The solution structure of an extended pleckstrin homology (PH) domain from the β-adrenergic receptor kinase is obtained by high resolution NMR. The structure establishes that the β-adrenergic receptor kinase extended PH domain has the same fold and topology as other PH domains, and there are several unique features, most notably an extended C-terminal α-helix that behaves as a molten helix, and a surface charge polarity that is extensively modified by positive residues in the extended α-helix and the C terminus. These observations complement biochemical evidence that the C-terminal portion of this PH domain participates in protein-protein interactions with Gβγ subunits. This suggests that the C-terminal segment of the PH domain may function to mediate protein-protein interactions with the targets of PH domains.

G protein-coupled receptor kinases (GRKs) are a unique family of serine-threonine kinases, which are responsible for activator-dependent phosphorylation of G protein receptors and provide rapid desensitization of the agonist occupied receptors (1). The GRKs are recognized to have three functional components: an N-terminal section believed to interact directly with the α-adrenergic receptor kinase-2), the C-terminal variable region contains a pleckstrin homology (PH) domain (2, 3), and a surface charge polarity due to mediate membrane association by various means. In GRK2 (also known as β-adrenergic receptor kinase-1) or GRK3 (β-adrenergic receptor kinase-2), the C-terminal variable region contains a pleckstrin homology (PH) domain (2, 3), conferring binding specificity to Gβγ proteins (reviewed in Ref. 1).

The PH domain family (reviewed in Refs. 4–6) appears to be a very large family of structurally homologous protein domains of moderate to low sequence similarity. The PH domain is believed to play a role in intracellular signal transduction, and the functional role of the PH domain has been characterized for several systems. In phospholipase Cβ, the PH domain has a high affinity (Kd < 1 μM) site for phosphatidylinositol 4,5-bisphosphate (PIP2) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) (7), which forms a crystallographically observed, well defined structural interaction (8). Other PH domains have different lipid specificities, and a well defined set of binding motifs does not readily emerge (9–15). One hypothesis is that PH domains present a framework with a polymorphic surface used for specific recognition, analogous to immunoglobulins (5, 9).

In the overall fold of the PH domain was observed to be common with that of the PTB (phosphotyrosine binding) domain (16, 17), a protein domain that shows little sequence homology to PH domains. In light of these developments, it is of significance to establish whether the nominal PH domain of GRK-2 (β-adrenergic receptor kinase (βARK-1)), which clearly binds (both in vivo and in vitro) to a protein partner, Gβγ subunits of the heterotrimeric G protein family (18), truly has the common structural motif of the PH/PTB domains, and what the relationship of putative lipid and protein binding sites might be in such a structure.

In this paper, we present the solution structure of an extended PH domain from human βARK1, determined at 0.4 Å r.m.s.d. by high resolution NMR using heteronuclear triple resonance methods. Although the overall fold and topology clearly establishes that the βARK1 extended PH domain is of the same family as other PH domains, there are several significant alterations (most notably an extension of the C-terminal α-helix, which in solution presents as a “molten helix” having a clear gradient of mobility) on the subnanosecond, as well as millisecond to microsecond, time scales, increasing toward the free C terminus. The polarity of the surface charge observed in other PH domains is altered by positively charged residues in the extended α-helix. This unusual clustering may be complemented by a highly negatively charged area on Gβγ subunits. Although a direct study of the Gβγ/PH domain complex is

A BINDING PARTNER OF Gβγ, SUBUNITS

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The solution structure and dynamics of the Pleckstrin Homology Domain of G Protein-coupled Receptor Kinase 2 (β-Adrenergic Receptor Kinase 1)

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Solution Structure of the PH Domain of GRK2 (βARK1)

Beyond the range of current NMR technology, the structure presented here supports a model in which the C-terminal portion of βARK PH domain in particular, and PH domains in general, participate in protein-protein interactions.

MATERIALS AND METHODS

Sample Preparation—Recombinant human βARK PH domain (hβARK1-(556–670)) was obtained by GST fusion expression from pGEX-2T (Pharmacia Biotech Inc.) in BL21 (DE3) Escherichia coli cells (Novagen, Madison, WI) and subsequent bacterial expression and protein purification as described previously (19) on a larger scale. The full-length hβARK1 cDNA clone was provided by Dr. Antonio DeBlasi (Mario Negri Sud, Santa Maria Imbaro, Italy). The sequence of the 119-residue construct used in the present study is shown in Fig. 1. It contains both the PH domain and the Gpβ,β'-binding motif (20). The first four residues are not from the natural sequence. Uniform 15N and 13C labeling was achieved by growing the cells in M9 minimal medium using standard procedures.

Solutions used for NMR studies contained 1–2 mM protein in 10 mM acetate buffer at pH 4.5 (uncorrected for isotope effects), 0.02% sodium azide, 1 mM [U-15N]EdTA, 5 mM [U-2H]dithiothreitol, and 10% H2O in the H2O samples. These low salt and low pH conditions were necessary to prevent protein aggregation. CD data indicated no changes in the protein secondary structure, between the buffer used for NMR studies and phosphate-buffered saline, pH 7.2. The external 1H chemical shift reference used was sodium 2.2-dimethyl-2-silapentane-5-sulfonate, and indirect referencing was used for 15N (21) and 13C. Spectra were essentially identical among several preparations of the PH domain.

NMR Spectroscopy—NMR experiments were run on Bruker DMX-500 and DMX-600 spectrometers. Quadrature detection was achieved by the States or States-time proportional phase incrementation methods. Some of the pulse schemes implemented pulse field gradients for coherence selection (HCCH-TOSY, 13C-separated NOESY-HMQC), and some used the sensitivity enhancement method (HSQC, heteronuclear NOE) (22). The water signal was suppressed either by the WATERGATE method (23) or by using selective on-resonance irradiation during a relaxation delay of ~1.3 s. Experiments were run at 35 °C with sweep widths of 8000 and 2000 Hz for 1H and 15N (at 600 MHz), respectively, unless indicated otherwise.

The homonuclear experiments, HOHAHA and NOESY, were run in both H2O and H2O using standard pulse sequences and phase cycling. A range of t1 increments from 200 to 512, each consisting of 2048 complex points, was typically acquired with 32–128 scans/increment. The heteronuclear experiments consisted of two-dimensional HSQC ([1H,15N] and [13C,13C]), HSQC-J, and HTQC, three-dimensional CBCA(CO)NH, CBCA(NH), HC(NH), CBCANH, HN(NO)CA, HNCO, HCCH-TOSY, and 13C-separated NOESY-HMQC, and two- and three-dimensional 15N-separated NOESY-HMQC (in H2O and in D2O) and HOHAHA-HMQC. The mixing time in the NOESY-HMQC experiments was 100 and 150 ms, and the spin lock duration was 30 ms in the HOHAHA-HMQC and 19 ms and 6 ms in the HCCH-TOSY. The three-dimensional spectra were recorded with a 32 × 100 × 100 hypercomplex matrix, with 32 scans/increment. The degree of amide hydrogen protection was assessed (a) by measuring hydrogen-deuterium exchange rates by following the intensity of cross-peaks in HMQC experiments after exchanging a fully protonated, lyophilized sample with 99.996% H2O, and (b) by comparison of cross-peak intensities in two HSQC experiments, with and without water presaturation (24). The three-bond H–H coupling was assessed by the method of (25). Heteronuclear 15N[1H] NOEs were measured using standard methods as described elsewhere (26). Two-dimensional H2O-selective heteronuclear 2H-edited ROEHSY experiments (27) were performed to map those amide hydrogens in the βARK PH domain that are exposed to and interacting with water molecules. Signal processing and assignment were done as discussed previously (26, 28).

Structure Calculation—Structure calculations used DIANA with REC- DAC strategy (29) or DYANA (30) with ECEPP stereochemistry, with structurally significant constraints of 1956 upper and 76 lower distance bounds (from ~3000 NOEs), 38 hydrogen bonds chosen within strands or helix with slowed exchange, 99 φ-angle constraints derived from 1H,15N coupling constants, and 99 ψ-angle constraints (derived from 15N chemical shift data) corresponding to conservative ranges of allowed torsion angles, in those regions of strand or helix that were well defined (Fig. 2). All peptide bonds were assumed to be trans. A final selection of 20 structures from 400 starting structures was done by using the lowest target functions (the ensemble statistics are shown in Table I). DIANA and DYANA use no assumptions about protein energetics, other than van der Waals repulsion; structures are unrefined and only adjusted by rotation/translation for comparison purposes. Structures were aligned using XPLOR or in-house software written in MATLAB (MathWorks) and displayed and analyzed with the INSIGHTII package ( Biosym) or with MOLMOL (31).

Table I: Table I

| Selected residues | Selected atoms | r.m.s.d. |
|-------------------|----------------|----------|
| All 119 residues  | Backbone heavy atoms | 3.80 ± 0.65 |
| Residues 560–658, floppy tails clipped off | Backbone heavy atoms | 1.08 ± 0.40 |
| Residues 560–658, floppy tails clipped off | All heavy atoms | 1.38 ± 0.41 |
| All elements of secondary structure | Backbone heavy atoms | 0.38 ± 0.09 |
| All elements of secondary structure | All heavy atoms | 0.82 ± 0.16 |
| All elements of secondary structure | All atoms | 1.20 ± 0.17 |
| All β-strands | Backbone heavy atoms | 0.44 ± 0.11 |
| α-Helix | Backbone heavy atoms | 0.31 ± 0.10 |

* Strands β1 through β7 and the α-helix. There were no NOE violations of upper limits >1 Å, and eight in the ensemble of 20 structures >0.5 Å, and eight dihedral angle violations >5°.

Fig. 1. The sequence of the protein construct used in the present work and its relation to the nominal PH domain and to the Gpβ,β'-binding region of βARK1. At the top is the nominal length PH domain section; below is the domain demonstrated previously (50) to be sufficient and optimal for Gpβ, binding, below which is the construct used here, which has the same Gpβ, binding. The lowercase “gshm” residues are from the GST construct, and are not further referred to. At the bottom, the complete sequence of hβARK1 PH domain, and the similar hβARK2 are compared, with the secondary structural elements of hβARK1 superimposed in color. The more flexible region of the C-terminal α-helix is shown in light blue.
Alignment of the PH/PTB Domains—Pairwise comparison of the PH/PTB domain structures was performed by superposition of the backbone heavy atoms (N, Ca, C, and O) of the residues from regions of regular secondary structure, as indicated by boxes in Table II. The α-helical insertions in the loop regions of the PH domains were not taken into account. The alignment was done by direct calculation of r.m.s.d. values and optimized by relative shift of the protein sequences within each secondary structure element (β1–β7 strands, α-helix), as well as by adding or removing individual residues. The resulting alignment and r.m.s.d. values are presented in Tables II and III, respectively.

Protein Backbone Dynamics—The backbone dynamics were assessed via $^{15}$N spin relaxation studies comprising $T_1$, $T_2$, and heteronuclear steady state NOE measurements using previously described protocols (26). Fifteen two-dimensional spectra with the relaxation delays of 4 ($\times 2$), 200, 400 ($\times 2$), 600, 900 ($\times 2$), and 1200 ms (positive initial $^{15}$N magnetization), and 4, 200 ($\times 2$), 400, 600 ($\times 2$), and 900 ms (negative initial $^{15}$N magnetization) were acquired in the alternate-sign $\Delta$ experiment (duplicate experiments are indicated by $\times 2$) (26). Eleven two-dimensional spectra were collected for the $T_2$ measurements, with the relaxation delays of 8 ($\times 2$), 16, 32 ($\times 2$), 48, 64 ($\times 2$), 80, 96 ($\times 2$), 112, 128 ($\times 2$), and 160 ms. The heteronuclear ($^1$H)$^{15}$N steady state NOEs were assessed as a ratio of cross-peak intensities in the experiments with and without proton presaturation. The relaxation data analysis was performed using programs RELAXFIT and DYNAMICS (26), tended to include anisotropic character of the overall motion of the protein (32).

RESULTS

hβARK-PH domain corresponding to residues 556–670 of human βARK1 (Fig. 1) was produced in E. coli and isolated as a GST fusion protein, cleaved, and purified. Solubility and stability limited observation to a narrow range of conditions, and the majority of studies were conducted in 20 mM acetate buffer at pH 4.5, 35 °C. Under these conditions, binding of the construct to G$_{\beta\gamma}$ is maintained (data not shown). The 546–670 ARK-PH domain corresponding to residues 556–670 of human βARK1 (Fig. 1) was produced in E. coli and isolated as a GST fusion protein, cleaved, and purified. Solubility and stability limited observation to a narrow range of conditions, and the majority of studies were conducted in 20 mM acetate buffer at pH 4.5, 35 °C. Under these conditions, binding of the construct to G$_{\beta\gamma}$ is maintained (data not shown). The 546–670 ARK-PH domain corresponding to residues 556–670 of human βARK1 (Fig. 1) was produced in E. coli and isolated as a GST fusion protein, cleaved, and purified. Solubility and stability limited observation to a narrow range of conditions, and the majority of studies were conducted in 20 mM acetate buffer at pH 4.5, 35 °C. Under these conditions, binding of the construct to G$_{\beta\gamma}$ is maintained (data not shown). The 546–670 ARK-PH domain corresponding to residues 556–670 of human βARK1 (Fig. 1) was produced in E. coli and isolated as a GST fusion protein, cleaved, and purified. Solubility and stability limited observation to a narrow range of conditions, and the majority of studies were conducted in 20 mM acetate buffer at pH 4.5, 35 °C. Under these conditions, binding of the construct to G$_{\beta\gamma}$ is maintained (data not shown). The 546–670 ARK-PH domain corresponding to residues 556–670 of human βARK1 (Fig. 1) was produced in E. coli and isolated as a GST fusion protein, cleaved, and purified. Solubility and stability limited observation to a narrow range of conditions, and the majority of studies were conducted in 20 mM acetate buffer at pH 4.5, 35 °C. Under these conditions, binding of the construct to G$_{\beta\gamma}$ is maintained (data not shown). The 546–670 ARK-PH domain corresponding to residues 556–670 of human βARK1 (Fig. 1) was produced in E. coli and isolated as a GST fusion protein, cleaved, and purified. Solubility and stability limited observation to a narrow range of conditions, and the majority of studies were conducted in 20 mM acetate buffer at pH 4.5, 35 °C. Under these conditions, binding of the construct to G$_{\beta\gamma}$ is maintained (data not shown). The 546–670 ARK-PH domain corresponding to residues 556–670 of human βARK1 (Fig. 1) was produced in E. coli and isolated as a GST fusion protein, cleaved, and purified. Solubility and stability limited observation to a narrow range of conditions, and the majority of studies were conducted in 20 mM acetate buffer at pH 4.5, 35 °C. Under these conditions, binding of the construct to G$_{\beta\gamma}$ is maintained (data not shown).
Solution Structure of the PH Domain of GRK2 (βARK1)

Fig. 3. Three-dimensional structure of the βARK1 PH domain. A, view of the backbone (N, Ca, C') of 20 superimposed NMR-derived structures of the βARK1 PH domain. Parts of the backbone belonging to the elements of secondary structure are colored (β-strands, yellow; α-helix, blue) and labeled. The termini of the construct and some residues in the loops β2/β3 and β3/β4 are disordered (see also D and E). B, a ribbon representation of the tertiary solution structure of the βARK1 PH domain, the same orientation as in A. C, the structure of PLCδ H domain from x-ray diffraction (8). D and E, ribbon representation of the backbone of the βARK1 PH domain. The ribbon width in D represents local backbone r.m.s.d. values in the ensemble of 20 calculated structures and, in E, the amplitude of nanosecond-subnanosecond backbone motion (as inferred from 15N relaxation data). The 15N relaxation data indicate a dynamic character of structural disorder in the N and C termini (S2 < 0.5 for the backbone NH groups in residues 552–557 and 659–670) and in the loops β2/β3 and β3/β4. The backbone mobility in the elements of secondary structure is restricted (S2 > 0.85, local correlation time in a subnanosecond time range). The ratio of the principal components of the rotational diffusion tensor of the molecule is D/z = 1.50; the z axis of the diffusion tensor is tilted by ≈20° angle from the C-terminal helix axis. The termini of the construct are disordered and highly flexible, with large amplitudes of backbone motion on a nanosecond time scale (Fig. 3). The β2/β3 and β3/β4 loops are disordered and display increased amplitudes of backbone dynamics on a subnanosecond to nanosecond time scale, as well as motions on a millisecond to microsecond time range (data not shown).

Of specific note, the C-terminal α-helix is clearly extended by more than one turn compared with C-terminal α-helices of most previously determined PH domain structures. The position/orientation of the helix appears to be fixed by interactions with the protein core, namely by a hydrophobic strip formed by Leu-640, Trp-643, Leu-647, Ala-650, Tyr-651, and Ala-654, which are located on the side of the helix facing the β-sandwich and are involved in contacts with several residues in the first two β-strands. The aromatic ring of Trp-643, the only conserved residue among PH domains, is buried in the protein core and exhibits numerous NOE contacts to residues in β1 and β2. Another aromatic residue in the helix, Tyr-651, is also oriented toward the interior of the β-sandwich. The NOESY data indicate several close contacts between the aromatic ring of Tyr-651 and the residues in the β4/β5 loop and in the strand β5. Both the structure of this loop and the orientation of the Tyr-651 ring are well defined, as indicated by low r.m.s.d. values in these parts of the structure, and by chemical shift non-equivalence of all four ring hydrogens of Tyr-651.

DISCUSSION

Relation to Other PH/PTB Domain Structures—The hβARK1 PH domain has very low sequence similarity to other PH domains of known three-dimensional structure, and therefore cannot be satisfactorily homology-modeled from known structures. The structure-based alignment of the hβARK1 PH domain with these PH domains (Tables II and III) demonstrates the same overall topology of the protein fold. The expected range of r.m.s.d. values between sequences of the same structural class, but with varying degrees of homology, has been derived previously (33). The r.m.s.d. values between different members of the PH/PTB domain family (Fig. 7) are within the range expected for such homologous sequences of low identity. The charge distribution is, however, different among the PH domains, and the large positive charge associated with the C-terminal helix of βARK is unusual.

Validity of the Derived Structure of the βARK PH Domain—The low pH (4.5) required for this study is close to the pKa values for both glutamate and aspartate, so variations in the side chain charges of these residues compared with physiological conditions are expected. Since this might result in a perturbed structure in those regions containing negatively charged residues, a question arises of whether the NMR structure derived under these conditions represents the protein...
structure under physiological conditions. As mentioned above, the circular dichroism data indicate no changes in the protein structure as compared with the more physiological conditions in a phosphate-buffered saline pH 7.2. To address this issue in greater detail, the $^1$H–$^{15}$N correlation maps (HSQC) were also recorded for the PH domain dissolved in the phosphate-buffered saline (pH 6.0, temperature 25 °C) or in 0.1 M Tris buffer (pH 7.9, 35 °C). The minor chemical shift changes (up to 0.06 ppm in $^1$H and 0.6 ppm in $^{15}$N) are consistent with expectations of variations in pH, temperature, and buffer content. The absence of significant chemical shift perturbations in this fingerprint region suggests no significant changes in the protein structure. The βARK PH domain tertiary structure here is also generally similar to other PH domain structures measured in the range pH 6.0–9.0 (8, 11, 34–39). The high flexibility of the C terminus in the extended βARK PH domain construct reported here is also preserved at the more physiological conditions, as indicated by negative steady-state heteronuclear NOEs observed for the C-terminal residues (663–670) in phosphate-buffered saline (pH 6.0, 25 °C). The binding to G$_{bg}$ subunits is also retained at pH 4.5, with c. 100 nm affinity of the GST fusion protein at pH 4.5 and 7.5, from an immunoblotted Western assay (19).

Structure of the C-terminal Extension: Molten Helix—The C-terminal segment shows an unusual structural feature that, to our knowledge, has not been reported previously in proteins. NOEs characteristic for an α-helix are preserved for residues toward the C terminus despite the gradual loss of other NMR characteristics of helical structure (deviations from standard chemical shifts, heteronuclear $^{15}$N–$^1$H NOEs, $^3$J$_{HNH}$ coupling) (Fig. 2). Increased mobility, indicated by both relaxation and solvent exchange/accessibility data, suggesting that the C-terminal part of the α-helix is present as a "molten helix" in solution. Molecular dynamics calculations (40) of α-helical melting appear to be qualitatively consistent with our NMR observations.

The hydrophobic residues C-terminal to Gln-656 (Leu-657, Val-658, and Val-661) are located at proper sequence positions to extend the already existing hydrophobic strip on the helix surface. However, being extended beyond possible interaction with the protein core’s β-sandwich and therefore exposed to solvent, these residues lack proper hydrophobic contacts to other residues that might stabilize the helix formation. It is possible, however, that the C-terminal extension of the helix becomes structured under certain conditions, e.g. in the presence of a binding partner such as G$_{bg}$ subunits. It is possible also, that the apparent "molten helix" is a consequence of the expression of the C-terminal region of βARK in the absence of its native protein context, and therefore might be stabilized through tertiary contacts with residues N-terminal to the PH domain. It is unlikely, however, that the thermodynamic stability of the protein is a result of special conditions of this study (low salt, pH4.5), since both the CD and NMR data (see above) indicate no significant perturbations in the βARK PH domain structure upon exchange into phosphate buffer.

In the human SOS PH domain, residues N-terminal to the normal PH domain (in the Dbl homology domain) make well defined structural contacts with the PH domain, (41) and in the Btk PH domain, the "Btk motif" C-terminal to its nominal PH domain packs against the C-terminal β-sheet (39).

Binding of Phosphatidylinositides and Inositol Phosphates—A general hypothesis that has been advanced in the literature is that the PH domain recognizes specifically and with high affinity highly anionic phospholipids, especially phosphatidylinositol (4, 5) bisphosphate (42). This has been illustrated for the PLCδ PH domain, in which the binding of PI(4,5)P$_2$ and Ins(1,4,5)P$_3$ is submicromolar, and a well defined structural interaction with the PH domain has been characterized (8). However, other PH domains appear to have significantly weaker affinity (11, 12, 42). There is also sensitivity to the isomer identity of the inositide, since the PH domain of Akt (13, 14) binds to phosphatidylinositol 3,4,5-bisphosphate, and a newly identified GRP1 binds to phosphatidylinositol 3,4,5-trisphosphate (43). It is evident that the basic residues in the N-terminal section involved in the PLCδ PH domain/ligand complex (8) are not generally present in the structure-based sequential alignment of Table II. The physiological relevance of phosphatidylinositol phosphates and phosphoinositides bind-
TABLE II
Structure-based alignment of the PH/PTB domains

Domains are as follows (top to bottom): GRK-2 (this work); human dynamin PH domain (35); PH domain of human SOS (41); PH domain of mouse β-spectrin (36); PH domain of Drosophila β-spectrin (37); N-terminal PH domain from pleckstrin (38); PLC-δ PH domain (8); PTB domain of Shc (16); PTB domain of IRS (17). The alignment was done by pair-wise superposition of the structures and direct calculation of aligned RMSD (Table III), based on the elements of regular secondary structure, as described in the text. Residues belonging to the elements of secondary structure used for the alignment are enclosed in boxes, labeled on the top. Numbers indicate residue positions within the corresponding secondary structure element. Conserved hydrophobic residues are colored green. The only conserved residue in PH domains, Trp α1–7, is colored blue and underlined.

In the Shc PTB domain, residues 58–114, belonging to an insertion in the β1/β2 loop are omitted.

TABLE III
Pairwise root-mean-square deviations (in Å) between the PH/PTB domain structures

The elements of regular secondary structure of the proteins used for the comparison and their alignment are indicated in Table II. Numbers shown above the diagonal were obtained with only Cα atoms selected for the alignment and r.m.s.d. evaluation, whereas numbers below the diagonal correspond to all heavy backbone atoms (N, Cα, Cζ, and O) in the selected core elements taken into account. The percent of sequence identity between core elements of the compared proteins is indicated in the parentheses. PH/PTB domain notation is the same as in Table 2. Protein atom coordinates were obtained from the Protein Data Bank, PDB entries 1DYN (dynamin), 1AWE (hSOS), 1PLS (pleckstrin), 1BTN (β-spectrin, mouse), 1DRO (β-spectrin, Drosophila), 1MAI (PLC-δ), 1SHC (Shc PTB), and 1IRS (IRS PTB).

*Dyn (A) and (B) refer to the crystal structures of the two monomers in the dynamin PH domain dimer observed in the crystallographic studies (35).
being to PH domains remains unresolved for the PH domains of β-spectrin, N-pleckstrin, dynamin, and βARK.

**Possible PI(4,5)P₂ Binding Site on βARK-1 PH Domain and Its Significance**—Using Ins(1,4,5)P₃ as a model compound for PI(4,5)P₃, the ¹⁵N and ¹H spectral perturbations upon titration were mapped for hβARK-1 PH domain using a previously published procedure (12). Under the experimental conditions, the βARK PH domain binds Ins(1,4,5)P₃ with Kᵣ of 207 μM, according to our protein fluorescence titration measurements using the protocol described in Ref. 12. The data (not shown) indicated maximal shift perturbations at residues Gly-569, Trp-576, Arg-578, Tyr-580, and Ala-596, located in the N-terminal segment of the domain, a pattern seen similar to, but different in detail from, other PH domains. The amide ¹H chemical shift of Asp-635 is also perturbed (0.02 ppm), that is probably caused by variation in the distance between this site and the closely located (in the unbound state) positively charged side chain of Arg-578.

The association of βARK with membranes is complex. It has been suggested (44) that the high affinity binding of βARK to microsomal membranes depends on a segment of the N terminus of βARK, distinct from the putative PH domain. However, other investigators have shown that the βARK PH domain binds with moderate specificity to PI(4,5)P₃ and suggest that synergistic interactions of binding to both PI(4,5)P₃ and to Gₓₐ proteins via the PH domain are required for activation of βARK (45). This synergism was not observed in a model system of higher turnover, where PI(4,5)P₃ was inhibitory (46).

Residues perturbed by the Ins(1,4,5)P₃ binding are located mostly in the β1/β2 loop and in the N-terminal part of the β2-strand. This rather flexible loop is relatively distant from both the putative Gₓₐ binding site and the area of hydrophobic contacts between the α-helix and the protein core. The present data provide no direct structural evidence for a possible relationship between the phospholipid and Gₓₐ binding regions.

**Protein Interaction**—The clustering of positive residues in the extended C-terminal helix creates a positively charged site on the βARK PH domain surface (Fig. 4, a–e), in addition to a cluster of positive charges at the opening of the β-barrel, a site implicated for phospholipid binding (11) in other PH domains (Fig. 4f). This causes a different polarity of the surface charge in the case of βARK PH domain, so that in the fully extended construct, the dipole moment of the molecule is aligned at ~30° angle relative to the helical axis (Fig. 5). Truncation of a few C-terminal residues causes a 2.5-fold reduction in the protein dipole moment and alters the orientation of the dipole vector to close to perpendicular to the helix axis. Further truncation causes only minor variations of the dipole vector. This reduction could explain the observed differences of Gₓₐ, binding to truncated βARK1 PH domains (19).

A depression on the βARK PH domain surface between the α-helix and the β-strand, flanked by positively charged side chains of Lys-644, Lys-645, Arg-648, and Arg-652 (hexil); Lys-623 (β6); and Arg-625 (β6/β7) (Fig. 4) resembles the site in the PTB domains involved in the phosphopeptide binding of that protein. This topological similarity suggests that these residues might be involved in electrostatic interaction with a negatively charged cluster on the Gₓₐ surface formed by Glu-226, Asp-228, Asp-246, and Asp-247 of the WD5 and Asp-267, Asp-290, and Asp-291 of the WD6 subunits (Fig. 6). With the exception of the Asp-247 and Asp-291, which are highly conserved among the WD40 subunits and are involved in the formation of an interand intra-blade hydrogen bonds (47, 48), other negatively charged residues in this cluster are unique for WD5 and WD6, and highly conserved in eukaryotes. Asp-228 and Asp-246 are directly involved in ionic interactions with the switch-II region (α-Lys-210, Fig. 6) of Gₓₐ, which plays a critical role in G protein heterotrimer formation (47). It is possible that in the absence of Gₓₐ, the PH domain of βARK interacts (via its positively charged C terminus) with the same residues on the top of Gₓₐ surface and therefore either directly or indirectly interferes with Gₓₐ binding to Gₓₐ.

The βARK2 (GRK-3) PH domain has a different receptor and Gₓₐ selectivity, but possesses a similar pattern of basic residues (Lys-644, Lys-645, Lys instead of Arg at 625 and 652, and Arg instead of Lys at 623) (Fig. 1). The difference of sequence in the C-terminal segment of the βARK2 PH domain as compared with βARK1 (Arg/Asn-648, Gln/Arg-656, and Gln/Arg-659) results in an increased total positive charge and thus a larger polarization of the PH domain. Consistent with the above electrostatic model of the βARK - Gₓₐ interaction, a recent Gₓₐ binding assay (49) involving the C-terminal-peptide sequences (corresponding to the segments 643-670 and 648-665) indicates a higher potency of the PH domain of βARK2 compared with that of βARK1.

The structure of the βARK PH domain allows rationalization of the results of βARK truncation studies (50). The modifica-
tions in βARK that affect the Gβγ binding in that assay can be explained either by deletion of one or more core elements, hence causing a disruption of the overall fold of the PH domain, or by deletion of positively charged residues on the C terminus (see below).

Several fusion proteins, containing sequences encompassing a PH domain (from Ras-GRF, Ras-GAP, OSBP, β-spectrin, IRS-1, and others), were shown to bind Gβγ in vitro with varying affinities, and some of these PH domains were able to compete with the βARK PH domain and Gα for binding to Gβγ (18). The C-terminal extended PH domains of Btk (51), IRS-1, and Dbl (19) were also demonstrated to interact with the Gβγ heterodimer in vitro. The results of indirect assays (51, 52) suggest that some of these PH domain constructs may also interact with Gβγ in vivo. The C-terminal parts of all these proteins, in particular region corresponding to residues 644–670 in βARK (Figs. 1 and 4), are rich in basic residues, and this supports the proposed βARK-Gβγ interaction model. Other literature data are also consistent with this model. Mutation of the last four of the five basic residues in the highly charged C terminus (Arg-660, Lys-663, Lys-665, Lys-667, and Arg-669) to acidic ones leads to almost complete loss of the Gβγ binding (20), as does a deletion of the last nine residues (662–670) (50). A truncated fragment, ending with Asn-666, does retain an

FIG. 6. Electrostatic polarization of the Gβγ heterodimer. A, molecular surface of the Gαβγ complex colored by the electrostatic potential, from red (−10 kT/e) to blue (+10 kT/e). A fragment of Gα (green) contains the switch II region (α1 helix), which is intimately involved in a direct contact with the top of the Gαβ propeller in the heterotrimer (47, 48). Those positively charged residues of Gα (Lys-209, Lys-210) in the Gαβγ complex less than −3 Å between side chain heavy atoms from Asp-228 and Asp-246 of Gα are labeled. The highly negatively charged area around this position may serve as a complementary charged surface to the βARK1 PH domain. B, a diagrammatic representation of the location of negative charges on the Gα surface. The surface corresponding to the highly negatively charged area in A is indicated by a broken line. The general sequence consensus of a WD motif and location of negatively charged residues in the sequences of WD5 and WD6 subunits are also shown (B, top). The residues in the WD repeat sequence are marked: x, a non-conserved position; h, a conserved hydrophobic position; r, a conserved aromatic; p, a conserved polar position; t, a tight turn containing Gly, Pro, Asp, or Asn. The superscripts indicate the range of residues observed in the various known Gα subunits. Those acidic residues that are unique for the WD5 and WD6 subunits are shaded in gray. A was generated using GRASP (56); atom coordinates were extracted from PDB entry 1GG2; the schematic representation of Gβγ and the general sequence consensus of WD motifs were adopted from Ref. 59.

FIG. 7. Backbone r.m.s.d. values between the PH/PTB domains versus percent of sequence identity in the superimposed secondary structure elements. Data are taken from Table III. The solid line represents the relation: r.m.s.d. = 0.4 e^{0.37 H} − 0.1, where H is a fraction of identical residues (33), obtained for homologous proteins. Data corresponding to βARK1 PH domain are indicated by solid symbols. The leftmost data point (open square) corresponds to r.m.s.d. between DynA and DynB (see Table III).
intermediate level of $G_{bg}$ activation, whereas further truncation up to Val-661 leads to a significantly lower amounts of $G_{bg}$ binding compared with the full-length construct (50). On the other hand, the results of deletion analysis (19) indicate that the C-terminal extension beyond Glu-656 is not absolutely required for binding to $G_{bg}$: the full-length C-terminal extension (553–689 construct) dramatically increases the maximal extent of $G_{bg}$ binding although does not significantly alter the binding affinity. A 18-residue peptide comprising the C-terminal amino acids 648–665 was recently shown to bind $G_{bg}$, also suggesting that the critical C-terminal extension in $\beta$ARK1 PH domain required for $G_{bg}$ binding might be shorter than initially suggested by (50). However, other functions may be associated with the extended domain.

REFERENCES

1. Premont, R. T., Inglese, J., and Lefkowitz, R. J. (1995) FASEB J. 9, 175–182
2. Mayer, B. J., Ren, R., Clark, K. L., and Baltimore, D. (1993) Cell 73, 629–630
3. Haslam, R. J., Koide, H. B., and Hemmings, B. A. (1993) Nature 363, 309–310
4. Prowse, T. (1995) Nature 373, 573–580
5. Cohen, G. B., Ren, R., and Baltimore, D. (1995) EMBO J. 14, 237–248
6. Shaw, G. (1996) BioEssays 18, 35–46
7. Lemmon, M. A., Ferguson, K. M., O’Brien, R., Sigler, P. B., and Schlessinger, J. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 10472–10476
8. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) Cell 83, 1037–1046
9. Cowburn, D., and Kuriyan, J. (1996) in Signal Transduction (Heldin, C.-H., and Purton, M., eds), pp. 127–142, Chapman & Hall, London
10. Harlan, J. E., Yoon, H. S., Hajduk, P. J., and Fesik, S. W. (1995) Biochemistry 34, 9859–9864
11. Hyvonen, M., Macias, M. J., Nilges, M., Oschkinat, H., Saraste, M., and Ochs, K. H. (1994) Nature 369, 675–677
12. Zheng, J., Cahill, S. M., Lemmon, M. A., Fushman, D., Schlessinger, J., and Cowburn, D. (1995) J. Mol. Biol. 253, 573–580
13. Haslam, R. J., Koide, H. B., and Hemmings, B. A. (1993) J. Biol. Chem. 268, 245–253
14. Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell. Biol. 17, 538–544
15. Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Panayotou, G. (1996) EMBO J. 15, 6241–6250
16. Zhou, M. M., and Fesik, S. W. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 695–700
17. Eck, M. J., Dhe-Paganon, S., Trub, T., Nolte, R. T., and Shoelson, S. E. (1996) Cell 85, 695–705
18. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 10217–10220
19. Mahadevan, D., Thanik, N., Singh, J., McPhie, P., Zangrilli, D., Wang, L. M., Guerrero, C., LeVine, H., III, Humphre, C., Saldanha, J., Gutkind, J. S., and Najmabadi-Hakse, T. (1995) Biochemistry 34, 9111–9117
20. Touhara, K., Koch, W. J., Hawes, B. E., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 17069–17075
21. Live, D. H., Davis, D. G., Agosta, W. C., and Cowburn, D. (1984) J. Am. Chem. Soc. 106, 1939–1941
22. Palmer, A. G., Cavanaugh, J., Wright, P. E., and Rance, M. (1991) J. Magn. Reson. 93, 151–170
23. Sklenar, V., Firotto, M., Leppik, R., and Sandek, V. (1993) J. Magn. Reson. 99, 241–245
24. Morelli, M. A., Stier, G., Gibson, T., Joseph, C., Musco, G., Pastore, A., and Trave, G. (1995) FEBS Lett. 358, 193–198
25. Kay, L. E., and Bax, A. (1990) J. Magn. Reson. 86, 110–126
26. Fushman, D., Cahill, S., and Cowburn, D. (1997) J. Mol. Biol. 266, 173–194
27. Daum, C., and Hommel, U. (1995) J. Biol. Chem. 270, 309–310
28. Fushman, D., Cahill, S., Lemmon, M. A., Schlessinger, J., and Cowburn, D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 14304–14308
29. Guentert, P., and Wuthrich, K. (1991) J. Biol. Chem. 266, 447–456
30. Guentert, P., Mumenthaler, C., and Wuthrich, K. (1997) J. Mol. Biol. 273, 816–820
31. Fushman, D., Cahill, S., Lemmon, M. A., Schlessinger, J., and Cowburn, D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 30340–30344
32. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) Nature 371, 168–170
33. Harlan, J. E., Yoon, H. S., Hajduk, P. J., and Fesik, S. W. (1995) Structure 3, 1185–1195
34. Zheng, J., Cahill, S. M., Lemmon, M. A., Fushman, D., Schlessinger, J., and Cowburn, D. (1996) J. Mol. Biol. 255, 1–21
35. Chothia, C., and Lesk, A. M. (1986) EMBO J. 5, 823–828
36. Shaw, G. (1996) J. Mol. Graph. 14, 51–55
37. Johnna, N., Szabo, A., and Bax, A. (1996) J. Am. Chem. Soc. 118, 6896–6891
38. Boxton, J., and De Blasi, A. (1997) EMBO J. 16, 3396–3404
39. Somam, K. V., Karimi, A., and Case, D. A. (1991) Biopolymers 31, 1351–1361
40. Zheng, J., Chen, R.-H., Corbalan-Garcia, S., Cahill, S. M., Bar-Sagi, D., and Cowburn, D. (1997) J. Biol. Chem. 272, 30340–30344
41. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) Nature 371, 168–170
42. Tsukada, S., Simon, M. I., Witte, O. N., and Katz, A. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1256–1260
43. Luteri, L. M., Hawes, B. E., Touhara, K., van Biesen, T., Koch, W. J., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 12984–12989
44. Winhart, D., Sykes, B. D., and Richards, F. M. (1992) Biochemistry 31, 12647–12651
45. Winhart, D. S., and Sykes, B. D. (1994) Methods Enzymol. 239, 363–392
46. Evans, V. S. (1993) J. Mol. Graphics 11, 134–138
47. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Biopolymers 31, 281–286
48. Barlow, D. J., and Thornton, J. M. (1986) Biopolymers 25, 1717–1723
49. Antosiewicz, J. (1995) Biophys. J. 69, 1344–1354
50. Nee, E. J., and Smith, T. F. (1996) Cell 84, 175–178

Solution Structure of the PH Domain of GRK2 (βARK1)

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The Solution Structure and Dynamics of the Pleckstrin Homology Domain of G Protein-coupled Receptor Kinase 2 (β-Adrenergic Receptor Kinase 1): A BINDING PARTNER OF G βγ SUBUNITS
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