Identification of the Region in Actin-binding Protein that Binds to the Cytoplasmic Domain of Glycoprotein Ibα*

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Actin-binding protein (ABP-280) is a component of the submembranous cytoskeleton and interacts with the glycoprotein (GP) Ibα subunit of the GP Ib-IX complex in platelets. In the present studies, we have identified the binding site for GP Ibα in ABP-280. A melanoma cell line lacking ABP-280 was stably transfected with the cDNAs coding for GP Ib-IX, then transiently transfected with cDNA coding for various carboxyl-truncates of ABP-280. Immunocapture assays and co-immunoprecipitation experiments from detergent-lysed cells showed that deletion of residues 2099 through 2136 within repeat 19 abolished binding, but deletion of residues 2099 through 2136 within repeat 19 abolished binding. In the yeast two-hybrid system, an ABP-280 fragment comprising repeats 17–19 bound GP Ibα. Deletion from either end abolished binding. Individual or multiple repeats of ABP-280 were expressed as fusion protein in bacteria and purified; structural folding was evaluated, and binding to GP Ib-IX was assessed. Binding depended on the presence of repeats 17–19. None of the individual repeats were able to bind to GP Ib-IX. These findings demonstrate that residues 1850–2136 comprising repeats 17–19 contain the binding site for GP Ib-IX.

The activation and association of membrane receptors with the submembranous cytoskeleton is of key importance in regulating cellular functions such as adhesion, motility, transmembrane signaling, receptor distribution, and receptor function. A major component of the submembranous cytoskeleton is actin-binding protein-280 (ABP-280), also known as filamin. In platelets, ABP-280 cross-links actin filaments and anchors the membrane skeleton to the plasma membrane by interacting with the cytoplasmic tail of the von Willebrand factor receptor, the glycoprotein (GP) Ib-IX complex (1–6). GP Ib-IX contains a calpain cleavage site. Calpain acts initially at the site between repeats 23 and 24 to generate 90-kDa and a carboxyl-terminal fragment of 100 kDa (7, 8). These glycoproteins are each transmembrane proteins (9–11). The interaction of the GP Ib-IX complex with ABP-280 is mediated by a region in the central portion of the cytoplasmic domain of GP Ibα (5, 6, 12). In other cells of myeloid lineage that do not express GP Ib-IX, ABP-280 binds the high affinity IgG receptor (FcγRI) and the β2 integrin (13, 14).

Biochemical and structural analysis of human ABP-280 and cloning of the human endothelial ABP-280 cDNA reveals a protein of 2647 amino acids with three functional domains (15). ABP-280 subunits self-associate head to head (16) using the most carboxyl repetitive element (15, 17). The opposing amino-terminal end of each subunit contains an actin-binding domain (15, 17–19). The bulk of ABP-280 forms a semiflexible rod domain and is composed of 24 repeats, each about 96 residues long, that are predicted to fold into 8 β-sheets. The rod domain of ABP-280 is interrupted twice by short sequence inserts of 20–40 residues between repeats 15 and 16 and repeats 23 and 24. These regions are postulated to be flexible hinges, and they both contain a calpain cleavage site. Calpain acts initially at the region between repeats 15 and 16 to generate an amino-terminal fragment of 190 kDa and a carboxyl-terminal fragment of 100 kDa (20). The 100-kDa fragment is subsequently cleaved at the site between repeats 23 and 24 to generate 90- and 10-kDa subfragments. Previous reports showed that in platelet lysates in which ABP-280 has been cleaved, GP Ib-IX co-immunoprecipitates with the 100-kDa fragment and to a lesser extent with the 90-kDa fragment (4, 6, 21), suggesting that the binding site for GP Ibα is located in the carboxyl-terminal portion of ABP-280.

In the present studies, using three different approaches, we characterized the region of ABP-280 that interacts with GP Ibα. In the first approach, a melanoma cell line that lacks ABP-280 (22) was stably transfected with the cDNAs coding for the three subunits of the GP Ib-IX complex, then transiently transfected with cDNAs coding for ABP-280 lacking increasing numbers of amino acids from the carboxyl-terminal end; the ability of the truncated forms of ABP-280 to associate with GP Ib-IX was assessed. An ABP-280 fragment truncated at residue 2136 at the border of repeats 19 and 20 (yielding a fragment lacking 511 carboxyl-terminal residues) retained GP Ibα binding. In contrast, a fragment truncated at residue 2099 failed to bind. These results suggest that repeats 20–24 are not required for binding of GP Ibα but that removal of residues 2099 to 2136 of the carboxyl-terminal third of repeat 19 abolishes binding. As a second approach, the yeast two-hybrid system was used (23–27). In this system, an ABP-280 fragment of 286 amino acids, spanning residues 1850–2136 comprising repeats 17–19, bound GP Ibα. Fragments with deletions at either end of this fragment were unable

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to bind. Lastly, repeats 17–19 were expressed in bacteria individually or as multiple repeats. Repeats expressed individually or in tandem were unable to bind to GP Ib-IX; as in the two-hybrid system, a fragment consisting of repeats 17–19 (residues 1850–2136) was required for binding to GP Ib-IX.

**MATERIALS AND METHODS**

Characterization of the Binding Domain by the Two-hybrid System—

Yeast plasmids containing the GAL4 DNA binding domain (pGBT9) (26) and the GAL4 activation domain (pGAD424) (26) were obtained from Clontech Laboratories (Palo Alto, CA). The yeast strain SFY526 (28) (Clontech) was used to assay protein–protein interactions. All yeast manipulations as well as β-galactosidase enzyme activity assays were performed as recommended by the supplier (Clontech).

For preparation of a GP Ib-IX cDNA-containing plasmid, a 0.58-kb DNA fragment containing the sequence coding for the cytoplasmic domain of GP Ib (nucleotides 1637–2218) (9) was cloned into pGBT9 (pGBT9-GP Ib-IX). For preparation of plasmids containing cDNA encoding fragments of ABP-280, a 3.44-kb DNA fragment (nucleotides 4927–8313) coding for the carboxy-terminal part of ABP-280 (aa 1585–2647) (15) was inserted into pGAD424 (pGAD-ABP3.4). P.GAD-ABP3.4 was cut with BglII (all restrictions enzymes used were purchased from New England Biolabs, Inc., Beverly, MA), and the resultant 8.7-kb fragment was isolated and ligated. This new plasmid (pGAD-ABP2.1) contained the ABP cDNA (nucleotides 4927–7017) coding for aa 1585–2282. pGAD-ABP3.4 was also digested with PstI; the 8-kb fragment was isolated and religated. The resulting plasmid (pGAD-ABP1.4) contained the ABP cDNA (nucleotides 4927–6630) coding for aa 1585–2066. Additional constructs were generated by digesting pGAD-ABP1.2 with BglII and producing deletions using exonuclease III (U. S. Biochemical Corp.). The fragments were verified by DNA sequencing analysis.

**Expression of ABP-280 Fragments in Melanoma Cells**—

The melanoma cells used in these studies were from the human melanoma cell line M2 lacking ABP-280 (22). The cells were stably transfected with the cDNAs encoding GP Ib-IX, GP Ib-IX, and GP IX, as described previously (29). Subsequently, cells were transiently transfected with the calcium phosphate method (28) (Clontech) was used to transfect the melanoma cells with either full-length or truncated ABP-280. The cells were harvested 48 h after transfection.

To prepare ABP-280 constructs for transfection into the melanoma cells, full-length or fragments of cDNA coding for ABP-280 were inserted into pCDM8 (31). ABP-280 cDNA in the Bluescript SK vector was digested with XbaI, and carboxy-terminal truncations were obtained by incrementally deleting the 3′ end with exonuclease III. A synthetic oligonucleotide, containing stop codons in all three reading frames, was added. After digestion, the truncated ABP-280 cDNA fragments were subcloned into pCDM8. The clones were sequenced to determine the sizes of the deletions. Three clones were selected. pGAD-ABP1.8 contained the cDNA sequence (nucleotides 4927–6753) coding for aa 1585–2194; pGAD-ABP1.6 (nucleotides 4927–6580) coding for aa 1585–2136; and pGAD-ABP1.5 (nucleotides 4927–6469) coding for aa 1585–2099. A 0.2-kb fragment isolated after digesting pGAD-ABP1.6 with PstI was introduced into pGAD424 to generate pGAD-ABP0.2 (nucleotides 6370–6580 coding for aa 2066–2136). Various fragments of DNA coding for ABP were generated by PCR, and the resultant PCR products were inserted into pGAD424 to generate pGAD-ABP0.3 (nucleotides 6232–6580 coding for aa 2020–2136), pGAD-ABP0.5 (nucleotides 6022–6580 coding for aa 1950–2136), and pGAD-ABP0.8 (nucleotides 5722–6580 coding for aa 1850–2136). The sequences of the amplified fragments were verified by DNA sequencing analysis.

**Expression of ABP-280 Repeats as Fusion Protein in Bacteria**—ABP-280 cDNAs corresponding to the desired amino acid sequences were generated by PCR and ligated into a pGEX vector (Pharmacia Biotech Inc.). All 3′ primers contained stop codons in all three reading frames to insure proper termination of translation. The sequences of the amplified fragments were verified by DNA sequencing analysis.

**Binding of ABP-280 to GP Ib-IX in Melanoma Cells Does Not Require Repeats 20–24 of the ABP-280 Subunit**—As a first approach to identifying the region of ABP-280 that interacts with GP Ib-IX, we used the melanoma cell line M2 deficient in ABP-280 (22). M2 cells were stably transfected with the cDNAs encoding GP Ib-IX, GP Ib-IX, and GP IX. Even in the absence of ABP-280, the three subunits were expressed, formed a complex, and were inserted into the plasma membrane. Fig. 1
demonstrates the surface expression of GP Ib-IX in cells stained with an antibody against GP Ib-IX. Similar results were obtained with an antibody against GP IX (data not shown).

The GP Ib-IX-expressing cells were then transiently transfected with the constructs shown in Fig. 2 containing cDNAs coding for ABP-280 lacking increasing numbers of amino acids from the carboxyl-terminal end. The amount of ABP-280 carboxyl truncates expressed in the cells transfected with the different constructs was very similar (data not shown). To determine whether GP Ib-IX associated with the various truncated forms of ABP-280, the transfected cells were lysed in a Triton X-100-containing buffer. The ABP-280 fragments were captured with an anti-ABP antibody absorbed onto microtiter plates, and the amount of GP Ib-IX associated with the fragments was determined. Fig. 3A shows that like full-length ABP-280 in stably transfected cells (construct 1), ABP-280 truncates expressed in cells transiently transfected with constructs 2–5 (lacking 27 to 257 carboxy-terminal amino acids contained within the last third of repeat 22 through repeat 24) were recovered in association with GP Ib-IX. Similarly, a protein lacking repeats 20–24 (deletion of 511 carboxy-terminal amino acids), expressed in cells transiently transfected with construct 6, was able to bind to GP Ib-IX just as well as full-length ABP-280 (Fig. 3B). In contrast, an ABP-280 fragment missing 548 amino acids (lacking repeats 20–24 plus the carboxy-terminal third of repeat 19), expressed just as efficiently as the fragment lacking 511 carboxy-terminal amino acids (Fig. 3, inset, compare lanes 7 and 6), did not bind to GP Ib-IX (Fig. 3B, column 7). Similar results were obtained when GP Ib-IX was captured with an anti-GP Ib-IX antibody and the amount of ABP-280 associated was measured (data not shown).

The ability of the ABP-280 fragments to interact with GP Ib-IX in melanoma cells was also assessed by immunoprecipitating ABP-280 from detergent-lysed cells. In this experiment, GP Ib-IX-expressing cells were transiently transfected with the cDNA encoding full-length or truncated ABP-280. Full-length ABP-280 (Fig. 4B, lane 2), as well as proteins missing 511 and 548 amino acids from their carboxy termini (Fig. 4B, lanes 3 and 4), were immunoprecipitated using an anti-ABP antibody. GP Ib-IX co-immunoprecipitated with full-length ABP-280 and with ABP-280 lacking 511 carboxy-terminal amino acids (Fig. 4A, lanes 2 and 3). However, GP Ib-IX did not co-immunoprecipitate with ABP-280 lacking 548 carboxy-terminal amino acids (Fig. 4A, lane 4).

These results suggest that repeats 20–24 of ABP-280 are not required for binding of ABP-280 to GP Ib-IX, but that residues 2099–2136 in the carboxyl-third of repeat 19 are essential.

**Further Characterization of the GP Ib-IX Binding Site in the Yeast Two-Hybrid System**—As a second approach to identifying the GP Ib-IX binding site on ABP-280, we used the two-hybrid system (23–27). Based on previous findings, suggesting that the binding site for GP Ib-IX was located in the carboxy-terminal third of ABP-280 (4, 21), the cDNA encoding the carboxy-terminal third of ABP-280 (aa 1585–2647, consisting of the middle of repeat 14 through repeat 24) was fused to the GAL4 activation domain. The cDNA encoding the cytoplasmic domain of GP Ib-IX was fused to the GAL4 DNA binding domain. The recombinant plasmids were co-transformed into the SFY526 yeast strain. The interaction between GP Ib-IX and ABP-280 reconstituted the function of GAL4; β-galactosidase activity was induced, resulting in the generation of blue colonies (Fig. 5). Neither fusion protein alone was able to activate the transcription of the reporter gene. These results confirmed that the binding site for GP Ib-IX was contained within the carboxyl-terminal third of the molecule. As in the melanoma cells, deletion of the last 511 carboxy-terminal amino acids of the ABP-280 subunit (aa 1585–2136, consisting of the middle of repeat 14 through repeat 19) did not affect its binding to GP Ib-IX in the two-hybrid system (Fig. 5). Binding was lost, however, when just 37 more residues were deleted (deletion of the last 548 carboxy-terminal amino acids).

The results presented thus far demonstrate that a fragment of ABP-280 consisting of the middle of repeat 14 through repeat 19 (residues 1585–2136) can bind GP Ib-IX. To determine which part of the amino-terminal portion is required for binding, constructs containing cDNA coding for ABP-280 fragments with increasing deletions from the amino-terminal end were prepared. A truncated fragment of 286 amino acids containing residues 1850–2136 (comprising repeats 17–19) was still able to bind to GP Ib-IX (Fig. 5). However, further deletions of this fragment from its amino-end abolished the binding to GP Ib-IX (Fig. 5); a truncate consisting of residues 1950–2136 of ABP-280 was expressed, blue colonies could be detected after 30 min, whereas a 15–18-h incubation was necessary to detect an interaction with a trun-

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![Figure 1](image1.png)

**Fig. 1.** Expression of GP Ib-IX on the surface of transfected melanoma cells lacking ABP-280. Nontransfected melanoma M2 cells (dotted line) or cells stably transfected with the cDNAs encoding GP Ib-IX, GP Ib-IX, and GP IX (solid line) were incubated with MAAb Ib-23, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG and analyzed by flow cytometry.

![Figure 2](image2.png)

**Fig. 2.** Schematic representation of the ABP-280 carboxyl truncates expressed in melanoma cells. The horizontal bars represent the full-length ABP-280 and the different ABP-280 carboxyl truncates expressed in the experiments shown in Figs. 3 and 4 (labeled 1–7, on the left of the horizontal bars). The numbers on the right of the horizontal bars indicate the carboxyl-terminal amino acid. The numbers in the horizontal bars correspond to the repeat number. Calpain 1 and 2 show sites at which ABP-280 is known to be cleaved by calpain.
Fig. 3. Immunocapture assays showing which of the ABP-280 truncates associate with GP Ib-IX. The GP Ib-IX-expressing cells, deficient in ABP-280, were transiently transfected with one of the six cDNAs coding for carboxyl termini ABP-280 truncates (shown in Fig. 2). The cells were harvested 48 h after transfection and lysed in a Triton X-100-containing lysis buffer (see "Materials and Methods"). ABP-280 fragments from 25,000 to 160,000 cells were captured with an anti-ABP-280 antibody, and the amount of GP Ib-IX associated with ABP-280 was determined. The numbers on the top of each column correspond to the expressed ABP-280 carboxyl truncates as defined in Fig. 2. The unlabeled columns represent the signal obtained with cells deficient in ABP-280, and the columns labeled 1 show the signal obtained with cells stably transfected with full-length ABP-280 cDNA. The results shown in A and B were obtained in two different experiments, and both are representative of three independent experiments. The inset in B is a Western blot showing the amount of ABP-280 fragments 6 and 7 expressed in comparable numbers of cells.

Fig. 4. Co-immunoprecipitation experiments determining which of the ABP-280 fragments bind to GP Ib-IX. GP Ib-IX-expressing cells, deficient in ABP-280, were transiently transfected with the full-length ABP-280 cDNA or the ABP-280 cDNA coding for aa 1–2136 or for aa 1–2099. The cells were lysed 48 h after transfection in a Triton X-100-containing buffer (see "Materials and Methods"). The Triton-soluble fractions were incubated with MAb ABP-4-coupled immunobeads. Immunoprecipitated proteins were electrophoresed through a 7.5% SDS-polyacrylamide gel under reducing conditions and then transferred to nitrocellulose. The blots were first probed with a monoclonal antibody against GP Ibα (MAb Ib-4) (A), followed by a monoclonal antibody against ABP (MAb ABP-4) (B). Lane 1, GP Ib-IX-expressing cells deficient in ABP-280; lane 2, GP Ib-IX cells transfected with full-length ABP-280 cDNA (fragment 1 in Fig. 2); lane 3, GP Ib-IX cells transfected with the ABP-280 cDNA coding for aa 1–2136 (fragment 6 in Fig. 2); lane 4, GP Ib-IX cells transfected with the ABP-280 cDNA coding for aa 1–2099 (fragment 7 in Fig. 2).

**DISCUSSION**

The goal of the present study was to identify the domain on ABP-280 that binds GP Ibα. Three different approaches have lead to the same conclusion that the binding site for GP Ibα is located within repeats 17–19 of ABP-280. In the first approach, melanoma cells deficient in ABP-280 but expressing GP Ib-IX were transfected with various cDNAs directing the synthesis of carboxyl-deleted ABP-280. ABP-280 truncates lacking carboxyl-terminal residues from 27 to 511 associated with GP Ib-IX in these cells, but further deletion of 37 residues abrogated GP...
in repeats 17–19 (residues 1850–2136). This is somewhat surprising because a great degree of conformity to the consensus repeat exists in repeats 17 and 19, and we would expect the binding site to be located in a less conserved region. A bigger divergence exists in repeat 18, where the regular pattern is interrupted by a 9-residue deletion at the initial portion of the repeat. This divergence from the consensus sequence may play a role in the binding of GP Ib.

The rod domain of ABP-280 is interrupted at two points by sequence inserts that contain a calpain cleavage site. Earlier studies showed that in platelet lysates in which ABP-280 was cleaved by calpain, GP Ib-IX remained associated with the 100-kDa carboxyl portion of the polypeptide chain and to a lesser extent with its 90-kDa calpain-generated subfragment (4, 6, 21). This suggested that GP Ib-IX might bind near the calpain cleavage site in the hinge just before repeat 24 (15). Alternatively, calpain cleavage could perturb the conformation of the 90-kDa subfragment and thereby diminish GP Ib-IX binding. The location of the GP Ib binding site determined in the present study suggests the latter mechanism to explain the decreased amount of GP Ib associated with the 90-kDa subfragment.

ABP-280 has proved difficult to dissociate from the GP Ib-IX complex in detergent-lysed platelets (1–4, 34). This observation stands in marked contrast to the $1 \times 10^{-7}$ m$^{-1}$ affinity of ABP-280 for GP Ib-IX measured in vitro (5). An experimental observation may shed some light on this discrepancy. The interaction between the fragment of ABP-280 formed by repeats 17–19 (residues 1850–2136) and GP Ib was relatively strong in the two-hybrid system compared to the one obtained with larger fragments from ABP-280 (Fig. 5). This might suggest that binding to GP Ib can be modulated by flanking sequence on both ends of repeats 17–19. Many other factors could, of course, contribute to the weaker signal obtained in the two-hybrid system with larger fragments; for example, these fragments may be folded inappropriate as repeats, or there
may be differences in the accessibility of the interacting domains to each other or accessibility of the activation domain to the transcriptional machinery.

Human GP Ib-IX has been expressed in hamster and mouse nonhematopoietic cells in a form that associated with ABP-280 (35). Thus, the domain on ABP-280 that associates with the cytoplasmic domain of GP Ibα appears to have been conserved. ABP-280 is a prominent component of many different cell types (36, 37), and it now appears that the rod of this protein may act as a scaffolding to collect and tether diverse molecules at the cytoplasmic surface of the plasma membrane. Membrane association is conferred in platelets and megakaryocytes by the binding of repeats 17–19 to GP Ib-IX complexes. Other membrane receptors that bind to ABP-280 are the IgG Fc receptor I (FcγRI) (13) and integrin β2 (14). Sequence comparison between the cytoplasmic tails of GP Ibα, FcγRI, and integrin β2 has, however, revealed no sequence conservation (9, 14, 38). Thus, the domain on ABP-280 implicated in the interaction with FcγRI and integrin β2 is likely to be different from the one interacting with GP Ibα. Other proteins now known to bind along ABP-280 include small GRP-binding proteins, the endoproteinase furin,2 and the transcription factor 

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