A Novel Ligand-binding Site in the \( \zeta \)-Form 14-3-3 Protein Recognizing the Platelet Glycoprotein Ib\( \alpha \) and Distinct from the c-Raf-binding Site*

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We reported previously that the \( \zeta \)-form 14-3-3 protein (14-3-3-\( \zeta \)) binds to a platelet adhesion receptor, glycoprotein (GP) Ib-IX, and this binding is dependent on the SGHSL sequence at the C terminus of GPIb. In this study, we have identified a binding site in the helix I region of 14-3-3-\( \zeta \) (residues 202–231) required for binding to GPIb-IX complex and to the cytoplasmic domain of GPIb\( \alpha \). We also show that phosphorylation-dependent binding of c-Raf to 14-3-3-\( \zeta \) requires helix G (residues 163–187) but not helix I. Thus, the GPIb-IX-binding site is distinct from the binding sites for RSXpSXP motif-dependent ligands. Furthermore, we show that wild type 14-3-3-\( \zeta \) has a higher affinity for GPIb-IX complex than recombinant GPIb\( \alpha \) cytoplasmic domain. Deletion of helices A and B (residues 1–32) disrupts 14-3-3-\( \zeta \) dimerization and decreases its affinity for GPIb-IX. Disruption of 14-3-3-\( \zeta \) dimerization, however, does not reduce 14-3-3-\( \zeta \) binding to recombinant GPIb\( \alpha \) cytoplasmic domain. This suggests a dual site recognition mechanism in which a 14-3-3-\( \zeta \) dimer interacts with both GPIb\( \alpha \) and GPIb\( \beta \) (known to contain a phosphorylation-dependent binding site), resulting in high affinity binding.

A platelet receptor for von Willebrand factor, the glycoprotein (GP) Ib-IX-V complex (GPIb-IX-V), mediates initial platelet adhesion to the subendothelial matrix and triggers platelet activation under high shear rate conditions (for reviews, see Refs. 1 and 2). GPIb-IX-V also binds thrombin and is important in thrombin-induced platelet activation (3–5). GPIb-IX-V consists of four different transmembrane subunits as follows: disulfide-linked GPIb\( \alpha \) and GPIb\( \beta \) forms a 1:1 complex with GPIX (6); the GPIb-IX complex (GPIb-IX) forms a 2:1 complex with GPV which may dissociate in certain detergents such as Triton X-100 (7). Accumulating evidence indicates that ligand binding to GPIb-IX-V triggers transmembrane signaling events including activation of protein kinase C (8, 9) and tyrosine kinases (10), elevation of intracellular calcium (8, 11, 12), synthesis of thromboxane A\( _2 \) (8), and activation of phosphoinositide 3-kinase (10), leading to activation of ligand binding function of the responsive adhesion receptor, integrin \( \alpha_{\text{IIb}} \beta_3 \). We found that GPIb-IX is physically associated with an intracellular signaling protein, the \( \zeta \)-form 14-3-3 protein (14-3-3-\( \zeta \)) (13), suggesting a potential role for 14-3-3-\( \zeta \) in GPIb-IX-V mediated signaling. We further identified a C-terminal 5-residue sequence of GPIb\( \alpha \) (SGHSL) that is critical for the binding of 14-3-3-\( \zeta \) (14), a result confirmed by Andrew et al. (15). Interestingly, in addition to the C-terminal sequence of GPIb, GPIb-IX binding to 14-3-3-\( \zeta \) also involves a protein kinase A (PKA)-phosphorylated binding site in the cytoplasmic domain of GPIb\( \beta \) (15, 16).

14-3-3-\( \zeta \) belongs to the 14-3-3 family of highly conserved intracellular proteins (17). The 14-3-3 proteins are dimeric; each monomer is composed of nine anti-parallel \( \alpha \)-helices forming a large ligand binding groove as revealed by crystal structure analysis (18, 19). The 14-3-3 proteins bind and regulate a variety of intracellular signaling molecules, including various protein kinase C isoforms (17, 20), c-Raf (21–24), Bcr (25), middle T antigen (26), c-Cbl (27), cdc25 (28), and the cell death factor BAD (29). The 14-3-3 proteins have also been implicated in the assembly of protein kinase complexes (30). Thus, it is possible that binding of GPIb-IX to 14-3-3-\( \zeta \) links GPIb-IX to intracellular signaling pathways. In addition, the 14-3-3-binding site in GPIb\( \alpha \) has been shown (31) to be important in regulating the lateral movement of GPIb-IX-V in plasma membrane, suggesting its possible involvement in regulating GPIb\( \alpha \) interaction with the cytoskeleton. Thus, structural definition of the 14-3-3-\( \zeta \)-GPIb-IX interaction may serve as a basis for understanding the role of 14-3-3 in signaling mediated by GPIb-IX.

Many of the 14-3-3-binding proteins contain an Arg-Ser-X-phosphoserine-X-Pro (RSXpSXP) motif, originally found in c-Raf (32). At least two of the RSXpSXP motif-containing ligands c-Raf and tryptophan hydroxylase can be induced to bind 14-3-3 by PKA-catalyzed phosphorylation (32, 33). Other serine protein kinases have also been shown to induce 14-3-3 binding to various proteins (27, 34). Recently, several different 14-3-3 binding sequences have been identified in 14-3-3 ligands including RX(Y/F)XpSXP, RX(X)pS(pT)Xp (where (pT) is phosphothreonine) (15, 27, 35). In the cytoplasmic domain of GPIb\( \alpha \), a 5-residue sequence, SGHSL, is both necessary and sufficient for interaction with 14-3-3-\( \zeta \) (14, 15). Interestingly, in contrast to RSXpSXP-containing ligands c-Raf and tryptophan hydroxylase, the cytoplasmic domain of GPIb\( \alpha \) appears to be a poor PKA substrate in intact platelets (36, 37). Thus, characterization of the structural basis of the GPIb\( \alpha \) interaction with 14-3-3 will clarify the differences between RSXpSXP-containing 14-3-3 ligands and GPIb\( \alpha \) and further our understanding of how 14-3-3 regulates functions of different types of ligand proteins.

In this study, we have identified a binding site in 14-3-3-\( \zeta \) in the helix I region of 14-3-3-\( \zeta \) encompassing residues 202–231 that interact with the GPIb\( \alpha \) cytoplasmic domain and the intact GPIb-IX complex. We also show in vitro that c-Raf binds to...
14-3-3ζ in a PKA-dependent manner and that this binding requires helix G but not helix I. Thus, the 14-3-3ζ binding site in 14-3-3ζ is distinct from the binding site for RSxPSPx-containing ligand c-Raf. Furthermore, we show that 14-3-3ζ dimerization is required for high affinity binding to GPIb-IX complex, suggesting that a dual site recognition mechanism involving GPIb and β subunits and dimerized 14-3-3ζ.

MATERIALS AND METHODS

Recombinant 14-3-3ζ, 14-3-3ζ Mutants, and Recombinant GPIb Cytoplasmic Domain—Cloning of the cDNA encoding wild type 14-3-3ζ was described previously (14). The 14-3-3ζ cDNA was subcloned into pmalC2 vector (New England Biolabs, Beverly, MA). The construct (pmαl1433ζ) encodes a fusion protein with the N-terminal region corresponding to the Escherichia coli maltose-binding protein (MBP) and C-terminal region corresponding to 14-3-3ζ. Mutagenesis of pmαl1433ζ was performed using PCR techniques (38). In all mutants except T3, stop codons were introduced into reverse primers at designated sites of the 14-3-3ζ. The stop codon in mutant T3 was introduced inadvertently by PCR error. The mutants were subcloned into a pmalC2 vector at the EcoRI and XhoI sites. Correct sequences were verified by automated sequencing. The wild type 14-3-3ζ and 14-3-3ζ truncation mutants were purified by affinity chromatography using a cross-linked amylene-Sepharose 4B (New England Biolabs, Beverly, MA). Equivalent amounts of 14-3-3ζ or MBP were conjugated onto cyrogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech), respectively. Coupling efficiencies in all cases were better than 99% as assessed by optical density at 280 nm wave length.

The cDNA encoding GPIbα in a pBlueScript vector was a generous gift from Dr. Jerry Ware at the Scripps Research Institute, La Jolla, CA. The cDNA fragment encoding the cytoplasmic domain of GPIbα (residues 518–610) was generated by PCR with EcoRI and XhoI sites incorporated in the forward and reverse primers, respectively. The PCR product was subcloned into the pmalC2 vector. The correct sequence was verified by automated sequencing. The protein was expressed and purified as described previously (38).

Binding of Platelet GPIb-IX and Mal-βC to 14-3-3ζ—Specific binding of the platelet GPIb-IX complex to 14-3-3ζ-conjugated beads has been described previously (14). Briefly, washed platelets were resuspended in Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5.6 mM Na-glucose, 3.3 mM NaH₂PO₄, 3.8 mM Hepes, pH 7.35) and solubilized by adding an equal volume of the solubilization buffer (2% Triton X-100, 0.1 mM Tris, 0.01 mM EDTA, 0.15 M NaCl, and 1 mM dithiothreitol, pH 7.4) containing 0.2 mM E64 (Boehringer Mannheim) and 1 mM phenylmethylsulfonyl fluoride (13). In some experiments, platelets were solubilized in the presence of 1 mM CaCl₂ but in the absence of EGTA and E64 to allow calpain cleavage of GPIbα and thus generation of the C-terminal domain of GPIb-IX complex. After centrifugation at 100,000 × g for 30 min, the lysates (200 μl) were incubated with 25 μl (50% of total) MBP-conjugated control beads or 14-3-3ζ-conjugated beads at 4°C for 1 h. The beads were then washed three times in a 1:1 mix of Hepes buffer and solubilization buffer. Bound proteins were extracted with SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by Western blotting with a rabbit anti-peptide antibody against the cytoplasmic domain of GPIbα (14). Reactions with antibodies were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech), and Kodak X-Omat AR film. In some experiments, reactions were visualized using SuperSignal chemiluminescence substrate (Pierce) and quantitated with a phosphor-sensor using a Bio-Rad phosphorimager and chemiluminescence substrate (Pierce) visualized using SuperSignal chemiluminescence substrate (Pierce).

RESULTS

Truncation Mutagenesis of 14-3-3ζ—In order to identify the sequence within 14-3-3ζ that is responsible for its interaction with the GPIb-IX complex, we made various 14-3-3ζ truncation mutants (Fig. 1). Mutants T1 to T6 were truncated from the C-terminal end of 14-3-3ζ. Mutants T7–T9 were truncated progressively from the N-terminal end of the protein. T11–T309 encompasses helices G and H, and T12 contains helices H and I. T13 (188–209) contains a single helix H that was implicated in cryptophan hydroxylase binding (34). T14 (202–231) containing helix I was generated when results obtained with T1–T13 indicated the location of the GPIb-binding site (see below). As 14-3-3ζ is composed of nine anti-parallel α-helices (A–I), truncation sites in all these mutants are located between two neighboring α-helices as determined by the published crystal structure to avoid disruption of each of these helical structures (Fig. 1). These mutant proteins were expressed as fusion proteins with maltose-binding protein. Equivalent amounts of the purified wild type and mutant 14-3-3ζ were conjugated to cyanogen bromide-activated Sepharose 4B as described previously (38).

A Binding Site for the GPIb-IX Complex Is Located between Residues 188 and 231 of 14-3-3ζ—We first examined the binding of Triton X-100-solubilized platelet GPIb-IX to the above described Sepharose beads conjugated with 14-3-3ζ truncation mutants (T1–T13). MBP-conjugated beads were used as a neg-
GPIb washes, bead-bound GPIb-IX complex was separated by SDS-PAGE (246), T11-(136–209), T12-(188–231), and T13-(188–209). After three mutants T5-(1–209), T6-(1–231), T7-(33–246), T8-(136–246), T9-(188–209) (mutant T5), which removes the helix I (the first 14-3-3-zues 231 and 246 is not required. Further truncation at residue 231 (mutant T6) did not negatively affect GPIb-IX binding (Fig. 2). Furthermore, none of the truncation mutants that lack helix I bound to GPIb-IX (T5, T11, T13, Fig. 2; T1-T4, data not shown). Thus, the helix I region encompassing residues 209–231 of 14-3-3-z contains a binding site for GPIb-IX complex. Similar results were also obtained when bead-bound GPIb-IX was detected with an antibody against the C-terminal region of GPIb, anti-IbaC (data not shown).

High Affinity Interaction Requires Dimerized 14-3-3-z and More Than One Subunit of GPIb-IX—Fig. 2 also shows that truncation mutants of 14-3-3-z lacking the N-terminal domain (T7, T8, T9, and T12) binds significantly less GPIb-IX in comparison with wild type 14-3-3-z. Since similar amounts of proteins were conjugated to these beads (Fig. 2C), this result indicates that GPIb-IX bound to these mutants with reduced affinity. In particular, the mutant T7 lacking only 33 residues in helices A and B (1–33) showed dramatically reduced binding to GPIb-IX (7%) in comparison with wild type 14-3-3-z and GPIb-IX. However, this reduction in GPIb-IX binding affinity is partially “rescued” by further truncations from the N terminus that removed helices A–E (T8, 20% binding compared with wild type 14-3-3-z or helices A–G (T9, 50% binding compared with wild type) (Fig. 2). It is not clear why these further truncations rescued the loss of binding affinity. One of the possibilities is that further truncations resulted in increased accessibility of GPIb-IX to the binding site in helix I. Nevertheless, this result suggests that the N-terminal domain, while important for high affinity, is not required for binding to GPIb-IX.

Previously reported crystal structural analysis of 14-3-3-z has revealed that the N-terminal helices (A and B) are involved in the formation of 14-3-3-z dimers (18, 19). Thus, it is possible that the reduced affinity for GPIb-IX is caused by disruption of dimerization. To examine this possibility, we determined the molecular mass of the MBP-14-3-3-z fusion proteins by gel filtration chromatography under non-denaturing conditions. The 14-3-3-z monomer has a molecular mass of ~30 kDa (40). MBP has a molecular mass of ~40 kDa. The molecular mass for the recombinant MBP-14-3-3 fusion protein is ~70 kDa as analyzed by SDS-PAGE. We found that the MBP-wild type 14-3-3-z fusion protein has a molecular mass of ~160 kDa as determined by gel filtration chromatography (Fig. 3A). Thus, the recombinant wild type 14-3-3-z indeed exists as a dimer even though its N terminus is fused to MBP. In contrast, the fusion proteins encoding 14-3-3 mutants (T7, T9, and T12) lacking the N-terminal domain showed a molecular mass of approximately 70, 55, and 45 kDa by gel filtration (Fig. 3A), indicating that they are monomers. Thus, the N-terminal domain deletion disrupted dimerization of 14-3-3-z. Taken together, these results suggest that a dimeric structure of 14-3-3-z is required for high affinity binding to GPIb-IX.

Previous studies suggest that 14-3-3 interaction with GPIb-IX may involve two binding sites in GPIb-IX located in the C-terminal domain of GPIb-α and GPIb-β, respectively (14–16). To examine whether the high affinity binding of dimerized 14-3-3-z to GPIb-IX may involve interaction with the cytoplasmic domains of both GPIb-α and GPIb-β, we compared binding of 14-3-3-z to the recombinant GPIb-α cytoplasmic (C-terminal) domain alone and to the C-terminal domain of the GPIb-IX complex (composed of the C-terminal domain of GPIb-α, GPIb-β and GPIX (6, 14)) (Fig. 4). As we reported previously (14), the C-terminal domain of GPIb-IX maintained the high affinity 14-3-3-z binding function (Fig. 4). In contrast, the recombinant cytoplasmic domain of GPIb-α bound much more weakly to 14-3-3-z, indicating that in addition to GPIb-α, other subunits of the GPIb-IX complex (probably GPIb-β) are involved in high affinity binding to 14-3-3-z dimer.

Helix I of 14-3-3-z (Residues 202–231) Contains a Binding Site for the GPIbα Cytoplasmic Domain—We showed previously that the binding of the GPIb-IX complex to 14-3-3-z is dependent on the interaction between 14-3-3-z and the C-terminal se-
quence of the GPIbα cytoplasmic domain (14). It is thus possible that the above identified GPIbα-IX-binding site of 14-3-3ζ contains a binding site for GPIbα. To examine this possibility, we tested the binding of recombinant GPIbα C-terminal domain MBP fusion protein (Mal-IbαC) to wild type 14-3-3ζ and various 14-3-3 mutants. Fig. 5 shows that wild type Mal-IbαC protein specifically binds to the 14-3-3ζ main MBP fusion protein (Mal-IbαC) to the monomeric truncation mutants T7, T9, and T12. Reversal of the C-terminal tail of 14-3-3ζ was also observed by truncation at residue 231 (mutant T6) did not negatively affect Mal-IbαC binding, whereas truncation at residue 209 (mutant T5) to remove helix I abolished Mal-IbαC binding. In addition, binding of other truncation mutants that lack the helix I to Mal-IbαC was also disrupted. Thus, the same helix I region (residues 209–231) of 14-3-3ζ that is required for GPIbα-IX binding is also required for 14-3-3ζ binding to the GPIbα cytoplasmic domain. In contrast to the results obtained with the GPIb-IX complex, however, the binding of Mal-IbαC to the monomeric truncation mutants T7, T8, T9, and T12 (lacking the N-terminal dimerization site) was not significantly reduced but rather a little stronger than the dimeric wild type 14-3-3ζ. This suggests that the dimeric structure of 14-3-3ζ is not required for binding to the recombinant GPIbα cytoplasmic domain. It is not clear why Mal-IbαC bound to the small truncation mutants stronger than to wild type 14-3-3ζ. It is unlikely that this is caused by the variation in the amounts of bead-conjugated proteins as the wild type and mutant 14-3-3ζ-MBP fusion protein bound identical amounts of anti-MBP antibody (see Fig. 2C). It is possible that truncations caused increased exposure of the ligand-binding site in the helix I of 14-3-3ζ to the recombinant GPIbα cytoplasmic domain.

To verify further that helix I is indeed a binding site for the GPIbα cytoplasmic domain, we made an additional 14-3-3ζ...
truncation mutant, T14-(202–231), containing only helix I. This mutant strongly bound to Mal-IbC (Fig. 5B), indicating that the 30-residue helix I (202–231) region of 14-3-3α is sufficient for binding to the GPIbo cytoplasmic domain.

**PKA-dependent c-Raf Binding to 14-3-3α Requires Helix G** (Residues 162–187) but Not Helix I (Residues 209–231)—To determine whether the GPIbo-binding site of 14-3-3α is also required for the binding of other 14-3-3 ligands, we examined PKA-dependent binding of c-Raf (a 14-3-3 ligand with the RSXpSXP binding motif (32)) to the 14-3-3α truncation mutants. Fig. 6A shows that, in the absence of PKA pretreatment, c-Raf-MBP fusion protein bound weakly to 14-3-3α-beads, comparable with control MBP-conjugated beads. After PKA-pretreatment, however, c-Raf bound to 14-3-3α strongly. This binding was not affected by truncation of 14-3-3α that removed helix I (T5) or both helices H and I (T4) from the C terminus, indicating that the GPIbo-binding site in helix I is not required for the interaction of 14-3-3α with c-Raf (Fig. 6B). However, further truncation from the C terminus to residue 162 to remove helix G (T2) abolished specific c-Raf binding. The truncation mutant T8 lacking the N-terminal helices A–E still bound to c-Raf, but the mutant T9, which contains C-terminal helices H and I but lacks helices A–G, lost c-Raf binding capacity (Fig. 6B). Thus, binding of the RSXpSXP motif-containing ligand c-Raf to 14-3-3α requires helix G but not helix I.

**DISCUSSION**

In this study, we have further characterized the interaction between GPIbo-IX and 14-3-3α by analyzing the GPIbo-IX binding function of a series of truncation mutants of 14-3-3α. We show that a binding site for the GPIbo cytoplasmic domain is located in the helix I of 14-3-3α encompassing residues 202–231 in the C-terminal domain (Fig. 5). This binding site is also required for 14-3-3α binding to intact GPIbo-IX complex (Fig. 2). The GPIbo-binding site is distinct from the binding sites for RSXpSXP motif-dependent 14-3-3 ligands, as we show that PKA-dependent binding of c-Raf to 14-3-3α requires helix G but not helix I (Fig. 6). Previous studies also showed that the RSXpSXP-containing ligand tryptophan hydroxylase bound to a fragment of 14-3-3α containing helices G and H but not I (34). Furthermore, crystal structure data (35) and mutagenesis studies (41) suggest that phosphorylation-dependent binding of RSXpSXP motif containing ligands may involve the interaction of phosphoserine (pS) with Lys-49 and Arg-56 in helix C and Arg-127 and Tyr-128 in helix E, which are not required for binding to GPIbo cytoplasmic domain (Fig. 5). In fact, the presence of the helices C–E is inhibitory to the interaction between GPIbo-IX and monomeric 14-3-3α mutants (Fig. 2). Thus, our data suggest that different types of 14-3-3 ligands preferentially interact with 14-3-3 at different sites in the large ligand binding groove surrounded by helices C, E, G, and I (18, 19, 35). It is likely that GPIbo represents a different class of the 14-3-3 ligands which preferentially recognize the binding site in the helix I of 14-3-3 proteins, whereas RSXpSXP-like ligands preferentially interact with helix G. Andrews et al. (15) aligned several 14-3-3-binding proteins that have sequences similar to the GPIbo binding sequence (including Cdc25a, Cdc25b, c-Raf, and c-Cbl). A striking feature in these 14-3-3 ligands is the presence of an HSL tripeptide sequence. It would be interesting to investigate further whether this HSL motif is responsible for the binding of this class of 14-3-3 ligands to helix I of 14-3-3.

Interestingly, while our manuscript was in revision, Petosa et al. (42) reported crystal structural data indicating that an unphosphorylated non-RSXP peptide ligand of 14-3-3α (WDLDE) obtained by screening a phage display library binds by amphipathic interaction to sites within the ligand binding groove overlapping with but distinct from c-Raf PSXPXP peptide-binding sites, supporting the notion that different ligands may interact with different sites in the groove. Our data provide first evidence that the interaction with the helix I region is both required and sufficient for 14-3-3α binding to a physiolog-
We show that the GPIb-IX complex has a higher affinity for 14-3-3-ζ than the recombinant GPIb cytoplasmic domain (Fig. 4), suggesting that other subunits of GPIb-IX complex in addition to GPIb may be involved in the interaction with 14-3-3-ζ. This result is consistent with the previous findings that high affinity binding to 14-3-3-ζ involves both GPIbα (14, 15) and GPIbβ which contains a phosphorylation-dependent 14-3-3-ζ-binding site (15, 16). Wild type 14-3-3-ζ exists as a homodimer via the interaction between the N-terminal domains (helices A and B) of each monomer (18, 19). Thus, we investigated the possibility that high affinity interaction involves simultaneous binding of GPIbα and GPIbβ to a 14-3-3 dimer. We show that the 14-3-3 mutants lacking the dimerization site in helices A and B bound to the GPIb-IX complex with significantly reduced affinity compared with the dimeric wild type 14-3-3-ζ (Fig. 2). Gel filtration chromatography confirmed that deletion of helices A and B disrupted dimerization of 14-3-3-ζ (Fig. 3), suggesting that the high affinity binding of 14-3-3-ζ to GPIb-IX requires 14-3-3-ζ dimerization. Furthermore, since the binding of the recombinant GPIbα cytoplasmic domain and the phosphorylated c-Raf to these monomeric 14-3-3-ζ mutants was not decreased compared with dimeric wild type 14-3-3-ζ (Figs. 5 and 6), the reduced binding of GPIb-IX is unlikely to result from the disruption of ligand-binding sites in these monomeric mutants. Thus, 14-3-3-ζ binding to GPIb-IX appears to involve the interaction of 14-3-3-ζ dimer to GPIbα and an additional binding site in other GPIb-IX subunits. As GPIbβ contains a PKA-catalyzed phosphorylation-dependent binding site for 14-3-3-ζ, it is likely that high affinity binding of intact GPIb-IX involves interaction of GPIbα and GPIbβ to each monomer of the 14-3-3-ζ dimer. As GPIbγ phosphorylation has been found to be dynamically regulated in platelets by increase in intracellular c-AMP level (36, 37, 43), this dual site binding mechanism may enable the 14-3-3-GPIb-IX interaction to be dynamically regulated in platelets and thus may participate in the GPIb-IX-coupled intracellular signaling and cytoskeleton regulation.

We show that helix G of 14-3-3-ζ is critical for binding to the RSXpSXP motif containing ligand c-Raf (Fig. 6). The published crystal structural data, however, indicate that the critical phosphoserine in RSXpSXP motif is in the proximity of the residues Lys-49, Arg-56, Arg-127, and Tyr-128 in helices C and E, suggesting that helix G may not be the recognition site for the phosphoprotein in the RSXpSXP motif. While our manuscript is under revision, Petosa et al. (42) and Wang et al. (44) reported crystal structural data, however, indicate that the critical phosphoserine in RSXpSXP motif (32), whereas Wang et al. (44) used the yeast two-hybrid system, in vitro translated protein, or cell lysates with unknown phosphorylation status. Furthermore, binding of c-Raf to 14-3-3-ζ in their assays did not appear to be influenced by stimulation of c-Raf phosphorylation (44), which is similar to GPIb-IX binding to 14-3-3-ζ (13, 14). Thus, it is possible that in the assay by Wang et al. (44), c-Raf binding to 14-3-3-ζ may involve a GPIbα-like motif in addition to the RSXpSXP motifs. An HSL sequence similar to the 14-3-3 binding sequence of GPIbα was indeed found in c-Raf (15). Thus, we speculate that c-Raf binding to dimeric 14-3-3-ζ in vivo may be similar to GPIb-IX binding, involving a dual binding site mechanism.

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