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BINDING OF PH DOMAINS OF ß-ADRENERGIC RECEPTOR KINASE AND ß-SPECTRIN TO WD40/ß-TRANSDUCIN REPEAT CONTAINING REGIONS OF THE ß-SUBUNIT OF TRIMERIC G-PROTEINS

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SUMMARY: Pleckstrin homology (PH) domains are found in numerous proteins important in signal transduction and cytoskeletal function. Several PH domains are now known to contain a binding site for the ßγ subunits of trimeric G-proteins (Gßγ), a finding which naturally raises the question of where on the Gßγ complex these PH domains bind. Here we demonstrate binding of the PH domains of ß-adrenergic receptor kinase and ß-spectrin to the Gß subunit and not the Gγ subunit in a nitrocellulose gel replica assay. Furthermore, the C-terminal tryptic fragment of Gß containing only 5 WD40/ß-transducin (WD40) repeats also binds thesetwo PH domains. Finally, constructs containing only WD40 repeats of Gß were shown to bind to ß-ARK and ß-spectrin PH domains in solution. These findings suggest that WD40 repeats of Gß are ligands for PH domains and have interesting implications for other proteins containing WD40 sequences.

The pleckstrin homology (PH) domain is a roughly 100 amino acid segment found in a variety of molecules, many of which are important in signal transduction and cytoskeletal function (1-4). Many of the proteins containing PH domains also contain *src* homology 2 and 3 domains (SH2 and SH3), and it is widely supposed that PH domains, like SH2 and SH3 domains, have an important role in cellular function. Based on the finding that the PH domain of the ß-adrenergic receptor kinase (ß-ARK) coincides with a previously described binding site for the ßγ subunits of trimeric G proteins (Gßγ), it has been proposed that one of the functions of PH domains is to bind Gßγ (3,4). This proposal now has experimental verification for the PH domains of several different proteins (5). An obvious question raised by these results is exactly where on Gßγ PH domains bind. The answer to this question has some important implications: since the ligands for SH2 and SH3 domains are found in a variety of proteins, it is possible that PH domain binding sites similar

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to those of Gβγ may be found in other molecules also, giving all three domain types a comparable versatility. As a first step towards answering this question we provide evidence that the PH domains of two different molecules bind to the C-terminal region of Gβ in a region containing the so-called WD40 or β-transducin repeats. These data suggest a function for the WD40 repeats of Gβ and raise the question of whether WD40 sequences in molecules other than Gβ also bind PH domains.

MATERIALS AND METHODS

Bacterial constructs: The β-ARK C-terminal fusion protein was derived from the rat β-ARK$_1$ cDNA (GenBank accession number M87854) and encodes the C-terminal 224 amino acids (amino acids 465-689) fused to glutathione-S-transferase (GST). It was constructed by polymerase chain reaction (PCR) based mutagenesis and incorporated into the prokaryote expression vector pGEX-2T (Pharmacia). This construct contains the β-ARK PH domain and a total of about 120 amino acids of flanking sequence and was a kind gift of Drs. R. Lefkowitz and J. Inglese. Polymerase chain reaction of this construct was performed to amplify and incorporate 5' *BamHI* and 3' *EcoRI* linkers into a nucleic acid segment encoding 123 amino acids including the entire β-ARK PH domain and a few flanking amino acids (547-669, here referred to as β-ARK PH domain). This PCR product was ligated into the pGEM-T vector (Promega), subcloned and inserted into *BamHI* and *EcoRI* cleaved pGEX-3X (Pharmacia).

The β-spectrin C-terminal fusion protein was derived from the *SmaI* restriction fragment of a cDNA encoding the non-erythroid isoform of human β-spectrin described by Winkelmann et al. (cDNA 8B, GenBank accession number M37884, ref. 6). This construct includes the entire PH domain and about 110 amino acids of flanking sequence. The cDNA was inserted into the *SmaI* site of pGEX-2T (amino acids 1061-1274, referred to as β-spectrin C-terminus). In the same way as for β-ARK the β-spectrin PH domain was PCR amplified and mutagenized to produce a 109 amino acid segment (1125-1233, here referred to as β-spectrin PH domain). PCR products were ligated into the pGEM-T vector, cloned and then inserted into *BamHI* and *EcoRI* digested pGEX-3X (Pharmacia). The correctness of the two new PH domain constructs was assured by full nucleic acid sequencing and also by mass spectroscopy of the PH domain protein product following proteolytic removal of the GST. In the case of the β-ARK PH domain we have also performed circular dichroism studies and obtained, as expected, a primarily β structure with a small amount of α-helix (4).

PCR based mutagenesis was then used to amplify out the WD40 repeats of bovine Gβ$_1$, human Gβ$_2$ and human Gβ$_3$ and to include 5' *EcoRI* and 3' *Sal I* sites. Appropriate cDNAs were obtained from Dr. Henry Fong and correspond to GenBank accession numbers A25457, B26617 and A35096 respectively. The primers were designed to exclude the α-helical coiled-coil region encompassing the N-terminal 28 amino acids but to contain the entire C-terminus and all 7 WD40 repeats. Accordingly Gβ$_1$WD40 corresponds to amino acids 35-340, Gβ$_2$WD40 corresponds to 31-340 and Gβ$_3$WD40 is 36-340. The PCR products were ligated into pGEM-T and then excised and transferred to appropriately treated pGEX-4T.1 (Pharmacia) as described above. Proteins were expressed in *E. coli* using standard procedures and soluble fusion proteins were bound to glutathione-agarose affinity columns (Sigma). These columns were treated with thrombin to cleave off the GST component and WD40 constructs were then eluted from the columns. Thrombin was inactivated by addition of 1mM phenylmethylsulfonylfluoride.

Gβγ preparation: Gβγ protein was purified from 1.2 kg of frozen cow brains (Pel-Freeze) as described (7). Briefly DEAE-cellulose purified cholic acid (Sigma) was used to solubilize brain membrane proteins which were then resolved on a 1L DEAE-Sephacel column. G-protein content was assayed by GTP-γS^{35} binding, and positive fractions were pooled and concentrated prior to further resolution on a 1L Ultrogel AcA 34 column followed by again assaying for G-proteins. The Gα subunits were dissociated from Gβγ by treatment with AMF (buffers containing Al^{3+}, Mg^{2+} and F^-) and finally separated by hydrophobic interaction chromatography on Phenyl-Sepharose (Sigma) using a cholate gradient. The major protein band running at about 38kDa stains strongly with a polyclonal Gβ antibody raised against a peptide sequence which is highly conserved in all Gβ isotypes (Upstate Biotechnology Inc.), as shown in fig. 1. The Gγ subunits are also clearly visible, running, as expected, at about 9kDa. Trypsin cleavage of the Gβγ preparation was performed

to produce 14kDa and 27kDa Gß fragments as described (8). Identification of these two major Gß protein fragments was performed with the same Gß antibody since the trypsin cleavage site is included in the peptide immunogen used to make this antibody. Finally the amino acid compositions of intact Gß and the two fragments were determined and found to closely match the expected profiles using methods described previously (9).

Nitrocellulose gel replica assay: The Gßγ preparation was run out for immunoblotting and reacted with GST constructs or GST control. The various constructs and GST control were made up to a concentration of 150-200µg/ml using the Bradford protein assay and used at a range of dilutions up to 1:1000 from these solutions. Following extensive washing the blots were reacted with anti-GST monoclonal antibody diluted 1:1000 (Santa Cruz Biotechnology Inc.), then further washed prior to incubation with goat anti-mouse IgG/alkaline phosphatase conjugate diluted 1:1000 (Sigma). After a final extensive wash alkaline phosphatase reaction product was generated with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma).

Binding experiments: These experiments were performed as described (10, 11). Briefly, ß-ARK PH domain GST, ß-spectrin PH domain GST or GST control were bound to small glutathione-agarose columns and washed with phosphate buffer containing 0.01% polyoxyethylene 10 lauryl ether (lubrol, Sigma). a total of 2.5µg/ml of a mixture of the three WD40 constructs or native Gßγ were then applied to the column. Following extensive washing in buffer plus lubrol protein was eluted with SDS-PAGE sample buffer and the eluate processed for immunoblotting using the same Gß antibody described above as a probe for Gß and/or WD40 construct binding.

RESULTS

As shown in fig. 1 lane 1, the Gßγ preparation contains a major band with an apparent molecular weight of 38kDa and lower band running at about 9kDa, corresponding to the expected positions of Gß and Gγ respectively. Blotting with Gß polyclonal antibody confirmed that the 38kDa band was Gß (fig. 1 lane 2). Blots of this preparation with the ß-ARK PH domain construct revealed strong labelling of Gß but no labelling of Gγ (fig. 1 lane 3), and the same result was obtained with the ß-spectrin PH domain construct (fig. 1 lane 4). In contrast incubation with GST alone showed only faint background staining (fig. 1 lane 5). Other experiments showed that the two larger GST constructs containing the entire C-terminal regions of ß-ARK and ß-spectrin described above also stained Gß only, as did a construct containing only the C-terminal region of the ß-ARK PH domain (not shown). These results are consistent with previous studies (5, 10, 11) but extend these by showing that a major component of the PH domain binding site is found solely in Gß.

Encouraged by these results we treated the Gßγ preparation with trypsin to further map the PH domain binding region. As shown in figure 2, left panel lane 2, and consistent with previous work (8) trypsin produced two fragments of apparent molecular weight 27kDa and 14kDa. The identity of the two major fragments was verified by analysis of amino acid composition and using a polyclonal peptide antibody raised against a Gß peptide sequence which includes the trypsin cleavage site, and which would therefore be expected to stain both large tryptic fragments. The 14kDa band is therefore firmly identified as the N-terminal fragment and the 27kDa band as the C-terminal fragment. The 27kDa C-terminal fragment bound both ß-ARK and ß-spectrin PH domain constructs strongly, although some weaker staining of the 14kDa N-terminal fragment was also seen, particularly in the case of the ß-spectrin (fig. 2, upper left panel, ß-ARK is lanes 1-4, ß-spectrin is lanes 5-7 and GST control is lanes 8-10). As would be expected from previous

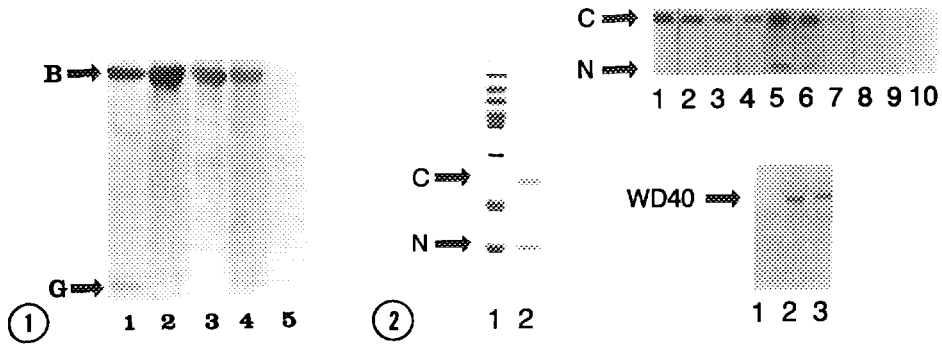

Figure 1. Lanes 1-5: Blotting of Gßγ preparations with GST-PH domain constructs. Lane 1 is Coomassie Brilliant Blue (CBB) stained protein. B shows the position of Gß (38kDa) and G shows the position of Gγ (9kDa). Lane 2 is an immunoblot stained with Gß antibody. Lanes 3 and 4 are similar immunoblots reacted with ß-ARK PH domain GST fusion protein and ß-spectrin PH domain GST fusion protein, and lane 5 was reacted with GST control. All proteins were used at 150-200µg/ml.

Figure 2. Left panel: CBB stained gel showing tryptic cleavage of Gßγ preparation. Lane 1 is molecular weight markers (Promega) corresponding to, from top to bottom, 97.4, 66.2, 55, 42.7, 40, 31, 21.5 and 14.4kDa. Lane 2 shows the two tryptic fragments of Gß of apparent molecular weight reported to be 27 and 14kDa (8). Right Top panel: Blotting of tryptic fragments of brain Gß with GST-PH domain constructs. C indicates position of the C-terminal 27kDa fragment and N indicates position of N-terminal 14kDa fragment. Lanes 1-4 were reacted with the ß-ARK PH domain construct at concentrations of 5µg/ml, 1.25µg/ml, 300ng/ml and 150ng/ml,respectively. Lanes 5-7 were reacted with the ß-spectrin PH domain construct at concentrations of 50µg/ml, 5µg/ml and 500ng/ml,respectively. Lanes 8-10 were reacted with 25µg/ml, 2.5µg/ml and 1.25µg/ml GST control,respectively. Bottom right panel: Binding of a mixture of $Gß_1$WD40, $Gß_2$WD40 and $Gß_3$WD40 to immobilized GST (lane 1), GST-ß-spectrin PH domain (lane 2) and GST-ß-ARK PH domain (lane 3). Position of constructs is indicated by WD40.

experiments the binding of the ß-spectrin PH domain to Gß appeared to be significantly weaker than that of the ß-ARK PH domain (5). The robustness of staining in these experiments was such that the ß-ARK PH domain constructs diluted to as little as 150ng/ml still showed strong staining for the C-terminal band, although staining for the N-terminal fragment became invisible at low dilutions, suggesting a marked preferential binding for the C-terminal fragment. As before essentially no staining was seen with the GST control. Also as before constructs containing the entire C-termini of ß-ARK and ß-spectrin and only the C-terminus of the ß-ARK PH domain all stained in the same way as shown for the ß-ARK and ß-spectrin PH domain constructs (not shown).

A final series of experiments were performed in order to address the possibility that the binding described above reflected non-physiological interactions found only in SDS denatured proteins. Accordingly constructs containing only the 7 WD40 repeats of $Gß_1$, $Gß_2$ and $Gß_3$ were produced as soluble GST fusion proteins in *E. coli.* and were treated with thrombin to remove the GST component. A mixture of these three WD40 constructs bound to ß-ARK and ß-spectrin PH domain GST fusion proteins immobilized on

glutathione-agarose but not to GST under similar conditions. (fig. 2, lower right panel lane 1 = GST, Lane 2 = ß-spectrin, lane 3 = ß-ARK).

DISCUSSION

The results presented here show that at least part of the PH domain binding interaction resides in the Gß molecule and does not require the Gγ subunit. Mammalian Gß molecules are 340 amino acids long and are built up of a short N-terminal segment containing an α-helical coiled-coil sequence which interacts with a complementary α-helical coiled-coil region of Gγ. The remainder of Gß is built up from 7 of the so-called WD40 or ß-transducin repeated sequences (12, 13). These repeats are about 40 amino acids long, usually contain the peptide Trp-Asp (hence WD) and are of unknown function. Trypsin cleavage of Gß produces an N-terminal 17kDa fragment containing the α-helical coiled-coil region and the first 2 WD40 repeats and a C-terminal 27kDa fragment which contains the remaining 5 WD40 repeats (8). Our data show strong binding of both ß-ARK and ß-spectrin PH domains to the Gß C-terminal region containing these 5 WD40 repeats. In addition both constructs also showed some less robust binding to the N-terminal fragment. Since the only known motifs in the C-terminal 27kDa fragment are these 5 WD40 repeats, and the only motifs shared by the two fragments are also WD40 repeats, the data presented here are in line with the suggestion that the PH domains tested bind to Gß WD40 repeats. This conclusion is also consistent with our demonstration that the PH domains of ß-ARK and ß-spectrin are able to specifically bind soluble Gß constructs containing only WD40 motifs. The data also suggest a degree of specificity conferred by the number or exact type of repeats found. It is also possible that some other currently unrecognized non-WD40 sequences, such as linkers between WD40 repeats, bind the PH domains although this seems much less likely. We therefore currently hypothesize that one of the functions of Gß WD40 repeats is to bind PH domains. Further studies, now in progress, will examine this question in more detail hopefully to fully understand the characteristics of Gß required for PH domain binding, as well as to examine the possibly different binding characteristics of different Gß isoforms.

The data presented here do not rule out a role for the N-terminal α-helical coiled-coil of Gßγ in binding ß-ARK as has been suggested by other workers (14). These workers proposed that the Gßγ binding region of ß-ARK, corresponding to the C-terminal α-helix of the PH domain, formed a triple-helical coiled-coil in association with the α-helical coiled-coil dimer formed by the N-termini of Gß and Gγ. However this hypothesis is based the computer algorithm of Lupas et al. (15) which indicates a 62% likelihood of a α-helical coiled-coil in a 29 amino acid peptide encompassing the Gßγ binding region of ß-ARK. The homologous peptides in other PH domains, which presumably also bind Gßγ, all score significantly lower with this algorithm, in some cases giving zero probability for α-helical coiled-coil propensity due to their content of proline residues. The experimental data presented here show that the putative α-helical coiled-coil interaction cannot be the sole mediator of ß-ARK Gßγ interactions. In preliminary experiments we have also

found that two 28 amino acid synthetic peptides corresponding to the α-helical coiled-coil region of $G\beta_2$ and $G\gamma_2$ have no effect on the ability of the ß-ARK PH domain GST construct to bind Gßγ, although it is of course possible that other Gßγ combinations would have a more significant inhibitory effect.

The findings presented here have some interesting implications. The ligands for SH2 and SH3 domains are phosphotyrosine containing peptides and proline-rich sequences respectively each of which are found in a variety of proteins (16, 17). The suggestion that PH domains can bind to the WD40 repeats of Gß naturally raises the question of whether PH domains might also bind WD40 sequences in other molecules. WD40 repeats are found in numerous vertebrate, *C. elegans*, *Drosophila*, yeast and *Dictyostelium* proteins, a wide cross-species distribution similar to that of PH domains (12, 13). WD40 repeat containing molecules have a variety of cellular roles including signal transduction and cytoskeletal function, as is the case with molecules containing PH domains. It is therefore possible that PH domain/WD40 interactions may be involved in a variety of important cellular processes. These speculations are now being examined experimentally.

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