Interaction of calmodulin with the cytoplasmic domain of platelet glycoprotein VI


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Supported by the National Health & Medical Research Council of Australia and by the British Heart Foundation.

RUNNING TITLE: Calmodulin binding to GPVI

SCIENTIFIC SECTION HEADING: Brief Report

WORD COUNT: Abstract: 149; Text: 1,198 (excluding figure legends and references)

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Abstract

The platelet collagen receptor, glycoprotein (GP)VI, and GPIb-IX-V that binds von Willebrand Factor, initiate platelet aggregation at low or high shear stress, respectively. We recently reported that positively-charged, membrane-proximal sequences within cytoplasmic domains of GPIbβ and GPV of GPIb-IX-V bind calmodulin [Andrews RK, Munday AD, Mitchell CA, Berndt MC. Blood. 2001;98:681-687]. We now show that GPVI also binds calmodulin: (1) calmodulin co-immunoprecipitated with GPVI from resting platelet lysates using an anti-GPVI IgG, but partially dissociated in platelets activated by collagen or collagen-related peptide; (2) calmodulin co-precipitated from platelet lysates with maltose-binding protein (MBP)-GPVI cytoplasmic domain fusion protein, but not MBP alone; (3) a GPVI-related synthetic peptide based on the membrane-proximal sequence, His269-Pro287, induced a shift in calmodulin migration on non-denaturing gels, an assay that identifies calmodulin-binding peptides. His269-Pro287 is analogous to the calmodulin-binding sequence in GPIbβ. The novel interaction of GPVI and calmodulin may regulate aspects of GPVI function.
Introduction

Platelet activation and aggregation in normal hemostasis or pathological thrombosis is initiated by engagement of specific adhesion receptors. At low shear stress, collagen receptors such as glycoprotein (GP)VI initiate platelet activation. At high shear, platelet adhesion is primarily dependent on the GPIb-IX-V complex binding von Willebrand Factor. Subsequent platelet aggregation involves elevation of cytosolic Ca$^{2+}$, and triggering of signaling pathway(s) leading to cytoskeletal rearrangements and activation of the integrin, αIIbβ3 (GPIIb-IIIa). Defining pathways by which ligand binding to GPVI and/or GPIb-IX-V activate αIIbβ3 may provide therapeutic targets for attenuating thrombosis. Recent studies have identified signaling molecules associated with the cytoplasmic domain of GPIb-IX-V, including 14-3-3ζ, the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and calmodulin. Consistent with their parallel physiological function, common elements involved in mechanisms of regulation and signaling are emerging for GPVI and GPIb-IX-V. For instance, a functional association with Fc receptor γ chain (FcRγ) has been implied for both receptors. In this study, we show that like GPIbβ and GPV, the cytoplasmic domain of GPVI binds calmodulin, an interaction that is decreased following platelet activation. We further show that a synthetic peptide based on the positively-charged, membrane-proximal sequence His269–Pro287 contains a conserved calmodulin recognition site, and interacts with purified calmodulin.

Materials and methods

MBP-GPVI fusion protein
A maltose-binding protein (MBP)-GPVI fusion protein was prepared from cDNA encoding the GPVI cytoplasmic sequence Glu266-Ser316 subcloned into a pMAL-c2x vector (New England Biolabs, Beverly, MA, U.S.A.) at EcoRI and XbaI sites. The construct encoded a fusion protein with the N-terminal region corresponding to \textit{E.coli} MBP and the GPVI sequence at the C-terminus. The correct sequence was verified by sequencing. MBP and MBP-GPVI expressed in \textit{E.coli} were purified on amylose-Sepharose according to the manufacturer’s instructions (New England Biolabs).

\textbf{GPVI-calmodulin association}

Washed platelets (2-5 x 10^8/mL) were resuspended in buffer containing 1 mM RGDS and 10 \(\mu\)M indomethacin and treated with 10 \(\mu\)g/mL collagen or collagen-related peptide, or 200 nM A23187, for 5-60 s at 37°C. Some samples were pre-incubated with 20 \(\mu\)M PP1 (Src-family kinase inhibitor) for 20 min at 37°C. Platelets were lysed by addition of an equivalent volume of ice-cold lysis buffer (2%[v/v] Triton X-100, 40 mM Tris-HCl, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/mL leupeptin, 10 \(\mu\)g/mL aprotinin, and 1 \(\mu\)g/mL pepstatin-A, pH 7.3), centrifuged at 15,000xg for 10 min, and pre-cleared with 30 \(\mu\)L of a 50% suspension of protein A-Sepharose in TBS-T buffer (20 mM Tris-HCl, pH 7.3, 136 mM NaCl, 0.1%[v/v] Tween 20). Lysates were immunoprecipitated using 3 \(\mu\)g/mL anti-GPVI antibody MM20411 and 30 \(\mu\)L of protein A-Sepharose for 2 h at 4 °C as previously described. MM20411 was characterized and provided by Dr Masaaki Moroi, Kurume University, Japan (unpublished results). Lysates were also precipitated with amylose-Sepharose beads and either MBP or MBP-GPVI fusion protein as described elsewhere.

\textbf{Blotting}
Immunoblotting with anti-calmodulin monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A) or anti-MBP IgG (New England Biolabs) was performed as described elsewhere.\textsuperscript{8,13-15} Ligand blotting with the GPVI-targeting snake toxin, convulxin, was performed as previously described.\textsuperscript{14,15}

**Gel shift assay**

The interaction of synthetic peptides corresponding to His269-Pro287 of GPVI\textsuperscript{12} or Lys529-Gly544 of GPV\textsuperscript{16} (Chiron Mimotopes, Clayton, Australia) with bovine calmodulin (Sigma, St Louis, MO, U.S.A.) was analyzed on non-denaturing gels.\textsuperscript{8,17-20} Calmodulin (0.3 nmol) in 0.1 M Tris-HCl, pH 7.5 containing 4 M urea was incubated with GPVI or GPV peptides for 30 min at 22 °C in the presence of either 1 mM Ca\textsuperscript{2+} or 10 mM EGTA.

**Results and discussion**

In this study, we found that the cytoplasmic domain of GPVI binds calmodulin. Firstly, immunoblotting with anti-calmodulin antibody showed that calmodulin co-immunoprecipitated with GPVI from resting platelet lysates (Figure 1). We previously showed that up to 25% of total platelet calmodulin was redistributed from the cytosol to the cytoskeleton following platelet stimulation.\textsuperscript{8} Following platelet stimulation with collagen or collagen-related peptide, there was decreased association of calmodulin with GPVI (Figures 1A and 1B). Ligand blotting with convulxin showed levels of immunoprecipitated GPVI were essentially unaffected on this time scale (Figure 1A). In different experiments with these agonists, dissociation occurred after a lag of 30-60 s (typically ~60% loss after 60 s), and this loss was maintained up to 90 s (Figure 1A). Dissociation of calmodulin from GPVI induced by collagen-related peptide was delayed relative to stimulation of tyrosine phosphorylation
(not shown), and was blocked by the Src-family kinase inhibitor PP1 (Figure 1B), suggesting an activation-dependent mechanism for the observed dissociation. In contrast to the GPVI agonists, ionophore A23187 that induces GPVI-independent platelet activation did not affect calmodulin-GPVI association (Figure 1B). Secondly, we used an MBP fusion protein containing the entire cytoplasmic sequence of GPVI, Glu266-Ser316 (MBP-GPVI), to specifically co-precipitate calmodulin from platelet lysates (Figure 1C). In contrast, calmodulin was not precipitated by MBP alone.

A membrane-proximal 16-residue sequence, His269-Pro287, within the cytoplasmic domain of GPVI was analogous to calmodulin-binding motifs found in GPIbβ, L-selectin and CBCP (Figure 2A). CBCP is a non-physiological calmodulin-binding control peptide representative of positively-charged, amphipathic α-helices typical of calmodulin recognition sites in other proteins. A helical-wheel representation of the calmodulin-binding sequences of GPVI and GPIbβ revealed an almost identical charge distribution between the two peptides (Figure 2B). To examine whether this sequence in GPVI bound calmodulin, we used a non-denaturing gel shift assay previously used to identify calmodulin-binding peptides. The peptide His269–Pro287 induced a concentration-dependent shift in calmodulin migration in the presence of Ca²⁺, but not in the presence of EGTA (Figure 2C). Maximal shift was observed at ~10-fold molar excess of peptide to calmodulin, suggesting the affinity of the interaction was comparable to that of the GPV-related peptide, and within the range reported for other calmodulin-binding peptides, 1:1 (K_D ~30 nM) or 50:1 (K_D ~2 µM). The location of the calmodulin shift for the GPVI-related peptide was different from that induced by the GPV peptide (Figure 2C). When both peptides were included in the assay at equimolar concentrations, there was ~50% of each GPVI-calmodulin and GPV-calmodulin complex, suggesting the peptides interacted with calmodulin at the same site and with comparable affinity. Like GPIbβ, GPV and other proteins, the Ca²⁺-dependence for the
interaction of GPVI-related peptide with calmodulin was in contrast with the co-association of calmodulin with immunoprecipitated GPVI or MBP-GPVI fusion protein containing the full length cytoplasmic domain which occurred in the presence of EGTA. However, decreased calmodulin association with GPVI in activated platelets (this study) and corresponding redistribution of calmodulin to the activated cytoskeleton,\(^8\) suggests elevation of cytosolic Ca\(^{2+}\) in activated platelets\(^2-4\) may potentially regulate calmodulin interactions with GPVI.

The cytoplasmic domain of GPVI consists of 51 residues.\(^12\) Arg249 within the transmembrane domain, together with elements within the cytoplasmic domain mediates association of GPVI with FcR\(\gamma\).\(^10\) FcR\(\gamma\), containing an ITAM (immunoreceptor tyrosine-based activation motif), is critical for GPVI-dependent platelet activation.\(^2,10\) Binding of calmodulin to a membrane-proximal sequence of GPVI raises the possibility that this interaction may regulate some aspect of the association of GPVI with FcR\(\gamma\) or FcR\(\gamma\)-independent events. It is presently unclear whether decreased calmodulin association following stimulation would be consistent with downregulation of functional GPVI-FcR\(\gamma\) association. In conclusion, this study has identified calmodulin as a GPVI–associated protein in platelets. Current studies aim to define functional consequences of the GPVI–calmodulin interaction.

**Acknowledgements**

We thank Ms Carmen Llerena and Ms Andrea Aprico for outstanding technical assistance. We also thank Drs Moroi and Jung for the antibody to GPVI.
REFERENCES


Figure Legends

Figure 1. Co-precipitation of GPVI and calmodulin from platelet lysates. (A) Immunoprecipitation by anti-GPVI antibody (MM20411) of untreated platelet lysates or lysates of platelets (5 x 10^8/mL) treated with 10 µg/mL collagen-related peptide. Samples were electrophoresed on 10 or 15% polyacrylamide gels under reducing conditions, and immunoblotted with anti-calmodulin IgG. The position of the antibody light chain is shown. Blots were visualized using horseradish peroxidase-coupled second antibody (Silenus, Hawthorn, Australia) and ECL detection (Amersham, Buckinghamshire, U.K.). Samples were also ligand blotted with convulxin (lower panel) to determine GPVI levels. (B) Immunoprecipitation by anti-GPVI IgG of untreated platelet lysates, or lysates of platelets treated with 10 µg/mL collagen or collagen-related peptide, or 200 nM A23187 for 90 s (upper panel) or 30 s (lower panel) at 37°C. Where indicated, platelets were pre-incubated with 20 µM PP1 prior to stimulation with collagen-related peptide. Samples were immunoblotted with anti-calmodulin IgG as described above. Experiments with collagen-related peptide were performed six times, and those with collagen three times. (C) Co-precipitation of calmodulin with amylose-Sepharose beads from platelet lysates in the presence of MBP or MBP-GPVI (Glu266-Ser316) fusion protein. Precipitates were electrophoresed on 5-20% SDS-polyacrylamide gels under reducing conditions, electrotransferred to nitrocellulose, probed with anti-calmodulin IgG or anti-MBP IgG, and visualized using the ECL reagent. Results are typical of three separate experiments.

Figure 2. GPVI cytoplasmic sequence and other calmodulin-binding sequences. (A) GPVI cytoplasmic sequence, His269-Pro287, compared with calmodulin-binding sequences in GPIbβ (Arg149–Leu167), a model calmodulin-binding control peptide CBCP, and L-selectin. Identical amino acids or conserved substitutions are boxed. (B) Helical wheel
representation of the GPIbβ and GPVI cytoplasmic sequences. (C) Non-denaturing gel shift of calmodulin (0.3 nmol) in the presence of increasing concentrations of GPVI peptide, His269–Pro287. The shift is shown relative to that induced by a peptide based on the GPV calmodulin-binding sequence Lys529–Gly544. Upper and lower panels were electrophoresed in the presence of 1 mM Ca²⁺ or 10 mM EGTA, respectively. Gels were stained with Coomassie blue.
Figure 1

MS#2001-11-0008
Figure 2

MS#2001-11-0008

(revision 1)
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