Receptor- and Nucleotide Exchange-independent Mechanisms for Promoting G Protein Subunit Dissociation*

Received for publication, June 18, 2003, and in revised form, July 11, 2003
Published, JBC Papers in Press, July 24, 2003, DOI 10.1074/jbc.C300271200

Mousumi Ghosh, Yuri K. Peterson, Stephen M. Lanier, and Alan V. Smrcka

From the Department of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 and the Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112

Mechanisms for heterotrimeric G protein activation that do not rely on G protein-coupled receptor activation are becoming increasingly apparent. We recently identified βγ subunit-binding peptides that we proposed bound to a "hot spot" on βγ subunits, stimulating G protein dissociation without stimulating nucleotide exchange and activating G protein signaling in intact cells. AGS3, a member of the activators of G protein signaling family of proteins, also activates G protein signaling in a nucleotide exchange-independent manner, and AGS3 homologues are involved in asymmetric cell division during development. Here we demonstrate that a consensus G protein regulatory (GPR) peptide from AGS3 and related proteins is sufficient to induce G protein subunit dissociation and that both the GPR and hot spot-binding peptides promote dissociation to extents comparable with a known G protein activator, AMF. Peptides derived from adenyl cyclase 2 and GRK2 prevented formation of the heterotrimeric complex but did not alter the rate of α subunit dissociation from βγ subunits. These data indicate that these nucleotide exchange-independent G protein activator peptides do not simply compete for interactions with βγ subunits, but actively promote subunit dissociation. Thus, we propose two novel mechanisms for nucleotide exchange-independent activation of G protein signaling, one that involves conformational changes in the α subunit and one that involves conformational changes in the βγ subunits.

Heterotrimeric G proteins activated by G protein-coupled receptors mediate a wide variety of cellular processes (1). The mechanisms by which G protein-coupled receptors activate G proteins have not been fully defined, but involve interactions between the activated receptor, G protein α subunits, and perhaps G protein βγ subunits. This interaction leads to the exchange of GDP for GTP on the G protein α subunit, leading to a conformational change resulting in dissociation of the βγ subunits from the α subunits (1, 2). The free αGTP and βγ subunits interact with downstream targets and regulate their activities.

Multiple mechanisms for G protein activation that do not rely on G protein-coupled receptors or even nucleotide exchange are becoming increasingly apparent. We recently identified a receptor independent mechanism for activation of G protein βγ subunit signaling by peptides derived from a random peptide phage display screen that we have proposed bind to a "hot spot" on βγ subunits (3, 4). Protein interaction hot spots are regions on protein surfaces through which unique characteristics suited to driving protein-protein interactions that are often selected for in random peptide screens (5, 6).

Activators of G protein signaling (AGS1–3 proteins) (7, 8) were isolated from a genetic screen in yeast to look for activation of the βγ-mediated mating pathway. AGS3 binds to α subunits and activates the signaling pathway without stimulating nucleotide exchange on the Go subunit (7). Sequences similar to a 25–30 amino acid repeat region in AGS3 were found in multiple other proteins and suggested to be a signature G protein regulatory (GPR) motif (7). This motif was also independently postulated to be a G protein-binding motif and termed the GoLoco motif (9). Synthetic peptides representing this motif inhibit GDP release from α subunits (10–12) and have been co-crystallized with G protein α subunits (13).

To determine whether the GPR peptides and βγ hot spot-binding peptides uniquely target critical sites on G protein subunits to promote subunit dissociation or if they are simply steric competitors of α-βγ binding, we analyzed and compared the effects of multiple peptides believed to interact at the βγ-α subunit interface for their ability to induce the α subunit dissociation from βγ subunits. We conclude that both the hot spot and GPR motif consensus peptides have the unique ability to dissociate heterotrimers by a mechanism that most likely involves conformational changes in the βγ and α subunits, respectively.

EXPERIMENTAL PROCEDURES

Peptides—SIGK, QEHA, and bARK-ct peptide (643–670) were synthesized by Alpha Diagnostics International, purified by high performance liquid chromatography to greater than 90% purity, and whose identity was confirmed by mass spectrometry analysis. The SIGK peptide was derived from the previously described SIRK (SIRKALNILGYDYNDYD) peptide using a doping mutagenesis and rescreening strategy (14). Since SIGK had an apparently higher affinity for βγ than SIRK but whose properties were otherwise similar to SIRK, this peptide was used throughout the studies described here. The sequences of these peptides were as follows: SIGK, SIGKAPKILGYPDYD; QEHA, QEHAQEPERQYMHICTMVEFAYALVGH; βARK-ct peptide, WKKELRDAYREAQQLVQRVPKMNKPR. The GPR consensus motif peptide, TMGEEDFDLLAKSQKRMDDQRVDLAG, was synthesized and purified by Biosynthesis, Inc. (Lewisville, TX). All the peptides were dissolved in water.

Preparation of Biotinylated βγ Subunits—The cDNA for rat β1 subunit was subcloned into a baculovirus transfer vector for expression of amino-terminal fusions of a bicistronic acceptor peptide and the biotinylated β subunit was expressed as described previously (7). Biotinylated
β1γ2 was purified from Sf9 cells using hexahistidine-tagged α1, following the procedure described previously (15).

**Measurement of α-βγ Interactions by Flow Cytometry**—Binding of fluorescein isothiocyanate-labeled myristoylated α1 (F-α1) to biotinylated βγ (b-βγ) subunits was measured using a flow cytometry assay (4, 16). F-α1 was kindly provided by Dr. Richard Neubig and was prepared by reacting purified myristoylated α1 with fluorescein isothiocyanate, followed by dialysis and repurification by βγ-agarose chromatography (16). The resulting F-α1 has a specific activity of 11 pmol of [35S]GTP·S bound per μg and 0.9 mol of dye/mmol of protein incorporated.

Biotinylated b-γ (50 pM final concentration) was mixed with streptavidin beads in HEDNMLG buffer (20 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 1.2 mM MgCl2, 0.1% CHES, 10 μM GDP) at room temperature. After 30 min, the beads were washed twice by centrifugation in a microcentrifuge with HEDNMLG buffer and resuspended in the same buffer at 107 beads/ml (50 pM b-γ). For α subunit dissociation experiments, the beads with bound 50 pM b-βγ subunits were preincubated with 300 pM F-α1 for 10 min prior to the addition of the different peptides or α1. For equilibrium binding measurements 300 pM F-α1 and peptides or α1 were added simultaneously. The amount of F-α1 bound to beads with b-βγ was assayed at the times indicated in the figure legends using a BD Biosciences FACs Calibur flow cytometer. Nonspecific binding, determined by the simultaneous addition of 300 pM F-α1, and 50 nM myristoylated α1 subunits to the b-βγ bound beads, was 10–20% of the total signal and was subtracted from the mean channel numbers from each experiment unless otherwise indicated.

**RESULTS**

**Comparison of the βγ Hot Spot-dependent Dissociation Mechanism with AMP-induced Subunit Dissociation**—We wanted to determine whether the βγ hot spot-dependent mechanism for subunit dissociation was similar in magnitude to classically described mechanisms for subunit dissociation. AMP (AlF4− plus Mg2+) is a well characterized mediator of G protein subunit dissociation. We chose AMP for the comparison rather than GTP or GTP·S, because the rate of dissociation of the G protein subunits by AMP is not limited by the GDP release rate.

To measure α1 binding and dissociation from βγ, we used a flow cytometry assay developed by Sarvazyan et al. (16) that measures protein α-βγ binding at concentrations of α and βγ near the Kd for their interaction. In this assay, biotinylated βγ (b-βγ) was immobilized on the surface of beads and fluorescein-labeled α1 (F-α1) was added. F-α1 that bound to b-βγ on the beads was detected by the flow cytometer. The binding was concentration-dependent, and on and off rates could be measured.

To demonstrate that the βγ hot spot-binding peptide (SIGK) and AMP could inhibit a subunit interactions with βγ, they were compared for their effects on the initial binding of F-α1 to b-βγ. Excess unlabeled myristoylated α1 was used to measure nonspecific binding. SIGK, AMP, and α1 all inhibited formation of the heterotrimeric F-α1βγ complex to comparable extents, indicating they were equally effective at preventing heterotrimer formation (Fig. 1A).

Next we measured the effects of SIGK on subunit dissociation. First we determined the concentration of SIGK required for dissociation of an F-α1βγ complex (Fig. 1B). Increasing concentrations of SIGK caused a progressive increase in disruption of the preformed complex with maximal dissociation observed between 20 and 30 μM peptide. To compare the SIGK-mediated dissociation rates with AMP, we compared a maximally effective concentration of SIGK with a standard concentration of AMP (30 μM AlCl3, 10 mM NaF, 10 mM MgCl2) that should be sufficient to activate all of the α subunits (17). Both SIGK and AMP enhanced the release of α subunits from βγ subunits relative to the intrinsic α1 off rate (measured by addition of a 50-fold excess of unlabeled myristoylated α1) (Fig. 1C). The koff with AMP was 0.62 min−1 and with SIGK was 0.5 min−1 compared with the intrinsic off rate of 0.1 min−1.

**Effect of MgCl2 on the Rate of a Subunit Dissociation from the Heterotrimeric Complex**—Magnesium is known to profoundly affect the interaction of α and βγ subunits, and dissociation by activation of the α subunit with either GTP·S or AlF4− is strongly dependent upon Mg2+ concentration (1, 18). Magnesium stabilizes interactions of AlF4− and GTP·S at the nucleotide-binding site and directly influences subunit dissociation through an undefined lower affinity site. For the experiment in Fig. 1C, SIGK-mediated dissociation was measured with 1.2 mM MgCl2 and 1 mM EDTA (0.2 mM free Mg2+). To determine whether Mg2+ was required for subunit dissociation by SIGK, we measured SIGK-mediated dissociation in the absence of MgCl2 in the presence of 1 mM EDTA (Fig. 1D). SIGK was able
to significantly enhance subunit dissociation in the absence of Mg$^{2+}$, indicating Mg$^{2+}$ is not required for this dissociation mechanism. Addition of Mg$^{2+}$ at a free concentration of 10 mM increased the intrinsic off rate of F-$\alpha_1$ as expected from 0.1 min$^{-1}$ in the absence of Mg$^{2+}$ to 0.37 min$^{-1}$ with 10 mM Mg$^{2+}$. The rate of a subunit dissociation by SIGK increased from 0.43 min$^{-1}$ without Mg$^{2+}$ to 1.14 min$^{-1}$ with 10 mM Mg$^{2+}$.

Effect of $\beta\gamma$-Binding Peptides on the Interaction of F-$\alpha_1$, with $\beta\gamma$. Since SIGK and other hot spot-binding peptides enhance the off rate of a from $\beta\gamma$ subunits, it suggests they do not act simply by competition for reformation of spontaneously dissociating $\alpha$-$\beta\gamma$ complexes. Such a mechanism would not increase the dissociation rate relative to the intrinsic off rate of a dissociation from $\beta\gamma$. Based on this we have suggested that the peptides stabilize a conformation of $\beta\gamma$ subunits that has a lower affinity for $\alpha$ leading to an enhanced rate of $\alpha$ subunit dissociation (4). An alternative mechanism might be that a small peptide could compete for one of the two major contacts of $\alpha$ subunits with the sides and top of the $\beta$ subunit torus during transient separation of one of these contacts, thereby leading to an enhancement of the dissociation rate. If there were nothing unique about the hot spot-binding peptides, and it were simply a competitor at the $\alpha$-$\beta\gamma$ interface, this model would predict that any peptide that bound at the $\alpha$ subunit interface with $\beta\gamma$ should enhance the off rate.

To try to distinguish between these two mechanisms, we tested two $\beta\gamma$-binding peptides thought to bind to $\beta\gamma$ subunits at the $\alpha$ subunit interface. QEH$A$ is a 27-residue peptide derived from the second catalytic domain of adenyl cyclase 2 (amino acids 956–982) and inhibits $\beta\gamma$ regulation of several effectors including K$^+$ channels, phospholipase C-$\beta$, and adenyl cyclase (19). The IC$_{50}$ for QEH$A$ effects on most processes was 50–200 $\mu$m. Cross-linking of the QEH$A$ peptide to the $\beta$ subunit is prevented by the $\alpha$ subunit, suggesting it binds to $\beta\gamma$ within the $\alpha$ subunit-binding site (19, 20). Another peptide derived from the COOH-terminal region of GRK2 ($\beta$ARK-ct, 643–670) (21) also binds to $\beta\gamma$ subunits, has an IC$_{50}$ of 100 $\mu$m for its effects, and has properties consistent with binding at the $\alpha$ subunit-binding site on $\beta\gamma$ subunits. This notion is supported by the recently determined co-crystal structure of $\alpha$ subunits with GRK2 (22), demonstrating binding of the region of GRK2 corresponding to this peptide to a region on $\beta\gamma$ that overlaps with the $\alpha$ subunit-binding site.

To confirm that these peptides block $\alpha$-$\beta\gamma$ interactions, the ability of these peptides to block binding of F-$\alpha_1$ to $\beta\gamma$ was tested by flow cytometry. Both peptides inhibited the heterotrimer formation with QEH$A$ inhibiting by 90% and $\beta$ARK-ct peptide (300 $\mu$m) by more than 65% compared with 85% by SIGK (Fig. 2A). This supports the idea that QEH$A$ and $\beta$ARK-ct peptide can inhibit $\alpha$-$\beta\gamma$ interactions probably by directly competing for $\alpha$ binding to $\beta\gamma$.

To determine whether QEH$A$ and $\beta$ARK-ct peptides can stimulate release of $\alpha_2$ from the heterotrimer, we measured the off rates in the presence of these peptides at concentrations that significantly inhibited $\alpha$ binding to $\beta\gamma$. Neither of these peptides caused any significant enhancement of the rate of dissociation of the $\alpha$ subunits from the preformed complex (Fig. 2B). There are some minor differences in the rate of dissociation for QEH$A$, $\beta$ARK, and the intrinsic dissociation rate, but these differences were not consistently reproducible and were not significant. Thus, while being able to compete for $\alpha$-$\beta\gamma$ interactions they are unable to promote dissociation of $\alpha$ from $\beta\gamma$. These data strongly suggest that the $\beta\gamma$ hot spot-binding peptides act through a unique mechanism that does not involve simple steric occlusion of $\alpha$ binding sites on $\beta\gamma$ subunits.

**Effect of a GPR Consensus Motif Peptide on Association and Dissociation of Ga$\beta\gamma$**—A class of proteins that stimulate G protein $\beta\gamma$ subunit signaling by a nucleotide exchange-independent mechanism is the AGS proteins. AGS3 binds to $\alpha$ subunits, inhibits GDP release, and promotes $\beta\gamma$ subunit signaling in yeast and is involved in establishing $\beta\gamma$ and $\alpha$ subunit-dependent asymmetric cell division in *Caenorhabditis elegans* and *Drosophila*. A 28-amino acid (GPR) motif consensus pep-
tide derived from AGS3 and other related proteins is able to bind to α subunits and inhibit nucleotide exchange in a manner comparable with larger protein fragments of AGS3 (10, 23). Since a GPR-like peptide from RGS14 binds to α subunits near the α-βγ interface, it was proposed that these peptides enhance βγ subunit-dependent processes by competing for rebinding of α to βγ by steric occlusion of the βγ binding site on α (13). We directly tested whether the GPR peptide could inhibit α-βγ binding and/or promote subunit dissociation. 1 μM GPR peptide was able to inhibit α binding to βγ to an extent greater than that observed with 10 μM SIGK (Fig. 3A). The IC_{50} for inhibition of α-βγ interactions was 250 nM (Fig. 3B), comparable with that observed for the ability of the peptide to inhibit GDP dissociation from α subunits. Next we tested whether 1 μM GPR peptide could cause dissociation of a preformed α-βγ complex. The GPR peptide caused a rapid dissociation of the G protein subunits, about 2-fold faster (0.95 min^{-1}) than that observed with 25 μM SIGK (0.5 min^{-1}) and about 13-fold higher than the intrinsic off rate of the F-α_{3} subunit (Fig. 3C). The GPR peptide very potently promoted dissociation (Fig. 3D) and was about 10-fold more potent than SIGK (compare Fig. 3D with Fig. 1B). Thus, the GPR consensus peptide is a very potent and effective promoter of G protein subunit dissociation. Since they dramatically increase the dissociation rate of α from βγ, it strongly suggests that the GPR peptides act by a non-competitive mechanism.

**DISCUSSION**

We compared the effects of the GPR peptides and βγ hot spot-binding peptides with peptides that are thought to bind at the G protein βγ subunit-α subunit interface. We show that AC2- and βARK-derived peptides are capable of blocking βγ-α subunit interactions consistent with previous data, suggesting that they bind at the α-βγ interface. These competitor peptides were unable to enhance the rate of G protein subunit dissociation, while SIGK and GPR peptides significantly enhanced subunit dissociation. The enhanced rates of G protein subunit dissociation by SIGK and GPR peptides were comparable with a known activator of G proteins, AMF. This strongly suggests that neither the SIGK peptides nor the GPR peptides are simply acting by preventing reassembly of dissociated subunits to lead to G protein activation. The GPR- and βγ-binding peptides are unique in that they induce subunit dissociation, probably through conformational alterations of α or βγ subunits respectively.

In the x-ray crystallographic structural model of a GPR-like peptide from RGS14 in a complex with α_{3} (13), conformations of switch I and II are altered relative to heterotrimERIC α GDP βγ. These conformational differences could result in subunit dissociation via a mechanism analogous to the GTP or AIF_{1}-dependent conformational changes in the switch regions of the α subunit that contribute to subunit dissociation (24).

GPR proteins play important roles in regulation of asymmetric cell division in *Drosophila* (25) and in *C. elegans* (26). In particular, they appear to regulate the polarized distribution of free βγ and α subunits derived from heterotrimeric G proteins for correct orientation of mitotic spindles. In α subunit immunoprecipitates from *Drosophila* sensory organ precursor cells, Pins (an AGS3 homologue in *Drosophila*) and a peptide representing the GPR motif from Pins disrupted α-βγ interactions when added during the immunoprecipitation (25). Our results that directly measure subunit dissociation are consistent with these results.

The data presented also support a model where a conformational change in βγ subunits induced by hot spot-binding peptides results in destabilization of interactions with α subunits and an increase in the koff for subunit dissociation, βγ subunits have indeed been shown to undergo conformational changes upon binding of phosducin (27, 28), although the functional significance of these changes are not entirely understood. A mechanism for βγ-binding peptide-mediated enhancement of subunit dissociation, whatever the details of the mechanism, is clearly very distinct from other mechanisms that exist for promoting subunit dissociation by either GPR peptides or nucleotide binding that involve conformational alterations of the switch regions of the α subunits.

**Acknowledgment**—We thank Dr. Richard Neubig for both his advice on performing the flow cytometry experiments and for providing us with fluorescein labeled α_{1}.

**REFERENCES**

1. Gilman, A. G. (1987) *Annu. Rev. Biochem.* 56, 615–649
2. Hamm, H. E. (1998) *J. Biol. Chem.* 273, 669–672
3. Scott, J. K., Huang, S. F., Gangadhar, R. P., Samorski, G. M., Clapp, P., Gross, R. A., Taussig, R., and Smrcka, A. V. (2003) *EMBO J.* 20, 767–776
4. Goujard, F., Ghoosh, M., Maik, S., Yang, J., Hinke, P. M., Grindeland, K. K., Neubig, R. R., and Smrcka, A. V. (2003) *J. Biol. Chem.* 278, 19634–19641
5. Delano, W. L., Ultsch, M. H., de Vos, A. M., and Wells, J. A. (2000) *Science* 287, 1279–1283
6. Delano, W. L. (2002) *Curr. Opin. Struct. Biol.* 12, 14–20
7. Takesono, A., Cismowski, M. J., Ribas, C., Bernard, M., Chung, P., Hazard, S., Duzic, E., and Lanier, S. M. (1999) *J. Biol. Chem.* 274, 33202–33205
8. Blumer, J. R., and Lanier, S. M. (2000) *Receptor Channels* 9, 195–204
9. Siderovski, D. P., Diverse-Pierluisi, M., and De Vries, L. (1999) *Trends Biochem. Sci.* 24, 340–341
10. Peterson, Y. K., Bernard, M. L., Ma, H. Z., Hazard, S., Graber, S. G., and Lanier, S. M. (2000) *J. Biol. Chem.* 275, 33183–33186
11. Peterson, Y. K., Hazard, S., Graber, S. G., and Lanier, S. M. (2002) *J. Biol. Chem.* 277, 6767–6770
12. De Vries, L., Fischer, T., Tronchere, H., Brothers, G. M., Stockbichle, B., Siderovski, D. P., and Farquhar, M. G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 14364–14369
13. Kimple, R. J., Kimple, M. E., Betts, L., Sondak, J., and Siderovski, D. P. (2002) *Nature* 416, 878–881
14. Gallop, M. A., Barrett, R. W., Dower, W. J., Fodor, S. P., and Gordon, E. M. (1994) *J. Med. Chem.* 37, 1235–1251
15. Kanase, T., and Gilman, A. G. (1995) *J. Biol. Chem.* 270, 1734–1741
16. Sarvazyan, N. A., Remmers, E. A., and Neubig, R. R. (1998) *J. Biol. Chem.* 273, 7934–7940
17. Higashijima, T., Ferguson, K. M., Sternweis, P. C., Ross, E. M., Smigel, M. D., and Gilman, A. G. (1987) *J. Biol. Chem.* 262, 752–756
18. Katada, T., Northup, J. K., Bokoch, G. M., Ul, M., and Gilman, A. G. (1984) *J. Biol. Chem.* 259, 3578–3585
19. Chen, J., DeMuro, V., Dingus, J., Henry, A., Li, J., Sui, J., Carter, D. J., Blank, J. L., Eton, J. H., Stoffel, R. H., Inglese, J., Leukowitz, R. J., Logothetis, D. E., Hildebrandt, J., and Iyengar, R. (1995) *Science* 268, 1166–1169
20. Weng, G. Z., Li, J. R., Dingus, J., Hildebrandt, J. D., Weinstein, H., and Iyengar, R. (1996) *J. Biol. Chem.* 271, 26445–26448
21. Koch, W. J., Inglese, J., Stone, W. C., and Leukowitz, R. J. (1993) *J. Biol. Chem.* 268, 8256–8260
22. Lodowski, D. T., Pitcher, J. A., Capel, W. D., Leukowitz, R. J., and Tesmer, J. J. G. (2003) *Science* 300, 1256–1260
23. Naitochin, M., Lester, B., Peterson, Y. K., Bernard, M. L., Lanier, S. M., and Artymevy, N. O. (2000) *J. Biol. Chem.* 275, 40981–40985
24. Sprang, S. R. (1997) *Annu. Rev. Biochem.* 66, 639–678
25. Schaefer, M., Petronczi, M., Derner, D., Forte, M., and Knoeblich, J. A. (2001) *Cell* 107, 183–194
26. Gotta, M., Dong, Y., Petersen, Y. K., Lanier, S. M., and Ahringer, J. (2003) *Curr. Biol.* 13, 1029–1037
27. Loew, A., Ho, Y. K., Blandell, T., and Bax, B. (1998) *Structure (London)* 6, 1007–1019
28. Gaudet, R., Bohn, A., and Sigler, P. B. (1996) *Cell* 87, 577–588