinity chromatography HMGB1 has been captured as a ligand in other affinity column chromatographies. These studies have been performed using chromatographies where proteins, nucleic acids, lipids or carbohydrates have been used as baits coupled to solid matrices. Studies where HMGB1 has specifically been shown to bind to novel ligands include the use of Receptor for Advanced Glycation End Products (RAGE) [5], single stranded DNA [6], sulfatide [7] and carboxylated glycan [8] affinity columns.

2.2. HMGB1-column affinity chromatography

HMGB1 consists of three domains. Two of them, Box A and Box B, are DNA-binding domains, while the third is an acidic carboxy terminal domain that binds histones H1 and H3 [reviewed in 9 and 10]. The use of HMGB1 affinity column chromatographies in isolation of proteins has been described in the literature. HMGB1-Sepharose column was used as a negative control in a study where a specific Syndecan-3 binding partner was detected. Although Syndecan-3 has highly sulphated glycosaminoglycan side chains, it did not bind to the HMGB1-Sepharose [11]. HMGB1-domain affinity column chromatography has also been used to study other HMGB1 interactions. Thus, Hox proteins were discovered as HMGB1-binding proteins using HMGB-domain coupled Sepharose column chromatography [12]. In addition, the HMGB1-binding peptide of RAGE was determined using HMGB1-domain coupled -Sepharose chromatography [13].

2.3. HMGB1 as a modulator of innate immunity

Most of the HMGB1 studies performed during the last decade have been focused on its functions as a modulator of inflammation. The role of HMGB1 in the immune system was recognised more than a decade ago [14]. Since then additional studies have confirmed that elevated tissue levels of HMGB1 can serve as a general marker of inflammation or tissue damage [reviewed in 15,16 and 17].

In blood circulation, leukocytes [18], platelets [19] and endothelial cells [20] express HMGB1. In unactivated nuclear cells, HMGB1 localises to the nucleus, and in resting platelets, HMGB1 localises to the cytoplasm. After activation, HMGB1 localises towards the periphery of the cell and it is released to the extracellular space via an unconventional secretion pathway [18, 21]. Recently, HMGB1 was also detected in platelet-derived

microvesicles [22]. As a result, serum HMGB1-levels are elevated and the level of HMGB1 in the serum often correlates to the disease severity [23]. Further, the extent of the post translational modifications (acetylation of lysines, proteolytic cleavage or oxidation of cysteines) of HMGB1 correlates to various pathological states [24, 25, 26].

2.4. Platelets

Platelets mediate haemostasis when vessel wall is injured. During hemostasis, platelet receptors work in sequence by slowing down platelets via glycoprotein (GP) Ib/von Willebrand factor to bring them into contact with subendothelial matrix proteins e.g. collagen, then activating them via GPVI to release the contents of various granules and to express integrins in an active state. Thrombus forms when more platelets become incorporated via activated integrin αIIbβ3, which is bridged by several adhesive RGD-containing proteins, but other receptors also finetune the response. Incoming platelets are incorporated into the growing thrombus, until eventually, this activity decreases limiting the size of the thrombus. Simultaneously to the adhesive process, platelets engage in the procoagulant transformation and thrombin generation by changes in the plasma membrane phospholipids and by liberating coagulation factors, e.g. factor V/Va. Platelets also release microvesicles which may spread activation-promoting molecules or recruit heterogenic cell interactions. The final result is that the exposed subendothelium becomes protected by a non-thrombogenic platelet surface while maintaining blood flow. Subsequently, events of tissue repair are initiated to restore the vascular wall and finally to remove the thrombus. In contrast to normal hemostasis, pathological thrombosis manifests these events in an uncontrolled fashion yielding to cessation of blood flow.

Platelets have also recently been discovered to play a role in immunity [27], development [28] and neuroinflammation [29]. Therefore, various heterogenic cell interactions of platelets with other cells such as leukocytes or endothelial cells are of current research interest. Platelets have several interactive mechanisms ranging from the liberation of bioactive molecules to direct membrane-borne cell-cell interactions (e.g. CD62P, CD40L, JAM-A, GPIb) [30].

Platelets contain and liberate bioactive molecules including proteins, lipids and even mRNA and miRNA, which allow them to participate in and modulates diverse physiological functions, some of which at the first glance may seem to be contradictory e.g., pro-and anticoagulant functions. A proteomic analysis of the releasate from TRAP-activated platelets showed that in addition to the nearly 400 previously identified α-granule- or microvesicle-associated molecules, platelets liberated over 300 previously unrecognized molecules [31]. Many of these proteins have interactions with each other which further modulate their biological functions. Clearly, dynamics, mechanisms and selectivity of the different secretion processes must be carefully controlled in the platelets. Versatility seems to be a key property of the platelet.

Platelets bind to HMGB1 but the cell surface receptor mediating this interaction is unknown. Platelets express previously recognised HMGB1 receptors TLR2/4/9 [27], RAGE [32], transmembrane proteoglycans [33] and anionic lipids [19]. Whether these structures mediate HMGB1 binding to platelets has not been much studied. However, previously one study showed that RAGE and TLR2 could mediate HMGB1 binding to platelets [34]. Inhibition of

coagulation or platelet activation *in vivo* reduced serum HMGB1-levels in rat disease models suggesting that platelets are an important source of circulating HMGB1 [35, 36]. Further, circulating HMGB1 levels were shown to correlate with platelet activation markers in patients with hematologic malignancy and disseminated intravascular coagulation [37].

3. Materials and methods

3.1. HMGB1-column affinity chromatographies

3.1.1. Binding of bacterial DNA to HMGB1 affinity column

Recombinant HMGB1 was coupled to activated Sepharose 1 ml High-Trap –column (GE Healthcare, Little Chalfont, UK) according to manufactur´s protocols [38]. HMGB1-affinity chromatography of bacterial lysates and the quantitative analysis of eluted DNA were conducted as described [38].

3.1.2. Binding of histones H1 and H3 to HMGB1 affinity column

C6 glioblastoma cell [39] nuclear fraction was isolated as described [40] and analysed in HMGB1-Sepharose affinity column (1 ml High-Trap column) chromatography using ÄKTA Micro High-performance liquid chromatography station (GE Healthcare) with phosphate buffered saline (PBS) as a chromatography buffer. Flow rate in chromatography was 1 ml/min. HMGB1 column bound proteins were eluted using linear NaCl-gradient (0.15-2 M NaCl, 20 min). 0.5 ml fractions were collected. The protein-containing fractions were analysed in a histone ELISA. Briefly, MaxiSorp microwell plates (Nunc, Roskilde, Denmark) were coated with 2 μg/μl of anti-PAN-Histone antibody (Millipore Corporation, Billerica, MA, USA) in PBS overnight at 4 °C, and wells were blocked with bovine serum albumin (BSA, Sigma-Aldhrich, St. Louis, MO, USA). HMGB1 column eluted C6 cell nuclear protein fractions were diluted seven-fold with 10 mM Tris, pH 7.5, and samples were applied to the wells. After 1h incubation at 37 °C, wells were washed with PBS and the bound histones H1 and H3 were detected with 1/1000 dilution of rabbit anti-Histone H1 antibody (Active Motif, Carlsbad, CA, USA) or rabbit anti-Histone H3 antibody (Cell Signaling Technology, Danvers, MA, USA), followed by horseradish peroxidase -conjugated anti-Rabbit IgG (GE Healthcare) detection..

3.1.3. Isolation of HMGB1 binding component from platelets

Outdated platelet concentrates (Finnish Red Cross Blood Service, Helsinki, Finland) were lysed in lysis buffer [10 mM Tris-HCl, pH 8.5 containing protease inhibitor cocktail (Roche, Basel, Switzerland)] and centrifuged for 2 h at 20 000 g (4 °C). The pellet was extracted overnight at 4 °C with 50 mM octyl-glucoside (Sigma-Aldhrich) – PBS containing 10 μg/ml of aprotinin (Sigma-Aldhrich) and 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldhrich). The homogenate was centrifuged for 2 h at 20 000 g and the supernatant was diluted 10 times with PBS and applied to diethylaminoethanol-Sepharose –column (GE Healthcare). The column was

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washed with 5 mM octyl-glucoside – PBS containing 10 μg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride and bound proteins were eluted with 2 M NaCl 5 mM octyl-glucoside – PBS containing 10 μg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride. HMGB1-binding fractions were identified by a microwell binding assay. Briefly, diluted fractions were used to coat MaxiSorp plate wells and the HMGB1-binding capacity was measured by a recombinant HMGB1 binding assay as described [38].

The molecular weight of the HMGB1-binding platelet protein was determined using gel-filtration chromatography. Diethylaminoethanol-Sepharose –column eluted fractions that bound to HMGB1 were diluted five-fold with 5 mM octyl-glucoside in PBS and were applied to Mono Q column (GE Healthcare), washed with 0.15 M NaCl 10 mM phosphate, 5 mM octyl-glucoside, pH 7.5 and eluted with 0.15 – 2 M NaCl in the same buffer (0-100% gradient, 10 min, 1 ml/min, 1 ml fractions were collected) using ÄKTA Prime chromatography station (GE Healthcare). Absorbance at 280 nm and the relative conductivity of the eluate were monitored. The eluted protein-containing fractions were concentrated and fractionated using the ÄKTA Prime Superdex-75 gel filtration column chromatography. The mobile phase was 5 mM octyl-glucoside, 10 mM phosphate, 0.15 M NaCl, pH 7.5, at flow rate of 0.2 ml/min. 0.5 ml fractions were collected and their HMGB1-binding capacity was measured as described above.

In addition, HMGB1-binding platelet membrane anionic proteins were analysed using a HMGB1-affinity column chromatography and mass spectrometry. Anion exchange colum eluted fractions of platelet membrane proteins were diluted five-fold with 5 mM octyl-glucoside in PBS and applied to HMGB1-Sepahrose column. The column was washed with 5 mM octyl-glucoside in PBS and bound proteins were eluted with 5 mM octyl-glucoside, 2 M NaCl in PBS and analysed in SDS-PAGE. The gel was stained with silver. The major high molecular weight band in the silver stained gel was analysed using mass spectrometry in the Proteomics Unit of Institute of Biotechnology (University of Helsinki, Finland). The eluted non-reduced protein fractions were also analysed in Western blot assay using anti-MMRN1 mouse monoclonal antibody ab56890 (Abcam, Cambridge, UK) as a primary antibody.

3.2. Proximity ligation assay

Proximity ligation assay was performed using Duolink II in situ PLA Protein Detection Kit (Olink Bioscience, Uppsala, Sweden). Human Umbilical Vein Endothelial Cells (HUVEC) –cells were obtained from PromoCell (Heidelberg, Germany) and human platelets were obtained from 3.8% citrate anticoagulated blood donated by healthy volunteers after an informed consent had been obtained according to the Declaration of Helsinki. The following antibodies were used: anti-MMRN1 mouse monoclonal antibody (Abcam), anti-HMG1 rabbit IgG (Pharmingen Becton Dickinson Co, Franklin Lakes, NJ, USA), anti-influenza hemagglutinin -probe (HA-probe) mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-trimethyl-Histone H3 (Lys27) antibody (Millipore).

3.3. Platelet adhesion assay

Static adhesion of washed thrombin-activated mouse platelets to a protein coated microwell plate was measured as described [19, 41, 42].

3.4. Thrombin generation assay

Thrombin generation in solution was determined as described [43] using 80% phosphatidylcholine / 20 % phosphatidylserine lipid vesicles prepared by sonication from 10 mg/ml of lipids (Avanti, Alabaster, Alabama, USA) in 3 mM $CaCl_2$-containing PBS –buffer. For the thrombin generation assay, vesicles were diluted and preincubated for 3 min at 37 °C in buffer B (137 mM NaCl, 10 mM Hepes, 5 mM glucose, 2.7 mM KCl, 2 mM $MgCl_2$, 0.05 % BSA, pH 7.4) with 3 mM $CaCl_2$. Coagulation factor mix with 5 nM Bovine Factor X/Xa (FX and FXa, Enzyme Research Labs Inc., Swansea, UK) and 10 nM Bovine Factor V/Va (FV and FVa, Enzyme Research Labs Inc.) in buffer B with $CaCl_2$ was added and preincubated for 1 min at 37°C prior to initiating the reaction with 10 μM bovine prothrombin (Enzyme Research Labs Inc.). Thrombin formation was assessed by subsampling 40 μl aliquots at selected intervals. Thrombin generation was terminated by the addition of 10 μl of stop buffer (120 mM NaCl, 50 mM Tris, 20 mM EDTA, 0.05 % BSA, pH 7.4). Thrombin activity was measured using a chromogenic substrate S-2238 (Chromogenix, Mölndal, Sweden) and the color reaction was stopped with acid and absorbance was measured at 405 nm. Recombinant HMGB1 was preincubated at a concentration of 5-50 μg/ml with the coagulation factor mix on ice. Final prothrombinase-activating conditions were 3 mM $CaCl_2$, 1.0 nM factor V/Va, 0.5 nM factor X/Xa and 1.0 μM prothrombin.

Thrombin generation on adherent platelets was determined as described [44]. Human platelets were isolated from healthy volunteers who denied having any medication for the previous 10 days. Blood was collected into 1/6 volumes of ACD-buffer (39 mM citric acid, 75 mM sodium citrate, 135 mM D-glucose, pH 4.5) and centrifuged at 200 g for 12 min. Platelet-rich plasma (PRP) was supplemented with 1/10 vol of ACD-buffer and 1/1000 vol of 100 ng/ml prostaglandin E1 (Sigma-Aldhrich) for 15 min. Platelets were centrifuged for 15 min at 650 g, washed once and recentrifuged at 500 g for 15 min before their suspension into Tyrode's buffer (137 mM NaCl, 11.9 mM $NaHCO_3$, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.1 $MgCl_2$ mM, 5.6 mM D-glucose, pH 7.4) with 0.35 % BSA. Platelet concentration was measured spectrofotometrically at 405 nm assuming A 0.025 to correspond to 1 x 10^6 cells/ml. Platelets were adhered at 100 x 10^6 cells/ml on collagen-coated (10 μg/ml, Kollagenreagens Horm, Hormon Chemie, Munich, Germany) for 1 hr at room temperature and washed three times before adding 280 ml of buffer B with 3 mM $CaCl_2$ for 30 min at room temperature, and followed by the thrombin generation assay as described above.

3.5. Release of HMGB1 from thrombin-activated washed mouse platelets

Blood was collected from anesthetised mice using cardiac puncture method and the blood was anticoagulated with 10 mM EDTA and 2 μg/ml of prostaglandin E1. Blood was centrifuged at 120 g for 5 minutes and PRP was collected. Two hundred microliters of CFT-buffer (135 mM

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NaCl, 11.9 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 2.7 mM KCl, 5.6 mM Dextrose, pH 7.4) and 100 μl of Tyrode's buffer were added to rest of the blood and centrifuged again at 120 g for 5 minutes and PRP was collected. Again 200 μl of CFT-buffer and 100 μl of Tyrode's buffer was added to the rest of the blood. The blood was centrifuged at 120 g for 5 minutes and PRP was collected. Finally the PRP-fractions were pooled and 2 μg/ml of prostaglandin E1 was added. PRP was centrifuged at 2 000 g for 6 minutes and the cell pellet was washed twice with CFT - Tyrode's buffer containing prostaglandin E1. The washed platelet pellet was suspended to Tyrode's buffer containing 1 mM $CaCl_2$ and absorbance at 405 nm was measured to determine cell number as described above. Thrombin was added to cells (1 U/ml), cells were incubated at room temperature for 10 minutes and centrifuged at 2 000 g for 6 minutes. The platelet free supernatant was analysed with HMGB1 and mIL-6 ELISA Kits (IBL International GmbH, Hamburg, Germany).

4. Results

4.1. Binding of bacterial DNA to HMGB1-affinity chromatography column

The function of HMGB1-affinity column was validated by evaluating the column´s ability to bind bacterial DNA that is known to form proinflammatory complexes with HMGB1 [45]. Bacterial lysate was loaded to the column, the column was washed and the bound substances were eluted from the column with an increasing salt concentration. DNA in the eluted fractions was detected with a fluorescent DNA-dye. DNA bound strongly to the HMGB1-Sepharose column and did not bind at all to the control Sepharose-column lacking HMGB1 (Figure 1 A). We concluded that HMGB1-Sepharose affinity column in this study functioned in a similar way as the HMGB1-columns used in the previous studies [6, 46, 47].

4.2. Binding of histones H1 and H3 to HMGB1-affinity chromatography column

The function of HMGB1-affinity column was further validated by evaluating its ability to bind histones H1 and H3, since histones H1 and H3 have previously been shown to bind to HMGB1 in biochemical assays other than affinity chromatography [48, 49].

For affinity chromatography, nuclear extracts of C6 glioblastoma cells were loaded to a HMGB1-Sepharose column and the bound proteins were eluted with linear NaCl-gradient. Histones in elution fractions were analysed with ELISA. As a result histones H1 and H3 were detected among eluted proteins (Figure 1 B and C). In conclusion, it was shown that both histone H1 and H3 interact with HMGB1 and also affinity chromatography can be used to detect these interactions.

4.3. Identification of MMRN1-protein complex as a HMGB1-binding structure

Platelets are known to bind HMGB1, however, the cell surface receptors mediating the binding are poorly characterised. Here, we analysed the platelet membrane HMGB1-binding components using chromatographic, enzymatic, immunological and cell biological methods.

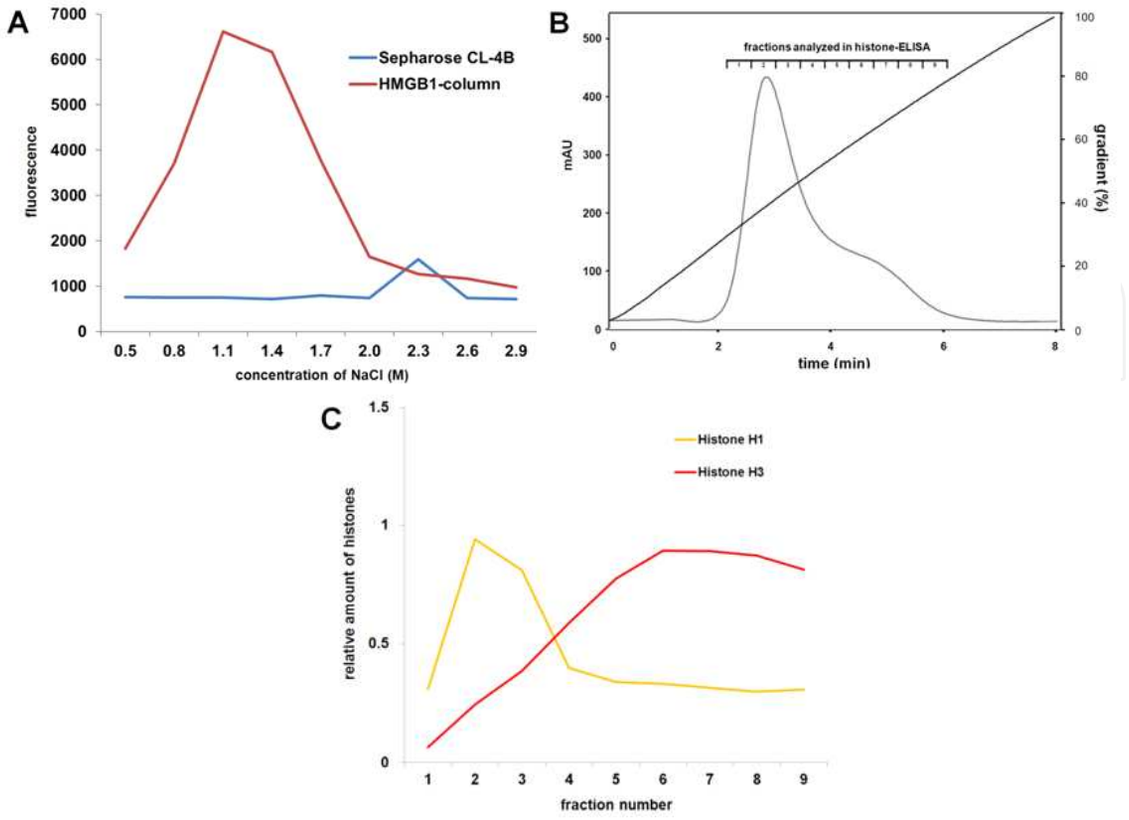

Figure 1. Binding of HMGB1 ligands to HMGB1-affinity chromatography column. A) Sepharose-coupled HMGB1 column was loaded with bacterial lysate and the bound substances were eluted with an increasing salt concentration. Eluted material contained DNA that was detected with a fluorescent DNA-dye. B) HMGB1-affinity column was loaded with a glioblastoma cell nuclear lysate and the bound proteins were eluted with linear NaCl-gradient. Dark grey line indicates salt gradient, light gray line indicates absorbance at 280 nm. C) Protein containing fractions from the chromatography shown in figure 1 B were analysed with histone ELISA. Histones H1 and H3 were eluted from the column with an increasing salt concentration. Histone H3 bound more strongly to the HMGB1-cloumn than did histone H1 (the fraction with the highest absorbance in each assay was determined as 1 and the relative values of other fractions were calculated. N=3, mean values are shown).

Human platelet membrane proteins were isolated with anion exchange chromatography and the isolated proteins were found to bind to recombinant HMGB1 in a microwell binding assay (data not shown). HMGB1-binding proteins were further fractionated using gel filtration chromatography and the binding of size-excluded proteins to recombinant HMGB1 was determined. A high molecular weight protein fraction was found to bind to HMGB1 (Figure 2 A and B).

A similar high molecular weight protein fraction was found to contain endogenous HMGB1 from mouse platelets (Figure 5 A and B). When the anionic human platelet membrane protein fraction was analysed in HMGB1-affinity chromatography, a high molecular weight protein was found to be eluted from the column with high salt buffer (Figure 2 B). Mass spectrometric analysis revealed that this protein had identical tryptic peptide sequences when compared to Multimerin-1 (MMRN1) (Figure 2 C). Western blot analysis of the HMGB1-column eluted

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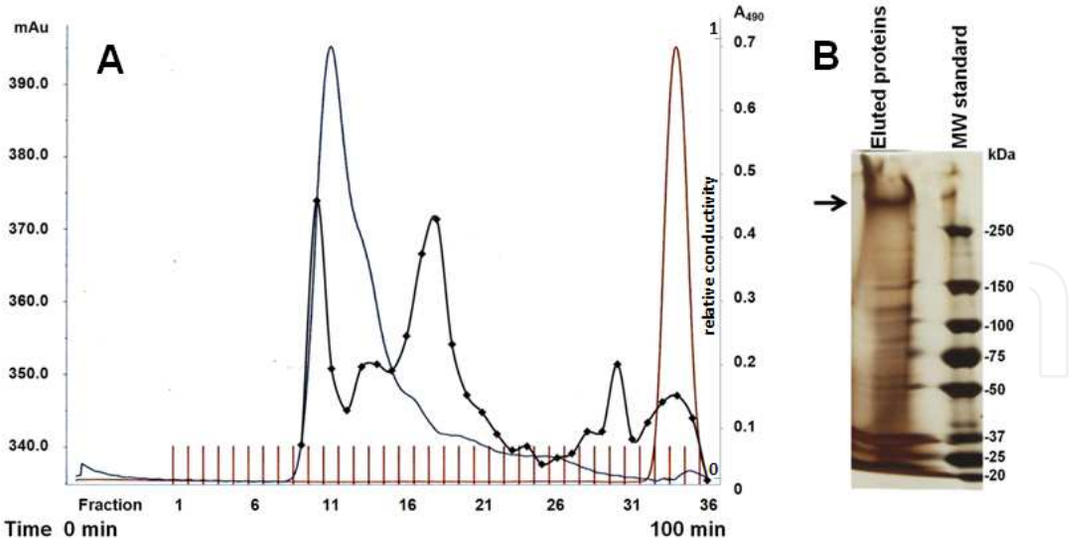

C

Match to: Q13201 Score: 39
Multimerin-1 OS=Homo sapiens GN=MMRN1 PE=1 SV=3

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
412 - 424	712.3538	1422.6930	1422.6838	6	0	K.TVSSLSEDLESTR.Q	(Ions score 32)
796 - 804	541.2983	1080.5820	1080.5968	-14	0	R.YNFVLQVAK.T	(Ions score 24)
951 - 961	646.3725	1290.7304	1290.7296	1	0	K.HSLPDIQLLQK.G	(Ions score 25)
989 - 999	542.8240	1083.6335	1083.6288	4	0	R.SLPGSLANVVK.S	(Ions score 22)

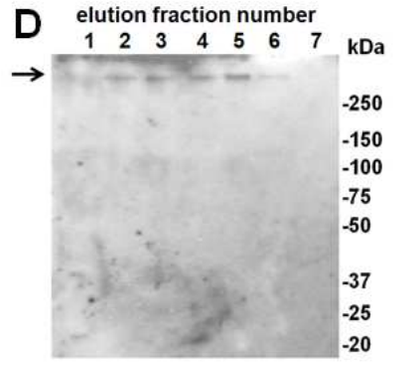

Figure 2. Isolation and identification of the HMGB1-binding component from platelets. A) Anionic platelet membrane proteins were fractionated with size-exclusion chromatography and the binding of recombinant HMGB1 to microwells coated with the chromatography fractions was analysed. The strongest HMGB1-binding was detected in the high molecular weight fraction. Black line in the chromatogram indicates absorbance at 490 nm in HMGB1-binding assay. Blue and red lines indicate absorbance at 280 nm (mAu) and relative conductivity, respectively. Fractions are indicated by short red vertical lines. B and C) Platelet membrane proteins were eluted from the HMGB1-affinity column and analysed by SDS-PAGE followed by mass spectrometry. The high molecular weight protein band indicated by the arrow was identified as Multimerin-1. D) A Western blotting analysis of platelet membrane proteins eluted with high salt from HMGB1-affinity column. The arrow points to the high molecular weight band detected with an anti-Multimerin-1 antibody.

anionic platelet membrane proteins with anti-MMRN1 monoclonal antibody confirmed the finding that MMRN1 bound to HMGB1-column (Figure 2 D).

Since both HMGB1 and MMRN1 are present on the surface of activated platelets and cultured endothelial cells, we studied the association of HMGB1 and MMRN1 on the cell surface using a proximity ligation assay. With the proximity ligation assay, it could be demonstrated that HMGB1 and MMRN1 are in close proximity of each other on both the activated platelet and the cultured endothelial cell surfaces (Figure 3).

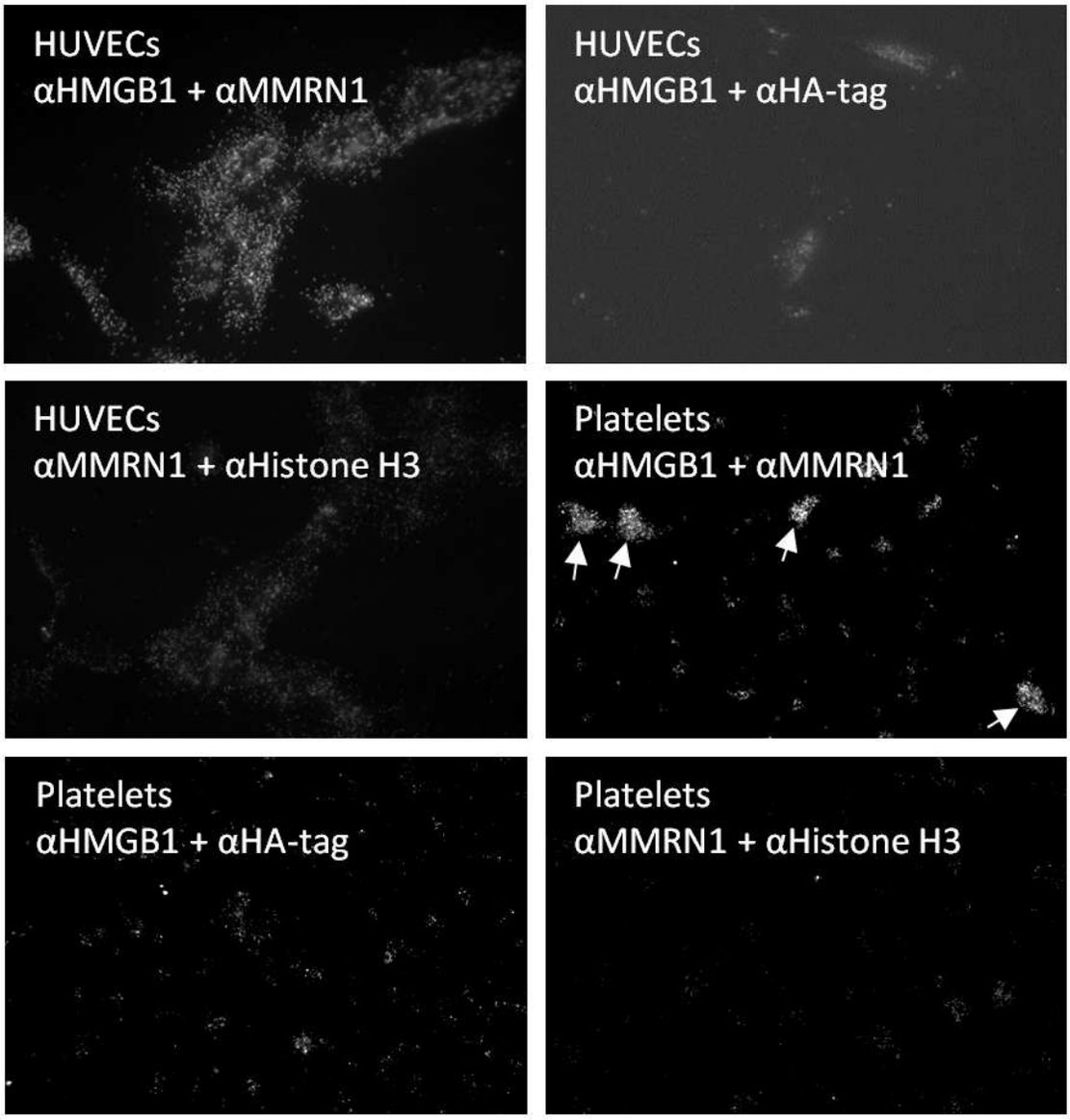

Figure 3. Endogenous HMGB1 associates with MMRN1. Proxomity ligation assay with anti-HMGB1 and anti-MMRN1 antibodies revealed a close association of HMGB1 and Multimerin-1 on both the endothelial cell and the activated platelet surfaces (arrows indicate positively stained platelets). Negative control stained cells with anti-trimethyl Histone H3 and anti-MMRN1 antibodies or with anti-HA-probe and anti-HMGB1 antibodies did not yield significant signals in the proxomity ligation assay.

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MMRN1 has been shown to enhance platelet adhesion to collagen. Next, we tested whether MMRN1 mediates static adhesion of activated platelets to HMGB1. We used both platelets derived from control C57BL/6JOlaHsd mice deficient in Multimerin-1 and α-synuclein genes and platelets derived from C57BL/N6 mice expressing Multimerin-1 and α-synuclein [50]. We could not observe any significant differences in platelet adhesion to HMGB1 between the two different mouse strains (data not shown).

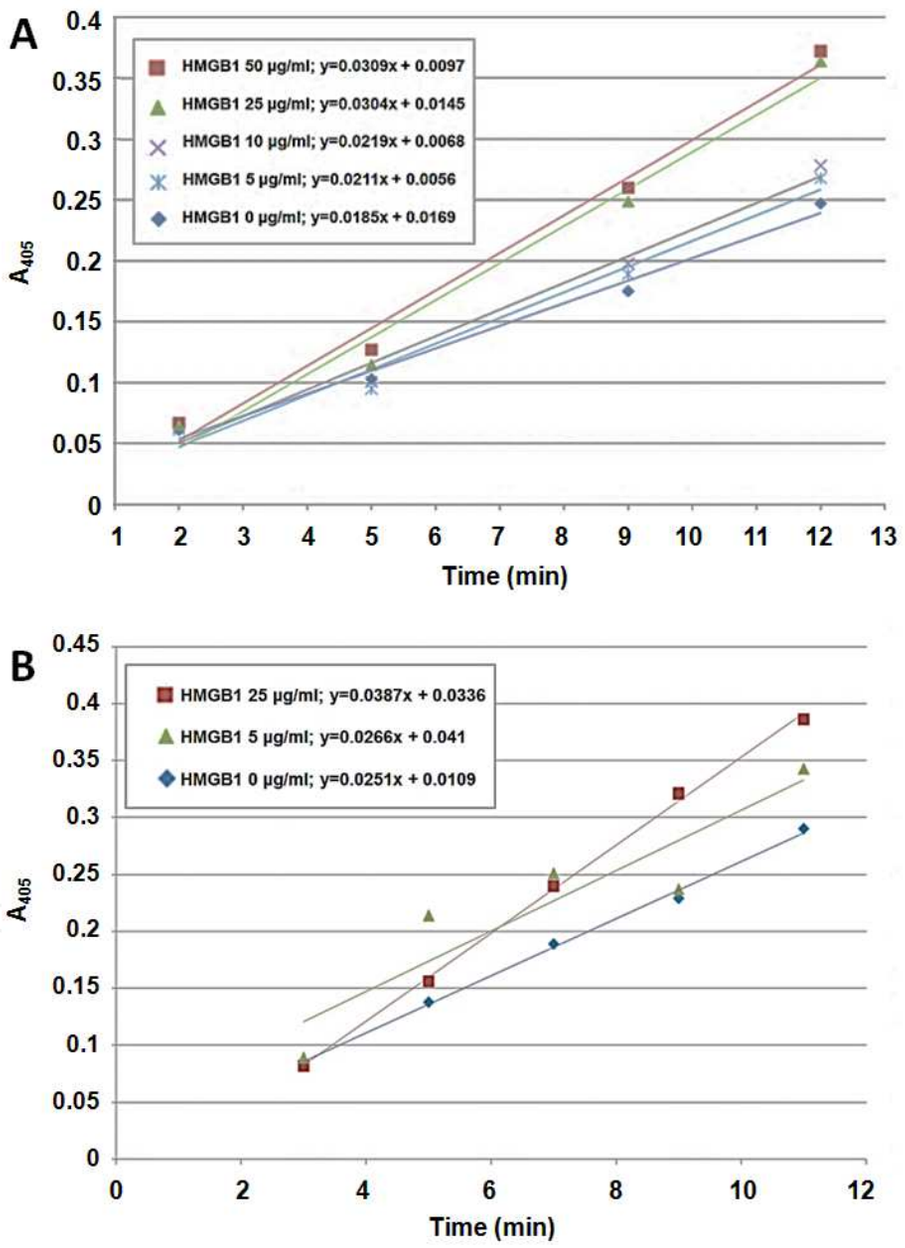

Figure 4. HMGB1 potentiates thrombin generation. Factor Va/Xa mediated thrombin generation was potentiated by recombinant HMGB1 in a similar way on both phospholipid vesicles (A) and the activated platelet surfaces (B). Potentiation occurred in both cases at ≥25 µg/ml concentration of recombinant HMGB1. 0 = control with PBS as a vehicle. A representative graph of three repetitions is shown.

Both MMRN1 and HMGB1 bind to phosphatidylserine [19, 38, 51]. Previously, it has been shown that HMGB1 enhances the effects of thrombin *in vivo* [52]. In contrast, MMRN1 has been shown to inhibit thrombin generation in plasma *in vitro* [53]. Therefore, we tested whether HMGB1 affects FXa- and FVa-catalysed thrombin generation on phosphatidylcholine/ phosphatidylserine vesicles and on activated human platelets. Addition of exogenous HMGB1 enhanced thrombin generation on both the vesicle and the activated platelet surfaces (Figure 4 A and B). The minimal concentration of HMGB1 needed for enhancement was 25 μg/ml on both surfaces suggesting that the potentiation of thrombin generation by HMGB1 is mediated by similar mechanism in both systems, ie. the potentiation requires a lipid surface, but it is independent of other structures, including MMRN1 present on the activated platelet surface.

We further evaluated the possible role of MMRN1 on the biology of HMGB1. Gel filtration assays of platelet protein lysates from mouse platelets lacking MMRN1 and α-synuclein genes and from mouse platelets having MMRN1 and α-synuclein genes revealed that the sendogenous HMGB1 is mainly complexed with high molecular weight structures indicating a MMRN1-independent high molecular weight HMGB1-complex formation (Figure 5 A and B). Finally, the release of HMGB1 from thrombin-activated washed mouse platelets was measured with an ELISA. The concentration of HMGB1 released from 1 U/ml of thrombin-activated washed C57BL/N6 mouse platelets was 5.7 ± 0.8 ng/ml (n=4). We observed a significant difference in the concentrations of the 1 U/ml of thrombin-activated platelet released HMGB1 between the mice lacking MMRN1 and α-synuclein and the mice having MMRN1 and α-synuclein (Figure 5 C). Platelets from the mice lacking MMRN1 and α-synuclein genes released more HMGB1 than platelets from the mice having MMRN1 and α-synuclein genes. Platelets from mice lacking MMRN1 and α-synuclein genes released more HMGB1 than platelets from mice having MMRN1 and α-synuclein genes (Figure 5 C). α-Synyclein regulates vesicle release in many cell types. However, measurement of IL-6 release did not show any difference between the two mouse strains, which is corroborated by the finding that the activation of both types of mouse platelets with 1 U/ml of thrombin induced similar release of P-selectin [54]. This suggests that the observed difference in HMGB1 release may not be due to a differential release response to a high concentration of thrombin. HMGB1 has been described to bind to the filamentous pathological form of α-Synyclein but not to monomers [55]. However, since α-synuclein occurs in a monomeric form in the platelets [56], the lack of α-synuclein hardly affects the release of HMGB1.

5. Conclusions

In this study, we isolated a MMRN1-complex with HMGB1-affinity chromatography from platelets. MMRN1 and HMGB1 were shown to exist at a close proximity of each other on both the endothelial cell and the activated platelet surface, and the release of HMGB1 from activated platelets from mice lacking MMRN1 and α-synuclein genes was increased. Further, HMGB1 potentiated FVa/FXa catalysed thrombin generation on both artificial anionic vesicles and the activated platelet surface.

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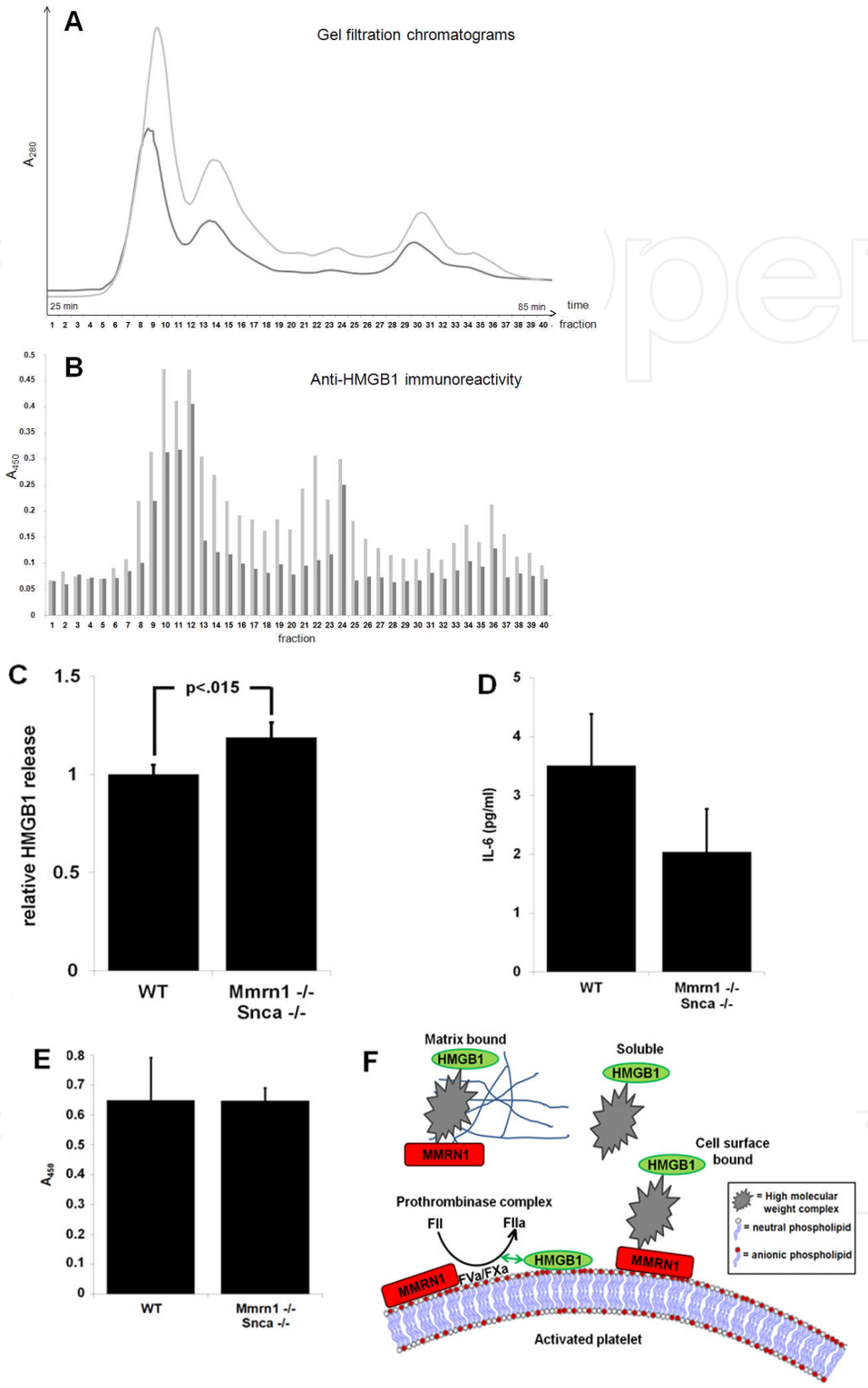

Figure 5. Size-exclusion chromatography and the secretion analyses of HMGB1 using washed platelets derived either from Mmrn1/Snca negative C57BL/6JOlaHsd mouse or C57BL/N6 mouse expressing MMRN1/SNCA. A) Gel filtration of mouse platelet anionic proteins. Light grey line indicates platelet proteins from C57BL/6JOlaHsd mouse and dark grey line indicates platelet proteins from C57BL/N6 mouse. B) Binding of anti-HMGB1 antibody to the microwells coated with the gel filtration eluted fractions. Light grey bars indicate the fractions from C57BL/6JOlaHsd mouse pla-

telets and dark grey bars indicate the fractions from C57BL/N6 mouse platelets. C) Higher concentration of HMGB1 is detected in platelet released material derived from 1 U/ml of thrombin-activated mouse platelets lacking MMRN1/SNCA when compared to released material derived from 1 U/ml of thrombin-activated mouse platelets expressing Mmrn1/Snca (n=6 C57BL/6JOlaHsd mice, n=8 C57BL/N6 mice; mean HMGB1 amount in C57BL/N6 derived samples was determined as 1 and the relative values were calculated). HMGB1 levels were at the linear range of ELISA detection. D) In contrast to HMGB1-release, there was no difference in interleukin-6 release from either type of platelets. E) Absorbance at 405 nm was equal in washed platelet fractions derived from either strain indicating the existence of equal number of cells (n=4). F) A hypothetical model of association of HMGB1 to the activated platelet surface and matrix. Activated platelets express phosphatidylserine on their surface. The prothrombinase complex consisting of coagulation factors FVa and FXa, MMRN1 and HMGB1 can all bind to phosphatidylserine. Phosphatidylserine surfaces catalyse the formation of thrombin (FIIa) from prothrombin (FII). HMGB1 can potentiate thrombin generation. Activated platelets release HMGB1 that can be found in the media either as a soluble molecule or bound to microvesicles. It can also bind to the extracellular matrix and the cell surfaces. The release of HMGB1 from activated platelets was found to be increased in the platelets derived from Mmrn1/Snca negative mice. Possible explanations for this phenomenon are that MMRN1 complex anchors HMGB1 to the cell surface or it interferes with HMGB1-ELISA detection like some other HMGB1 binding components (60).

MMRN1 is a variably-sized homopolymer belonging to the disulphide–linked multimeric proteins of the elastin microfibril interface located protein (EMILIN) family. Megakaryocytes, platelets and endothelial cells carry MMRN1 in their secretory granules, α-granules or Weibel-Palade bodies, respectively. MMRN1 size can range from trimers to large disulphide linked polymers which can exceed millions of Daltons [57, 58]. The prepro-MMRN1 molecule contains 1228 amino acids with a 19 amino acid signal peptide. MMRN1becomes only liberated upon cell activation and has not been detected as a free plasma molecule. MMRN1 has functions in both platelet adhesion and procoagulant activity. As an interesting link to immunity, MMRN1 has also been shown to mediate neutrophil binding [59].

The adhesive function of MMRN1 is manifested in its several molecular features (for a review see reference [60]). MMRN1 can assemble into fibrils and become associated to extracellular matrix proteins such as collagens type I and type III [62]. MMRN1 contains the RGD-sequence by which it can interact with e.g. integrins $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$, but integrin-independent binding can also occur via phosphatidyl serine [61]. MMRN1 has been shown to support platelet adhesion even at high shear rates thus resembling the function of von Willebrand factor [62]. Additionally, MMRN1 has been shown to increase von Willebrand factor -dependent platelet adhesion to collagen.

In addition to platelet adhesion, MMRN1 participates in procoagulant activity. Like FVa, MMRN1 binds to phospholipids [51]. MMRN1-lipid binding was enhanced by increasing phosphatidylserine content of phosphatidylserine:phosphatidylethanolamine membranes, and by increasing phosphatidylethanolamine and cholesterol content of low phosphatidylserine membranes [60]. Additionally, MMRN1 binds both inactive and active factor V with high affinity (Kd 2 and 7 nM), but surprisingly, its role in thrombin generation has been suggested to be inhibitory [53]. Exogenous MMRN1 has been shown to delay and reduce thrombin generation by plasma and platelets. In this capacity, MMRN1 could act as controller of unlimited thrombin generation upon vascular injury. However, the effect of MMRN1 on FVa function *in vivo* has not been explored.

MMRN1 gene and α-synuclein gene knockout mice were used to study Ferric chloride – induced thrombus formation with intravital microscopy. In these mice, platelet adhesion and

thrombus formation were impaired and the deficit could be corrected with exogenous MMRN1 [54].

No specific MMRN1 deficiency in humans or animals has been reported. MMRN1 deficiency is not lethal since a deletion combining the major part of MMRN1 gene and a-synuclein in mice renders them viable and without an obvious phenotype [50]. In humans, MMRN1 gene has been linked to Parkinson´s disease and neurodevelopmental disorders [63, 64]. In addition, a genetic multiplication of SNCA and MMRN1 locus in humans can lead to parkinsonism [65]. Whereas α-synuclein gene is highly expressed in the brain, the expression of MMRN1 gene can be detected in neural stem cells [65, 66].

Our results show that endogenous HMGB1 associates to a high molecular weight MMRN1-complex in human platelets. However, HMGB1 also associates to a high molecular weight complex in mouse platelets lacking MMRN1, suggesting that MMRN1 itself does not mediate the HMGB1-interactions within the complex. MMRN1 can instead mediate interactions of the complex with the activated platelet surface leading to a decreased amount of platelet released soluble HMGB1 (Figure 5). The mechanism of the HMGB1 induced potentiation of thrombin formation observed in this study remains unknown. Both the prothrombinase complex and HMGB1 bind to phosphatidylserine. However, whether HMGB1 is in direct contact with coagulation factors requires further investigation.

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