The role of calpain in the regulation of ADAM17-dependent GPIbα ectodomain shedding

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**Abstract**

There are evidence that both a disintegrin and metalloproteinase 17 (ADAM17) and calpain are involved in platelet glycoprotein (GPIbα) ectodomain cleavage. However, the relationship between the two enzymes in the shedding process remains unclear. Here we show that calcium ionophore A23187- and α-thrombin-induced GPIbα shedding is completely inhibited by the metalloproteinase inhibitor GM6001, whereas it is only partially inhibited by calpain inhibitors. Calpain activator dibucaine-induced GPIbα shedding was completely inhibited by both metalloproteinase and calpain inhibitors. On the other hand, calpain inhibitors did not inhibit GPIbα shedding induced by the reagents that specifically activate ADAM17. Furthermore, A23187-induced GPIbα shedding in Chinese hamster ovary cells expressing wild-type or mutant GPIb-IXα was also partially inhibited by calpain inhibitors and almost completely inhibited by GM6001. Therefore, these data indicate that calpain plays an important role in the regulation of ADAM17-dependent GPIbα ectodomain shedding in both platelets and nucleated cells.

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**Introduction**

The interaction of platelet glycoprotein (GP)Iβα with von Willebrand factor (vWF) exposed at the injured vessel wall initiates platelet adhesion, and simultaneously triggers intracellular signaling events such as elevation of intracellular calcium, and activation of multiple protein kinase pathways, consequently resulting in integrin activation, and platelet thrombus formation [1–3]. 1 In addition, GPIbα also contains binding sites for α-thrombin, P-selectin, and Mac-1 at the extracellular N-terminal 282 residues [2,3]. Therefore, GPIbα ectodomain shedding which down-regulates the surface expression of the functional receptor and results in the generation of glyocalcin (GC), a soluble N-terminal fragment of GPIbα that is released during ectodomain shedding, has important implications for thrombosis, and hemostasis.

GPIbα ectodomain shedding has been reported to occur commonly in platelets activated by chemical or physiological agonists, such as α-thrombin, calcium ionophore A23187, calmodulin inhibitor N-[6aminohexyl]-5-chloro-I-naphthalene sulfonamide (W7), phorbol 12-myristate-13-acetate (PMA), or N-ethylmaleimide (NEM) [4–8]. However, the mechanisms underlying the agonist-induced GPIbα ectodomain shedding remain obscure. Bergmeier, and coworkers reported recently that a disintegrin and metalloproteinase 17 (ADAM17) is expressed in platelets, and responsible for ectodomain shedding of GPIbα induced by platelet storage, mitochondrial injury or physiological activations [4,5]. In addition, a high concentration of aspirin was also shown to induce shedding of the GPIbα and GPV ectodomains via an ADAM17-dependent mechanism [6]. ADAM17 is a member of the metalloproteinase-disintegrin family, composed of an extracellular functional domain, a transmembrane domain, and a cytoplasmic tail [9,10]. Although these reports indicate that GPIbα is proteolyzed by ADAM17, the regulation of ADAM17-mediated-GPIbα shedding still remains to be elucidated.

Calpains are calcium-dependent thiol proteases highly expressed in human platelets [11], and play regulatory roles in both early events of platelet-mediated fibrin clot retraction [12–16]. GPIbα, integrin β3, actin binding protein (ABP), talin, and signaling proteins (Ilk and Fak) have been reported as the substrates of calpain in platelets activated by thrombin, collagen, or calcium ionophore A23187 [8,17–21]. Particularly, GPIbα can be cleaved directly by purified calpain [8]. Therefore, calpain had been thought to be the enzyme generating GC directly. However, it is generally accepted that calpain is not externalized when platelets are activated by agonists [22], and some calpain inhibitors such as calpeptin, (2S,3S)-trans-epoxysuccinyl-I-leucylamido-3-methylbutane ethyl ester (E64d), and leupetin had no effective inhibitory effects on...
the role of calpain in GPIb membrane-bound enzyme rather than calpain [25]. Up to now, the role of calpain in GPIb ectodomain shedding remains unclear.

Therefore, the aim of the current study is to investigate the role of calpain in ADAM17-mediated-GPIb ectodomain shedding. The data demonstrate that calpain plays an important role in regulating ADAM17-mediated GPIb ectodomain shedding in platelets induced by agonists. The regulatory role of calpain occurs commonly in both platelets and Chinese hamster ovary (CHO) cells.

**Materials and methods**

**Reagents**

Monoclonal antibody SZ2 against GPIbα was generous gift from Dr. Changgeng Ruan (Soochow University, Suzhou, China). Carbobenzoxy-valinyl-phenylalaninal (MDL28170), trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E64), calpain inhibitors I and II, calcium ionophore A23187, a broad metalloproteinase inhibitor GM6001, and N-ethylmaleimide (NEM) were purchased from Calbiochem (San Diego, CA). Dibucaine, phorbol 12-myristate-13-acetate (PMA), H2O2, α-thrombin and monoclonal anti-talin antibody were purchased from Sigma (St. Louis, MO). FITC-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against filamin A was purchased from Novocastra Laboratories (Newcastle, UK).

**Cell lines expressing recombinant proteins**

CHO cells expressing recombinant wild-type GPIbα-IX (1b9), and mutant GPIbα-IX with GPIbα ectodomain truncated at residue 551 (Δ551) had been established previously [26]. Stable cell lines were selected by cell sorting using the anti-GPIbα monoclonal antibody, SZ2.

**Preparation of washed platelets**

For studies involving human subjects, approval was obtained from the Beihang University institutional review board. Informed consent was provided according to the Declaration of Helsinki. Washed platelets were prepared as described previously [27], briefly, fresh blood from healthy volunteers was anti-coagulated with 1/7 volume of acid-citrate-dextrose (ACD, 2.5% trisodium citrate-13-acetate (PMA), H2O2, α-thrombin and PMA for 30 min; H2O2 for 60 min). The concentration of vehicle DMSO in all samples was 1%. Samples were centrifuged at 4000 rpm for 5 min to harvest the supernatants. Each supernatant was added with one-fourth volume of 5 × SDS sample buffer, and resolved in 8% SDS–PAGE, and immunoblotted with the anti-GPIbα N-terminal antibody SZ2 (1:1000).

**Flow cytometric analysis of GPIbα shedding in platelets**

Washed platelets were pre-incubated with GM6001 (100 μM), MDL28170 (100 μM), or vehicle control (DMSO) at RT for 10 min, and then incubated with or without (control) A23187 (5 μM) at 37 °C for 40 min or dibucaine (1 μM) at RT for 15 min. About 50 μl treated platelet suspensions were incubated with SZ2 (5 μg/ml) or mouse IgG (5 μg/ml) as negative control at RT for 30 min. After washing, platelets were further incubated with fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse IgG antibody in the dark at RT for 30 min, and then analyzed by flow cytometry (FACSCalibur, BD Biosciences).

**Western blot analysis of talin and filamin A cleavage**

Washed platelets (3 × 10^8/ml) were pre-incubated with MDL28170 (100 μM), calpain inhibitor I (100 μM), calpain inhibitor II (250 μM), GM6001 (100 μM) or vehicle (DMSO) at RT for 10 min, and then incubated with A23187 (5 μM), α-thrombin (0.5 μM under stir) and dibucaine (1 μM) at RT or 37 °C for different time. The concentration of vehicle (DMSO) in all samples was 1%. Then the platelets were lysed with an equal volume of lysis buffer containing 0.1 μM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1/100 aprotinin on ice for 30 min. Each sample was added with one-fourth volume of 5X loading buffer and analyzed by 6% SDS–PAGE, and immunoblotted with the anti-talin (1:2500) or anti-filamin A (1:500) monoclonal antibody.

**Flow cytometric analysis of GPIbα shedding in 1b9 and Δ551 cells**

CHO cells expressing wild-type and mutant GPIbα-IX were resuspended in modified Tyrode’s buffer as described previously [26,28]. The cells (2.5 × 10^6/ml) were pre-incubated with GM6001 (100 μM), MDL28170 (100 μM), or DMSO control, and then incubated with or without (control) A23187 (5 μM) at 37 °C for 40 min. About 50 μl treated cell solutions were incubated with SZ2 (5 μg/ml) or mouse IgG (5 μg/ml) control at RT for 30 min. After washing, cells were further incubated with FITC-labeled goat-anti-mouse IgG antibody in the dark at RT for 30 min, and then analyzed by flow cytometry.

**Statistical analysis**

The Western blot was scanned and optical density of each band from the same blot was quantitated with NIH Image software (Image Processing and Analysis in Java, National Institutes of Health). Data are presented as means ± SD. The statistical difference between groups was carried out using one-way ANOVA with posthoc Dunnett test. A P-value less than 0.05 was considered significant.

**Results**

A23187- and α-thrombin-induced GPIbα shedding is completely inhibited by ADAM17 inhibitor, whereas is only partially inhibited by calpain inhibitors

There are evidences that both ADAM17 and calpain are involved in GPIbα ectodomain shedding [4–8,19,20]. However, the relationship between ADAM17 and calpain in the shedding process
remains unclear. In order to investigate the exact roles of calpain and ADAM17 in GPIbα shedding, platelets were pre-incubated with calpain or ADAM17 inhibitors, and then incubated with A23187. As shown in Fig. 1A–D, A23187-induced GPIbα shedding was dose-dependently inhibited by MDL28170, and was also obviously inhibited by other cell-permeable calpain inhibitors. The inhibitory effects were further confirmed by the parallel flow cytometry analysis (Fig. 1E and F). However, although the calpain inhibitors completely inhibited calpain-dependent talin and filamin A cleavage (Fig. 1G and H), which indicated the complete inhibition of calpain activity, A23187-induced GPIbα shedding was only partially inhibited by the calpain inhibitors. On the other hand, the metalloproteinase inhibitor GM6001 completely inhibited A23187-induced GPIbα shedding (Fig. 1A–F). Thrombin activates calpain and incurs GPIbα ectodomain shedding under stirring conditions, which is similar to physiological conditions [12,29]. Therefore, the effects of calpain and ADAM17 inhibitors on α-thrombin-induced GPIbα shedding were investigated under stirring conditions. Consistent with A23187-induced GPIbα shedding, although calpain inhibitors completely inhibited calpain activity as detected by talin and filamin A cleavages, they only partially inhibited α-thrombin-induced GPIbα shedding (Fig. 2). On the other hand, GM6001 completely inhibited α-thrombin-induced GPIbα shedding (Fig. 2A). Taken together, these data suggest calpain is par-

Fig. 1. Calpain inhibitors partly inhibit A23187-induced GPIbα shedding. Platelets were pre-incubated with calpain inhibitors, GM6001, or vehicle (DMSO), and then incubated with or without (control) A23187 as described in Materials and methods. (A–D) Treated platelets were centrifuged at 4000 rpm for 5 min, and the supernatants were resolved on an 8% SDS–PAGE, and probed with SZ2. Results are representative of three separate experiments with different donators (A and B). The Western blot was scanned and optical density of each band was quantitated with NIH Image software. Relative amounts of GC from three independent experiments are shown (means ± SD) (C and D). Relative amount of GC equals arbitrary unit of inhibitor/arbitrary unit of DMSO × 100. (E) Treated platelets were analyzed for GPIbα surface expression by anti-GPIbα N-terminal antibody SZ2 and flow cytometry. Representative flow cytometric histograms of three separate experiments with different donators are shown. (F) Fluorescence geo mean of GPIbα surface expression are presented as means ± SD from three independent experiments. *P < 0.05 compared with control. (G and H) Treated platelets were lysed with equal volume of lysis buffer, and then the lysates were resolved on a 6% SDS–PAGE, and probed with anti-talin (G) and anti-filamin A (H) antibodies. Results are representative of three separate experiments with different donators. Platelet lysates, MDL28170, calpain inhibitor I, and calpain inhibitor II are labeled as Pls, MDL, CI I, and CI II, respectively.
pain inhibitors, inhibited PMA-induced GPIb shedding. As shown in Fig. 4, E64 did not inhibit agonists-inhibitor MDL28170, and then incubated with A23187, thrombin, ADAM17 directly, washed platelets were pre-incubated with cell-impermeable calpain inhibitor does not inhibit A23187-, thrombin-, and dibucaine-induced GPIb shedding.

Cell-impermeable calpain inhibitor does not inhibit A23187-, thrombin-, and dibucaine-induced GPIb shedding.

In order to exclude the possibility that calpain inhibitor inhibits ADAM17 directly, washed platelets were pre-incubated with cell-impermeable calpain inhibitor E64, or cell-permeable calpain inhibitor MDL28170, and then incubated with A23187, thrombin, or dibucaine. As shown in Fig. 4, E64 did not inhibit agonists-induced GPIb shedding. These data indicate that calpain inhibitor does not directly inhibit ADAM17.

Calpain inhibitors do not inhibit PMA, H$_2$O$_2$, and NEM induced GPIb shedding.

Next, we further characterized the roles of ADAM17 and calpain in GPIb ectodomain shedding induced by ADAM17 activators. Phosphorylation of ADAM17 at threonine 735 by protein kinase C (PKC) increases ADAM17 proteolytic activity [31]. PMA, a diacylglycerol analog that activates PKC, induces GPIb ectodomain shedding [7]. Therefore, firstly, we examined the effects of ADAM17 and calpain inhibitors on PMA-induced GPIb ectodomain shedding. As shown in Fig. 5A, ADAM17 inhibitor, but not calpain inhibitors, inhibited PMA-induced GPIb shedding. Reactive oxygen species (ROS) activates ADAM17 by reduction–oxidation modifications of its cysteiny1 sulphydryl groups [32]. Thus, we further investigated the inhibitors on ROS-induced GPIb shedding, and the results indicated calpain inhibitors do not inhibit H$_2$O$_2$-induced GPIb shedding (Fig. 5B). ADAM17 contains a Cys switch in the prodomain, allowing this proteinase to be directly activated by thiol-modifying reagents such as NEM [33]. So we also detected the effects of ADAM17 and calpain inhibitors on NEM-induced GPIb shedding. Consistent with the previous observations, ADAM17 inhibitor, but not calpain inhibitors, inhibited NEM-induced GPIb shedding (Fig. 5C). Taken together, these data demonstrate that calpain inhibitors have no effect on ADAM17 activation-dependent GPIb shedding.

Talin cleavage is earlier than GPIb shedding in dibucaine-treated platelets.

The above data suggest that calpain is upstream of ADAM17, and regulates the ADAM17-dependent GPIb shedding. To further confirm these observations, time course experiments of calpain and ADAM17 activations were performed. Platelets were incubated with calpain activator dibucaine, and then calpain and ADAM17 activations were determined by the cleavage of talin and GPIb, respectively. As shown in Fig. 6, talin cleavage began at 3 min after the addition of dibucaine, whereas, GPIb cleavage occurred at 6 min. Thus, these data further support that calpain is an upstream regulator of ADAM17 activation, and regulates the ADAM17-mediated-GPIb shedding.

Calpain regulates ADAM17 mediated-GPIb shedding in nucleated cells.

Calpain and ADAM17 exist widely in nucleated cells and serve as proteinases of many substrates involving in the regulation of cell adhesion, migration and signaling [34,35]. In order to investigate whether calpain regulates ADAM17 activation in nucleated cells, A23187-induced GPIb shedding was performed in CHO cells expressing wild-type GPIb-IX. Similar to the data obtained with platelets, A23187-induced GPIb shedding was only partially inhibited by MDL28170, whereas, was almost completely inhibited.
Filamin A associates with the cytoplasmic tail of GPIbα, anchoring the GPIb-IX–V complex to the cytoskeleton of platelets [1,36]. In addition, filamin A has been confirmed to be a substrate of calpain [18], raising the concern whether calpain regulates ADAM17-mediated GPIbα shedding through the cleavage of filamin A. Thus, we investigated the role of the association of filamin A with the cytoplasmic domain of GPIbα in GPIbα ectodomain shedding by examining A23187-induced GPIbα shedding in A551 cells which lack filamin A binding site in the cytoplasmic domain of GPIbα (Fig. 7A). As shown in Fig. 7, there is no obvious difference between 1b9 and A551 cells in A23187-induced GPIbα shedding and the effects of calpain and ADAM17 inhibitors on the shedding, thus excluding the possibility that calpain regulates GPIbα shedding through regulating the association of filamin A with the cytoplasmic domain of GPIbα.

**Discussion**

The data presented here demonstrate that calpain plays an important role in regulating ADAM17-dependent GPIbα ectodomain shedding. The regulatory role of calpain occurs commonly in both platelets and CHO cells.

GPIbα ectodomain shedding, which results in the generation of GC, has been reported to occur commonly in platelets activated by chemical or physiological agonists [4–8,37]. There are evidences by GM6001 (Fig. 7B and C). These data suggest that calpain regulates ADAM17 activation in nucleated cells.

**Fig. 3.** Calpain inhibitors inhibit dibucaine-induced GPIbα shedding. Platelets were pre-incubated with calpain inhibitors, GM6001, or vehicle DMSO, and then incubated with or without (control) dibucaine as described in Materials and methods. (A and B) Treated platelets were analyzed for GPIbα surface expression by anti-GPIbα N-terminal antibody SZ2 and flow cytometry. Representative flow cytometric histograms of three separate experiments with different donators are shown (A). Fluorescence geo mean of GPIbα surface expression are presented as means ± SD from three independent experiments (B). *P < 0.05 compared with control. (C–F) Treated platelets were centrifuged at 4000 rpm for 5 min, and the supernatants were resolved on an 8% SDS–PAGE and probed with SZ2. Results are representative of three separate experiments with different donators (C and D). Relative amounts of GC from three independent experiments are shown (means ± SD) (E and F). Relative amount of GC equals arbitrary unit of inhibitor/arbirtary unit of DMSO × 100. (G and H) Treated platelets were lysed with equal volume of lysis buffer, and then the lysates were resolved on a 6% SDS–PAGE and probed with anti-talin (G) and anti-filamin A (H) antibodies. Results are representative of three separate experiments with different donators. Platelet lysates, MDL28170, calpain inhibitor I, and calpain inhibitor II are labeled as Pls, MDL, Cl I, and Cl II, respectively.
that calpain is involved in the agonists-induced GPIbβ shedding [8,37]. However, calpain is not externalized when platelets are activated by agonists [22], and some calpain inhibitors, particularly impermeable calpain inhibitors such as calpeptin, E64, and leupeptin had no effective inhibitory effects on GC generation in some experiments [23–25]. Therefore, up to now, the role of calpain in GPIbβ ectodomain shedding remains unclear. It was reported recently that ADAM17 is expressed in platelets and responsible for ectodomain shedding of GPIbβ induced by platelet storage, mitochondrial injury or physiological activations [4–7], thus, raising the concern what is the relationship between the calpain and ADAM17 in GPIbβ ectodomain shedding.

In the current observations, several lines of evidence indicate that calpain plays an important role in regulating ADAM17-dependent GPIbβ ectodomain shedding: (1) A23187 and α-thrombin-induced GPIbβ shedding was partially inhibited by calpain inhibitors, whereas was completely inhibited by ADAM17 inhibitor; (2) calpain activator induced GPIbβ shedding was completely inhibited by both ADAM17 and calpain inhibitors; (3) calpain inhibitors did not inhibit PMA, H2O2, and NEM-induced GPIbβ shedding; and (4) calpain activation was earlier than ADAM17 activation. In addition, GPIbβ ectodomain shedding induced by protein kinase A inhibition was also completely inhibited by ADAM17 inhibitor, but only partially inhibited by calpain inhibitors in our previous studies [37].

Interestingly, although calpain indeed regulates ADAM17-dependent GPIbβ ectodomain shedding, the calpain inhibitors could not completely abolish agonists-induced GPIbβ shedding. The possible reason might be that agonists activate calpain, and simultaneously trigger other signals leading to ADAM17 activation directly. For example, the signaling cascades lead to the activation of PKC which results in ADAM17 phosphorylation and activation [31]. Thus, these data indicate that calpain presents as one of up-stream regulators regulating ADAM17-mediated-GPIbβ shedding, whereas, ADAM17 which is regulated by calpain or other regulators is responsible for GPIbβ shedding.

A23187 and dibucaine are both chemical agonists which never exist in physiological conditions. Thus in order to investigate the physiological relevance of the current observations, α-thrombin, a physiological agonist, was selected to examine the role of calpain in the GPIbβ shedding. The data indicated that α-thrombin-induced GPIbβ shedding was also partly inhibited by calpain inhibitors under stirring conditions. We also detected the role of calpain in α-thrombin-induced GPIbβ shedding under non-stirring conditions. As expected, α-thrombin only induced slight GPIbβ shedding which could not be inhibited by calpain inhibitors (data not shown). Furthermore, our previous data indicate that GPIbβ ectodomain shedding induced by the GPIbβ–VWF interaction under flow conditions was also completely inhibited by ADAM17 inhibitor, but only partially inhibited by calpain inhibitors [38]. Therefore, these data indicate that the current observations also have physiological implications.

Calpain and ADAM17 serve as sheddases of many substrates in nucleated cells [34,35]. To further confirm whether the regulation of ADAM17 by calpain exists in nucleated cell as a common mechanism in the process of ADAM17 activation, CHO cells expressing wild type GPIb-IX were selected and induced to GPIbβ shedding.
Fig. 6. Dibucaine-induced GPIbα and talin cleavage. Platelets were incubated with dibucaine at RT for different time, and then analyzed for GPIbα and talin cleavage. (A and B) Treated platelets were centrifuged at 4000 rpm for 5 min, and the supernatants were resolved on an 8% SDS–PAGE and probed with SZ2. Results are representative of three separate experiments with different donors (A). Relative amounts of GC from three independent experiments are shown (means ± SD) (B). Relative amount of GC equals arbitrary unit of each time point/arb. unit of 15 min × 100. (C and D) Treated platelets were lysed with equal volume of lysis buffer, and then the lysates were resolved on a 6% SDS–PAGE and probed with anti-talin antibody. Results are representative of three separate experiments with different donors (C). Relative amounts of talin fragments from three independent experiments are shown (means ± SD) (D). Relative amount of talin fragment equals arbitrary unit of each time point/arb. unit of 15 min for 190 kDa fragment or 1 min for 235 kDa fragment × 100. Platelet lysates and glycocalcin are labeled as Pls and GC, respectively.

Fig. 7. Calpain regulates ADAM17 mediated GPIbα shedding in CHO cells. (A) Schematic of the filamin A binding site and GPIb-IX mutant used in these studies. (B–E) CHO cells expressing wild type (B and C) or mutant GPIb-IX with GPIbα truncated at residue 551 (D and E) were pre-incubated with MDL28170, GM6001, or vehicle (DMSO) at RT for 10 min, and then incubated with or without (control) A23187 at 37 °C for 40 min. Treated cells were analyzed for GPIbα surface expression by anti-GPIbα N-terminal antibody SZ2 and flow cytometry. Representative flow cytometric histograms of three separate experiments are shown (B and D). Fluorescence geo mean of GPIbα surface expression are presented as means ± SD from three independent experiments (C and E). *P < 0.05 compared with control. MDL28170 is labeled as MDL.
The results indicated that inhibition of calpain also partially inhibited ADAM17-mediated-GPIbα shedding, suggesting that calpain regulates ADAM17 activity in nucleated cells. In addition, we also investigated the possibility that calpain regulates ADAM17-mediated-GPIbα shedding through the cleavage of filamin A. However, there is no obvious difference between B9 and Δ551 cells in A23187-induced GPIbα shedding and the effects of calpain and ADAM17 inhibitors on the shedding, thus excluding the role of the association of filamin A with the cytoplasmic domain of GPIbα in the process.

In conclusion, the data indicate that calpain presenting as one of up-stream regulators plays an important role in the regulation of shedding process, whereas, ADAM17 is responsible for GPIbα ectodomain shedding. The regulatory role of calpain occurs commonly under physiological conditions or in nucleated cells, thus the findings have important implications for both the regulation of GPIbα ectodomain shedding in vivo and the interaction of calpain and ADAM17 in nucleated cells.

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