Structural Studies on the PH Domains of Dbl, Sos1, IRS-1, and βARK1 and Their Differential Binding to $G_{\beta\gamma}$ Subunits

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ABSTRACT: Pleckstrin homology (PH) domains are ~110 amino acid residues in length and are structurally conserved in a number of intracellular signaling proteins. A role for these domains has been postulated for βARK, which binds to $G_{\beta\gamma}$ subunits. We have quantified the binding of individual $(His)_6$-tag PH domains of human Dbl, human Sos1, rat IRS-1, human βARK, and human βARK with an extra 33-residue C-terminal extension (βARK+C) to $G_{\beta\gamma}$ subunits. Our *in vitro* binding studies show that all of the PH domains (apart from Sos1), bind $G_{\beta\gamma}$ subunits in a dose-dependent manner, but βARK+C binds 4 times as much $G_{\beta\gamma}$ at saturation as the others. The IRS-1 PH domain has a similar half-maximal concentration of $G_{\beta\gamma}$ binding (18 nM) to βARK+C (30 nM), suggesting that the IRS-1 PH domain has sufficient determinants for $G_{\beta\gamma}$ binding. The βARK PH domain alone has a half-maximal value of 45 nM but a drastically reduced extent of $G_{\beta\gamma}$ binding, suggesting that both the PH domain and the C-terminal 33 residues are necessary for maximal binding. Dbl has a half-maximum concentration of $G_{\beta\gamma}$ binding of 45 nM and a maximal extent of binding similar to that of βARK, but it is difficult to demonstrate saturable binding of $G_{\beta\gamma}$ to Sos1. Since it was previously predicted that the C-terminal PH domain of Pleckstrin [Tyers, M., et al. (1988) *Nature 333*, 470−473] contains a potential calcium binding site, we have tested the different PH domains for calcium binding. Only the PH domain of Dbl bound $^{45}Ca^{2+}$ with a K_d of 10 μM. CD spectroscopy of the purified recombinant PH domains indicated that they are predominantly β-sheet structures. Furthermore, the CD spectrum of the Dbl PH domain was significantly altered in the presence of 10 μM Ca^{2+}.

Platelet and leukocyte C kinase substrate (Pleckstrin) is a 47 kDa protein that is a major substrate of protein kinase C in platelets. There is a significant internal similarity in Pleckstrin between residues 1−106 and 239−350, defining two copies of a domain positioned N- and C-terminally (Tyers et al., 1988). A number of intracellular signaling proteins (71 sequences to date) contain Pleckstrin homology (PH)[1] domains of approximately 110 residues each within their polypeptide chains (Mayer et al., 1993; Haslam et al., 1993; Musacchio et al., 1993; Shaw et al., 1993; Parker et al., 1994; Gibson et al., 1994). PH domains are present in several isoforms of G-protein receptor Ser/Thr kinases (βARK1 and βARK2), isoforms of phospholipase C ($PLC_{\beta,\delta,\gamma}$), GTPase activating proteins (GAP), nucleotide exchange proteins (Ras-GRF, Dbl, and Sos1), and a substrate of the insulin receptor (IRS-1). The PH domain, like the well-characterized Src homology 2 and 3 (SH2 and SH3) domains (Pawson & Gish, 1992; Fry et al., 1993), appears to be noncatalytic and may be involved in protein−protein interactions proximal to the membrane cytoskeleton. Sequence alignment and secondary structure prediction have suggested that PH domains are β-sheet proteins comprising 7−8 β-strands with a single C-terminal α-helix (Musacchio et al., 1993). The three-dimensional structures of the N-terminal PH domains of Pleckstrin and β-spectrin determined by NMR spectroscopy (Yoon et al., 1994; Macias et al., 1994) and that of dynamin determined by X-ray crystallography (Ferguson et al., 1994) have confirmed the preceding prediction (Musacchio, 1993).

Functionally, the direct interaction of a PH domain with the βγ-subunits of the heterotrimeric G-protein includes the βARK PH domain together with a C-terminal extension of 25 residues. This complex is involved in β2-adrenergic receptor desensitization (Koch et al., 1993). PLC_β is activated by a direct interaction with $G_{\beta\gamma}$ complex, but a

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[1] Abbreviations: IRS-1, insulin receptor substrate 1; βARK, β-adrenergic receptor kinase; PLC_γ, phospholipase C_γ; GAP, GTPase activating protein; Ras-GRF, Ras-guanine nucleotide releasing factor; Sos1, son of sevenless; $G_{\beta\gamma}$, G-protein βγ-subunits; PKC, protein kinase C; CD, circular dichroism; PH, Pleckstrin homology.

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specific interaction with its PH domain has not yet been demonstrated (Parker et al., 1994). Nine different PH domains expressed as glutathione *S*-transferase fusion proteins (βARK, Grf, Gap, Plc, Atk, Osbp, IRS1, spectrin, and Racβ) bound *in vitro* to purified $G_{\beta\gamma}$-subunits to varying extents (Touhara et al., 1994), suggesting that this interaction may be functionally relevant. Apart from $G_{\beta\gamma}$ binding, the PH domain of Bruton tyrosine kinase (Btk) was shown to physically associate with calcium-dependent and -independent isoforms of protein kinase C (PKC). This association led to phosphorylation of Btk and its down-regulation (Yao et al., 1994). The PH domain of Rac has been shown to bind to PKC isoform ξ (Konishi et al., 1994).

The PH domains of Dbl, Sos1, and IRS-1 were selected for study since there is evidence for their interaction with other proteins directly (Dbl with rho/rac; Sos1 with ras) and indirectly (IRS-1 with Ras) [for a review, see Boguski and McCormick (1993)]. Dbl encodes a 66 kDa phosphoprotein that is partitioned between the cytosol and the plasma membrane associated with the cytoskeletal matrix (Srivastava et al., 1986). Some PH domains have been demonstrated to bind phosphatidylinositol 4,5-biphosphate (Harlan et al., 1994), which could determine their membrane localization. Sos1 is a cytoplasmic guanine nucleotide exchange catalyst that interacts with p21 ras (Bonfini et al., 1993), but the function of its PH domain remains to be elucidated. IRS-1 is a cytoplasmic protein that associates with and is tyrosine-phosphorylated by the insulin receptor. Its PH domain has been implicated in mediating associations with other proteins involved in insulin signaling (Myers et al., 1994).

We have performed structural and functional studies on the βARK, Dbl, Sos1, and IRS-1 PH domains expressed as $(His)_6$-tag fusion proteins and show that these domains possess similar secondary structures and have the capacity to differentially bind to purified $G_{\beta\gamma}$-subunits. *In vitro*, the Dbl PH domain is shown to bind Ca^{2+}, affecting its structure. The physiological significance of this binding remains to be determined.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids. The DNA sequences corresponding to the PH domains of human Dbl ($hDbl^{762-871}$) (proto-Dbl cDNA; Ron et al., 1988), human Sos1 ($hSos1^{450-569}$) (Sos1 from λ ZAP human brain cDNA library, Stratagene), rat IRS-1 ($rIRS\text{-}1^{1-119}$) (rat liver cDNA library, Stratagene), human βARK ($h\beta ARK^{553-656}$), and human βARK plus the C-terminal 33 residue extension ($h\beta ARK{+}C^{553-689}$) (Chuang et al., 1992) were synthesized using the polymerase chain reaction (PCR), with a *Bam*HI and a *Hin*dIII site at the 5′- and 3′-ends, respectively. The DNA fragments were cloned into a pQE30 plasmid (type 4) containing a $(His)_6$-tag (Qiagen). DNA sequencing confirmed the presence of the correct DNA sequences.

Bacterial Expression and Protein Purification. The pQE30 plasmid containing the hDbl, hSos1, rIRS-1, hβARK, and hβARK+C PH domains was transfected into competent M15 *Escherichia coli* cells carrying the plasmid pREP4 (Qiagen). Bacterial expression and protein purification were performed as previously described (Mahadevan et al., 1994), except that the proteins were eluted with 250 mM imidazole at pH 6.3. The purified proteins were further purified by FPLC using a Mono S column (Pharmacia) and concentrated using Centricon 10 membranes (Amicon).

Circular Dichroism Spectroscopy. The far-UV CD spectra (195−260 nm) of each PH domain from hDbl, hSos1, rIRS-1, hβARK, and hβARK+C were recorded at 22 °C at a concentration of 0.25 mg/mL with a JASCO J-500C spectropolarimeter, using settings previously described (Mahadevan et al., 1994). CD studies were conducted in 20 mM Tris-HCl (pH 7.4). Secondary structure analysis was performed using the method of Provencher and Glockner (1981).

Detection of $G_{\beta\gamma}$ Binding to PH Domains Using the ECL Western Blot Method. The FPLC-purified $(His)_6$-tag PH domains were used to assess direct binding to purified bovine brain $G_{\beta\gamma}$ subunits (Sternweis & Pang, 1990). Purified $G_{\beta\gamma}$-subunits at varying concentrations (0−360 nM) were incubated with 1 μM each of the PH domains (Dbl, Sos1, IRS-1, βARK, and βARK+C) in 50 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl, and 0.01% Lubrol (TSL buffer) in a final volume of 60 μL for 20 min on ice. The reaction mixture was then incubated with 20 μL of TSL buffer-equilibrated Ni^{2+}−NTA−agarose (Qiagen) for an additional 15 min on ice with gentle mixing. As a control experiment, $G_{\beta\gamma}$-subunits in the absence of PH domains and heat-denatured $G_{\beta\gamma}$-subunits or PH domains (65 °C for 10 min) were used to determine nonspecific binding to Ni^{2+}−NTA−agarose beads. The beads were centrifuged at 500*g* for 5 min and the supernatant was removed. The beads were then washed three times with 0.4 mL of TSL buffer. The samples were separated by 12% SDS−PAGE (NOVEX) in a Tris−glycine buffer system and transferred to nitrocellulose paper (0.45 μm). The nonspecific binding sites were blocked with 5% (w/v) nonfat dry milk in TTBS buffer (20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 0.05% Tween-20), and the blot was incubated with pan-specific antibodies recognizing all subtypes of G_β-subunits at 0.1 μg/mL (rabbit polyclonal IgG, Santa Cruz Biotech) overnight at 4 °C. The blot was washed with TTBS and incubated for 1 h with donkey anti-rabbit HRP IgG at 1:2500 dilution (Amersham). Subsequently, the blot was washed with TTBS, incubated with ECL detection reagents (Amersham), and then exposed to Kodak X-OMAT-LS film for variable times (1−30 min) at room temperature. The films were developed in an automated film processor (Kodak) under standard conditions. All blots from a given experiment were exposed on the same piece of film for several different time periods. Film showing a linear response to exposure time was used to determine $G_{\beta\gamma}$ binding. The bands were quantified using a BioImager (Millipore) or an LKB laser densitometer; results are reported after correction for nonspecific binding.

EC50's were determined as the concentration (nanomolar) of $G_{\beta\gamma}$ at which one-half of the saturation value of binding was obtained for that particular PH domain. If binding was not saturable, then no EC_{50} value was reported.

Calcium Binding Assays. (a) $^{45}Ca^{2+}$ Overlay Assay. The detection of Ca^{2+} binding to the above-mentioned PH domains was carried out as described by Maruyama et al. (1984), as modified according to Kawasaki et al. (1985). The PH domains (2.5 μg) were bound to nitrocellulose paper in a Mini Fold II slot blot system (Schleicher and Schuell, Keene, NH). The nitrocellulose filters were removed and washed twice for 10 min each at room temperature with overlay buffer (10 mM imidazole (pH 6.8) and 100 mM NaCl) (Gailit & Rouslahti, 1988) containing 10 mM EDTA to remove any protein-bound divalent cations. The filters were then washed four times with overlay buffer to remove

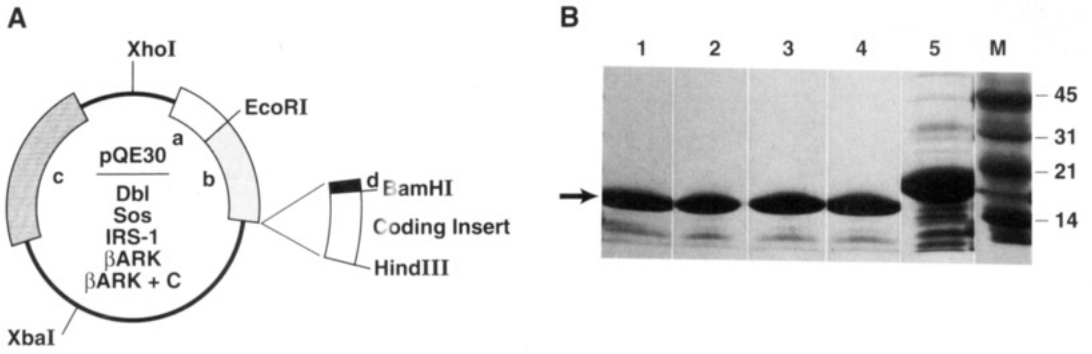

FIGURE 1: (A) Expression vector pQE30 (Qiagen) constructed for the hDbl, hSos1, rIRS-1, hβARK, and hβARK+C PH domains. The vector possesses the following features: (a) a regulatable promoter/operator, (b) a synthetic ribosomal binding site (RBSII), (c) β-lactamase, and (d) a His_6-tag placed N-terminally. (B) The purified PH domains of Dbl, Sos1, IRS-1, βARK, and βARK+C-terminal 33 residues electrophoretically separated by 4−20% SDS−PAGE and stained with Coomassie Blue R-250.

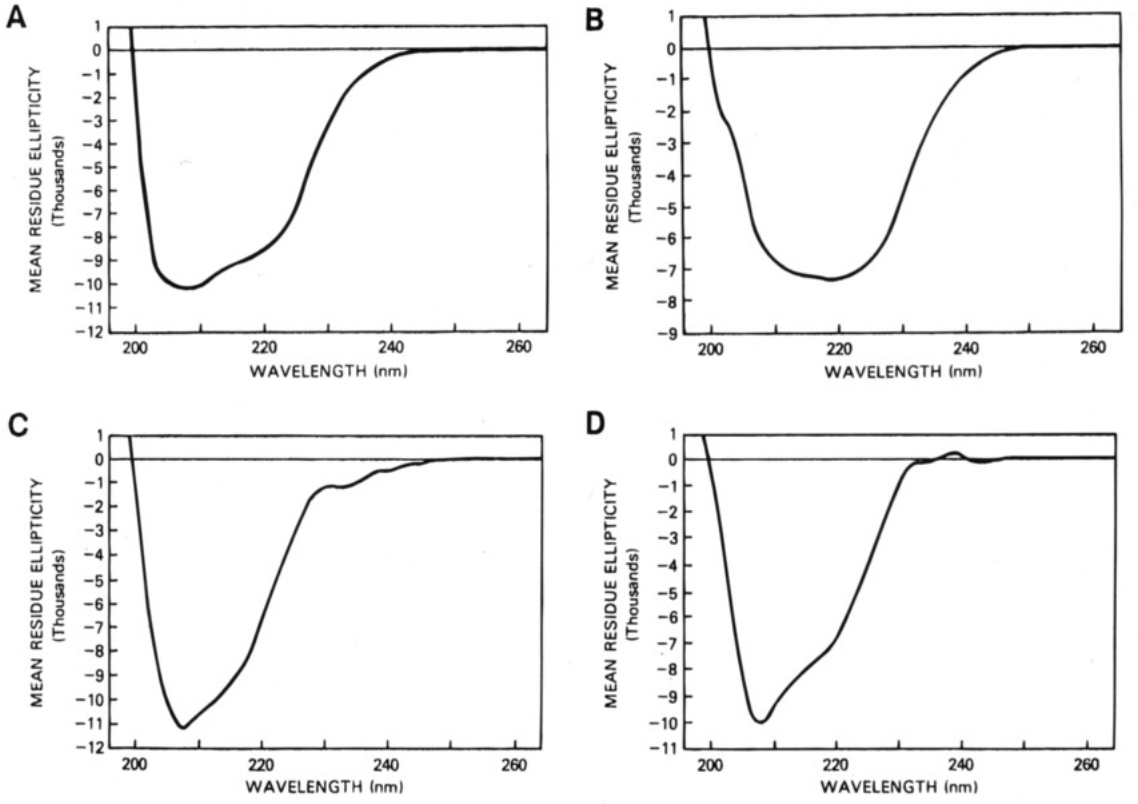

FIGURE 2: Far-UV CD spectra of the purified PH domains of (A) hDbl, (B) hSos1, (C) rIRS-1, and (D) hβARK at a protein concentration of 0.25 mg/mL in 20 mM Tris-HCl (pH 7.4).

the EDTA. The $^{45}Ca^{2+}$ (Amersham) overlay was performed by incubating the filters for 30 min at room temperature in overlay buffer containing 0.44 μCi/mL (2 μL in 2 mL) $^{45}Ca^{2+}$ (50 nM) in the absence or presence of unlabeled calcium chloride (0.1−100 μM). The filters were rinsed rapidly with 50% ethanol in water, air-dried, and exposed to Kodak XAR-5 X-ray film for 24 h at room temperature. For quantitative analysis, the filters were exposed to a phosphor screen and analyzed by a phosphoimager (Molecular Dynamics).

(b) Circular Dichroism Spectroscopy. The far-UV CD spectra (195−260 nm) of the PH domains of Dbl, Sos1, IRS-1, βARK, and βARK+C were measured in the presence of 10 μM Ca^{2+} ions, and the secondary structure was determined as described earlier.

(c) Ca^{2+}-Dependence of $G_{\beta\gamma}$ Binding to PH Domains. The $G_{\beta\gamma}$ binding assay described earlier was performed with 183 nM $G_{\beta\gamma}$ with either 1 mM EGTA or varying concentrations of added calcium ions (0.1−100 μM). As one control, $G_{\beta\gamma}$-subunits alone were incubated in the presence of calcium ions and Ni^{2+}−NTA−agarose to determine nonspecific binding. The results were analyzed by the ECL method as described earlier and corrected for nonspecific binding. A second control with Ca^{2+} was included in which both the amount of Dbl PH domain bound to the Ni^{2+}−NTA beads and the amount of $G_{\beta\gamma}$ associated with the beads were determined by Coomassie Blue and silver staining after SDS−PAGE analysis.

RESULTS

Expression and Purification of PH Domains. In order to elucidate the structural mechanism(s) of interaction of PH domains with $G_{\beta\gamma}$-subunits, we bacterially expressed and purified the His_6-tagged PH domains of hDbl, hSos1, rIRS-1, hβARK, and hβARK+C. The first 110 residues of IRS-1 is a PH domain, while in Dbl it is more C-terminal in the

Table 1: Half-Maximal Values of PH Domain Binding to $G_{\beta\gamma}$-Subunits Calculated from Figure 3B

PH domain	half-maximal $G_{\beta\gamma}$ binding (nM)
βARK+C	30
IRS-1	18
Dbl	45
βARK	45
Sos1	not saturable

protein. The PH domain of Sos1 is positioned between the CDC24 and 25 homology domains, and the βARK PH domain is positioned C-terminal to the kinase domain. Figure 1A shows the expression vector pQE30 constructed for the PH domains. Figure 1B shows the SDS−PAGE profile of the FPLC-purified PH domains of Dbl, Sos1, IRS-1, βARK, and βARK+C. All of the PH domains migrate at a relative molecular weight of 17 000, except for the βARK+C domain which migrates at a relative molecular weight of 19 000. The purity of the preparations is estimated to be >95%. All of the preceding PH domains are basic molecules with calculated p*I*'s in the range 8−11.

PH Domains Are β-Sheet Proteins. The far-UV CD spectrum of each of the highly purified PH domains indicates that they are essentially β-sheet proteins (Figure 2 A−D). The Sos1 PH domain is mainly β-sheet, with a calculated β-sheet content of 58%, and little or no α-helix as indicated by the diagnostic trough at 215 nm. The PH domains of Dbl and IRS-1 have β-sheet contents of 60% and α-helical contents of 10−16%. However, the βARK PH domain is different in that it has an α-helical content of 23% and a β-sheet content of 37%. Despite the variability in sequence within the PH domain family, our results support the idea that a conserved β-barrel-like three-dimensional structure may form the structural basis for many PH domains (Yoon et al., 1994; Macias et al., 1994; Ferguson et al., 1994).

Differential Binding of PH Domains to $G_{\beta\gamma}$-Subunits. The binding of individual PH domains (1 μM) of Dbl, Sos1, IRS-1, βARK, and βARK+C to purified $G_{\beta\gamma}$-subunits (0−360 nM) is shown in Figure 3A. All PH domains (apart from Sos1) show dose-dependent binding, which is saturable (βARK+C saturates at >400 nM, data not shown). Binding is rapid, reaching a maximum by 10 min, and is stable through 30 min (data not shown). Interestingly, the maximal amount of $G_{\beta\gamma}$ binding to βARK+C is more than 4-fold higher than that to the other PH domains. The reason for

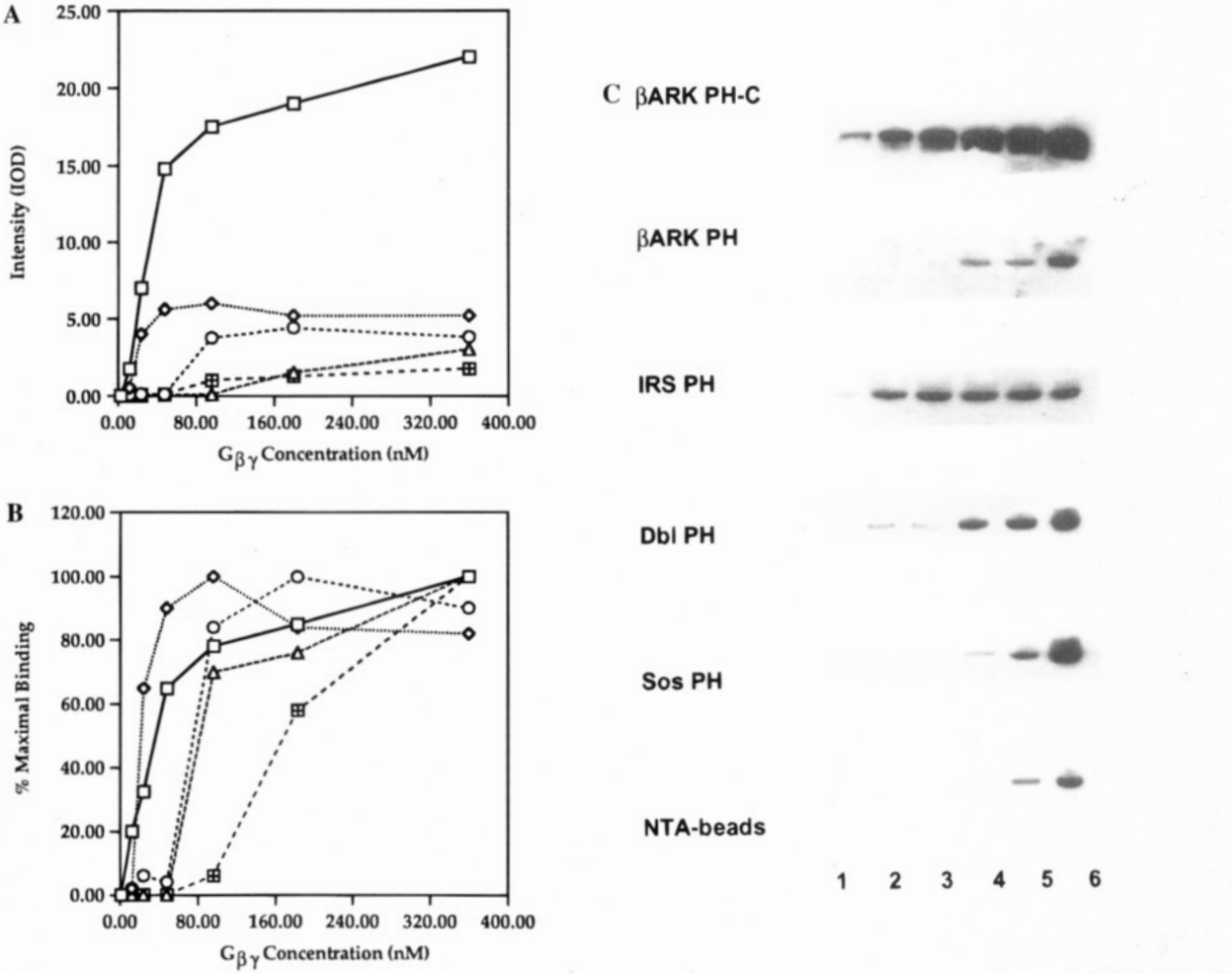

FIGURE 3: (A) Binding of the individual PH domains to $G_{\beta\gamma}$-subunits: βARK+C (square), IRS-1 (diamond), Dbl (circle), βARK (crossed square), and Sos1 (triangle). The PH domains at a concentration of 1 μM were incubated with increasing concentrations (0−360 nM) of purified $G_{\beta\gamma}$-subunits and processed as indicated in Experimental Procedures. The results are presented as the integrated optical density (IOD) of G_β immunoreactivity as a function of $G_{\beta\gamma}$ concentration. Results are representative of four experiments. (B) Determination of half-maximal binding of $G_{\beta\gamma}$ to PH domains. The binding of $G_{\beta\gamma}$ to the individual PH domains is expressed as a percent of the maximal binding observed for each PH domain. Data and symbols are the same as in panel A. (C) Western blot analysis of $G_{\beta\gamma}$ binding to PH domains. Raw ECL data from a 2 min exposure of blots to film. Concentration of $G_{\beta\gamma}$ in the assay: lane 1, 11.25 nM; lane 2, 22.5 nM; lane 3, 45 nM; lane 4, 90 nM; lane 5, 180 nM; lane 6, 360 nM.

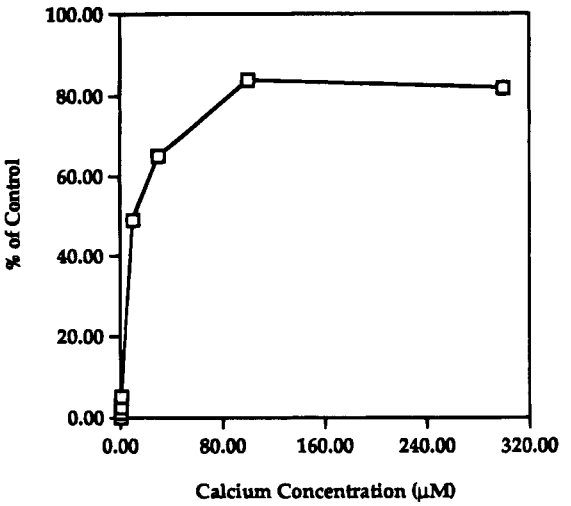

FIGURE 4: Binding of $^{45}Ca^{2+}$ to the Dbl PH domain. $^{45}Ca^{2+}$ (50 nM) was titrated against unlabeled Ca^{2+} (0.1−100 μM), as described in Experimental Procedures. The data are presented as the percent of the binding of $^{45}Ca^{2+}$ observed in the absence of the added unlabeled Ca^{2+} as a function of added unlabeled Ca^{2+}.

the higher level of binding of βARK+C in comparison to other PH domains is not clear.

To assess the relative affinities of the PH domains independent of the saturation level, we determined the concentration of $G_{\beta\gamma}$ required to achieve half-maximal binding (Figure 3B). The results indicate that, apart from Sos1, the PH domains show similar half-maximal affinities (EC_{50}'s) for binding to $G_{\beta\gamma}$ (βARK+C, 45 nM; βARK, 30 nM; IRS-1, 18 nM; Dbl, 45 nM) (Table 1). These results suggest that the βARK C-terminal 33 residues mainly contribute to the level of binding, rather than to the affinity for $G_{\beta\gamma}$. The low level of Sos1 binding, combined with the absence of saturation with increasing concentrations of $G_{\beta\gamma}$, implies that this PH domain has a weak affinity for $G_{\beta\gamma}$ and that Sos1 PH−$G_{\beta\gamma}$ interactions are nonspecific. The binding of the $G_{\beta\gamma}$-subunits alone to the Ni^{2+}−NTA−agarose was negligible. The $G_{\beta\gamma}$-subunits in the absence of PH domains and heat-denatured $G_{\beta\gamma}$ or heat-denatured PH domains (65 °C for 10 min) showed no binding to Ni^{2+}−NTA−agarose beads. Heat-denatured $G_{\beta\gamma}$-subunits failed to bind to the βARK+C PH domain. Raw data from the ECL-detected Western blots of $G_{\beta\gamma}$ binding are presented in Figure 3C.

Effect of Calcium on the Conformation of the Dbl PH Domain. The $^{45}Ca^{2+}$ overlay showed that only the Dbl PH domain binds $^{45}Ca^{2+}$ under the conditions of the assay. A K_d value of 10 μM was determined by displacement with unlabeled Ca^{2+} (Figure 4). This suggests the presence of a calcium binding site within the Dbl structure with an affinity comparable to that determined for S-100-like intracellular calcium binding proteins, which have K_d values in the 10−30 μM range (Gibbs et al., 1994). Since the three-dimensional structures of three PH domains are now known, we can infer the structures of the PH domain family. The PH domain sequences of Dbl, Sos1, IRS-1, and βARK were aligned with respect to the N-terminal PH domain of Pleckstrin (Pln) (Yoon et al., 1994) to ascertain a potential calcium binding site(s) within the Dbl PH domain (Figure 5). In β-spectrin following β-strand 3, there is an 8-residue α-helix (Macias et al., 1994) that is absent from Pln. In the Dbl PH domain, this region has an insertion similar to that of β-spectrin with the following sequence, ESGEGSD, which we suggest as the potential calcium binding site. Since calcium binding sites require 6 or 7 ligand atoms arranged regularly around the Ca^{2+}, the two serine residues, the polypeptide backbone carbonyls, and the surrounding water molecules are suggested to contribute to this site. When the other aligned PH domains (Musacchio et al., 1993; Gibson et al., 1994), were analyzed for the presence of potential calcium binding sites like that observed for Dbl, none could be identified.

We examined whether the binding of Ca^{2+} to the Dbl PH domain would lead to a conformational change. CD spectroscopy is suitable as a probe for measuring secondary structural differences. The addition of 10 μM Ca^{2+} to the Dbl PH domain changed its conformation significantly (Bayley, 1973): from 10% α-helix/60% β-sheet to 1% α-helix/70% β-sheet (Figure 6).

Since the Dbl PH domain is able to bind Ca^{2+} and alter its conformation, we investigated the ability of Ca^{2+} to modulate PH domain binding to $G_{\beta\gamma}$-subunits. The purified Dbl PH domain (1 μM) was assayed in the presence of varying added Ca^{2+} concentrations (0−100 μM; 0 = no added Ca^{2+} or 1 mM EGTA), in the presence of 183 nM $G_{\beta\gamma}$-subunits. The binding of $G_{\beta\gamma}$-subunits to Ni^{2+}−NTA−agarose in the absence of the Dbl PH domain and in the presence of increasing Ca^{2+} concentrations was negligible (data not shown). An apparent increase in $G_{\beta\gamma}$ binding to PH−NTA−agarose was correlated with an increase in Dbl PH domain binding to Ni^{2+}−NTA−agarose. This artifact precluded determination of the effect of Ca^{2+} on the Dbl−PH domain interaction with $G_{\beta\gamma}$. This change in the binding of the resin with Ca^{2+} was not observed for any other PH domains.

DISCUSSION

Pleckstrin homology (PH) domains are present in a number of intracellular signaling proteins. Currently the only documented function is for the βARK PH domain, which mediates translocation of this kinase to the plasma membrane by binding to membrane-associated $G_{\beta\gamma}$-subunits (Inglese et al., 1993). The present work is the first quantitation of the interaction of $G_{\beta\gamma}$-subunits with PH domains from several different proteins lacking a GST fusion protein partner. Half-maximal binding of $G_{\beta\gamma}$ to the PH domain occurred at 30, 45, 18, and 45 nM for βARK+C, βARK, IRS-1, and Dbl, respectively. Binding of $G_{\beta\gamma}$ to the Sos1 PH domain did not saturate with increasing $G_{\beta\gamma}$ concentrations, and thus can be considered to be nonspecific. The EC_{50}'s we report for the different PH domains are for the fraction of $G_{\beta\gamma}$ binding to PH domain that we can observe. When the binding is saturable, the apparent EC_{50}'s indicate the similarity of the binding of $G_{\beta\gamma}$ to the PH domains. The βARK+C domain (residues 553−689) binds more than 4-fold more $G_{\beta\gamma}$ at saturation than the other PH domains. Truncation of the C-terminal 33 amino acids following the predicted βARK PH domain dramatically decreases the maximal extent of $G_{\beta\gamma}$ binding without significantly affecting its apparent affinity of the remaining binding. Thus, the C-terminal 33-amino acid extension mainly affects the apparent stoichiometry of $G_{\beta\gamma}$ binding to the βARK+C domain.

We have investigated possible reasons for the observed low stoichiometry of $G_{\beta\gamma}$ binding to isolated PH domains. Estimation of the $G_{\beta\gamma}$ remaining in the supernatant of the

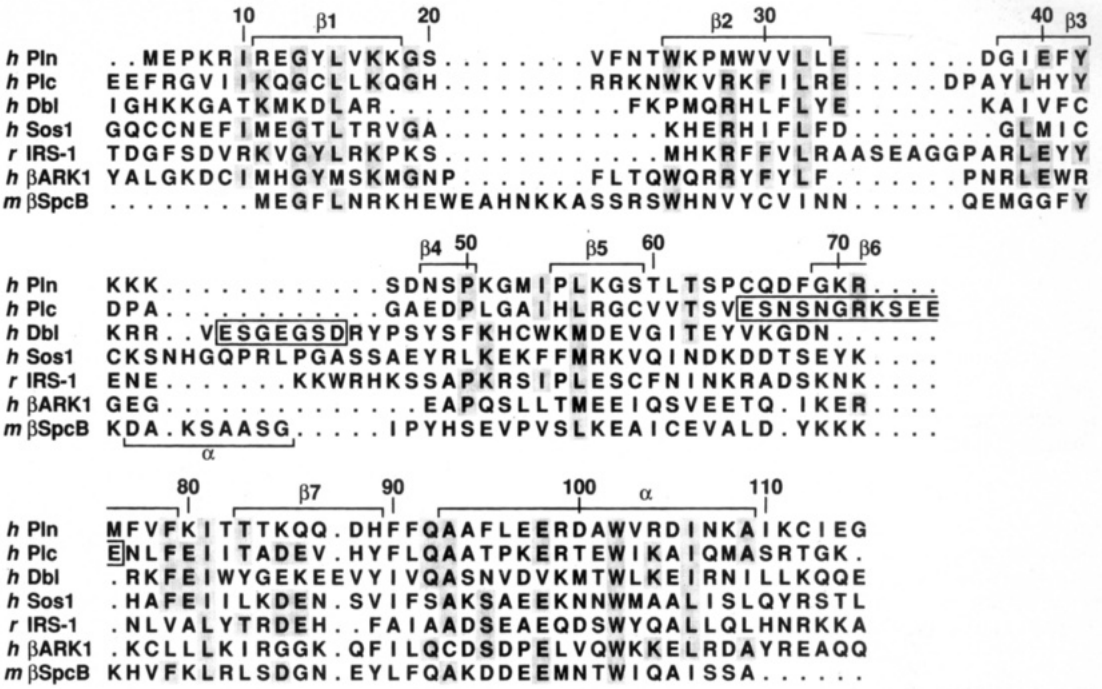

FIGURE 5: Structural alignment of the PH domain. Sequences of Dbl, Sos1, IRS-1, βARK, and the C-terminal domain of Pleckstrin (Plc) are aligned with those of the N-terminal domain of pleckstrin (Pln) and β-spectrin, for which three-dimensional structures are available (Yoon et al., 1994; Macias et al., 1994). The shaded areas indicate identical residues if present in more than three sequences. The secondary structure is shown along the sequence indicated as β-strand or α-helix. β-Spectrin has an additional α-helix between β-strands 3 and 4. The predicted calcium binding site in the Dbl PH domain, ESGEGSD, is present in a loop between β-strands 3 and 4 and is boxed. The predicted calcium binding site on Plc is also boxed.

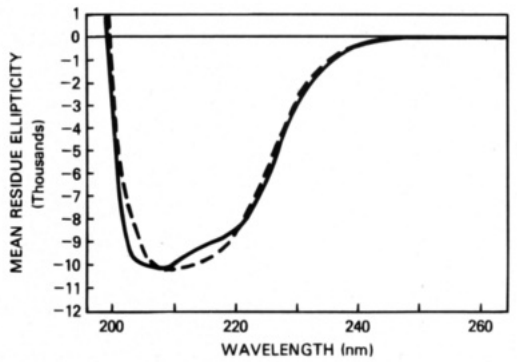

FIGURE 6: Far-UV CD spectrum of the Dbl PH domain (0.25 mg/mL) in 20 mM Tris -HCl (pH 7.4), in the absence (solid line) and presence (dashed line) of 10 μM Ca^{2+}.

binding assay by Western blot suggests that $G_{\beta\gamma}$ binding to immobilized βARK+C domain attains a maximum of about 10% of the amount expected for a 1:1 stoichiometry with the immobilized domain. No free PH domain is detectable in the supernatant from the beads. This substoichiometric binding is a consistent finding over several preparations of PH domains. Similar results are obtained with direct [^{3}H]-propionyl-$G_{\beta\gamma}$ binding to the βARK+C domain. The $G_{\beta\gamma}$ preparation is at least 80% functional, as indicated by experiments with repeated absorption with the βARK+C domain and by $G_{\beta\gamma}$-supported pertussis toxin-catalyzed ADP-ribosylation of $G\alpha_{i,o}$ (data not shown). Thus, the $G_{\beta\gamma}$ partner does not limit binding. Binding of $G_{\beta\gamma}$ is the same whether the PH domains are preimmobilized on the Ni^{2+}−NTA−agarose beads or whether the complex absorbed after the proteins have interacted in solution.

Another possible explanation is that refolding of the different PH domains may not be equally efficient. However, the primarily β-sheet conformation determined by CD for the $(His)_6$-tagged PH domains tested here indicates that stable protein folding units have been formed. The inferred secondary structure is consistent with that found for those PH domains where three-dimensional solution structures have been determined.

Our results show that the PH domains tested, apart from Sos1, bind saturably to $G_{\beta\gamma}$ with comparable apparent affinities. Interesting possibilities are raised by the observed interaction of two of the PH domains with $G_{\beta\gamma}$ that have not previously been recognized. IRS-1 is a cytoplasmic protein that associates with and is tyrosine-phosphorylated by the insulin receptor. Its PH domain is positioned N-terminally, and removal of this domain from IRS-1 appears to impair insulin signaling (Myers et al., 1994). Microinjection studies of the IRS-1 PH domain into *Xenopus* oocytes in the absence and presence of insulin indicate that meiotic maturation induced by insulin is inhibited by 40% compared to the insulin control (P. Aroca and D. Mahadevan, unpublished results). Elucidation of the mechanism of action of the IRS-1 PH domain will be of interest in the light of its ability to interact with $G_{\beta\gamma}$-subunits. Dbl is a protooncogenic protein that elicits nucleotide exchange for some members of the *ras*-related family of GTP binding proteins, which appear to regulate cytoskeletal activities such as the maintenance of cell polarity, shape, and cytokinesis (Ridley et al., 1992). The Dbl PH domain bound $^{45}Ca^{2+}$ with a K_d of 10 μM, which is comparable to that of S-100-like intracellular calcium binding proteins (Gibbs et al., 1994). The enhanced binding of the Dbl PH domain to the resin in the presence of Ca^{2+} prevented us from assaying the effects of Ca^{2+} on the Dbl PH−$G_{\beta\gamma}$ interaction.

Sos1 is a cytoplasmic guanine nucleotide exchange protein that interacts with p21 ras (Bonfini et al., 1993), but the significance of its PH domain is currently unknown, although

it has been shown that ras-dependent activation of the MAP kinase pathway is mediated by $G_{\beta\gamma}$-subunits (Crespo et al., 1994). The Sos1 PH domain binds to $G_{\beta\gamma}$-subunits nonsaturably and to a minimal extent compared to the other PH domains. The CD spectrum of the Sos1 PH domain shows that it has little or no α-helix, unlike the CD spectra of the other PH domains studied here. It may be significant that $G_{\beta\gamma}$ and βARK are postulated to interact through α-helical coiled-coil structures (Simond et al., 1993). Not all PH domains may bind to isolated $G_{\beta\gamma}$-subunits.

The three-dimensional structures determined for three distinct PH domains indicate that they are composed of a β-barrel made up of seven β-strands with a C-terminal α-helix (Yoon et al., 1994; Macias et al., 1994; Ferguson et al., 1994). The PH domain of β-spectrin has an additional α-helix following the third β-strand (Macias et al., 1994). Here, we show that although the PH domains of Dbl, Sos1, IRS-1, and βARK exhibit considerable primary sequence variability, they are mainly β-sheet proteins with variable α-helical content. This result is consistent with the two NMR structures, which show 42% β-sheet and 10−15% α-helical contents for the pleckstrin and β-spectrin PH domains. In the βARK and Ras-GRF PH domains, the C-terminal α-helix is thought to play an important role in $G_{\beta\gamma}$ binding (Touhara et al., 1994). However, in contrast to SH2 and SH3 domains, PH domains are highly variable in primary sequence, which may reflect the fact that these domains may be performing functions in addition to $G_{\beta\gamma}$ binding. For example, the *xid* mutation in the Btk PH domain, where arginine 26 has been mutated to a cysteine, does not appear to perturb the structure but may affect the binding of this PH domain to another protein, such as serine/threonine-phosphorylated PKC (Gibson et al., 1994; Yao et al., 1994). $G_{\beta\gamma}$ binding was shown to require a different part of the protein: subdomains 5 and 6 of the PH domain and the conserved tryptophan in subdomain 6 (Tsukada et al., 1994).

In conclusion, we have demonstrated that four distinct PH domains bind differentially to $G_{\beta\gamma}$-subunits, and this effect is quantified with respect to the βARK PH+C domain, which is a known functional target for these G-proteins. Although the relevance of the βARK PH domain−$G_{\beta\gamma}$ interactions for G-protein-coupled receptor phosphorylation is well accepted, the physiological significance of the *in vitro* determined $G_{\beta\gamma}$ interactions with the Dbl, Sos1, and IRS-1 PH domains remains to be determined.

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