Binding of Purified 14-3-3 ζ Signaling Protein to Discrete Amino Acid Sequences within the Cytoplasmic Domain of the Platelet Membrane Glycoprotein Ib-IX-V Complex†

Robert K. Andrews,* Simon J. Harris, Tracy McNally, and Michael C. Berndt

Hazel and Pip Appel Vascular Biology Laboratory, Baker Medical Research Institute, Prahran, Victoria, Australia, 3181

Received April 17, 1997; Revised Manuscript Received September 3, 1997⊗

ABSTRACT: The glycoprotein (GP) Ib-IX-V complex constitutively expressed on the platelet plasma membrane mediates initial adhesion of circulating platelets to vessel wall matrix at high shear, and shear-induced platelet aggregation. In both cases, this involves binding of GP Ib-IX-V to the adhesive glycoprotein, von Willebrand Factor (vWF). vWF binding to GP Ib-IX-V rapidly induces platelet activation, leading to cytoskeletal rearrangement, shape change, and secretion that enables αIIbβ3 integrin (GP IIb-IIIa)-dependent platelet aggregation. All these events are critical in (patho)physiological thrombus formation. The recent discovery that the signaling protein, 14-3-3 ζ, copurifies with the GP Ib-IX complex (minus GP V) [Du, X., Harris, S. J., Tetaz, T. J., Ginsberg, M. H., & Berndt, M. C. (1994) *J. Biol. Chem. 269*, 18287−18290] indicated a potential mechanism for vWF-dependent signaling. The aim of the present study was to identify discrete amino acid sequences that bind 14-3-3 ζ within the cytoplasmic domain of the receptor. As an initial screening assay, overlapping synthetic peptides based on the cytoplasmic domains of GP Ibα (100 residues), GP Ibβ (34 residues), GP IX (5 residues), and GP V (16 residues) were immobilized and assessed for the ability to bind purified 14-3-3 ζ. The C-terminal sequence GHSL of GP Ibα was identified as one 14-3-3 ζ interactive sequence, consistent with previous results [Du, X., Fox, J. E., & Pei, S. (1996) *J. Biol. Chem. 271*, 7362−7367]. Binding of ^{125}I-labeled 14-3-3 ζ to GHSL-containing peptides was inhibitable by unlabeled 14-3-3 ζ and by anti-14-3-3 ζ IgG. Ala-walking through the GHSL sequence suggested all residues were necessary for optimal binding. In addition, 14-3-3 ζ bound with lower affinity to a peptide based on the central region of the GP Ibα cytoplasmic domain (Arg-557−Gly-575), whereas peptide sequences within the cytoplasmic domains of GP Ibβ (Arg-160−Arg-175) and GP V (Lys-529−Gly-544) bound 14-3-3 ζ with comparable affinity to the GHSL-containing peptide. Soluble GHSL-containing peptides, GP Ibβ- and GP V-based peptides semidissociated 14-3-3 ζ from GP Ib-IX-V or GP Ib-IX in platelet extracts as analyzed by immunoprecipitation, suggesting these sequences, at least partially, mediate the GP Ib-IX-V−14-3-3 ζ interaction in cells. Further, phosphorylation of the GP Ibβ peptide at a site corresponding to a protein kinase A phosphorylation site (Ser-166) enhanced the affinity of 14-3-3 ζ binding by approximately 8-fold, suggesting phosphorylation as a potential mechanism for regulating 14-3-3 ζ association with the GP Ib-IX-V complex.

Many vascular cell adhesion receptors not only mediate cell-cell or cell-matrix interactions, but are also able to trigger ligand-induced cell signaling that regulates postadhesion cellular events. In thrombotic disease and normal hemostasis, the glycoprotein (GP)[1] Ib-IX-V complex on the surface of circulating platelets mediates adhesion to the multimeric adhesive glycoprotein, von Willebrand Factor (vWF). The vWF-GP Ib-IX-V complex interaction regulates both the initial adhesion of platelets to the subendothelium at high shear and shear stress-induced platelet aggregation at sites of turbulence in occluded vessels, leading to thrombus formation (Weiss, 1995). Binding of vWF to platelets also induces cell signaling that initiates the rapid physiological responses of activated platelets (De Marco & Shapiro, 1981; Kroll et al., 1991; Ikeda et al., 1993; Clemetson, 1995). These responses include secretion from storage organelles, reorganization of cytoskeletal actin filaments associated with shape change and spreading, and activation and expression of other adhesive receptors, such as the αIIbβ3 integrin (GP IIb-IIIa) and P-selectin, that mediate platelet−platelet or platelet−leukocyte interactions involved in thrombosis (Clemetson, 1995; Andrews et al., 1997).

The GP Ib-IX-V complex consists of the disulfide-linked α and β chains of GP Ib which form a 1:1 tightly associated noncovalent complex with GP IX (Berndt et al., 1985a; Du et al., 1987). On the platelet plasma membrane, GP Ib-IX exists as a 2:1 complex with GP V (Li et al., 1995;

† This work was supported by the National Heart Foundation, Australia.

* Address correspondence to Baker Medical Research Institute, P.O. Box 348, Prahran, Victoria, Australia, 3181. Tel: 61-3-9522-4333. Fax: 61-3-9521-1362.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1997.

[1] Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; Da, Dalton; GP, glycoprotein; PI3-kinase, phosphatidyl inositol 3-kinase; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; vWF, von Willebrand Factor.

S0006-2960(97)00893-3 CCC: $15.00
Published on Web 01/13/1998

Modderman et al., 1992) forming a receptor referred to as the GP Ib-IX-V complex. All of the components of the GP Ib-IX-V complex are members of the leucine-rich protein family. vWF binds to at least two sites within the ~40-kDa N-terminal peptide domain of GP Ibα (His-1−Glu-282) (Ward et al., 1996; Andrews et al., 1996). One vWF recognition site is an anionic/sulfated tyrosine sequence, Tyr-275−Glu-282 (Dong et al., 1994; Marchese et al., 1995; Ward et al., 1996). The potent platelet agonist, α-thrombin, also recognizes the Tyr-275−Glu-282 sequence (Marchese et al., 1995; Ward et al., 1996), and recent evidence suggests that optimal thrombin-dependent activation of platelets may involve the GP Ib-IX-V complex (Greco et al., 1996). In addition, a nonphysiological GP Ibα-binding ligand, 50-kDa alboaggregin, a venom protein from the white-lipped tree viper *Trimeresurus albolabris*, activates platelets coincident with its binding to the vWF-binding domain of the GP Ib-IX-V complex, while other GP Ibα-binding venom proteins do not appear to activate platelets (Peng et al., 1991, 1993; Andrews et al., 1996).

Du et al. (1994) recently reported the copurification of the signaling protein, 14-3-3 (ζ isoform), with GP Ib-IX from detergent extracts of human platelets, providing the first clue to a potential mechanism for vWF-dependent platelet activation. In the last three years, 14-3-3 proteins have been found to regulate the activity and assemblage of key signaling molecules that in turn regulate such diverse processes as mitogenesis, cell cycling, vesicular transport, and apoptosis. Proteins reported to bind 14-3-3 include the cell death agonist BAD, Raf-1, bcr, cbl, PKCε, PKCγ, cdc25a, and cdc25b phosphatases, the p85 subunit of PI3-kinase, tyrosine hydroxylase, tryptophan hydroxylase, and ADP ribosyltransferase [reviewed in Aitken (1996)]. Recent analysis of 14-3-3 binding to Raf-1 has identified two discontinuous amino acid sequences that bind 14-3-3 encompassing phosphorylation sites at Ser-259 and Ser-621 (Muslin et al., 1996). PKA-dependent phosphorylation of Raf-1 enhances 14-3-3 binding, while phosphatase treatment of either Raf-1 or the phosphorylated synthetic peptide containing Ser-259 markedly decreases binding of 14-3-3. Both sequences contain an RSXSXP motif, and this consensus sequence is contained in a number of other 14-3-3-binding proteins, including PKCε, cdc25b, bcr, and BAD. Phosphorylation of BAD also enhances 14-3-3 binding, attenuating the ability of BAD to form a complex with its physiological ligand, a bcl homologue (Zha et al., 1996).

Recently, Du et al. (1996) reported that the C-terminal sequence of the cytoplasmic domain of GP Ibα, Ser-Gly-His-Ser-Leu (SGHSL), constituted part of the 14-3-3 ζ-binding site, since transfected GP Ib-IX complex expressed in CHO cells containing recombinant truncated GP Ibα lacking this sequence failed to coimmunoprecipitate with 14-3-3 ζ. A synthetic 15-mer peptide containing this sequence also specifically bound recombinant 14-3-3 ζ (Du et al., 1996). In the present study, we have confirmed these results using a solid phase binding assay of purified 14-3-3 ζ to synthetic peptides based on the GP Ibα cytoplasmic sequence and shown that amino acid residues Gly-His-Ser-Leu at the C-terminus represent the minimal sequence required for 14-3-3 ζ binding. Further, we have extended this finding by examining overlapping synthetic peptides that together account for the entire cytoplasmic domain of the GP Ib-IX-V complex and identified amino acid sequences from GP Ibβ and GP V that may also participate in binding of the receptor complex to 14-3-3 ζ. In particular, the GP Ibβ peptide contains the RSXSXP-related sequence, RLSLTDP, suggesting that RXSX(S/T)XP may also represent a consensus motif for 14-3-3 ζ recognition.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) was purchased from Sigma, St Louis, MO. Chloramine T was obtained from Riedel-de Häen, Selze, Germany. Sephadex G25 PD10 columns were from Pharmacia, Uppsala, Sweden. Synthetic peptides based on the GP Ibα, GP Ibβ, GP IX, and GP V cytoplasmic domain sequences, in some cases containing an N-terminal cysteine residue to facilitate coupling, were purified by reverse-phase HPLC and characterized by mass spectroscopy (Chiron, Clayton, Australia or Auspep, Parkville, Australia). The synthetic peptide based on the GP Ibβ sequence, Arg-160−Arg-175, containing a phosphoserine at position Ser-166 was purchased from Chiron. Glycocalicin, the soluble extracytoplasmic proteolytic fragment of GP Ibα, was purified from human platelet concentrates as previously described (Andrews et al., 1989a).

Purification of 14-3-3 ζ. The purification of 14-3-3 ζ from human platelets has been reported in detail elsewhere (Du et al., 1994). Bovine 14-3-3 ζ was purified from fresh bovine brains by the same method. Briefly, three bovine brains on ice were trimmed of associated blood vessels and other tissue and stored frozen at −70 °C. After thawing overnight at 4 °C, brains were homogenized at ambient temperature and then extracted and treated exactly as described for purification of 14-3-3 ζ from lyophilized platelets (Du et al., 1994). Yield was typically ~20 mg 14-3-3 ζ from three brains. Purified 14-3-3 ζ was radioiodinated where appropriate with sodium [^{125}I]iodide (Australian Radioisotopes, Lucas Heights, Australia) using the chloramine T method (Berndt et al., 1985a) and separated from excess label by gel filtration on a Sephadex G25 PD10 column (Pharmacia).

Antibodies. WM23, a murine monoclonal antibody directed against the extracytoplasmic macroglycopeptide domain of GP Ibα has been characterized in detail elsewhere (Berndt et al., 1985a). Rabbit polyclonal antisera against purified 14-3-3 ζ and glycocalicin were raised using standard protocols (Berndt et al., 1985a). Anti-14-3-3 ζ IgG and anti-glycocalicin IgG were affinity purified on the appropriate purified protein coupled to a 1:1 mixture of Affi-gel 10 and 15 (1 mg of protein/10 mL of resin), according to the manufacturer's instructions (Bio-Rad, Richmond, CA). After washing with 0.01 M Tris and 0.15 M sodium chloride, pH 7.4 (TS buffer), bound IgG was eluted with 0.1 M glycine, pH 2.8, and peak fractions were immediately reneutralized by addition of one-fifth volume of 1 M Tris, pH 8.0. The antibodies were dialyzed into TS buffer. Rabbit nonimmune IgG was prepared as previously described (Berndt et al., 1985b).

Preparation of Synthetic Peptide-Coated Beads. Peptides were coupled to BSA (1 mg of peptide/10 mg of BSA) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Pierce, Rockford, IL), essentially as previously described for coupling peptides to hemocyanin (Lerner et al., 1981). Peptide-BSA conjugates and peptides alone were individually coupled to

a 1:1 mixture of Affi-gel 10 and 15 (1 mg of peptide/5 mL of resin and 10 mg of peptide-BSA/5 mL of resin), according to the manufacturer's instructions (Bio-Rad). Resins coupled to peptide and the corresponding peptide-BSA conjugate were pooled, washed in TS buffer, and resuspended as a 1:1 (v/v) suspension in TS buffer.

Binding of 14-3-3 ζ to Synthetic Peptide-Coated Beads. The binding of purified 14-3-3 ζ to synthetic peptide-coated Affi-gel 10/15 was measured by incubating 25% (v/v) resin and 0.5 μg/mL ^{125}I-labeled 14-3-3 ζ in a final volume of 0.1 mL TS buffer containing 0.1% (w/v) BSA. After 30 min at 22 °C, samples were centrifuged at 8750*g* for 2 min, and the radioactivity in aliquots of the supernatant was measured in a γ counter. Nonspecific binding was determined in a parallel assay by including a 50-fold excess of unlabeled 14-3-3 ζ protein. To assess the effect of antibodies, fluid-phase peptide or protein on binding of ^{125}I-labeled 14-3-3 ζ to peptide-coated beads, some assays included 50 μg/mL anti-14-3-3 ζ IgG or nonimmune IgG, 10−400 μM synthetic peptide or 1−100 μg/mL unlabeled 14-3-3 ζ. Standard assays were performed in triplicate or quadruplicate with a standard deviation from the mean of ≤5%.

Immunoprecipitation of Platelet Extracts with anti-14-3-3 ζ and anti-Glycocalicin Antibodies. Fresh human platelets were collected and washed as previously described (Berndt et al., 1985b), resuspended at 10^9/mL, and lysed by addition of an equal volume of ice-cold 0.01 M Hepes, 0.15 M sodium chloride, 5 mM EDTA, and 2% (v/v) digitonin, pH 7.4, containing 0.2 mM PMSF and 100 μg/mL leupeptin. Alternatively, the lysis buffer contained 2% (v/v) Triton X-100 in place of digitonin. After centrifugation at 16000*g* for 10 min, platelet extracts were incubated for 3 h at 22 °C with either nonimmune rabbit IgG or anti-glycocalicin IgG covalently coupled to tosyl-activated magnetic beads according to the manufacturer's instructions (Dynal, Melbourne, Australia). The effect of the synthetic peptides, A7-10 (VSIRYSGHSL), A7-5 (SGHSL), GP V (KIGQLFRK-LIRERALG), and B2 (RAAARLSLTDPLVAER), alone or in combination, on immunoprecipitation was determined by including 100 μM (final concentration) of the peptide in parallel assays. The beads were washed extensively with the lysis buffer, solubilized by boiling for 10 min in SDS−polyacrylamide gel running buffer and electrophoresed on SDS 5 to 20% exponential gradient polyacrylamide gels under reducing conditions (Laemmli, 1970). Proteins were electrotransferred to nitrocellulose, immunoblotted with either affinity-purified rabbit anti-14-3-3 ζ IgG or the murine anti-GP Ibα monoclonal antibody, WM23 (Berndt et al., 1985b), and visualized using the appropriate horseradish peroxidase-coupled secondary antibody (Silenus, Melbourne, Australia) and the ECL detection system (Amersham, U.K.).

RESULTS

Binding of Purified 14-3-3 ζ to Synthetic Peptide-Coated Beads. The cytoplasmic domain of the platelet membrane GP Ib-IX complex has previously been shown to associate with the cytoplasmic signaling protein, 14-3-3 ζ, since these proteins were copurified on immunoaffinity chromatography and were coimmunoprecipitated from platelet extracts (Du et al., 1994). On the platelet plasma membrane, GP Ib-IX forms a 2:1 complex with GP V (Li et al., 1995; Modderman et al., 1992) giving a complex consisting of seven transmembrane polypeptide chains: two copies each of GP Ibα, GP Ibβ, and GP IX and one copy of GP V (López, 1994). As an initial approach to screening amino acid sequences from the cytoplasmic domain of the GP Ib-IX-V complex that mediate binding to 14-3-3 ζ, we developed an *in vitro* assay involving binding of purified 14-3-3 ζ to immobilized synthetic peptides based on sequences of the cytoplasmic domains of GP Ibα (López et al., 1987), GP Ibβ (López et al., 1988), GP IX (Hickey et al., 1989), and GP V (Hickey et al., 1993; Lanza et al., 1993) that together constitute the entire cytoplasmic domain of the GP Ib-IX-V complex. The sequences of these peptides are shown in Figure 1. There was substantive binding of purified ^{125}I-labeled 14-3-3 ζ to synthetic peptide A4- and A7-coated beads based on the GP Ib α-chain, but none or only marginal binding to other GP Ibα peptides tested (Figure 2). Binding of ^{125}I-labeled 14-3-3 ζ to A4- and A7-coated beads was specific, since it was inhibitable by excess unlabeled 14-3-3 ζ (Figure 3A), and binding to A7-coated beads was significantly blocked by rabbit anti-14-3-3 ζ IgG (Figure 3B). In contrast, nonspecific binding to A1-coated beads (Figure 3, panels A and B) or the other GP Ibα peptide-coated beads (not shown) was unaffected by either excess unlabeled 14-3-3 ζ or polyclonal anti-14-3-3 ζ IgG. Specific binding of ^{125}I-labeled 14-3-3 ζ to A7-coated beads was saturable (Figure 4A), with an apparent dissociation constant (K_d) by Scatchard analysis of ∼81 nM (Figure 4A, inset). Binding of ^{125}I-labeled 14-3-3 ζ to A4-coated beads was of lower affinity ($K_d \approx 170$ nM) compared with A7-coated beads (Figure 4B). Two overlapping peptides based on the GP Ibα sequences Arg-557−Phe-568 and Arg-564−Gly-575 that encompass the A4 peptide (Arg-557−Gly-575) showed no measurable inhibition of binding of ^{125}I-labeled 14-3-3 ζ to A4-coated beads when included in the routine assay at a final concentration of 400 μM (not shown). The A3 and A4 peptides overlap a sequence Thr-535−Phe-568 in the central region of the GP Ibα cytoplasmic tail (Figure 1) previously identified as a binding site for actin-binding protein (Andrews & Fox, 1992), although the presence or absence of actin-binding protein had no effect on binding of 14-3-3 ζ to recombinant GP Ib-IX complex expressed in a mammalian cell line (Du et al., 1996).

The observation that 14-3-3 ζ specifically bound to A7-coated beads (Gly-592−Leu-610), compared with no detectable binding to the overlapping peptide A6-coated beads (Arg-585−Ile-603) suggested that the GP Ibα sequence Arg-604−Leu-610 may be critical for binding of 14-3-3 ζ (Figure 2). To test this possibility, a series of synthetic peptides representing N-terminal truncations of the A7 peptide were analyzed for their ability to inhibit specific binding of ^{125}I-labeled 14-3-3 ζ to A7-coated beads (Figure 5). The tetrapeptide corresponding to the C-terminal residues of GP Ibα, Gly−His−Ser−Leu, and all of the longer peptides containing this sequence inhibited 14-3-3 ζ binding to the A7-coated beads with similar potency (Figure 5). There was no measurable inhibition by the tripeptide sequence, His-Ser-Leu. Further, replacing any of the C-terminal four residues in the pentamer peptide, Ser−Gly−His−Ser−Leu, with Ala abolished the ability of the peptide to inhibit binding of 14-3-3 ζ to A7-coated beads (Figure 6).

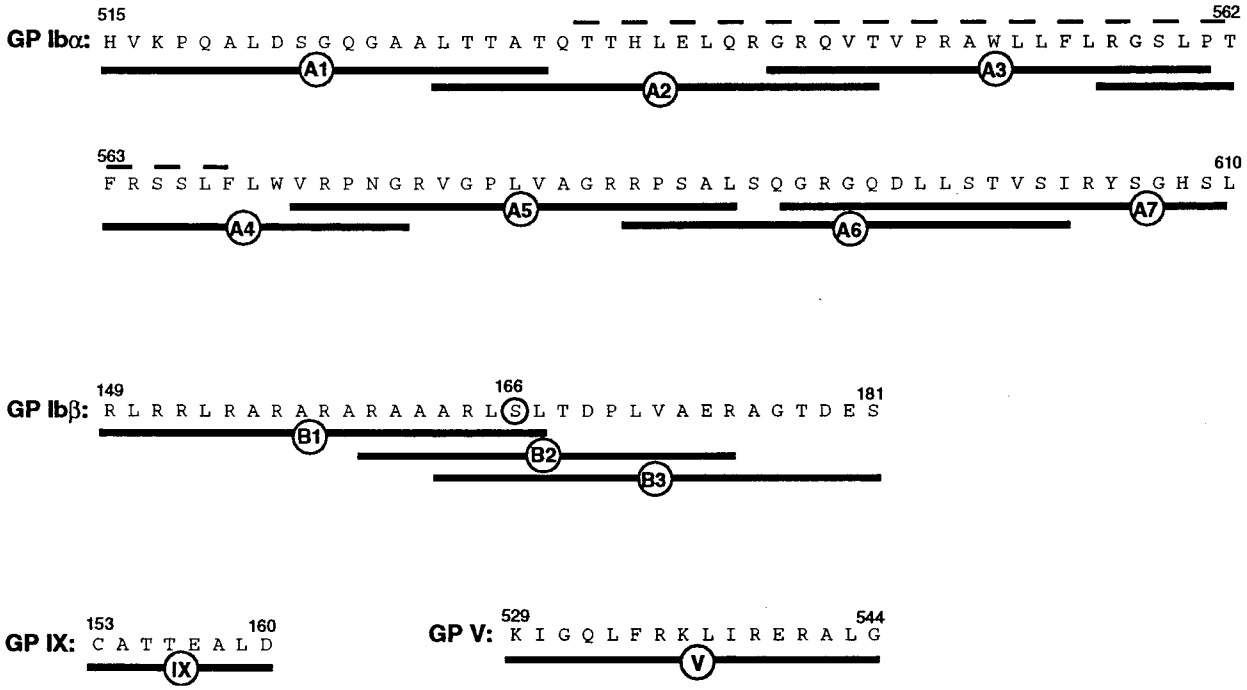

FIGURE 1: Synthetic peptides based on the amino acid sequences (solid lines) of the cytoplasmic domains of GP Ibα (López et al., 1987), GP Ib*β* (López et al., 1988), GP IX (Hickey et al., 1989), and GP V (Hickey et al., 1993; Lanza et al., 1993) that together constitute the cytoplasmic domain of the GP Ib-IX-V complex. The PKA phosphorylation site at Ser-166 of GP Ib*β* is circled. The dashed line represents amino acid sequence of GP Ibα (Thr-535−Gly-575) implicated in binding actin-binding protein (Andrews & Fox, 1992).

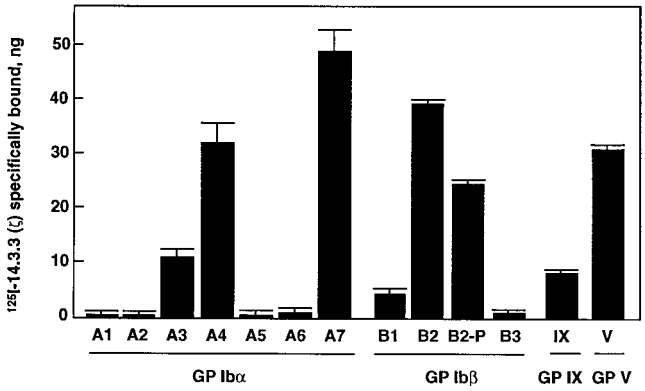

FIGURE 2: Specific binding of purified ^{125}I-labeled 14-3-3 ζ (1 *μ*g/mL, final concentration) to synthetic peptide-coated beads in 30 min at 22 °C. Synthetic peptide sequences: A1, His-515−Thr-533; A2, Leu-529−Thr-547; A3, Gly-543−Pro-561; A4, Arg-557−Gly-575; A5, Val-571−Leu-589; A6, Arg-585−Ile-603; A7, Gly-592−Leu-610; B1, Arg-149−Leu-167; B2, Arg-160−Arg-175; B2-P, Arg-160−Arg-175 containing a phosphoserine at Ser-166; B3, Ala-163−Ser-181; IX, Cys-153−Asp-160; V, Lys-529−Gly-544. Error bars are ± 1 × SDM (n = 4). Results are representative of two separate experiments.

Interestingly, other regions of the GP Ib-IX-V cytoplasmic domain bound to 14-3-3 ζ in addition to the sites identified within the cytoplasmic tail of GP Ibα (Du et al., 1996; this study). ^{125}I-labeled 14-3-3 ζ specifically bound to a peptide, B2, based on sequence within the cytoplasmic tail of GP Ib*β* and to a peptide based on the cytoplasmic tail of GP V (Figure 2) with similar affinities to the A7 peptide (K_ds of ∼70 and ∼61 nM, respectively) (Figure 4, panels C and D, respectively). Binding to the GP V peptide- and B2-coated beads was inhibitable by a 50-fold excess of unlabeled 14-3-3 ζ (Figure 3A). In contrast, there was no appreciable specific binding of ^{125}I-labeled 14-3-3 ζ to overlapping GP Ib*β* peptides, B1 and B3, or to the short cytoplasmic sequence of GP IX (Figure 2). The GP Ib*β* B2 peptide, Arg-160−Arg-175, overlaps a protein kinase A (PKA) phosphorylation site at Ser-166 (Wardell et al., 1989). Serine phosphorylation at a site corresponding to Ser-259 of a synthetic peptide based on the Raf-1 sequence, Leu-251−Met-265, has previously been demonstrated to enhance binding of 14-3-3 ζ (Muslin et al., 1996). Similarly, serine phosphorylation of the cell death agonist protein, BAD, also enhanced binding of 14-3-3 protein (Zha et al., 1996). We therefore examined a synthetic peptide based on the GP Ib*β* B2 peptide, Arg-160−Arg-175, containing a phosphoserine corresponding to Ser-166. In the initial screening assay, the level of binding of 14-3-3 ζ at 1 *μ*g/mL to the phosphorylated Arg-160−Arg-175 peptide (B2-P) was comparable to the unphosphorylated B2 peptide (Figure 2). The difference in the level of binding to the different beads probably reflects differences in coupling efficiency of the peptides. Unfortunately, we were unable to reliably quantitate the extent of peptide coupling to beads. The high affinity for 14-3-3 ζ binding to B2-P precluded quantitative binding analysis. However, a displacement assay where binding of ^{125}I-labeled 14-3-3 ζ to B2- or phosphorylated B2 (B2-P)-coated beads was competed by increasing concentrations of unlabeled 14-3-3 ζ (Figure 7), suggested that phosphorylation increased the affinity of the 14-3-3 ζ−peptide interaction by approximately 8-fold, since much higher concentrations of unlabeled 14-3-3 ζ were required for displacement of ^{125}I-labeled 14-3-3 ζ from phosphorylated B2−coated beads.

Finally, cross-blocking experiments were employed to address, in part, whether the amino acid sequences from within the cytoplasmic domain of the GP Ib-IX-V complex represented discrete 14-3-3 ζ binding sites. Binding of ^{125}I-labeled 14-3-3 ζ at 1 *μ*g/mL to A7-coated beads in the routine assay was competed by soluble A7 peptide at 250 *μ*M, but not by any of the other peptides tested at this concentration

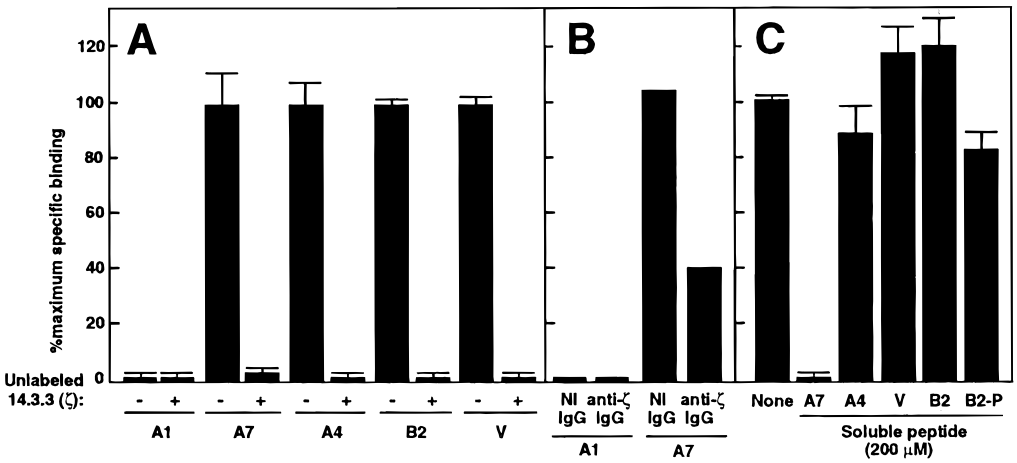

FIGURE 3: (A) Effect of a 50-fold excess of unlabeled 14-3-3 ζ on specific binding of ^{125}I-labeled 14-3-3 ζ (1 μg/mL, final concentration) to synthetic peptide A7-, A4-, B2-, and V-coated beads. Error bars are ± 1 × SDM (n = 3). Results are representative of two separate experiments. (B) Effect of rabbit nonimmune (NI) IgG and anti-14-3-3 ζ IgG (both at 50 μg/mL, final concentration) on specific binding of 1 μg/mL ^{125}I-labeled 14-3-3 ζ to A1- and A7-coated beads. (C) Effect of soluble synthetic peptides (200 μM, final concentration) on specific binding of ^{125}I-labeled 14-3-3 ζ (1 μg/mL) to A7-coated beads. Error bars are ± 1 × SDM (n = 4).

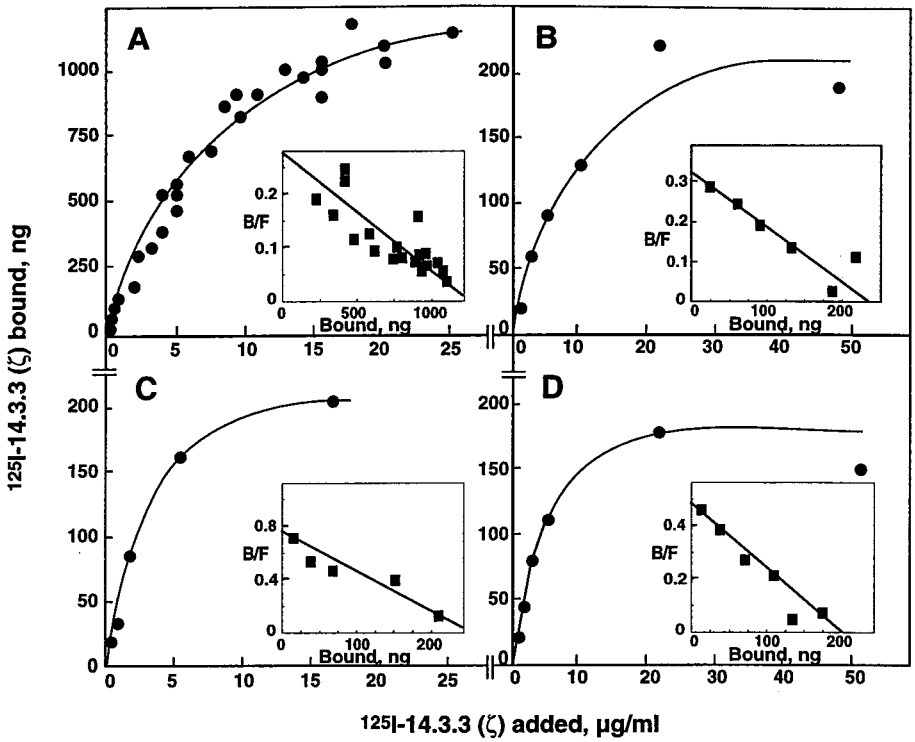

FIGURE 4: Dose-dependent specific binding of ^{125}I-labeled 14-3-3 ζ (1 μg/mL) to (A) A7-coated beads, (B) A4-coated beads, (C) B2-coated beads, and (D) GP V peptide-coated beads in 30 min at 22 °C. Nonspecific binding was determined using a 50-fold excess of unlabeled 14-3-3 ζ or A1-coated beads as control beads. Scatchard analysis is shown inset. Peptide sequences are as shown in Figure 1.

(Figure 3C). In related experiments, binding of ^{125}I-labeled 14-3-3 ζ (1 μg/mL) to B2-coated beads was partially competed by ~63% in the presence of soluble B2 peptide at 250 μM, but was not inhibited (≤12%) by the A7, A4, or V peptides at 250 μM (data not shown). This suggests that 14-3-3 ζ could bind to multiple sites within the receptor, and is consistent with the effect of peptides on the coprecipitation of 14-3-3 ζ with GP Ib-IX-V from platelet extracts described below.

Immunoprecipitation of 14-3-3 ζ and GP Ibα from Platelet Extracts. Studies demonstrating binding of purified 14-3-3 ζ to synthetic peptides *in vitro* suggested multiple sequences within the cytoplasmic domain of GP Ib-IX-V potentially mediated 14-3-3 ζ binding. To examine whether these sequences may be involved in the interaction between the intact proteins, synthetic peptides were tested for their ability to inhibit coimmunoprecipitation of 14-3-3 ζ with GP Ib-IX-V from platelet lysates. Platelets were extracted using the detergent, digitonin; under these conditions, GP V remains associated with the GP Ib-IX complex. Coprecipitation of 14-3-3 ζ by anti-GP Ibα IgG was partially (~50%) inhibited by the GP Ibβ peptide (B2) that bound 14-3-3 ζ, whereas the GP Ibα GHSL-containing peptide (A7-10) or the GP V peptide alone were essentially noninhibitory (Figure 8, upper panel). However, a combination of B2, A7-10 and the GP V peptide was most effective at inhibiting coprecipitation of 14-3-3 ζ by anti-GP Ibα IgG (Figure 8, upper panel), implying that each of these sequences may be

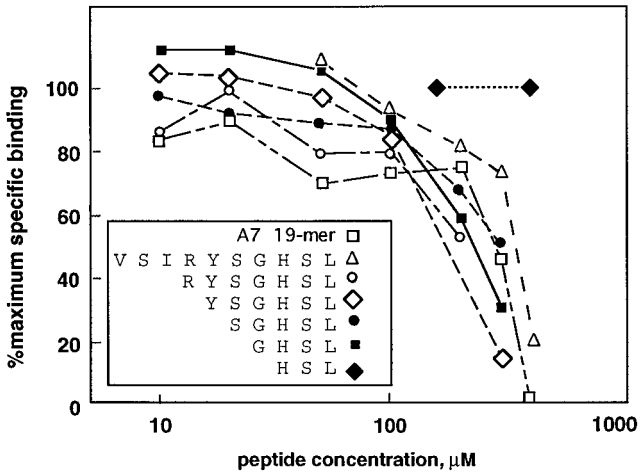

FIGURE 5: Effect of fluid-phase synthetic peptides on the specific binding of ^{125}I-labeled 14-3-3 ζ (1 μg/mL) to A7-coated beads in 30 min at 22 °C. Sequences of the truncated peptides based on the A7 peptide sequence are shown inset.

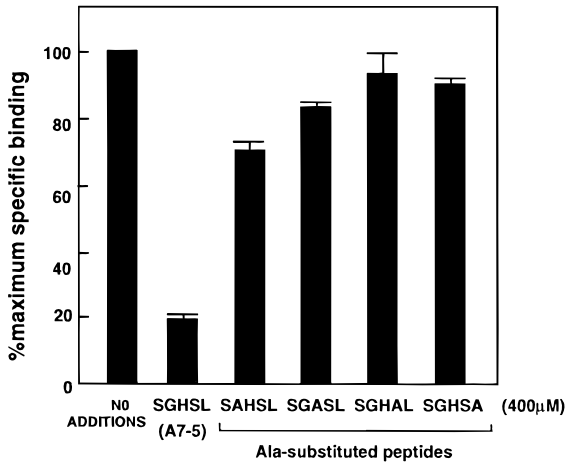

FIGURE 6: Effect of Ala-substitution of amino acid residues of the synthetic peptide, Ser-Gly-His-Ser-Leu, on the ability to inhibit the specific binding of ^{125}I-labeled 14-3-3 ζ (1 μg/mL) to A7-coated beads in 30 min at 22 °C. Error bars are ± 1 × SDM (n = 2). Results are representative of two separate experiments.

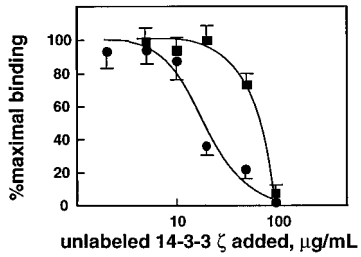

FIGURE 7: Effect of increasing concentrations of unlabeled 14-3-3 ζ on the specific binding of ^{125}I-labeled 14-3-3 ζ (1 μg/mL) to B2-coated beads (circles) or B2-P-coated beads (squares) in 30 min at 22 °C. Peptide sequences for B2 (Arg-160−Arg-175) and B2-P (Arg-160−Arg-175 containing a phosphoserine at Ser-166) are shown in Figure 1.

involved in 14-3-3 association with the receptor and that binding to individual sites is additive. Semiquantitation of the amount of 14-3-3 ζ coprecipitated with the GP Ib-IX-V complex in the presence or absence of peptides is shown in Figure 8 (lower panel). Du et al. (1994) reported that 14-3-3 ζ coprecipitated with GP Ib-IX from platelet extracts or purified from platelets appeared as a closely spaced doublet on SDS−polyacrylamide gels with apparent molecular

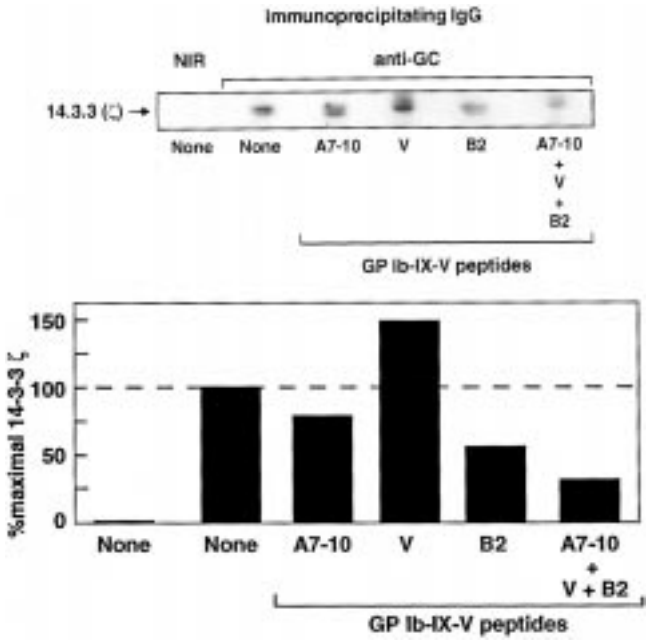

FIGURE 8: Effect of synthetic peptides (100 μM, final concentration), alone or in combination, on the coimmunoprecipitation of 14-3-3 ζ by rabbit anti-glycocalicin IgG from digitonin extracts of platelets. Precipitates were electrophoresed on SDS 5 to 20% exponential gradient polyacrylamide gels, electrotransferred to nitrocellulose, probed with rabbit anti-14-3-3 ζ IgG, and visualized using peroxidase-coupled secondary antibody and the ECL substrate (upper panel). The relative amounts of 14-3-3 ζ coimmunoprecipitated in the absence or presence of synthetic peptides was semiquantitated by laser densitometry (lower panel).

masses of ∼30 and ∼29 kDa, respectively. Both species of purified 14-3-3 ζ had identical internal amino acid sequences, and the relative intensity of the 30 and 29 kDa bands on immunostaining with an anti-14-3-3 ζ antibody was ∼1:2 (Du et al., 1994). Interestingly, the relative precipitation of the 30 kDa *versus* the 29 kDa species of 14-3-3 ζ appears to be differentially inhibited by soluble peptides (Figure 8, upper panel), suggesting the possibility that different sequences in the receptor may preferentially associate with different species of 14-3-3 ζ. These isoforms of 14-3-3 ζ may represent different phosphorylated (Aitken et al., 1995b) and/or otherwise post-translationally modified forms of 14-3-3. Further analysis of the nature of these interactions is beyond the scope of the present study.

The results on the effect of peptides on dissociation of 14-3-3 ζ from the GP Ib-IX-V complex were in apparent contradiction of an earlier study, demonstrating that the C-terminal residues Gly-607−Leu-610 of GP Ibα mediated the interaction of recombinant GP Ib-IX complex (minus GP V) with 14-3-3 ζ in heterologous cells (Du et al., 1996). In order to address this anomaly, we determined whether the Gly−His−Ser−Leu sequence identified by Du et al. mediated the GP Ib-IX−14-3-3 ζ interaction in solubilized platelets. Platelets were therefore lysed using Triton X-100 instead of digitonin, conditions where GP V is dissociated from GP Ib-IX (Berndt et al., 1985a). Samples were immunoprecipitated with either nonimmune IgG or affinity-purified polyclonal IgG directed against the extracytoplasmic region of GP Ibα in the presence or absence of the Ser-606−Leu-610 (A7-5) peptide (SGHSL). The immunoprecipitates were analyzed by Western blotting with an anti-GP Ibα monoclonal antibody (WM23) or anti−14-3-3 ζ IgG

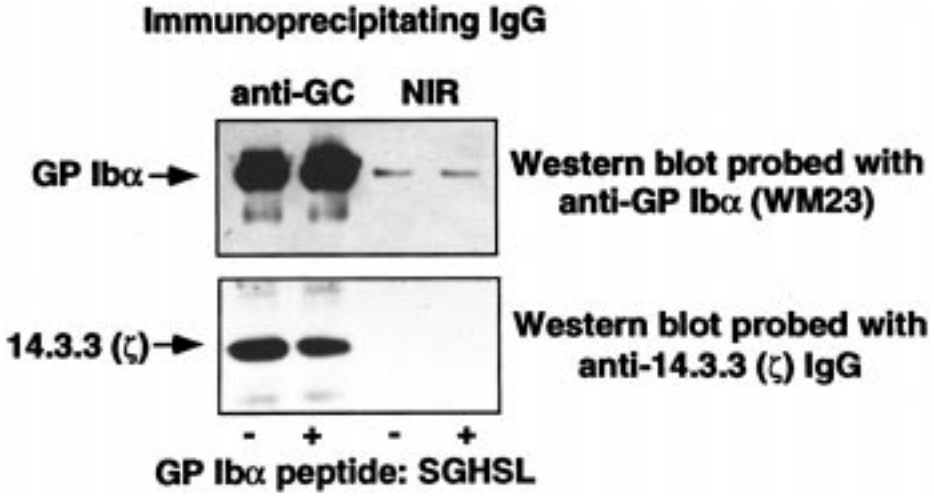

FIGURE 9: Effect of synthetic peptide A7-5 (100 μM, final concentration) on the coimmunoprecipitation of 14-3-3 ζ by nonimmune rabbit IgG (NIR) or rabbit anti-glycocalicin (soluble GP Ibα) IgG from Triton X-100 extracts of human platelets. Precipitates were electrophoresed on SDS 5 to 20% exponential gradient polyacrylamide gels, electrotransferred to nitrocellulose, probed with either a murine anti-GP Ibα monoclonal antibody (WM23) or rabbit anti-14-3-3 ζ IgG as indicated, and visualized using peroxidase-coupled secondary antibody and the ECL substrate.

(Figure 9). In the presence of peptide, there was a partial, but consistent, decrease in the precipitation of 14-3-3 ζ by the anti-GP Ibα antibody even though there was no decrease in precipitation of GP Ibα, consistent with the results of Du et al. (1996). Together, these results imply that (1) the C-terminal Gly−His−Ser−Leu sequence is involved, at least partially, in 14-3-3 ζ association with GP Ib-IX (Du et al., 1996; this study), and further, that (2) other amino acid sequences within the cytoplasmic domain of the GP Ib-IX-V complex, including the B2, GP V and possibly A4 sequences, may contribute to, or regulate, binding of 14-3-3 ζ to the intact GP Ib-IX-V complex in platelets.

DISCUSSION

Binding of multimeric von Willebrand Factor (vWF) to its adhesive receptor on the platelet surface, the glycoprotein (GP) Ib-IX-V complex, whether induced by shear stress, or by the vWF modulators, ristocetin or botrocetin, transmits an intracellular signal and activates platelets, as measured by elevation of intracellular Ca^{2+} and phosphorylation of cytoplasmic proteins (De Marco & Shapiro, 1981; Kroll et al., 1991, 1993; Ikeda et al., 1993; Clemetson, 1995; Ozaki et al., 1995). In shear-induced platelet aggregation, platelet activation enables a secondary phase of GP IIb-IIIa complex-mediated aggregation that stabilizes the platelet aggregate. Although binding of vWF to platelets is known to induce breakdown of phophatidylinositol 4,5-bisphosphate, phosphatidic acid generation, activation of protein kinase C, thromboxane A_2 synthesis, and the cytoskeletal association of activated phosphatidyl inositol (PI) 3-kinase and pp60src (Kroll et al., 1991; Fox et al., 1993; Clemetson, 1995; Jackson et al., 1995), the early events in the GP Ib-IX-V-dependent signaling pathway are currently undefined. Two cytoplasmic proteins are known to directly associate with the cytoplasmic domain of the GP Ib-IX-V complex: the cytoskeletal protein, actin-binding protein (Andrews & Fox, 1992; Fox, 1993), and the signaling protein, 14-3-3 ζ (Du et al., 1994). One or both of these proteins are candidates for regulating, at least partially, early stages in the vWF-dependent signaling pathway(s). The objective of the present work was to map potential binding sites for 14-3-3 ζ on the GP Ib-IX-V complex as the basis for defining the mechanism of GP Ib-IX-V-dependent platelet activation.

14-3-3 ζ is an ~29-kDa protein of 245 amino acid residues which is a member of the 14-3-3 protein family [reviewed in Aitken (1995, 1996)]. 14-3-3 proteins bind a number of cytoplasmic proteins involved in signaling or mitogenesis, including the serine/threonine kinase Raf-1, PKC, cbl, bcr, polyoma middle T antigen, cdc25a, cdc25b, and PI3-kinase (Aitken, 1995, 1996; Muslin et al., 1996; Liu et al., 1996). Muslin et al. (1996) showed that phosphorylation at a specific site in the Raf-1 sequence (Ser-259) enhanced 14-3-3 ζ binding to a synthetic peptide overlapping this site. Phosphorylation at Ser-621 also enhanced 14-3-3 ζ binding. Both of these serine residues were within a conserved RSXSXP motif, also found in a number of other 14-3-3-binding proteins including the cell death agonist, BAD (Aitken, 1996; Zha et al., 1996). Binding of 14-3-3 ζ to the phosphoserine-259-containing Raf-1 peptide or Raf-1 itself was reversed by phosphatase treatment (Muslin et al., 1996), implying that activation of the cAMP-dependent PKA that phosphorylates Raf-1 at Ser-259 could be involved in regulation of 14-3-3 ζ binding. BAD is also phosphorylated *in vivo* by a PKA-like kinase and phosphorylated BAD shows enhanced association with 14-3-3 (Zha et al., 1996). Liu et al. (1997) recently reported that phosphorylation of c-cbl was involved in 14-3-3 τ binding. It was proposed that two $RX_{1-2}SX_{2-3}S$ consensus sequences may represent novel 14-3-3 recognition motifs, analogous to, but distinct from the conserved RSX-SXP sequence indentified in Raf-1 (Muslin et al., 1996).

Interestingly, both Raf-1 and c-cbl contain more than one site implicated in mediating association with 14-3-3 proteins (Muslin et al., 1996; Liu et al., 1997). Our analysis of synthetic peptides based on cytoplasmic sequences of the GP Ib-IX-V complex suggests there may also be multiple 14-3-3 ζ binding sites in this receptor. Identification of one of these sites at the C-terminus of GP Ibα is consistent with previous findings of Du et al. (1996), who showed that C-terminal truncation of recombinant GP Ibα after Tyr-605 abolished 14-3-3 ζ binding and that a 15-mer synthetic

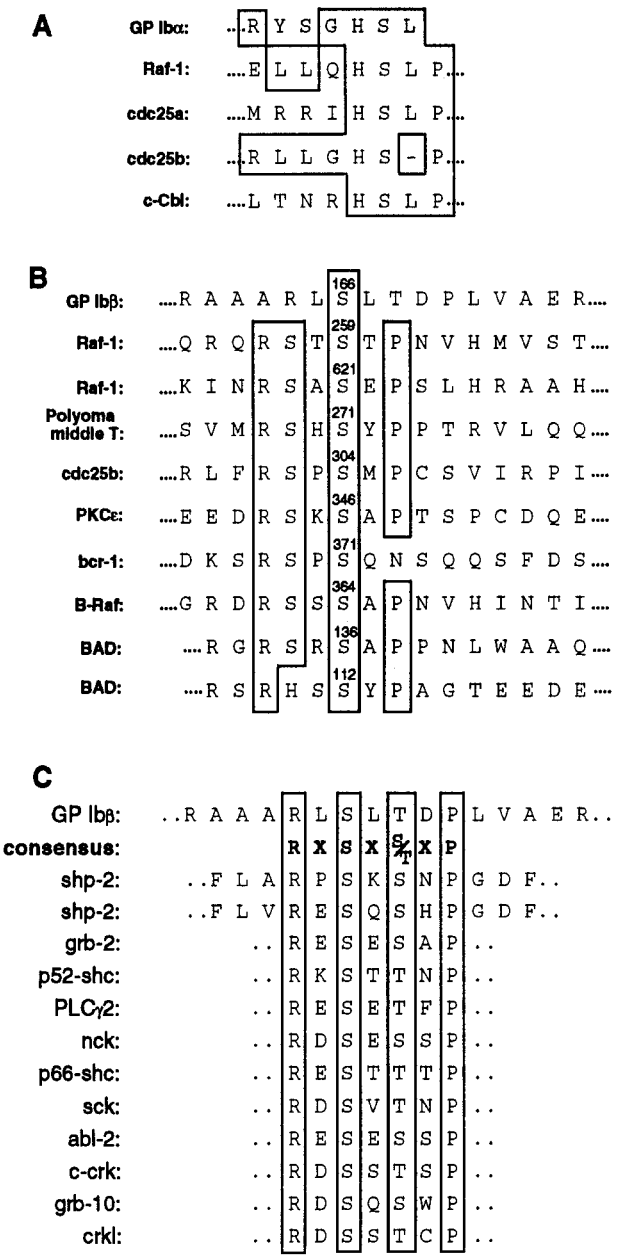

FIGURE 10: (A) Amino acid sequence comparison of the 14-3-3 ζ-binding Ser-Gly-His-Ser-Leu (SGHSL) sequence from the cytoplasmic domain of GP Ibα with analogous sequences from other proteins. (B) Comparison of the phosphoserine-containing sequences from the cytoplasmic domain of GP Ibβ, from Raf-1 and from other 14-3-3-binding proteins. The consensus residues of the RSXSXP sequence identified in Raf-1 (Muslin et al., 1996) and BAD (Zha et al., 1996) are boxed. (C) Comparison of the phosphoserine-containing 14-3-3 ζ-binding sequence from the cytoplasmic domain of GP Ibβ (B2) with sequences from the SH2 domains of other proteins. The consensus residues of the RXSX(S/T)XP sequence are boxed. Sequences were obtained from the SwissPro or GenBank protein sequence data banks.

peptide based on the GP Ibα C-terminus (596−610) bound 14-3-3 ζ. We have extended these findings by definitively localizing a 14-3-3-binding site to the C-terminal four residues (GHSL) by analysis of a series of truncated synthetic peptides incorporating this sequence, and alanine-substitution of individual residues within GHSL-containing peptides. Sequences analogous to the C-terminal GP Ibα sequence, Gly-His-Ser-Leu (GHSL), were conserved in a number of other 14-3-3-binding proteins (Figure 10A). Unlike GP Ibα, the postulated 14-3-3-binding residues in these other proteins are not at the C-terminus, but in all of these examples there is a conserved turn-inducing proline immediately C-terminal to the GHSL-like sequence (Figure 10A). Interestingly, the GHSL-like sequence in c-cbl encompassing Ser-619 (RHSLP) overlapped the $RX_{1-2}SX_{2-3}S$ consensus sequence (RHSLPFS) identified as being important in binding 14-3-3 (Liu et al., 1997). Our results together with those of Du et al. (1996) suggest that, like both RSXSXP in Raf-1 and BAD and $RX_{1-2}SX_{2-3}S$ in c-cbl, the GHSL sequence in GP Ibα may represent an analogous 14-3-3 recognition site, but without a requirement for phosphorylation. In platelets, GP Ibβ is a major substrate for PKA, and is phosphorylated in response to agents such as prostaglandin E_1 that elevate cytoplasmic cAMP (Fox & Berndt, 1989). Phosphorylation of GP Ibβ correlates with the specific inhibition of actin polymerization (Fox & Berndt, 1989). In this study, a peptide Arg-160−Arg-175 overlapping the Ser-166 residue specifically bound 14-3-3 ζ with an affinity ($K_d \approx 61$ nM) comparable to peptides based on the GP Ibα C-terminal sequence and GP V. Phosphorylation of the residue corresponding to Ser-166 within the Arg-160−Arg-175 peptide increased 14-3-3 ζ binding affinity by almost an order of magnitude. This effect of phosphorylation on a 14-3-3 ζ-binding sequence in GP Ibβ hints at an additional effect of PKA-dependent phosphorylation on regulating platelet activation. Since GP Ibβ phosphorylation specifically inhibits actin polymerization, the increased avidity for 14-3-3 ζ may reflect a role for this protein in the control of actin polymerization. GP Ibβ lacks the conserved RSXSXP phosphorylation site found in Raf-1, and conserved in other proteins including BAD (Figure 10B). The RLSLTDP sequence in GP Ibβ suggests that RXSX(S/T)XP may also represent a sequence for 14-3-3 recognition. Interestingly, this sequence is found within the src homology-2 (SH2) domains of many signaling molecules (Figure 10C), suggesting that it may mediate interaction of these domains with members of the 14-3-3 family. Finally, actin-binding protein, which is directly associated with GP Ibα (Andrews & Fox, 1992; Fox, 1993), contains a related potential PKA phosphorylation site, RAPSVAN (Gorlin et al., 1990), raising the possibility that this sequence may also interact with 14-3-3 ζ.

An α-thrombin-binding site on the GP Ib-IX-V complex has been localized to the anionic/sulfated tyrosine sequence within the N-terminal extracytoplasmic domain of GP Ibα that also binds vWF (De Marco et al., 1994; Marchese et al., 1995; Ward et al., 1996). In addition, GP V is a thrombin substrate (Berndt & Phillips, 1981; Berndt et al., 1986). Association of 14-3-3 ζ with the cytoplasmic sequence of GP V, Lys-529−Gly-544, could potentially be involved in thrombin-induced activation *via* GP Ib-IX-V. Although beyond the scope of this study, important questions are whether thrombin cleavage of GP V affects association/dissociation of 14-3-3 ζ with GP Ib-IX-V and whether 14-3-3 is involved in formation and/or regulation of the complex between GP Ib-IX and GP V.

Currently, the potential role of 14-3-3 ζ in GP Ib-IX-V-dependent signaling remains obscure. The effect of these peptides on 14-3-3 ζ binding to the C-terminal GP Ibα peptide and to the GP Ib-IX-V complex in platelet lysates together suggests that discrete sequences may represent

distinct 14-3-3 ζ recognition sites. Since 14-3-3 proteins are dimeric, it is conceivable that they may simultaneously bind to two or more ligands, such as GP Ib-IX-V and other signaling proteins like Raf-1, thereby regulating at least some of the signaling events downstream of the receptor. 14-3-3 ζ has previously been shown to bind and, in some circumstances, activate PKC (Robinson et al., 1994; Aitken, 1995, 1996; Aitken et al., 1995a). In platelets, activation of PKC results in phosphorylation of substrates including pleckstrin (p47) and myosin light chain (Scholey et al., 1980; Kroll et al., 1993). A possible role in PKC activation for 14-3-3 ζ associated with the GP Ib-IX-V complex would be consistent with the observed pleckstrin and myosin light chain phosphorylation induced by GP Ibα ligands, vWF, α-thrombin and 50-kDa alboaggregin (Kroll et al., 1991; Ikeda et al., 1993; Clemetson, 1995; Andrews et al., 1996). In addition, in platelets activated by vWF binding to GP Ib-IX-V, PI3-kinase is activated and redistributed to the cytoskeleton together with pp60src kinase (Jackson et al., 1994). Since 14-3-3 ζ associates with PI3-kinase, this interaction may also regulate GP Ib-IX-V-dependent signaling. Finally, the ability of 14-3-3 to bind to a number of diverse proteins, apparently through different amino acid sequence recognition sites (within Raf-1 and c-cbl for example) as well as GP Ib-IX-V, raises the possibility that 14-3-3 is involved in the assembly/regulation of signaling complexes. The mechanism for this signaling process, may ultimately be resolved in greater detail by determining (1) how 14-3-3 ζ association with GP Ib-IX-V changes on platelet activation or under conditions where platelet PKA becomes activated, and (2) which recognition sites specifically mediate the interaction in intact cells.

In conclusion, this study describes two major observations on the association of 14-3-3 ζ with the cytoplasmic domain of the GP Ib-IX-V complex that may be critical to understanding the mechanism of GP Ib-IX-V-dependent platelet activation in response to vWF and/or α-thrombin. Firstly, binding of purified 14-3-3 ζ to synthetic peptides corresponding to amino acid sequences within the cytoplasmic domains of GP Ibα, GP Ibβ, and GP V suggests there may be multiple binding sites per receptor for 14-3-3 ζ. Secondly, the effect of phosphorylation of the residue corresponding to Ser-166 within the GP Ibβ peptide, Arg-160−Arg-175, on 14-3-3 ζ binding raises the possibility that GP Ib-IX-V-dependent platelet activation may be, at least partly, regulated by cAMP-dependent PKA. Extending these studies to intact platelets should provide the basis for understanding the mechanisms of signaling critical to the onset and progression of thrombotic disease.

ACKNOWLEDGMENT

We are grateful to Carmen Llerena for providing superb technical assistance.

REFERENCES

Aitken, A. (1995) *Trends Biochem. Sci. 20*, 95−97.
Aitken, A. (1996) *Trends Cell Biol. 6*, 341−347.
Aitken, A., Howell, S., Jones, D., Madrazo, J., Martin, H., Patel, Y., & Robinson, K. (1995a) *Mol. Cell Biochem. 149/150*, 41−49.
Aitken, A., Howell, S., Jones, D., Madrazo, J., & Patel, Y. (1995b) *J. Biol. Chem. 270*, 5706-5709.
Andrews, R. K., & Fox, J. E. B. (1992) *J. Biol. Chem. 267*, 18605−18611.
Andrews, R. K., Booth, W. J., Gorman, J. J., Castaldi, P. A., & Berndt, M. C. (1989a) *Biochemistry 28*, 8317−8326.
Andrews, R. K., Gorman, J. J., Booth, W. J., Corino, G. L., Castaldi, P. A., & Berndt, M. C. (1989b) *Biochemistry 28*, 8326−8336.
Andrews, R. K., Kroll, M. H., Ward, C. M., Rose, J. W., Scarborough, R. M., Smith, A. I., López, J. A., & Berndt, M. C. (1996) *Biochemistry* 35:12629−12639.
Andrews, R. K., López, J. A., & Berndt, M. C. (1997) *Int. J. Biochem. Cell Biol.* (in press).
Berndt, M. C., & Phillips, D. R. (1981) *J. Biol. Chem. 256.*
Berndt, M. C., Gregory, C., Kabral, A., Zola, H., Fournier, D., & Castaldi, P. A. (1985a) *Eur. J. Biochem. 151*, 637−649.
Berndt, M. C., Chong, B. H., Bull, H. A., Zola, H., & Castaldi, P. A. (1985b) *Blood 66*, 1292−1301.
Berndt, M. C., Gregory, C., Dowden, G., & Castaldi, P. A. (1986) *Ann. NY Acad. Sci. 485*, 374−386.
Clemetson, K. J. (1995) *Thromb. Haemostasis 74*, 111−116.
De Marco, L., & Shapiro, S. S. (1981) *J. Clin. Invest. 68*, 312−328.
De Marco, L., Mazzucato, M., Masotti, A., & Ruggeri, Z. M. (1994) *J. Biol. Chem. 269*, 13946−13953.
Dong, J.-F., Li, C. Q., & López, J. A. (1994) *Biochemistry 33*, 13946−13953.
Du, X., Beutler, L., Ruan, C., Castaldi, P. A., & Berndt, M. C. (1987) *Blood 69*, 1524−1527.
Du, X., Harris, S. J., Tetaz, T. J., Ginsberg, M. H., & Berndt, M. C. (1994) *J. Biol. Chem. 269*, 18287−18290.
Du, X., Fox, J. E., & Pei, S. (1996) *J. Biol. Chem. 271*, 7362−7367.
Fox, J. E. B. (1993) *Thromb. Haemostasis 70*, 884−893.
Fox, J. E. B., & Berndt, M. C. (1989) *J. Biol. Chem. 264*, 9520−9526.
Fox, J. E. B., Lipfert, L., Clark, E. A., Reynolds, C. C., Austen, C. D., & Brugge, J. S. (1993) *J. Biol. Chem. 268*, 25973−25984.
Gorlin, J. B., Yamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwiatkowski, D. J., & Hartwig, J. H. (1990) *J. Cell Biol. 111*, 1089−1105.
Greco, N. J., Tandon, N. N., Jones, G. D., Kornhauser, R., Jackson, B., Yamamoto, N., Tanoue, K., & Jamieson, G. A. (1996) *Biochemistry 35*, 906−914.
Hickey, M. J., Williams, S. A., & Roth, G. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6773−6777.
Hickey, M. J., Hagen, F. S., Yagi, M., & Roth, G. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8327−8331.
Hourdille, P., Heilmann, E., Combrie, R., Winckler, J., Clemetson, K. J., & Nurden, A. T. (1990) *Blood, 76*, 1503−1508.
Ikeda, Y., Handa, M., Kamata, T., Kawano, K., Kawai, Y., Watanabe, K., Kawakami, K., Sagai, K., Fukuyama, M., Itagaki, I., Yoshioka, A., & Ruggeri, Z. M. (1993) *Thromb. Haemostasis 69*, 496−502.
Jackson, S. P., Schoenwaelder, S. M., Yan, Y., Rabinowitz, I., Salem, H. H., & Mitchell, C. A. (1994) *J. Biol. Chem. 269*, 27093−27099.
Kroll, M. H., Harris, T. S., Moake, J. L., Handin, R. I., & Schafer, A. I. (1991) *J. Clin. Invest. 88*, 1568−1573.
Kroll, M. H., Hellums, J. D., Guo, Z., Durante, W., Razdan, K., Hroblich, J. K., & Schafer, A. I. (1993) *J. Biol. Chem. 268*, 3520−3524.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lanza, F., Morales, M., de la Salle, C., Cazenave, J.-P., Clemetson, K. J., Shimomura, T., & Phillips, D. R. (1993) *J. Biol. Chem. 268*, 20801−20807.
Lerner, R. A., Green, N., Alexander, H., Liu, F.-T., Sutcliffe, J. G., & Shinnick, T. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3403−3407.
Li, C. Q., Dong, J.-F., Lanza, F., Sanan, D. A., Sae-Tung, G., & López, J. A. (1995) *J. Biol. Chem. 270*, 16302−16307.
Liu, Y.-C., Elly, C., Yoshida, H., Bonnefoy-Berard, N., & Altman, A. (1996) *J. Biol. Chem. 271*, 14591−14595.
Liu, Y.-C., Liu, Y., Elly, C., Yoshida, H., Lipkowitz, S., & Altman, A. (1997) *J. Biol. Chem. 272*, 9979−9985.
López, J. A. (1994) *Blood Coag. Fibrinol. 5*, 97−119.

López, J. A., Chung, D. W., Fujikawa, K., Hagen, F. S., Papayannopoulou, T., & Roth, G. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5615−5619.

López, J. A., Chung, D. W., Fujikawa, K., Hagen, F. S., Davie, E. W., & Roth, G. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2135−2139.

Marchese, P., Murata, M., Mazzucato, M., Pradella, P., De Marco, L., Ware, J., & Ruggeri, Z. M. (1995) *J. Biol. Chem. 270*, 9571−9578.

Michelson, M. D., Ellis, P. A., Barnard, M. R., Matic, G. B., Viles, A. F., & Kestin, A. S. (1991) *Blood 77*, 770−775.

Modderman, P. W., Admiraal, L. G., Sonnenberg, A., & von dem Borne, A. E. G. Kr. (1992) *J. Biol. Chem.* 267, 364−369.

Muslin, A. J., Tanner, J. W., Allen, P. M., & Shaw, A. S. (1996) *Cell 84*, 889−897.

Ozaki, Y., Satoh, K., Yatomi, Y., Miura, S., Fujimura, Y., & Kume, S. (1995) *Biochim. Biophys. Acta 1243*, 482−488.

Peng, M., Lu, W., & Kirby, E. P. (1991) *Biochemistry 30*, 11529−11536.

Peng, M., Lu, W., Niewarowski, S., & Kirby, E. P. (1993) *Blood 81*, 2321−2328.

Robinson, K., Jones, D., Patel, Y., Martin, H., Madrazo, J., Martin, S., Howell, S., Elmore, M., Finnen, M. J., & Aitken, A. (1994) *Biochem. J. 299*, 853−861.

Scholey, J. M., Taylor, K. A., & Kendrick-Jones, J. (1980) *Nature 287*, 2333−2335.

Ward, C. M., Andrews, R. K., Smith, A. I., & Berndt, M. C. (1996) *Biochemistry 35*, 4929-4938.

Wardell, M. R., Reynolds, C. C., Berndt, M. C., Wallace, R., & Fox, J. E. B. (1989) *J. Biol. Chem. 264*, 15656−15661.

Weiss, H. J. (1995) *Thromb. Haemostasis 74*, 117−122.

Zha, J., Harada, H., Yang, E., Jockel, J., & Korsmeyer, S. J. (1996) *Cell 87*, 619−628.

BI970893G