Structural Determinants for Interaction with Three Different Effectors on the G Protein β Subunit*

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In the yeast two-hybrid system, a 100-residue fragment (β1A) from the N terminus of the β subunit interacts with domains specific to adenylyl cyclase 2 (AC2), the muscarinic atrial potassium channel (GIRK1), and phospholipase C-β2 (PLC-β2). Based on the crystal structure of the G protein, β1A is composed of an N-terminal α helix, a loop, and five β strands in which the C-terminal four β strands form a β sheet, the first of seven sheets that make up the propeller structure of the β subunit. A mutant of β1A (L4P, L7P, and L14P), in which the α helix was potentially destroyed, interacted poorly with the G protein γ subunit but effectively with domains of AC2, GIRK1, and PLC-β2. In contrast, another mutant of β1A (S72A, D76A, and W82A), in which a network of hydrogen bonds was disrupted, interacted poorly with GIRK1 and PLC-β2 domains, but effectively with the γ subunit and the AC2 domain. These results suggest that the proper folding of the first five β strands in the G protein β subunit is a requirement for appropriately positioning residues that interact with GIRK1 and PLC-β2. Furthermore, since mutations that potentially disrupted the folding of these β strands did not affect interaction with AC2, the structural determinants on the G protein β subunit for interaction with various effectors may be different.

The G protein βγ complex plays an important role in modulating the function of a variety of effectors in cellular signaling. The effectors regulated by the βγ complex include adenylyl cyclases, phospholipase C-β2, and potassium channels (1–5). The βγ complex directly interacts with several effectors, and binding domains for the βγ complex have been identified in these effectors (6–11). Since the β and γ subunits form a tight complex in mammalian cells, individual roles of the β and γ subunits in effector regulation are unclear. Using the yeast two-hybrid system, we have been investigating interaction of the β and γ subunits with effectors. We previously demonstrated that it was the β subunit that interacts with domains specific to adenylyl cyclase type 2 (AC2) and the muscarinic atrial potassium channel (GIRK1) and that a 100-residue fragment (β1A) from the N terminus of the β subunit interacted with these effector domains as effectively as the whole β subunit (12). These results imply that the β subunit is an important element in regulating effector activity. The identification of a subdomain within the N-terminal 100-residue fragment of the β1 subunit will provide further insight into the mechanisms by which the βγ complex regulates effector function.

To examine interaction between the βγ complex and effectors, we have specifically used the yeast two-hybrid system because it allows us to directly measure the effect of mutations in a protein on its ability to bind another protein in vivo. Biologically relevant information about the structural basis of interactions in multiprotein complexes has been obtained through deletion and mutational analysis in the two-hybrid system (e.g., Refs. 13–15). We have also successfully used the yeast two-hybrid system to demonstrate that different members of the β and γ subunit families have differential abilities to form a complex and also to show that an N-terminal domain on the β subunit interacts with domains specific to two effectors (12, 16). We showed that the formation of the βγ complex as fusion proteins in yeast cells activated a reporter gene, and that the reporter activity was directly related to the amount of the βγ complex formed in yeast cells (16). Our finding using the two-hybrid system that the first 100 residues are important for interaction with AC2 have been supported by recent results obtained from cross-linking and modeling experiments with the βγ complex and the AC2 domain (17). In this report, we have used the two-hybrid system to analyze interaction of the βγ subunits with AC2 and GIRK1 as well as phospholipase C-β2 (PLC-β2). A domain (resides 580–641) of PLC-β2 has recently been shown to directly bind to the G protein βγ complex, and a fragment containing this domain has been shown to inhibit the βγ complex-mediated activation of phospholipase C-β2 (9).

The crystal structures for the G protein heterotrimer and the βγ complex indicate that the N-terminal 100 residues of the β subunit (β1A) that interacts with AC2 and GIRK1 is made up of distinct domains in terms of secondary structure (18–20). It consists of an N-terminal α helix, a loop, and five β strands. The first of these five strands is the outermost strand of the seventh β sheet in the β subunit. The C-terminal four β strands of β1A form the first β sheet. Based on the crystal structure, hydrogen bonding is suggested between residues in the loops that connect particular β strands and residues within specific β strands. These hydrogen bonds are important for stabilizing the folded structure of the multiple β strands of the β subunit. We have introduced mutations into the first 100 residues of the β subunit that potentially disrupt either the α helix or the hydrogen bonds that are important for stabilizing the folding of these five β strands. The mutants have been examined for interaction with both the γ subunit and the effector domains.

MATERIALS AND METHODS

Strains—Yeast strain Y180 and the plasmids pAS1 and pACTII have been described before (12, 16).

Constructs—pAS1 contains the Gal4 binding domain, and pACTII contains the Gal4 activation domain. cDNAs encoding a protein or a protein domain were subcloned downstream of either the Gal4 binding domain in pAS1 or the activation domain in pACTII. cDNAs for the γ subunits were subcloned in pAS1 and cDNAs for the β subunits, and

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1 The abbreviations used are: AC2, adenylyl cyclase 2; GIRK1, muscarinic atrial potassium channel; PLC-β2, phospholipase C-β2.
**RESULTS AND DISCUSSION**

Since the G protein β subunits were targets for domains specific to two effectors (12), we wanted to know whether the β subunits were also able to bind to a domain specific to another effector, phospholipase C-β2. PLC62 is a domain with 62 residues specific to phospholipase C-β2. This domain was able to bind to the βγ complex directly as a fusion protein (9). We first examined the specificity of the PLC62 domain to interact with the G protein β subunits. In our study, PLC62 was a hybrid with the RNA binding domain of Gal4 transcription factor, and the β subunits were hybrids with the activation domain of Gal4. These hybrids were co-expressed in a yeast strain containing a reporter gene that encodes β-galactosidase. If PLC62 interacts with the β subunits, the Gal4 transcription factor domains will be brought together to activate the expression of the reporter gene. When the PLC62 domain was co-expressed with the β subunits in yeast cells, the reporter gene was significantly activated, with higher reporter activities in cells expressing β1, β2, and βγ subunits (Fig. 1A). This is consistent with reports that a βγ complex containing either β1 or β2 was able to activate phospholipase C-β2 (4, 21). The different reporter activities elicited by PLC62 with different β subunit types may be due to differential interaction between PLC62 and the β subunit types. Co-expression of PLC62 with different γ subunit types did not significantly activate the reporter gene (Fig. 1B). We then tried to identify a region of the β subunit that interacts with PLC62 by co-expression of this effector domain with various regions of the β1 subunit. The β1 subunit was split into four different fragments using an appropriate restriction site as described before (12). Fragments included the following regions of the protein: β1A, residues 1–100; β1B, residues 101–187; β1C, residues 188–261; and β1D, residues 262–340 (12). Unrelated proteins, Grr1 and Tat, served as negative controls (16). The bars represent mean value of β-galactosidase activity from three different transformants examined in each of two independent experiments. In several cases where activity was very low, error bars are too small to be shown. β1 activity from panel A is shown again in panel C as reference.

β1A–β1D were subcloned in pACTII, as described previously (12, 16). A synthetic oligonucleotide cassette encoding AC2Q (residues 956–982 of adenyl cyclase 2) was subcloned into NcoI/BamHI cut pAS1 (12). DNA encoding AC2Q (residues 1–83 of GIRK1) was amplified by polymerase chain reaction using specific primers and subcloned into NcoI/BamHI cut pAS1 (12). DNA encoding the PLC62 domain (residues 580–641 of PLC2) was amplified by polymerase chain reaction using specific primers and subcloned into both pAS1 and pACTII which were cut with NcoI/BamHI. DNAs encoding two mutants of β1A fragment, β1Am1 (L4P, L7P, and L14P) and β1Am2 (S72A, D76A, and W82A), were amplified by polymerase chain reaction using specific primers and subcloned into NcoI/BamHI cut pACTII. Correct reading frames and nucleotide sequence after polymerase chain reaction were confirmed by nucleotide sequencing. Control plasmids expressing BD-Tat and AD-Gal4 were subcloned into pAS1 (12). DNA encoding the PLC62 domain (residues 580–641 of PLC2) was subcloned into NcoI/BamHI and each of the effector domains in yeast cells. The reporter gene was sig-nificantly activated, with higher reporter activities in cells expressing the PLC62 domain with various regionsoftheβγcomplex(Fig.1C).β1Aalsointeractedmoreeffectivelywithγ subunits than the entire β1 subunit (Ref. 12 and data not shown). Although PLC62 did not interact with other regions of the β1 subunit, this does not rule out the possibility that these fragments may be folded improperly in yeast. Together with our previous finding (12), these results strongly indicate that the N-terminal 100 residues from the β1 subunit contain subdomains that interact with three different effectors. Are these putative subdomains in β1A distinct structural determinants or overlapping regions?

Based on the crystal structure of the G protein, the β subunit is a propeller composed of seven β sheets with an N-terminal α helix. β1A, a 100-residue fragment from the N terminus of the β1 subunit, encompasses an N-terminal α helix, five β strands, and a loop connecting the α helix and the β strands (18, 20). The N-terminal α helix of the β1 subunit forms the beginnings of a parallel coiled-coil with the N-terminal α helix of the γ subunit. The first of the β strands is the outer strand of the seventh β sheet in the propeller structure of the β1 subunit. The next four β strands form the first β sheet. Both the α helix and/or the β strands could be involved in interaction with these effector domains.

To test if the N-terminal α helix is involved in interaction with effectors, we introduced mutations into β1A that have the potential to destroy the α helix. The α helix is stabilized by hydrogen bonds between the NH and CO groups of the main chain. The proline side chain is different from others in that its side chain is bonded to both the nitrogen and α-carbon atoms to form a ring structure. This prevents the N atom of proline from participating in hydrogen bonding and should form ster- obstacles to the formation of an α-helix. The α helix situated at the N terminus of the β subunit is amphipathic and forms part of a coiled coil with a similar amphipathic helix from the N terminus of the γ subunit. In the first mutant of β1A, β1Am1, we therefore chose to substitute three leucines in the α helix along the hydrophobic surface of the helix (Fig. 2A). These leucines (Leu-4, Leu-7, and Leu-14) in β1A were substituted with prolines. We then co-expressed β1Am1 with the γ subunit and each of the effector domains in yeast cells. The reporter activities induced by β1Am1 in combination with the γ subunit or the effector domains are shown in Fig. 2, B–E. As expected based on the three-dimensional structure for the βγ complex,
the ability of β1Am1 to interact with the γ subunit was dramatically decreased (Fig. 2B). The reporter activity stimulated by co-expression of β1Am1 and γ5 is about one-tenth of that seen in cells co-expressing β1A and γ5. It is highly unlikely that this result is due to altered expression of the mutant protein since we have repeatedly examined the expression of a variety of hybrids and found them to be expressed at similar levels (12, 16). The reduced interaction could result from both the loss of leucines at the binding surface and the disruption of the helix due to proline substitutions. We do not have direct biophysical results that show disruption of the N-terminal α helix of the β subunit, but, based on the effect of proline substitutions on the formation of an α helix, it is most likely that the helix is disrupted. Although the N-terminal α helix that is required for interaction with the γ subunit was potentially destroyed by the proline substitutions, the ability of β1Am1 to interact with AC2Q, GKN, and PLC62 was decreased less than 20% (Fig. 2, C–E). This indicates that the regions of β1A that interact with the effector domains were not significantly affected by potential disruption of the amphipathic α helix. The ability of the mutations in β1Am1 to affect interaction with the γ subunit is consistent with previous results from mutant analysis of β-γ interaction (22).

Since the results above indicated that residues in the N-terminal α helix of β1A were unlikely to be involved in the interaction with AC2Q, GKN, and PLC62, we investigated whether the appropriate folding of the β strands in β1A was a requirement for interaction with the effector domains. To address this question, we made the second mutant of β1A–β1Am2. The β subunit is made up of seven WD repeats of about 40 amino acids each (23). Based on the crystal structure of the β subunit, four residues that are largely conserved within each of the seven WD repeats have been implicated in forming hydrogen bonds that are important for stabilizing the characteristic folded structure of β strands (18, 20). In the mutant β1Am2, three of these residues, Ser-72, Asp-76, and Trp-82, which form hydrogen bonds were replaced with alanines. In the crystal structure of the βγ complex, the β strands 2–5 of the β subunit form the first β sheet in the G protein β subunit. Ser-72 is within the third β strand, Trp-82 is within the fourth β strand, and Asp-76 is within the loop connecting the third and the fourth β strand (Fig. 3A). Hydrogen bonding occurs between Asp-76 and His-54 which is located in the loop connecting the first and second β strands (Fig. 3A). Thus, a turn in the first β sheet is coupled to the first β strand. Ser-72 is hydrogen-bonded with Trp-82 as well as His-54. This bonding couples the third with the fourth β strand as well as the loop downstream of the first strand. Trp-82 is also in the hydrophobic core of the first β sheet and in contact with several residues. The folded structure formed by the five β strands in β1A is therefore likely to be disturbed significantly by altering Ser-72, Asp-76, and Trp-82 to alanines in the mutant β1Am2.

We first tested whether the β1Am2 mutant was able to interact with the γ subunit by co-expressing β1Am2 with γ5 in yeast cells. β1Am2 interacted with γ5 as effectively as β1A (Fig. 3B), indicating that the mutations in the region of the β strands do not affect complex formation with the γ subunit. Combined with the results from the β1Am1 mutant, this implies that residues in the α helical region of β1A are major determinants for interaction with the γ subunit. Wild type β1A elicited significant reporter activity with the three effector domains, AC2Q, GKN, and PLC62 (Fig. 3, C, D, and E). The magnitude of activity induced by different combinations was very different, consistent with our previous results (12). The higher activity induced by β1A with AC2Q compared to the other two domains is most likely due to differential binding. This is based on our previous results that show direct relationship between reporter activity elicited by a particular combination of hybrids and efficiency of complex formation between the same hybrids as determined by immunoprecipitation (16). Furthermore, the lower reporter activity elicited by β1A with GKN and PLC62 should result from significant protein-protein interaction because these effector domains do not show significant reporter activity when co-expressed with a variety of control proteins, unrelated proteins, γ subunit types, or the β1B-D fragments. The differences in reporter activities induced by the GKN and PLC62 domains with β1A in comparison with a variety of controls are clearly apparent in filter assays where yeast cells have been examined for β-galactosidase activity (Ref. 12 and data not shown). When the mutant, β1Am2, was co-expressed with GKN or PLC62 in yeast cells, β1Am2 was significantly affected in its interaction with these two effector domains, and the efficiency of interaction was decreased 3–4-fold (Fig. 3, D and E). As in the case of β1Am1, it is highly likely that this result is due to altered expression of the mutant protein since we have examined the expression of a variety of hybrids and found them to be expressed approximately at similar levels (12, 16). It is possible that the residues (Ser-72, Asp-76, and Trp-82) themselves are directly involved in binding to both GKN and PLC62. Altering these residues in β1A would therefore result in defective interaction with the effector domains. However, we think it is more likely that
interaction of β1Am2 with GKN and PLC62 is affected because the folding of the β strands in β1A is disrupted. This inference is based on the following. (i) Ser-72 and Trp-82 are located within the β sheet formed by β strands 2–5 where they are unlikely to be accessible to an effector domain. (ii) It seems unlikely that we could have at random mutated precisely those residues that are critical for binding with GKN and PLC62. Moreover, the insignificant effect of the L4P, L7P, and L14P mutations on β1Am1 binding with GKN and PLC62 show that mutational alterations of β1A do not in general lead to gross misfolding that renders the protein incapable of interaction with other proteins. The effective interaction of β1Am2 with AC2Q (Fig. 3C) further emphasizes the specificity of the effect of mutations in β1A on effector domain interactions.

Thus, the results from the analysis of the β1Am2 mutant imply that residues in β1A that interact with GKN and PLC62 require appropriate folding of the first five β strands of the β subunit to be able to bind these effector domains. Based on the ability of the α subunit to disrupt binding of an effector with the βγ complex, candidates for such residues are those that are positioned in the bends between the β strands 1-2, 3-4, and 5-6 as well as the exposed portions of the β strands located at the same surface of the βγ complex which interacts with the α subunit.

We have identified an N-terminal fragment of 100 residues from the G protein β subunit that is able to interact with domains specific to three different effectors, AC2, GIRK1, and PLC-β2. Within this fragment, the structural determinant for interaction with AC2 seems to be different from that for interaction with GIRK1 and PLC-β2 because of the following reason. Potential disruption of the folded structure of the β strands that should occur in the β1A fragment of the β subunit has no significant effect on the interaction of the mutant β1A with AC2Q. But potential disruption of the folded structure of the β strands in the mutant β1Am2 significantly affects interaction of this mutant β1A with GKN and PLC62. It is still likely that the structural determinant(s) for interaction with AC2Q are in the exposed surfaces formed by the folding of the five β strands. A domain (residues 52–100) of the β1 subunit that contains β strands 2–5 (which form the first β sheet) was able to interact with AC2Q in the two-hybrid system, although not as effectively as β1A (data not shown). Cross-linking and modeling implicate residues 75–165 of the β subunit in binding with the AC2Q peptide (17). A peptide specific to residues 86–105 of the β1 subunit has also been shown to inhibit interaction of the βγ complex with adenyl cyclase 2 in a sequence-specific manner. These results further confirm a role for residues in the structure formed by the first five β strands in interaction with AC2. The simplest interpretation of our results is that these residues do not require the formation of the folded structure of interacting β strands to be able to bind AC2Q.

The presence of different structural determinants for AC2Q versus GKN/PLC62 in β1A suggests that this may be one mechanism that the βγ complex uses to discriminate between structurally distinct effectors. Similarly, mutations at the C terminus of the β subunit affected PLC-β2 activation but not activation of the mitogen-activated protein kinase pathway (24). The specificity of interaction of the βγ complex with GIRK1 and PLC-β2 could be affected by domains other than those identified here. It was reported that two domains of GIRK1 were able to bind to the βγ complex, an N-terminal domain and a C-terminal domain (7, 8). GKN is the N-terminal domain of GIRK1 that is able to bind to the N-terminal fragment (β1A) of the β1 subunit. The C-terminal domain of GIRK1 could bind to another site on the βγ complex that has not been identified yet. This second site on the βγ complex may be important for specific interaction between the βγ complex and GIRK1.

We have begun to understand the individual roles of the G protein β and γ subunits in cellular signaling. It is likely that the γ subunit plays an important role in receptor interaction (25, 26). The results here together with our previous findings (12) indicate an important role for the β subunits in effector interaction. Results from other laboratories also support this role. The AC2Q peptide was chemically cross-linked to the β subunit (17), and the extreme C-terminal domain of the β subunit was important for activation of phospholipase C-β2 in COS cells (24). Since prenylation of the γ subunit is essential for appropriate interaction with effectors (e.g. Refs. 4 and 27), the lipid group at the C terminus of the γ subunits could also directly interact with a site on the effector molecules.

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