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Development and Characterization of Monoclonal Antibodies Against Protease Activated Receptor 4 (PAR4)

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Abstract

Background—Protease activated receptor 4 (PAR4) is a G protein coupled receptor (GPCR) which is activated by proteolytic cleavage of its N-terminal exodomain. This generates a tethered ligand that activates the receptor and triggers downstream signaling events. With the current focus in the development of anti-platelet therapies shifted towards PARs, new reagents are needed for expanding the field's knowledge on PAR4. Currently, there are no PAR4 reagents which are able to detect activation of the receptor.

Methods—Monoclonal PAR4 antibodies were purified from hybridomas producing antibody that were generated by fusing splenocytes with NS-1 cells. Immunoblotting, immunofluorescence, and flow cytometry were utilized to detect the epitope for each antibody and to evaluate the interaction of the antibodies with cells.

Results—Here, we report the successful generation of three monoclonal antibodies to the N-terminal extracellular domain of PAR4: 14H6, 5F10, and 2D6. We mapped the epitope on PAR4 of 14H6, 5F10, and 2D6 antibodies to residues (48–53), (41–47), and (73–78), respectively. Two of the antibodies (14H6 and 5F10) interacted close to the thrombin cleavage and were sensitive to α -thrombin cleavage of PAR4. In addition, 5F10 was able to partially inhibit the cleavage of PAR4 expressed in HEK293 cells by α -thrombin.

Conclusions—These new antibodies provide a means to monitor endogenous PAR4 expression and activation by proteases on cells.

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Addendum: M. M. Mumaw and M. T. Nieman conceived and designed the experiments. M. M. Mumaw, M. de la Fuente, A. Arachiche, and J. K. Wahl III performed the experiments. M. M. Mumaw and M. T. Nieman analyzed the data. M. M. Mumaw and M. T. Nieman wrote the manuscript.

Keywords

protease activated receptor 4; monoclonal antibody; G-protein coupled receptor; thrombin receptor; platelets

Introduction

Protease activated receptors (PARs) are a unique family of seven transmembrane receptors, G-Protein Coupled Receptors (GPCRs), that are activated by proteolysis of their N-terminus [1]. Once cleaved, the newly exposed N-terminus serves as a tethered ligand that activates the receptor by binding extracellular loop 2 [2–4]. There are four members of the PAR family (PAR1–4), which are widely expressed and activated by multiple proteases [1, 5]. PARs are capable of initiating signaling through G_i , G_q , and $G_{12/13}$ depending on the activating protease and cellular context [6–10]. PAR1 and PAR2 have been the most thoroughly studied, however recent advances have renewed interest in PAR4.

The functional roles of PAR4 have primarily been elucidated on platelets. PAR4 is traditionally thought of as a low-affinity thrombin receptor that serves as a redundant, back up receptor to PAR1. This view is fueled by the overlapping signaling functions of PAR1 and PAR4. However, several recent studies demonstrate that PAR4 has distinct signaling properties [11–13]. A key feature that distinguishes PAR4 is its ability to form hetero-oligomers with both PAR1 and the ADP receptor P2Y₁₂, which allows PAR4 to influence both thrombin and ADP initiated signaling [14–17]. For example, the rate of PAR4 cleavage is significantly enhanced by coexpression of PAR1 and PAR4 through hetero-oligomerization [17–20]. Although it remains to be determined if PAR1-PAR4 hetero-oligomers initiate distinct signaling pathways. In contrast, the interaction between PAR4 and P2Y₁₂ is directly linked to arrestin-2 recruitment and AKT signaling [15, 16]. The lateral associations of PAR4 with PAR1 and P2Y₁₂ place PAR4 at the center of platelet signaling. These interactions take on clinical significance in the context of current antiplatelet therapies that target PAR1, P2Y₁₂, or both in which case thrombin signaling will be funneled exclusively through PAR4 [21].

It is well known that there are multiple genetic risk factors for cardiovascular disease. Among these is a heritable inter-individual variation in platelet reactivity, which is greater in black than white individuals [22]. Recent studies directly link these differences to PAR4 [23–25]. Edelstein and colleagues provide direct genetic evidence for PAR4 by identifying polymorphisms that change amino acids in PAR4 at positions 120 (Ala/Thr) and 296 (Phe/Val) [24]. The polymorphism at 120 is common and is distributed by race. PAR4–120A exhibited a lower reactivity and was found primarily in white individuals, whereas, PAR4–120T was hyper reactive to agonists, resistant to a PAR4 antagonist, and found primarily in black individuals. The precise mechanism by which these PAR4 mutants effect PAR4 activation still needs to be examined.

Although PAR4 has been primarily studied in platelets, it has physiologic roles in other tissues and disease states. PAR4 inhibition has cardioprotective effects against myocardial ischemia/reperfusion injury [26]. In rodents, PAR4 has been shown to play a role in joint

pain and inflammation [27–29]. In addition, PAR4 expression is enhanced in high glucose stimulated human vascular smooth muscle cells [30]. And most recently, Pavic and colleagues demonstrated that PAR4 expression was upregulated in diabetes and plays a role in diabetic vasculopathy [31]. The sum of these studies demonstrates that PAR4 has emerging roles beyond platelets that need to be explored.

Currently, there is a paucity of good reagents for studying PAR4. Here, we report the successful generation of three monoclonal antibodies to the extracellular N-terminus of PAR4: 14H6, 5F10, and 2D6. We have mapped the epitope of these antibodies and each interacts with a unique region on PAR4. Two of the antibodies (14H6 and 5F10) interact near the thrombin cleavage site and are sensitive to α -thrombin cleavage of PAR4. Further, 5F10 partially blocks thrombin cleavage of PAR4. This new panel of PAR4 antibodies will allow experiments that monitor the expression and activation of PAR4 on cells without the use of exogenous epitope tags. These antibodies provide the field with essential tools to determine the roles of PAR4 in greater detail.

Materials and Methods

Reagents and Antibodies

Human α -thrombin was purchased from Haematological Technologies (specific activity 3200–3400 U/mg) (Essex Junction, VT). The secondary antibody IRDYE 800CW donkey anti-mouse IgG was purchased from LI-COR Biosciences (Lincoln, NE). HA-Tag (6E2) mouse mAb (Alexa Fluor® 647 Conjugate) was purchased from Cell Signaling Technology (Beverly, MA). Poly-L-Lysine and fibrinogen were purchased from Sigma-Aldrich (St. Louis, MO). VECTASHIELD mounting medium was purchased from Vector Laboratories, Inc. (Burlingame, CA). All cell culture reagents, zeocin, hygromycin B, and secondary antibodies Alexa Fluor® 647 donkey anti-mouse IgG and Alexa Fluor® 488 goat anti-mouse IgG were purchased from Invitrogen.

Cell Culture

NS-1 cells and J7774A.1 cells were grown in HY media (DMEM, NCTC, insulin, oxalacetic acid, and pyruvic acid) supplemented with 20% FBS and 1% Penicillin-Streptomycin. Flp-In™ T-REx™ 293 cells were purchased from Invitrogen and were cultured in DMEM supplemented with 10% fetal bovine serum, 1% Pen/Strep, and Zeocin. To generate Flp-In™ T-REx™ 293 cells expressing PAR4, cells were transfected with pOG44 vector and pcDNA5/FRT containing PAR4 using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells stably expressing PAR4 were selected with Hygromycin B (200 μ g/ml). PAR4 expression was induced with tetracycline (1 μ g/ml) for 36 hr.

Production and Purification of Recombinant Proteins

The full-length cDNA for human PAR4 was purchased from UMR cDNA resource center. Maltose binding protein-PAR4 fusion proteins (MBP-PAR4(18–78), MBP-PAR4(41–66), MBP-PAR4(48–72), and MBP-PAR4(54–66) (see Figure 1) were generated by amplifying the region of interest with PCR and subcloning into pMAL-c2 as an EcoRI-HindIII fragment. The specific primer sequences are available upon request. The MBP-PAR4 fusion

proteins were expressed in BL-21 *E. coli* and were purified by affinity chromatography on an amylose column. Briefly, the lysate was loaded onto an amylose column pre-equilibrated with column buffer (20 mM Tris-HCl pH 7.4, 0.2M NaCl, 1mM EDTA) at 4°C. The column was washed with 12 column volumes of column buffer, and the protein was eluted in 1 ml fractions with elution buffer (20 mM Tris-HCl pH 7.4, 0.2M NaCl, 1mM EDTA, 10 mM maltose). The concentration of purified protein was determined using BioRad protein assay kit.

Animals

F2RL3^{-/-} mice (referred to as PAR4^{-/-}) were obtained from the Mutant Mouse Regional Resource Center (MMRRC) (Chapel Hill, NC). All animal studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University School of Medicine.

Mouse Immunization and Antibody Production

The method was modified from the previously described protocol by James K. Wahl, III [32]. Five week old C57BL/6 F2RL3^{-/-} mice were subcutaneously injected with 150 µg of antigen (MBP-hPAR4(18–78)) in complete Freud's adjuvant. The mice were administered 150 µg of antigen via intraperitoneal injections every 2 weeks for 6 weeks. Two weeks after the final injection, additional boosts were given daily for three days prior to harvesting the splenocytes. Mouse primary splenocytes were isolated and fused with the myeloma cell line NS-1 using polyethylene glycol. The fusion was plated into 96 well plates and treated the following day with aminopterin to remove unfused NS-1 cells. The hybridoma supernatants were screened by immunoblotting against maltose binding protein (MBP) and MBP-PAR4 antigen. Positive hybridomas were selected by limiting dilution and maintained in HY media.

Antibody Purification

The antibody supernatant was isolated from hybridoma cells by centrifugation (200×g for 5 min). The supernatant was clarified by centrifuging at 14000×g for 10 min at 4°C. The supernatant was loaded onto a Protein A column pre-equilibrated with 20 mM sodium phosphate buffer pH 7.0. The column was then washed with 20 mM sodium phosphate buffer pH 7.0. The antibody was eluted from the column with 0.1 M Glycine-HCl pH 2.5 and the pH was immediately raised to 8.0 with 1 M Tris pH 9.0. The purified antibody was placed in dialysis buffer (0.1 M NaHCO₃ pH 8.5, 0.5 M NaCl) overnight. The antibody concentration was quantitated using a NanoDrop ND-1000 spectrophotometer (molar extinction coefficient 210,000 M⁻¹ cm⁻¹).

Monoclonal Antibody Isotype

The purified antibodies were diluted (100 ng/ml) and loaded on to isotyping cassettes (Thermo Scientific Pierce Rapid Mouse Antibody Isotyping Kit). The isotype for each antibody was determined (14H6: IgG2b, kappa; 5F10: IgG2b, lambda; 2D6: IgG1, kappa).

Western Blot Analysis

Immunoblotting was used to determine the epitope for each antibody. MBP, MBP-PAR4(18–78), MBP-PAR4(41–66), MBP-PAR4(48–72), and MBP-PAR4(54–66) (5 µg) were loaded and resolved by SDS-PAGE and transferred onto nitrocellulose. The membranes were incubated with purified primary monoclonal antibody (anti-MBP, 14H6, 5F10, or 2D6) for 1 hr. Followed by incubation with secondary antibody IRDYE 800CW donkey anti-mouse IgG for 1 hr. The membranes were developed using the Odyssey Infrared Imaging System.

For HEK293 cells expressing PAR4 and human platelets, the cells were lysed in RIPA buffer (1% NP-40, 0.5% Deoxycholate, 0.1% SDS) on ice. Following lysis, the lysate was collected and quantitated using the Biorad DC protein assay. 100µg of total protein was loaded and resolved by SDS-PAGE and transferred onto nitrocellulose. The membranes were incubated with 50 µg primary antibody (anti-MBP, 14H6, 5F10, or 2D6) for 1 hr, secondary antibody IRDYE 800CW donkey anti-mouse IgG for 1 hr, and developed using the Odyssey Infrared Imaging System.

Flow Cytometry Analysis

Flp-In™ T-REx™ 293 cells were treated with 1 µg/ml tetracycline for 36 hrs to induce expression of HA-PAR4. Cells (2×10^5) were washed in suspension twice with PBS; some cells were treated with α -thrombin (100 nM) for 5 min at 37°C prior to staining. For staining, the cells were incubated in PBS containing 2% BSA and treated with 2 µg of primary antibody (14F6, 5F10, 2D6) for 1hr, washed with PBS twice, treated with anti-mouse 647 secondary antibody (10 µg/ml) in PBS with 2% BSA for 30 min, and washed twice with PBS. The cells were analyzed using the BD LSRFortessa cell analyzer.

For measuring the effect on 14H6 and 5F10 on PAR4 cleavage, the experiment was carried out the same as described above except the cells were pretreated with 14H6 or 5F10 (2–50 µg) for 10 minutes and then activated with 100 nM α -thrombin for 5 min at 37°C. Following activation the cells were labeled with anti-HA-Tag Alexa Fluor 647 (2.5 µg/ml, clone 6E2, Cell Signaling). Data are expressed as percent cleavage, which was calculated by subtracting the mean fluorescence intensity from uninduced cells and setting it to 0%. Induced cells without thrombin were set to 100%.

Human Platelet Isolation

Human platelets were obtained from healthy donors. These studies were approved by the Case Western Reserve University Institutional Review Board and informed consent was obtained from all donors. Whole blood was collected into the anticoagulant acid citrate dextrose (ACD) (2.5% sodium citrate, 71.4 mM citric acid, 2% D-glucose) and centrifuged at $200 \times g$ for 10 minutes at room temperature. The top PRP layer was removed and centrifuged at $400 \times g$ for 10 minutes at room temperature to pellet the platelets. The platelet pellet was resuspended in HEPES-Tyrode buffer pH 7.4 (10 mM HEPES, 12 mM NaHCO₃, 130 mM NaCl, 5 mM KCl, 0.4 mM Na₂HPO₄, 1 mM MgCl₂, and 5 mM glucose). The number of platelets was quantitated using a Hemavet 950FS (Drew Scientific Inc, Waterbury, CT, USA).

Immunofluorescence Microscopy

Flp-In™ T-REx™ 293 cells adherent to coverslips coated with Poly-L-Lysine were treated with 1 µg/ml tetracycline for 36 hrs to induce expression of PAR4. The cells were fixed using 4% formaldehyde, blocked (1% BSA and 40 µg/ml non-immune human IgG in PBS), incubated with 20 µg primary monoclonal antibody (14H6, 2D6, 5F10), and labeled with secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG). The coverslips were mounted on standard glass slides using VECTASHIELD mounting medium. For human platelets, washed coverslips were coated with fibrinogen and subsequently blocked with 5% BSA in PBS. 0.5 ml of washed human platelets (1×10^7 platelets/ml) in Medium 199 was added to the coverslips and let adhere for 30 min at 37°C. The platelets were activated with thrombin (1 nM) for 2 hrs at 37°C. The platelets were then treated the same as HEK293 cells described above.

Results and Discussion

Generation of PAR4 Monoclonal Antibodies

Protease activated receptors are activated by cleavage of their N-terminus and antibodies targeted to this region have the potential to monitor receptor cleavage and internalization on primary cells. In this study we aimed to generate monoclonal antibodies against the N-terminal extracellular domain of PAR4. We used the entire mature exodomain of human PAR4 fused to maltose binding protein (MBP) as an antigen (MBP-PAR4(18–78)) (Figure 1). To maximize the immune response from the MBP-PAR4 fusion protein, we used PAR4^{-/-} mice as the host for antibody production. The hybridomas resulting from the splenocyte/NS-1 fusion were screened by immunoblotting for MBP and MBP-PAR4(18–78) simultaneously. The antibodies that recognized both recombinant proteins were discarded as they were directed toward the MBP region of the fusion protein.

Identification of the Antibody Epitopes on PAR4

The initial screening and epitope mapping resulted in antibodies that fell into 3 classes; the respective hybridomas were selected by serial dilution and further analyzed. Our preliminary screen against a panel of MBP-PAR4 fusion proteins identified three classes of antibodies. One antibody from each class (14H6, 5F10, and 2D6) were chosen to map the epitope on PAR4 in greater detail (Figure 1). Equal loading on the gel was verified by blotting with MBP antibody (Figure 2A). 14H6 interacted with MBP-PAR4(18–78), MBP-PAR4(41–66), and MBP-PAR4(48–72), but not MBP-PAR4(54–66) indicating that the PAR4 antibody 14H6 recognizes residues 48–53 of PAR4 (Figure 1, Figure 2B). 5F10 interacted with MBP-PAR4(18–78) and MBP-PAR4(41–66), but not MBP-PAR4(48–72) or MBP-PAR4(54–66) indicating that the PAR4 antibody 5F10 interacts with amino acids 41–47 of PAR4 (Figure 1, Figure 2C). 2D6 only recognized MBP-PAR4(18–78) (Figure 2D) indicating the epitope is at either end of the PAR4 exodomain (amino acids 18–40 or 73–78). The epitope was ultimately mapped to amino acids 73–78 (see below and Figure 3).

Detection of PAR4 Cleavage

Two of the antibodies (14H6 and 5F10) mapped epitopes near the thrombin cleavage site. Antibodies sensitive to PAR4 cleavage by thrombin would be useful reagents to monitor endogenous PAR4 activation on cells. To test if 14H6 and 5F10 recognized cleaved PAR4, MBP-PAR4(18–78) was proteolyzed with α -thrombin (100 nM) and the resulting MBP-PAR4(18–47) fragment was resolved by SDS PAGE. Neither 14H6 nor 5F10 recognized the thrombin cleaved PAR4 indicating that the epitope was disrupted following proteolysis (Figure 3). Our preliminary characterization of 2D6 mapped the epitope to either the N- or C-terminus of the PAR4 exodomain (Figure 2). The epitope was further mapped using thrombin cleaved MBP-PAR4(18–78). Following proteolysis, the 2D6 epitope was lost indicating that 2D6 binds C-terminal to the cleavage site; the 30 amino acid fragment (48–78) is too small to be detected on the gel (Figure 3). The combined mapping experiments determined the 2D6 epitope to be amino acids 73–78, the extreme C-terminus of the PAR4 exodomain.

In this study we focused on α -thrombin activation of PAR4. However, numerous other proteases (trypsin, plasmin, cathepsin) also activate PAR4 and therefore, cause platelet activation [33–35]. Plasmin, trypsin, and α -thrombin all cleave PAR4 at Arg⁴⁷. Therefore, 14H6 and 5F10 will be able to detect PAR4 activation by all of the PAR4 activating proteases in endogenous cells.

Recognition of PAR4 on Cells

To determine if the antibodies are able to detect PAR4 expressed on cells, we used Flp-In™ 293 cells engineered to express PAR4 or HA-tagged PAR4 (HA-PAR4) when treated with tetracycline (1 μ g/ml). 14H6, 5F10, and 2D6 were able to detect PAR4 expressed on HEK293 cells by immunofluorescence (Figure 4A). HA-tag and secondary antibody were used as positive and negative controls, respectively. By immunoblotting, 14H6 and 5F10 were able to detect PAR4 in HEK293 expressing PAR4 cells; however, 2D6 was not able to recognize PAR4 (Figure 4B). Our mapping studies with recombinant proteins show that 14H6 and 5F10 bind near the thrombin cleavage site. We next determined if these antibodies are able to detect the cleavage of PAR4 on cells with flow cytometry. The interaction of 14H6 and 5F10 with PAR4 was disrupted following activation by α -thrombin (Figure 4C). Consistent with previous studies, the maximum cleavage of PAR4 was ~50% [17, 36]. Expression of HA-PAR4 was confirmed by flow cytometry using a HA-tag antibody.

Recognition of PAR4 on Platelets

For a more physiological readout we examined if 14H6 and 5F10 were able to detect endogenous PAR4 expressed on human platelets. 14H6 and 5F10 both interacted with PAR4 expressed in unstimulated human platelets (Figure 5A). Following activation by α -thrombin (1, 10, 100 nM), 14H6 and 5F10 were able to detect a decreased level of uncleaved PAR4 as a result of the epitope for 14H6 and 5F10 being cleaved from the receptor. This effect was dependent on the concentration of α -thrombin used to activate the human platelets. A similar effect was observed when examining PAR4 expression on the surface of human platelets. Using immunofluorescence, 14H6 was able to detect PAR4 expressed on the surface of human platelets and following activation with thrombin, there was a decrease in

the interaction between PAR4 and 14H6 (Figure 5B). This indicates that on human platelets, 14H6 and 5F10 only interact with unactivated and uncleaved PAR4.

Inhibition of PAR4 Cleavage

Based on the location of the PAR4 interaction site of 14H6 and 5F10, we investigated if the antibodies were able to inhibit PAR4 cleavage. Flp-In™ 293 cells induced to express HA-tagged PAR4 were pretreated with 14H6 or 5F10 (2–50 µg) and activated with α-thrombin (100 nM) for 10 min; loss of the N-terminal HA-tag was used as the readout for detecting PAR4 cleavage. To quantify the amount of PAR4 cleavage, background staining from uninduced cells was subtracted from all samples (Figure 6). The antibody 14H6 did not interfere with thrombin cleavage up to 50 µg total antibody. However, 5F10 was able to partially inhibit PAR4 cleavage at 50 µg total antibody. These data are consistent with our mapping studies in which 5F10 mapped to the P7-P1 residues (nomenclature of Schechter and Berger [37]) corresponding to Ser⁴¹ to Arg⁴⁷ that are important for thrombin recognition of its substrates [38, 39]. In contrast, 14H6 maps to P1'-P7' residues (Gly⁴⁸-Val⁵³). We have previously described an antibody to PAR4 that blocks platelet thrombin-induced activation of human platelets and delays thrombosis in mouse models *in vivo* [36]. Therefore we tested the ability of 14H6 and 5F10 to block human platelet activation. Neither 14H6 nor 5F10 were able to block thrombin-induced aggregation at concentrations up to 10 µg/ml. The most plausible explanations for these results are that 14H6 and 5F10 bind with an affinity that is too low to overcome PAR1 activation, which is also present on the platelet surface.

Conclusions

In the current study, we generated three classes of monoclonal antibodies that recognize unique regions on human PAR4. Two of the antibodies (14H6, 5F10) were able to detect PAR4 expression in HEK293 cells by immunoblotting, immunofluorescence, and flow cytometry and were found to be sensitive to PAR4 activation. In addition, 5F10 could partially inhibit PAR4 cleavage by α-thrombin. Importantly, 14H6 and 5F10 were able to detect endogenous PAR4 expression in human platelets. The third antibody, 2D6, was able to detect PAR4 expressed on cells by immunofluorescence microscopy. The studies described in this current report demonstrate the development of three useful tools for advancing our understanding of PAR4 in platelets and less well studied tissues.

In studies moving forward examining genetic variations of PAR4 in human platelets and the role of PAR4 in other tissues, these antibodies provide novel tools, which provide the invaluable ability to detect PAR4 in endogenous tissues/cells and to measure the initial step in PAR4 activation, PAR4 cleavage. These antibodies fill a gap in the currently available reagents for examining PAR4 and enable further advances in understanding the function and role of PAR4 in newly appreciated tissues and disease conditions.

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References

1. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000; 407:258–264. [PubMed: 11001069]
2. Coughlin SR. How the protease thrombin talks to cells. *Proc Natl Acad Sci U S A*. 1999; 96:11023–11027. [PubMed: 10500117]
3. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*. 1991; 64:1057–1068. [PubMed: 1672265]
4. Nanevicz T, Ishii M, Wang L, Chen M, Chen J, Turck CW, et al. Mechanisms of thrombin receptor agonist specificity. Chimeric receptors and complementary mutations identify an agonist recognition site. *J Biol Chem*. 1995; 270:21619–21625. [PubMed: 7665575]
5. Steinhoff M, Buddenkotte J, Shpacovitch V, Rattenholl A, Moormann C, Vergnolle N, et al. Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. *Endocr Rev*. 2005; 26:1–43. [PubMed: 15689571]
6. Keularts IM, van Gorp RM, Feijge MA, Vuist WM, Heemskerk JW. α (2A)-adrenergic receptor stimulation potentiates calcium release in platelets by modulating cAMP levels. *J Biol Chem*. 2000; 275:1763–1772. [PubMed: 10636873]
7. Varga-Szabo D, Braun A, Nieswandt B. Calcium signaling in platelets. *J Thromb Haemost*. 2009; 7:1057–1066. [PubMed: 19422456]
8. Woulfe DS. Platelet G protein-coupled receptors in hemostasis and thrombosis. *J Thromb Haemost*. 2005; 3:2193–2200. [PubMed: 16194198]
9. Kim S, Jin J, Kunapuli SP. Relative contribution of G-protein-coupled pathways to protease-activated receptor-mediated Akt phosphorylation in platelets. *Blood*. 2006; 107:947–954. [PubMed: 16223779]
10. Kim S, Foster C, Lecchi A, Quinton TM, Prosser DM, Jin J, et al. Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. *Blood*. 2002; 99:3629–3636. [PubMed: 11986217]
11. Holinstat M, Voss B, Bilodeau ML, McLaughlin JN, Cleator J, Hamm HE. PAR4, but not PAR1, signals human platelet aggregation via Ca^{2+} mobilization and synergistic P2Y₁₂ receptor activation. *J Biol Chem*. 2006; 281:26665–26674. [PubMed: 16837456]
12. Voss B, McLaughlin JN, Holinstat M, Zent R, Hamm HE. PAR1, but not PAR4, activates human platelets through a Gi/o/phosphoinositide-3 kinase signaling axis. *Mol Pharmacol*. 2007; 71:1399–1406. [PubMed: 17303701]
13. Holinstat M, Voss B, Bilodeau ML, Hamm HE. Protease-activated receptors differentially regulate human platelet activation through a phosphatidic acid-dependent pathway. *Mol Pharmacol*. 2007; 71:686–694. [PubMed: 17151288]
14. Leger AJ, Jacques SL, Badar J, Kaneider NC, Derian CK, Andrade-Gordon P, et al. Blocking the protease-activated receptor 1–4 heterodimer in platelet-mediated thrombosis. *Circulation*. 2006; 113:1244–1254. [PubMed: 16505172]
15. Li D, D'Angelo L, Chavez M, Woulfe DS. Arrestin-2 differentially regulates PAR4 and ADP receptor signaling in platelets. *J Biol Chem*. 2011; 286:3805–3814. [PubMed: 21106537]
16. Khan A, Li D, Ibrahim S, Smyth E, Woulfe DS. The physical association of the P2Y₁₂ receptor with PAR4 regulates arrestin-mediated Akt activation. *Mol Pharmacol*. 2014; 86:1–11. [PubMed: 24723492]
17. Arachiche A, Mumaw MM, de la Fuente M, Nieman MT. Protease-activated Receptor 1 (PAR1) and PAR4 Heterodimers Are Required for PAR1-enhanced Cleavage of PAR4 by α -Thrombin. *J Biol Chem*. 2013; 288:32553–32562. [PubMed: 24097976]
18. Jacques SL, LeMasurier M, Sheridan PJ, Seeley SK, Kuliopulos A. Substrate-assisted catalysis of the PAR1 thrombin receptor. Enhancement of macromolecular association and cleavage. *J Biol Chem*. 2000; 275:40671–40678. [PubMed: 11005807]

19. Jacques SL, Kuliopulos A. Protease-activated receptor-4 uses dual prolines and an anionic retention motif for thrombin recognition and cleavage. *Biochem J.* 2003; 376:733–740. [PubMed: 13678420]
20. Nieman MT. Protease-activated receptor 4 uses anionic residues to interact with alpha-thrombin in the absence or presence of protease-activated receptor 1. *Biochemistry.* 2008; 47:13279–13286. [PubMed: 19053259]
21. Mumaw MM, Nieman MT. Race differences in platelet reactivity: is protease activated receptor 4 a predictor of response to therapy? *Arterioscler Thromb Vasc Biol.* 2014; 34:2524–2526. [PubMed: 25411106]
22. Bray PF, Mathias RA, Faraday N, Yanek LR, Fallin MD, Herrera-Galeano JE, et al. Heritability of platelet function in families with premature coronary artery disease. *J Thromb Haemost.* 2007; 5:1617–1623. [PubMed: 17663734]
23. Edelstein LC, Simon LM, Montoya RT, Holinstat M, Chen ES, Bergeron A, et al. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. *Nat Med.* 2013; 19:1609–1616. [PubMed: 24216752]
24. Edelstein LC, Simon LM, Lindsay CR, Kong X, Montoya RT, Tourdot BE, et al. Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. *Blood.* 2014 in press.
25. Tourdot BE, Conaway S, Niisuke K, Edelstein LC, Bray PF, Holinstat M. Mechanism of Race-Dependent Platelet Activation Through the Protease-Activated Receptor-4 and Gq Signaling Axis. *Arteriosclerosis, thrombosis, and vascular biology.* 2014
26. Strande JL, Hsu A, Su J, Fu X, Gross GJ, Baker JE. Inhibiting protease-activated receptor 4 limits myocardial ischemia/reperfusion injury in rat hearts by unmasking adenosine signaling. *J Pharmacol Exp Ther.* 2008; 324:1045–1054. [PubMed: 18055876]
27. McDougall JJ, Zhang C, Cellars L, Joubert E, Dixon CM, Vergnolle N. Triggering of proteinase-activated receptor 4 leads to joint pain and inflammation in mice. *Arthritis and rheumatism.* 2009; 60:728–737. [PubMed: 19248120]
28. Russell FA, Veldhoen VE, Tchitchkan D, McDougall JJ. Proteinase-activated receptor-4 (PAR4) activation leads to sensitization of rat joint primary afferents via a bradykinin B2 receptor-dependent mechanism. *J Neurophysiol.* 2010; 103:155–163. [PubMed: 19889854]
29. Russell FA, Zhan S, Dumas A, Lagarde S, Pouliot M, McDougall JJ. The pronociceptive effect of proteinase-activated receptor-4 stimulation in rat knee joints is dependent on mast cell activation. *Pain.* 2011; 152:354–360. [PubMed: 21238854]
30. Dangwal S, Rauch BH, Gensch T, Dai L, Bretschneider E, Vogelaar CF, et al. High glucose enhances thrombin responses via protease-activated receptor-4 in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 2011; 31:624–633. [PubMed: 21164077]
31. Pavic G, Grandoch M, Dangwal S, Jobi K, Rauch BH, Doller A, et al. Thrombin receptor protease-activated receptor 4 is a key regulator of exaggerated intimal thickening in diabetes mellitus. *Circulation.* 2014; 130:1700–1711. [PubMed: 25239438]
32. Wahl JK 3rd. Generation of monoclonal antibodies specific for desmoglein family members. *Hybrid Hybridomics.* 2002; 21:37–44. [PubMed: 11991815]
33. Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem.* 2000; 275:6819–6823. [PubMed: 10702240]
34. Xu WF, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, et al. Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci U S A.* 1998; 95:6642–6646. [PubMed: 9618465]
35. Quinton TM, Kim S, Derian CK, Jin J, Kunapuli SP. Plasmin-mediated activation of platelets occurs by cleavage of protease-activated receptor 4. *J Biol Chem.* 2004; 279:18434–18439. [PubMed: 14973136]
36. Mumaw MM, de la Fuente M, Noble DN, Nieman MT. Targeting the anionic region of human protease-activated receptor 4 inhibits platelet aggregation and thrombosis without interfering with hemostasis. *J Thromb Haemost.* 2014; 12:1331–1341. [PubMed: 24888424]

37. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun.* 1967; 27:157–162. [PubMed: 6035483]
38. Nieman MT, Schmaier AH. Interaction of thrombin with PAR1 and PAR4 at the thrombin cleavage site. *Biochemistry.* 2007; 46:8603–8610. [PubMed: 17595115]
39. Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. *Proc Natl Acad Sci U S A.* 2000; 97:7754–7759. [PubMed: 10869434]

Highlights

- Three monoclonal antibodies were generated to the N-terminus of human PAR4.
- The antibodies can detect PAR4 expression on HEK293 cells and on human platelets.
- Two antibodies are sensitive to PAR4 cleavage, which provides a readout for PAR4 activation.
- 5F10 is able to partially inhibit PAR4 activation by α -thrombin.

Figure 1. Schematic of PAR4 extracellular constructs fused to MBP

PAR4 extracellular fragments were subcloned into pMAL-C2 to generate MBP-PAR4(18–78), MBP-PAR4(41–66), MBP-PAR4(48–72), and MBP-PAR4(54–66), which were used for generating and screening PAR4 monoclonal antibodies. The interaction for each antibody is illustrated.

Figure 2. Characterization of extracellular PAR4 interaction sites

E. coli were transformed, cultured, lysed, and the fusion proteins (MBP, MBP-PAR4(18–78), MBP-PAR4(41–66), MBP-PAR4(48–72), and MBP-PAR4(54–66)) were purified by affinity chromatography. The epitopes for each PAR4 monoclonal antibody were determined by immunoblotting. (A) Control blot with MBP to show that all of the recombinant PAR4 sections were purified and are present on the gel. (B) 14H6 interacted with MBP-PAR4(18–78), MBP-PAR4(41–66), and MBP-PAR4(48–72) protein fragments. (C) 5F10 recognized MBP-PAR4(18–78) and MBP-PAR4(41–66). (D) 2D6 interacted with only the complete extracellular section, MBP-PAR4(18–78).

Figure 3. Monoclonal antibodies detect PAR4 cleavage by thrombin

The full exodomain of PAR4 (MBP-PAR4(18–78)) was cleaved with 100 nM α -thrombin (IIa) for 5 min and immunoblotted with 14H6 or 5F10 to determine if they recognize cleaved PAR4. The cleaved PAR4 exodomain was used to further map the 2D6 epitope to residues 73–78.

Figure 4. Detection of PAR4 on HEK293 cells

Following induction of Flp-In™ T-REx™ 293 cells with tetracycline (1 µg/ml) for 36 hrs, (A) immunofluorescence and (B) immunoblotting were used to determine the ability of 14H6, 5F10, or 2D6 to interact with PAR4 on cells. HA was used as a positive control. Secondary antibody was used as a negative control. (C) Flow cytometry was used to evaluate the ability of 14H6, 5F10, and 2D6 to interact with uncleaved HA-PAR4 and cleaved HA-PAR4 (100 nM α -thrombin (IIa)) on cells. HA-PAR4 induction was determined using a HA-tag specific antibody. Mouse IgG was used as a negative control.

Figure 5. Detection of PAR4 on platelets

(A) Human platelets (hPLT) were activated with α -thrombin (0, 1, 10, 100 nM) for 5 min at 37°C, lysed, and immunoblotted with 14H6 and 5F10 (50 μ g). HEK293 cells were used as a negative control. (B) Immunofluorescence examining the interaction of 14H6 (20 μ g) with unstimulated and α -thrombin (1 nM) stimulated human platelets.

Figure 6. Inhibition of PAR4 cleavage

Flp-In™ T-REx™ 293 cells were induced with tetracycline (1 mg/ml) for 36 hrs to express HA-PAR4. Following induction cells were treated with buffer, 14H6, or 5F10 (2–50 μg) for 10 min and activated with α-thrombin (100 nM) for 5 min at 37°C. Cleavage of PAR4 was measured by loss of an N-terminal HA-tag. % uncleaved PAR4 was normalized to not induced, not activated, and buffer pretreatment (0%) and induced, not activated, and buffer pretreatment (100%). **p<0.01