Selective Activation of Effector Pathways by 
Brain-specific G Protein $\beta_5^*$

(Received for publication, October 1, 1996, and in revised form, October 29, 1996)

Shiyeling Zhang, Omar A. Cosos‡, Chunghee Lee§, J. Silvio Gutkind‡, and William F. Simonds¶

From the Metabolic Diseases Branch, NIDDK, and the Molecular Signaling Unit, Laboratory of Cellular Development and Oncology, NIDR, National Institutes of Health, Bethesda, Maryland 20892

While multiple G protein $\beta$ and $\gamma$ subunit isoforms have been identified, the implications of this potential diversity of $\beta\gamma$ heterodimers for signaling through $\beta\gamma$-regulated effector pathways remains unclear. Furthermore the molecular mechanism(s) by which the $\beta\gamma$ complex modulates diverse mammalian effector molecules is unknown. Effector signaling by the structurally distinct brain-specific $\beta_5\gamma_5$ subunit was assessed by transient co-transfection with $\gamma_2$ in COS cells and compared with $\beta_1\gamma_2$. Transfection of either $\beta_1$ or $\beta_5$ with $\gamma_2$ stimulated the activity of cotransfected phospholipase C-$\beta_2$ (PLC-$\beta_2$), as previously reported. In contrast, cotransfection of $\beta_1$ but not $\beta_5$ with $\gamma_2$ stimulated the mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways even though the expression of $\beta_1$ in COS cells was evident by immunoblotting. The G protein $\beta_5$ expressed in transfected COS cells was properly folded as its pattern of stable C-terminal proteolytic fragments was identical to that of native brain $\beta_5$. The inability of $\beta_5$ to activate the MAPK and JNK pathways was not overcome by cotransfection with three additional $\gamma$ isoforms. These results suggest it is the G$\beta$ subunit which determines the pattern of downstream signaling by the $\beta\gamma$ complex and imply that the structural features of the $\beta\gamma$ complex mediating effector regulation may differ among effectors.

Both the heterotrimeric G protein $\alpha$ subunit and the $\beta\gamma$ complex transmit signals to effector molecules (1, 2). Multiple isoforms of $\beta$ and $\gamma$ subunits have been identified by cDNA cloning (3, 4), and the formation of $\beta\gamma$ heterodimers from particular combinations of $\beta$ and $\gamma$ subtypes may contribute to signaling specificity by the $\beta\gamma$ complex (5). G protein $\beta\gamma$-regulated effector molecules in vertebrates include inwardly-rectifying potassium channels (6), certain isoforms of adenylyl cyclase (7, 8) and phospholipase C-$\beta$ (PLC-$\beta$) (9–11), and as yet unidentified upstream targets in the mitogen-activated protein kinase (MAPK) (12–14) and c-Jun N-terminal kinase (JNK) (15) pathways.

The effector specificity of the recently described brain-specific $\beta_5$ subunit(3) was examined by transient cotransfection with $\gamma_2$ in COS cells and compared with $\beta_1$. We report here that while both $\beta_1$ and $\beta_5$ were found to activate PLC-$\beta_2$ in a $\gamma_2$-dependent fashion consistent with previous reports (3, 10), $\beta_5$, unlike $\beta_1$, did not stimulate the MAPK or JNK pathways. Cotransfection of different $\gamma$ isoforms failed to confer MAPK or JNK stimulatory ability to $\beta_5$. These results imply that the G$\beta$ subunit can define the pattern of downstream signaling mediated by the $\beta\gamma$ complex and suggest that distinct mechanisms mediate the activation of different $\beta\gamma$-responsive effectors.

EXPERIMENTAL PROCEDURES

cDNA Constructs—The cDNA for human phospholipase C-$\beta_1$ (16) (GenBank™ accession number M95768) (in pm1T2) was a gift from Dr. S. G. Rhee. Constructs encoding $\beta_1$, $\gamma_1$, and $\gamma_5$ in the vector pCDM8.1 (17) were described previously (18, 19). The expression constructs for hemagglutinin epitope-tagged (HA)-ERK2 and HA-JNK in pcDNA3 were described previously (12, 20). The cDNA for $\beta_5$ was obtained by PCR of mouse brain cDNA (Clontech) using specific primers (3) and the thermostable DNA polymerase Pyrococcus furiosus (Pfu) (Stratagene). The final construct contained two silent base changes (Ser130 TCt and Phe136 TTc) relative to the published sequence (4) (GenBank™ accession number U31382). A silent nucleotide substitution in codon Ala38 (GCt) was found. The cDNA for $\gamma_5$ (GB M99393) was obtained by PCR employing Ffu DNA polymerase from bovine brain cDNA (Clontech) and specific primers (22). The finished plasmid was verified by the chain termination method (23) using Sequenase 2.0 (U. S. Biochemical Corp.).

Protein Expression, Immunoblotting, and Proteolytic Analysis—Growth, maintenance, transfection (24), and fractionation of COS-7 cells was as described previously (18). Protein was determined by the method of Bradford (25) using bovine serum albumin as a standard. Membrane proteins or crude lysates were separated on 11% slab gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (26) and electro-transferred onto polyvinylidene difluoride membranes in Dunn’s buffer (27). For analysis of low molecular weight proteolytic fragments, the Tricine gel system of Schagger and von Jagow (28) was employed as indicated. Detection of G$\beta_5$ subunits employed the primary antibody SOS generated in rabbits against the synthetic dendocapetide SGSWDHLRW (conjugated to keyhole limpet hemocyanin) (29) corresponding to residues 342–353 at the C terminus of $\beta_5$. The antibody RA generated against a peptide corresponding to $\beta_5$ residues 256–265 has been described (30). Secondary detection employed 125I-Protein A followed by autoradiography on film (19) or a storage phosphor screen.
Selective Effector Activation by the G Protein β5 Subunit

RESULTS AND DISCUSSION

Functional Properties of β5 Cotransfected with γ2—The low degree of sequence homology between the brain-specific β5 subunit recently described by Simon and co-workers (3) and other Gβ subunits (e.g. only 53% amino acid identity with β1) suggests a possible specialization of β5 for interactions with Ga, Gγ, receptor, and/or effector molecules. The effector specificity of the β5 subunit was examined by cotransfection with γ2 in COS cells and compared with β1. As previously reported (3), transfection of the combination of β5 and γ2 produced a robust stimulation of cotransfected PLC-β2 (Fig. 1A). This stimulation was comparable with that of the β1/γ2 combination and was not seen with transfection of β5 or γ2 alone (Fig. 1A).

G protein β5 and β1 were then compared in their ability to activate the MAPK pathway in a γ-dependent fashion using a cotransfection paradigm employing HA-epitope-tagged ERK2 (12). The βγ complex of heterotrimeric G proteins activates the MAPK cascade in metazoans (12–14) as it does in yeast (35). Previous analysis by Hawes et al. (36) of Gβ subtypes 1–4 and multiple γ isoforms in transfected COS-7 cells showed a strong correlation between the ability of a specific βγ combination to activate PLC-β2 and its ability to activate the MAPK pathway. In contrast, we found that β5 was unable to stimulate MAPK activity in combination with γ2 despite the ability of β1 to do so under the same conditions (Fig. 1B and see below) and despite the efficacy of βγ/γ2 in the PLC assay (Fig. 1A). The inability of β5 to activate the MAPK pathway was not due to failure of expression of the β5 or HA-ERK2 proteins under the conditions of the MAPK assay as documented by immunoblots of the Triton lysates of transfected COS cells with specific antibodies (Fig. 1C and see below).

Analysis of Recombinant β5 by Immunoblotting and Limited Proteolysis—The ability of β5 to activate PLC-β2 in a γ-dependent fashion strongly implies its proper expression and assembly with γ2. To further exclude the possibility, however, that the failure of transfected β5 to activate the MAPK pathway was due to abnormal expression or folding of the recombinant polypeptide, additional analysis was performed using the anti-G protein antibody (Boehringer Mannheim).

Cleared cholate extracts of crude membrane preparations of transfected COS cells or BALB/c mouse brains were prepared by extraction with 1% (w/v) cholate, 10 mM EDTA, 50 mM Tris-Cl (pH 8.0) (buffer A) on ice for 30 min followed by centrifugation at 16,000 × g for 10 min. The preparation of bovine brain membrane cholate extract was as described (31, 32).

For limited proteolytic digestion of membrane detergent extracts, samples were diluted into buffer A and then incubated for 30 min at 37°C at a 1:30 or 1:40 (w/w) ratio of enzyme: extract protein. Reactions were terminated by the addition of denaturing sample buffer and boiling. The enzymes employed were 1:1-lysylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Sigma T-8642), endoproteinase Lys-C (Calbiochem 324715), and endoproteinase Glu-C (V8 protease) (Calbiochem 324713).

Phosphoinositol Phospholipase C Activity Assay—The PI-PLC activity of transfected cells was estimated by a modification of the procedure of Berridge et al. (33) as described previously (10, 34).

MAPK and JNK Activity Assays—The assays for MAPK and JNK activity were essentially as described by Crespo et al. (12) and Coso et al. (20), respectively. Approximately 2.5 × 10⁶ COS-7 cells were plated into 75-cm² flasks and incubated at 37°C overnight. On the following day, the cells were transfected by the DEAE-dextran method (24) using a total of 15 μg of DNA per cotransfection, typically including 5 μg of HA-ERK2 or HA-JNK (12, 20), 5 μg of Gγ, and 5 μg of Gβ. Vector DNA was added where necessary to keep the total amount of plasmid DNA per flask constant. The remainder of the assay was as described (12, 20) except that mouse monoclonal HA.11 (Berkeley Antibody Co.) was used for the immunoprecipitations (3 μl of HA.11 ascites fluid per 900 μl of detergent lysate).

**Fig. 1. Functional properties and expression of G protein β5 in COS cells.** A, PI-PLC stimulatory activity of G protein β1 and β5 compared in a cotransfection assay. COS cells in 75-cm² flasks were transfected with vector alone or with PLC-β1 (1.5 μg) either alone or in combination with β1 (10 μg), β5 (10 μg), γ2 (5 μg), and PI-PLC activity assayed as described under “Experimental Procedures.” The results shown represent the mean (± S.E.) value of triplicate determinations in a single experiment. Three additional experiments produced comparable results. B, MAPK stimulatory activity of G protein β1 and β5 compared in a cotransfection assay. COS cells in 75-cm² flasks were transfected with HA-ERK2 in combination with vector alone, or with PLC-β1 (1.5 μg) either alone or in combination with β1 (10 μg), β5, (10 μg), γ2 (5 μg), and β1/γ2. C, MAPK stimulatory activity of HA-ERK2 (HA-MAPK) and Gβγ in cotransfected COS cells. Aliquots (20 μg of protein) of the Triton detergent lysates of COS cells transfected under the conditions indicated in Fig. 1B were subjected to SDS-PAGE on 11% polyacrylamide gels and then immunoblotted with antibodies to the HA epitope (HA.11 mouse monoclonal) or the Gα C-terminal dodecapeptide (SGS rabbit polyclonal). Subsequent detection employed horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence as described under “Experimental Procedures.”
Selective Effector Activation by the G Protein β5 Subunit

Fig. 2. C-terminal Gβ5 antibody immunoblots of control and protease-treated membrane detergent extracts and comparison with Gβ1. A, left panel, immunoblot of cholate membrane extracts of control (Con) or transfected (β5 + γ2) COS cells (20 μg/lane), mouse (M), or bovine (B) brain (40 μg/lane) separated on an 11% SDS-PAGE gel and employing the Gβ5 C-terminal antibody SGS. Right panel, immunoblot of control and protease-treated bovine brain membrane cholate extract (50 μg/lane) separated on 11% SDS-PAGE gel and probed with either antipeptide antibody RA (against residues 256–265 of Gβ5), or antibody SGS as indicated. Samples were either kept on ice (Con) or treated with a 1:30 (w/w) ratio of TPCK-trypsin (Tryp), endoproteinase Lys-C (Lys), or endoproteinase Glu-C (V8) as described under "Experimental Procedures." Molecular mass of marker proteins (indicated in kilodaltons) are shown at the left of each panel. Detection of primary antibody was with 125I-Protein A followed by autoradiography. B, Tricine gel immunoblot of control and protease-treated cholate extracts from β5/γ2-transfected COS cells, mouse, and bovine brain with SGS antibody. Abbreviations are as in A. COS cell samples, 20 μg of protein/lane (3.5-h exposure), and brain samples, loaded at 60 μg of protein/lane (14-h exposure), were analyzed on the same Tricine gel (28). Digests were performed at a ratio of enzyme to extract protein of 1:40 (w/w) for 30 min at 37°C. Molecular masses of marker proteins (indicated in kilodaltons) are shown on the left as are positions of the top of the gel and the dye front (DF). Detection of primary antibody was with 125I-Protein A.

demonstrated by the ability of β5/γ2 and β5/γ7 to stimulate PLC-β2 activity above control levels in parallel functional assays (Fig. 3, lower right). In contrast to results with γ2, γ4, and γ7, cotransfection of γ3 with β5 produced no increment in PLC activity above control levels (Fig. 3, lower right).

Ability of β5 to Assemble with Different γ Isoforms and Activate PLC-β—Previous studies of βγ-responsive effectors comparing purified recombinant Gβγ complexes have found significant differences due to the γ component of the βγ heterodimer (40, 41). We therefore wondered if the inactivity of β5 in the MAPK assay was specific to the β5/γ2 combination and might be overcome by employing other γ isoforms. Because certain combinations of β and γ subunits fail to assemble as heterodimers (39, 42), we first compared three additional γ subunits with γ2 in their ability to assemble with β5 in cotransfected COS cells. Immunoblots of cholate extracts of COS cell membranes revealed no endogenous β5 immunoreactivity and no β5 signal resulting from transfection of any γ subunit alone (Fig. 3, upper panel), consistent with the results of Fig. 1C. Transfection of β5 alone or in combination with γ1 (transducin γ) produced only a faint SGS-reactive band at ~39-kDa which may reflect assembly of β5 with the endogenous pool of γ subunits (Figs. 1C and 3, upper panel). In contrast, cotransfection of β5 with γ2, γ4, or γ7 gave a reproducible increment of β5 immunoreactivity over that seen with β5 alone, with a rank order γ2 > γ4 = γ7 (Fig. 3). This increase in the steady state levels of membrane β5 seen with γ cotransfection presumably reflects increased stability and/or membrane targeting of the β subunit engendered by assembly with the exogenous γ. The reciprocal ability of cotransfected β to stabilize γ has been previously documented (43).

The functional association of β5 with γ4 and γ7 was also

body SGS generated against the C-terminal dodecapeptide of mouse β5. Like the N-terminal β5 antibody described by Watson et al. (3), SGS identified a major ~39-kDa band in immunoblots of detergent extracts of membranes prepared from β5/γ2 cotransfected but not control COS cells (Figs. 1C and 2A, left panel). This immunoreactive band comigrated with the major band in both mouse and bovine brain membrane extracts (Fig. 2A, left panel) consistent with previous reports (3). A faint upper SGS-reactive band of Mr ~90,000 was also noted in blots of mouse brain extract. The pattern of C-terminal fragments generated by limited proteolysis of bovine brain β5 was then compared with that of brain β1 by immunoblotting with specific antibodies (Fig. 2A, right panel). Such biochemical analysis has proven very useful in assessing the folding of native and recombinant G protein βγ complexes (19, 37–39). Whereas a stable ~26-kDa C-terminal fragment of β1 is identified by RA antibody (30) in a tryptic digest of bovine brain proteins, analysis of the same sample with the β5 C-terminal antibody SGS revealed no stable immunoreactive fragments (Fig. 2A, right panel). Limited digestion of the bovine brain samples with endoprotease Lys-C produced an SGS-reactive C-terminal fragment visible at the dye front, while digestion with V8 protease yielded a diffuse band centered at ~35 kDa. The RA immunoreactivity at ~36 kDa in the endoprotease Lys-C and V8 digests remained unchanged from control (Fig. 2A, right panel). Further analysis of the low molecular weight products on Tricine gels (28) revealed a major C-terminal SGS-reactive band of 12 kDa and a faint band of ~22 kDa in the endoprotease Lys-C digests of both mouse and bovine brains (Fig. 2B). In addition to the major 35-kDa band product, a minor product of ~11 kDa was noted in the Tricine gel blots of V8 protease digests of both brain samples (Fig. 2B). An identical pattern of SGS-reactive C-terminal fragments was seen upon limited proteolysis of detergent extracts of membranes prepared from COS cells cotransfected with β5 + γ2 (Fig. 2B). Taken together, these data demonstrate that the state of folding of the β5 polypeptide expressed in transfected COS cells inferred from selective proteolysis is indistinguishable from that of native β5 found in brain.
The G protein $\beta_5$ subunit fails to activate critical intermediates in the MAPK and JNK pathways in COS cells when complexed with $\gamma_2$, $\gamma_4$, $\gamma_5$, or $\gamma_7$ in $\beta\gamma$ heterodimers. In vertebrates, the G protein $\beta\gamma$ complex mediates effector regulation. The present results offer the strongest evidence to date that G protein $\beta$ subunits are sufficient to determine the effector selectivity of $\beta\gamma$ and suggest that the structural features of the $\beta\gamma$ complex mediating effector regulation may differ depending on the effector.
Selective Effector Activation by the G Protein βγ Subunit

...acting through Ras guanine nucleotide exchange factors including Sos (50) and Ras-guanine nucleotide releasing factor (51). The upstream mediators of the Ras- and Rac1-dependent activation of JNK by βγ subunits (15) remain unknown, but activation of a homologous pathway in S. cerevisiae appears to involve the direct interaction of βγ with the scaffolding protein Ste5p (52) and the guanine nucleotide exchange factor Cdc24p (53). It is tempting to speculate that the structure of βγ precludes its interaction with the mammalian counterparts of human phospholipase C-β, activation of JNK by Ras guanine nucleotide exchange factors involves the direct interaction of activation of a homologous pathway in S. cerevisiae. The inability of Gβ to activate JNK, a pathway linked to apoptosis in the inability of Gβ to activate JNK, a pathway linked to apoptosis in...