Interaction of calmodulin with the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX-V complex


Engagement of platelet membrane glycoprotein (GP) Ib-IX-V by von Willebrand factor triggers Ca\(^{2+}\)-dependent activation of αIIbβ3, resulting in (patho)physiological thrombus formation. It is demonstrated here that the cytoplasmic domain of GPIb-IX-V associates with cytosolic calmodulin. First, an anti-GPIb\(_x\) antibody coimmunoprecipitated GPIb-IX and calmodulin from platelet lysates. Following platelet stimulation, calmodulin dissociated from GPIb-IX and, like the GPIb-IX-associated proteins 14-3-3ζ and p85, redistributed to the activated cytoskeleton. Second, a synthetic peptide based on the cytoplasmic sequence of GPIb\(_x\), R149–L167 (single-letter amino acid codes), affinity-isolated calmodulin from platelet cytosol in the presence of Ca\(^{2+}\) as confirmed by comigration with bovine calmodulin on sodium dodecyl sulfate–polyacrylamide gels, by sequence analysis, and by immunoreactivity with the use of an anti-calmodulin antibody. The membrane-proximal GPIb\(_x\) sequence was analogous to a previously reported calmodulin-binding sequence in the leukocyte adhesion receptor, L-selectin. In addition, the cytoplasmic sequence of GPV, K529–G544, was analogous to a calmodulin-binding IQ motif within the α1c subunit of L-type Ca\(^{2+}\) channels. Calmodulin coimmunoprecipitated with GPV from resting platelet lysates, but was dissociated in stimulated platelets. A GPV-related synthetic peptide also bound calmodulin and induced a Ca\(^{2+}\)-dependent shift on non-denaturing gels. Together, these results suggest separate regions of GPIb-IX-V can directly bind calmodulin, and this novel interaction potentially regulates aspects of GPIb-IX-V-dependent platelet activation. (Blood. 2001;98:681-687)

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Materials and methods

Bovine serum albumin (BSA), bovine calmodulin, prostaglandin E\(_1\) (PGE\(_1\)), phenylmethylsulfonyl fluoride (PMSF), and leupeptin were purchased from Sigma (St Louis, MO). N-ethylmaleimide (NEM) was purchased from
Calbiochem (San Diego, CA). Synthetic peptides based on cytoplasmic sequences of GPIbβ17 or GPV,18 in some cases containing an N-terminal cysteine to facilitate coupling, were purified by reverse-phase high-pressure liquid chromatography (HPLC) and characterized by mass spectroscopy (Chiron Mimotopes) (Clayton, Victoria, Australia). A control peptide, CLKKLIRSPSIPHQY (single-letter amino acid codes), was obtained from the same supplier.

Antibodies

Rabbit polyclonal immunoglobulin G (IgG) against the soluble extracellular portion of GPIbα (glycocalcin) was raised and purified as previously described.8,9 A rabbit polyclonal antibody against human GPV was the kind gift of Dr David Phillips (COR Therapeutics, San Francisco, CA). Rabbit polyclonal IgG against human actin-binding protein was provided by Dr Dominic Chung (University of Washington, Seattle). Rabbit polyclonal antisera against the p85 subunit of PI-3 kinase and mouse monoclonal anticalmodulin IgG were obtained from Upstate Biotechnology (Lake Placid, NY).

Preparation of washed platelets and platelet subcellular fractions

Platelets were obtained from healthy volunteers and washed by means of a previously described method.19,20 Washed platelets (5 × 10^9/mL) were either left unstimulated or stimulated with 1 U/mL thrombin for 5 minutes at room temperature. Following thrombin activation, platelets were lysed with 1 vol Triton X-100 lysis buffer (200 mM Tris-HCl, pH 7.4, 10% Triton X-100, 50 mM ethylene glycol tetraacetic acid [EGTA], 4 mM leupeptin, 4 mM aprotinin, 2.5 mg/mL PMSF) to 9 vol platelets and rocked at 4°C for 1 hour. Under these conditions, GPV dissociates from GPIb-IX.8,9 Lysates were centrifuged at 15 400 g for 10 minutes to separate the Triton X-100 soluble and insoluble (actin cytoskeletal) extracts, and the platelet subcellular fractions were isolated as previously described.19,20 The cytoskeletal pellet was solubilized by incubation with 2 × radioimmunoprecipitation-assay buffer (20 mM NaH_2PO_4, pH 7.0, 0.15 M NaCl, 2 mM EDTA, 2 mM PMSF, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) for 1 hour at 4°C, followed by centrifugation at 15 400g for 10 minutes.

Immunoprecipitation of platelet lysates

Immunoprecipitation from lysates with rabbit nonimmune IgG, anti-GPIbα (glycocalcin) IgG, or anti-GPV IgG was carried out as previously described.8,13 For some experiments, platelets were pretreated with a final concentration of either 3 nM PGE_1 or 1 mM NEM prior to lysis and immunoprecipitation. For Western blotting, samples were electrophoresed on SDS–polyacrylamide gels under reducing conditions, electrophoresed to nitrocellulose, blocked with 5% skim milk powder in TS buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4), and immunoblotted with anti-GPIbα, anti-p85, anti–actin-binding protein, or anticalmodulin antibodies, also as described elsewhere.13,19 Blots were visualized by means of the appropriate horseradish peroxidase–coupled antirabbit or antimouse secondary antibody (Silenus, Hawthorn, Australia), and the electrogenerated chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, United Kingdom).

Preparation of peptide-affinity resins

Synthetic peptides corresponding to the GPIbβ sequence, R149–L167, the GPV sequence, K529–G544, or the control peptide CLKKLIRSPSIPHQY were coupled to BSA (0.25 mg peptide per 10 mg BSA) with N-maleimido-benzoyle-N-hydroxysuccinimide (Pierce, Rockford, IL) as previously described.13 The peptide–BSA conjugate or peptide alone was coupled to BSA (0.25 mg peptide per 10 mg BSA) with cysteine to facilitate coupling, were purified by reverse-phase high-pressure liquid chromatography (HPLC) and characterized by mass spectroscopy (Chiron Mimotopes), and immunoblotted with anti-GPIb antibody, Tris-HCl, 0.15 M NaCl, pH 7.4), and immunoblotted with anti-GPIb antibody, nitrocellulose, blocked with 5% skim milk powder in TS buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4), and immunoblotted with anti-GPIbα, anti-p85, anti–actin-binding protein, or anticalmodulin antibodies, also as described elsewhere.13,19 Blots were visualized by means of the appropriate horseradish peroxidase–coupled antirabbit or antimouse secondary antibody (Silenus, Hawthorn, Australia), and the electrogenerated chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, United Kingdom).

Peptide-affinity chromatography of platelet cytosol

Cytosol from 10 U day-old washed human platelets was prepared as previously described.8 The cytosol was dialyzed into TS buffer, CaCl_2 added to give a final concentration of 1 mM, and loaded at 25 mL/hr onto 10 × 1 cm GPIbβ-peptide, GPV-peptide, or control-peptide columns and washed with TS buffer containing 1 mM CaCl_2. Bound protein was eluted by 10 mM EGTA in TS buffer, and fractions (2 mL) were analyzed on SDS–5%–to-20% polyacrylamide gels under reducing conditions as described elsewhere.13

Nondenaturing gel shift assay

The effect that a synthetic peptide based on the GPV sequence, K529–G544, had on the migration of bovine calmodulin was analyzed on nondenaturing gels as previously described.21-23 Calmodulin (approximately 0.3 nmol) was mixed with 0 to 3.0 nmol GPV-related peptide or a control peptide (CLKKLIRSPSIPHQY) in 0.1 M Tris-HCl, pH 7.5, containing 4 M urea in the presence of either 1 mM Ca_2+ or 10 mM EGTA. After incubating for 30 minutes at 22°C, 0.5 vol 50% glycerol was added; samples were electrophoresed on 12.5% polyacrylamide gels containing 4 M urea at 50 mA for 3 hours; and gels were stained with Coomassie blue.

Amino acid sequence analysis

For sequence analysis, protein was dialyzed into distilled water and digested with trypsin (0.1 mg trypsin per milligram protein) overnight at 37°C. Tryptic fragments were separated by reverse-phase HPLC eluted by a linear 0% to 60% (vol/vol) acetonitrile gradient and sequenced as previously described.24

Results

Coprecipitation of calmodulin with GPIb-IX or GPV from platelet lysates

Prior to investigating whether the GPIb-IX-V complex bound calmodulin, platelet lysates were first immunoblotted with an anticalmodulin antibody. As shown in Figure 1, calmodulin was present in the cytosolic fraction of both untreated and thrombin-stimulated platelets. Interestingly, activation of platelets by thrombin resulted in a redistribution of cytosolic calmodulin and markedly increased association with the actin cytoskeleton (Figure 1). For comparison, Figure 1 also shows the same samples immunoblotted with an antibody against the p85 subunit of PI

**Figure 1. Translocation of platelet proteins to the activated cytoskeleton.** Platelets that were untreated or stimulated with α-thrombin (1 U/mL, final concentration) for 5 minutes at room temperature were lysed, and cytosolic and actin cytoskeletal (CYSK) fractions were isolated and immunoblotted with antibodies to p85, calmodulin, GPIbα, or GPV. Blots were visualized by means of a peroxidase–coupled second antibody and the ECL reagent. Results are typical of 3 separate experiments.
It has previously been shown that phosphorylation of GPIbβ at S166 enhances binding of 14-3-3ζ,13,15 Treatment of platelets with PGE1 results in protein kinase A (PKA)–dependent phosphorylation of platelet GPIbβ and enhanced 14-3-3ζ binding.15,25 We therefore assessed whether PGE1 treatment of platelets affected association of calmodulin. As shown in Figure 3A, the extent of calmodulin that coimmunoprecipitated with GPIb-IX from the Triton X-100 soluble fraction of platelet lysates was not significantly affected by PGE1 treatment, suggesting calmodulin association was not competing with 14-3-3ζ binding to GPIbβ.

It has also previously been shown that treatment of platelets with NEM causes dissociation of actin-binding protein from GPIbα.8 This is demonstrated by immunoblotting of anti-GPIb immunoprecipitates with an anti–actin-binding protein antibody in Figure 3B. Treatment of platelets with NEM also resulted in a reduction in the level of calmodulin associated with GPIb-IX (Figure 3B). The level of calmodulin associated with GPIb-IX in NEM-treated compared with untreated platelets, however, was reduced by 40% to 50% in 3 separate experiments. These data suggest that calmodulin association with the GPIb-IX complex is not dependent on coassociation of actin-binding protein but may be stabilized in part by its coassociation.

**Binding of calmodulin to a synthetic peptide based on the cytoplasmic sequence of GPIbβ**

To investigate potential calmodulin binding sites within GPIb-IX-V, we observed that a sequence within the cytoplasmic tail of GPIbβ (R149–L167) was analogous to calmodulin-binding motifs found in other proteins (Figure 4). A synthetic peptide corresponding to the GPIbβ sequence, R149–L167 (B1 peptide), was coupled to agarose. Platelet cytosol (Figure 5A) was loaded onto the affinity resin in the presence of 1 mM Ca+++, and bound protein was eluted.
by 10 mM EGTA. While no detectable proteins eluted from a control peptide (CLKKLIRSPSIPHQY) column (Figure 5B), a protein eluted from the B1 peptide column was approximately 22 kDa on SDS–polyacrylamide gel electrophoresis under both reducing (Figure 5C) and nonreducing (not shown) conditions. Two lines of evidence suggested that the protein eluted from the B1 column was calmodulin. First, it comigrated with bovine calmodulin on SDS–polyacrylamide gels and was immunoblotted by an anticalmodulin antibody (Figure 5D). Second, the amino acid sequence of a tryptic fragment of the purified protein revealed a sequence (R/K)XVMXNLGE that was conserved in human calmodulin (RHVMTNLGE).

Interaction of calmodulin with the cytoplasmic tail of GPV

The cytoplasmic tail of GPV also contained a sequence with similarity to an IQ motif (Figure 6) previously reported to interact with calmodulin in a Ca\(^{2+}\)-dependent manner. As described above for the GPIbβ peptide, an affinity column of the GPV peptide coupled to agarose specifically bound a 22-kd protein from platelet cytosol in the presence of Ca\(^{2+}\). The bound protein was eluted by EGTA, comigrated with bovine calmodulin on SDS–polyacrylamide gels, and was immunoblotted by an anticalmodulin antibody (Figure 5D). To further examine whether this sequence in GPV bound calmodulin, we also used a nondenaturing gel shift assay previously shown to specifically identify calmodulin-binding protein.
peptides. A synthetic peptide corresponding to the entire GPV cytoplasmic sequence, K529-G544, induced a concentration-dependent shift in calmodulin migration in the presence of Ca++, but not in the presence of EGTA (Figure 7). In contrast to the GPV-related peptide, a positively charged control peptide, CLKK-LIRSPSIPHQY, did not affect calmodulin migration in the gel shift assay when tested at concentrations up to a 10-fold molar excess over calmodulin (data not shown). Related gel shift experiments with the B1 peptide were not technically feasible owing to poor solubility of this peptide at high concentrations.

Discussion

The platelet membrane GPIb-IX-V complex binds to the adhesive ligand vWF and initiates platelet adhesion and aggregation at high-shear stress in flowing blood. Elevation of cytosolic Ca++ in activated platelets leads to actin polymerization and to cytoskeletal rearrangement and activation of αIibβ3, resulting in platelet aggregation and contraction. Recent studies have suggested that 14-3-3z, which directly binds to one or more sequences within the cytoplasmic domain of GPIb-IX-V, may be involved in linking engagement of GPIb-IX-V with activation of αIibβ3. The p85 subunit of PI 3-kinase has also been identified as being associated with GPIb-IX-V in platelets.

In the current study, we have found an additional association between the cytoplasmic domain of GPIb-IX-V and the cytosolic regulatory protein calmodulin. Immunoblotting with anticalmodulin antibody showed that calmodulin was present in the soluble fraction of both resting and thrombin-activated platelets, but as with 14-3-3z and p85, there was an increased association with the cytoskeleton following activation. Calmodulin coimmunoprecipitated with GPIb-IX or GPV from the Triton X-100 soluble fraction of resting platelets, but not thrombin-stimulated platelets, suggesting the GPIb-IX-V-calmodulin complex dissociates in activated platelets.

Within the cytoplasmic domain of GPIbβ, a membrane-proximal 16-residue sequence was similar to other reported calmodulin-binding sequences (Figure 4). In Figure 4B, the GPIbβ sequence is compared with CBCP, a nonphysiological calmodulin-binding control peptide that is representative of a positively charged, amphipathic α-helix typical of calmodulin-recognition sites in other proteins. In addition, a sequence comprising the 16-residue cytoplasmic tail of GPV resembled the IQ motif described in the α1c subunit of the L-type Ca++ channel (Figure 6). Calmodulin is a critical Ca++ sensor for both inactivation and facilitation of L-type Ca++ channels, and mutation of N11624 within this motif dramatically affects the Ca++-dependent feedback regulation of this channel. Conserved motifs of the type represented in either GPIbβ (Figure 4) or GPV (Figure 6) have been reported to specifically bind calmodulin in a range of different proteins. Both motifs are net positively charged, but whether they recognize identical or overlapping sites on calmodulin has not been determined. The GPIbβ- and GPV-related peptides both affinity-isolated calmodulin from platelet cytosol in the presence of Ca++. In contrast to calmodulin’s association with intact GPIb-IX or GPV in platelet extracts that contained EGTA, binding of calmodulin to synthetic peptides based on GPIbβ or GPV was observed only in the presence of Ca++. This suggests the Ca++ dependency and/or affinity of the interaction with calmodulin is different in the intact receptor. This is not inconsistent with other reports of calmodulin-binding peptides from RC3 (neurogranin). In this case, Ca++ sensitivity of interactions with calmodulin was conferred by specific residues immediately upstream of the calmodulin-binding site (Figure 6A). The GPV peptide also induced a shift in migration of bovine calmodulin on nondenaturing gels in the presence of Ca++. Maximal shift was observed at a 10-fold molar excess of peptide to calmodulin, suggesting the interaction was of lower affinity than that reported for the α1c-related peptide, which required equimolar peptide for maximal shift (dissociation constant [Kd], approximately 30 nM), but is of higher affinity than a peptide based on the α1 subunit of the PI(4,5)P2 ion channel where greater than 50-fold excess of peptide was required (Kd, approximately 2 μM).

Structure-function analysis has revealed several striking similarities between platelet GPIbβ and the neural cell–specific, cytoskeletal regulatory protein GAP-43 (Figure 8A). Both GPIbβ (as part of GPIb-IX) and GAP-43 bind calmodulin independently of Ca++ (this study and Baudier et al32), are palmitoylated at a site N-terminal to the calmodulin recognition sequence, and are serine-phosphorylated within or proximal to the calmodulin-binding site by PKA or protein kinase C (PKC), respectively. Interestingly, phosphorylation of GPIbβ by PKA inhibits the actin polymerization that normally occurs following platelet activation. In contrast, GAP-43 becomes concentrated at the cytoplasmic face of the membrane in growth cones during axon development and stabilizes long actin filaments when phosphorylated by PKC. Unphosphorylated GAP-43 reduces actin filament length, and calmodulin association potentiates this effect. It is postulated that association of calmodulin with GAP-43, myristylated alamin-rich C kinase substrates (MARCKS), and other proteins modulates their PKC-dependent phosphorylation. It has also been shown that the MARCKS-related calmodulin-binding peptide directly facilitates actin polymerization and bundle formation. In addition, GAP-43, MARCKS, and CAP23 have been shown to colocalize with PI(4,5)P2 and regulate actin polymerization at plasmalemmal rafts in neural cell lines. This process also involves colocalized P1-kinase, a signaling protein also specifically associated with GPIb-IX-V.

In a manner similar to calmodulin’s effect on PKC phosphorylation of GAP-43, calmodulin could regulate PKA access to GPIbβ and thereby influence activation-dependent actin reorganization in platelets. This possibility deserves future investigation. In this study, Ca++ sensitivity of interactions with calmodulin was conferred by specific residues immediately upstream of the calmodulin-binding site (Figure 6A). The GPV peptide also induced a shift in migration of bovine calmodulin on nondenaturing gels in the presence of Ca++. Maximal shift was observed at a 10-fold molar excess of peptide to calmodulin, suggesting the interaction was of lower affinity than that reported for the α1c-related peptide, which required equimolar peptide for maximal shift (dissociation constant [Kd], approximately 30 nM), but is of higher affinity than a peptide based on the α1 subunit of the PI(4,5)P2 ion channel where greater than 50-fold excess of peptide was required (Kd, approximately 2 μM).

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regard, the calmodulin-binding sequence of GPIbβ is immediately upstream of a conserved RXSXTXP motif that binds 14-3-3ζ and includes the PKA phosphorylation site (Figure 8A) (this study, Andrews et al., and Calverly et al.). Phosphorylation of this sequence inhibits actin polymerization (see above) and increases the affinity of GPIbβ for 14-3-3ζ. Our data suggest that calmodulin association with GPIb-IX is not affected by phosphorylation of GPIbβ, an event previously shown to increase 14-3-3ζ association with GPIbβ. This implies both calmodulin and 14-3-3ζ can potentially bind GPIb-IX. We also showed that NEM treatment diminished the level of GPIb-IX–associated calmodulin. This suggests not only that actin-binding protein and calmodulin can independently associate with GPIb-IX, but also that the calmodulin association with GPIb-IX may be in part stabilized by the coassociation with the GPIb-IX-V complex of actin-binding protein. Further studies are needed, however, to investigate whether NEM treatment may directly decrease the interaction of calmodulin with GPIb-IX independently of actin-binding protein association.

Like 14-3-3ζ, calmodulin binds a wide range of ligands, including calmodulin-dependent kinases I and II, and phosphatases such as calcineurin, in addition to cytoskeletal proteins. GPIb-IX-V, with high copy number (greater than 10,000 per platelet) and multiple sites for calmodulin and 14-3-3ζ (Figure 8B), could therefore be involved in submembranous localization of both calmodulin- and 14-3-3ζ-associated signaling proteins. Dissociation of the Ca2+/calmodulin-binding domain of calmodulin and GPIb-IX-V (this study), and elevation of cytosolic Ca2+ following platelet activation,1 may facilitate Ca2+/calmodulin activation of as-yet-undefined signaling or cytoskeletal proteins.

In summary, this study has identified calmodulin as a GPIb-IX–V–associated protein in platelets, and its specific recognition sequences might ultimately provide novel targets for therapeutic regulation of platelet activation. Further studies are currently underway to define the precise functional consequences of the GPIb-IX-V–calmodulin interaction.

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