A C-terminal Mutant of the G Protein β Subunit Deficient in the Activation of Phospholipase C-β*

(Received for publication, May 31, 1996, and in revised form, July 3, 1996)

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The molecular mechanism by which the G protein βγ complex modulates multiple mammalian effector pathways is unknown. Homolog-scanning mutagenesis of the G protein β subunit was employed to identify residues critical for the activation of phospholipase C-β2 (PLC-β2). A series of chimeras was made by introducing small segments of the Dictyostelium β subunit into a background of mammalian β2 and tested in COS cell cotransfection assays for their ability to activate PLC-β2 and assemble with mammalian γ2. A chimera that contained four Dictyostelium β substitutions within the C-terminal 14 residues was unable to activate PLC-β2 when cotransfected with γ2 despite its demonstrable expression in a γ2-dependent manner. Cotransfection of the mutant blocked m2 muscarinic receptor activation of PLC by a pertussis toxin-sensitive pathway. This C-terminal mutant retained the ability, however, to stimulate the mitogen-activated protein kinase pathway. These results imply that activation of different βγ-responsive effectors is mediated by distinct domains.

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cells were harvested after 2 days, and the cell pellets were kept at -80°C until further analysis.

Upon thawing, the cells were lysed and fractionated into cytosolic and crude membrane fractions as described previously (27), and protein was determined by the method of Bradford (33) using bovine serum albumin as a standard. Cytosolic proteins were separated on 11% gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (34) and electrotransferred onto polyvinylidene difluoride membranes in Dunn's buffer (35). Detection of Gβ subunits employed primary antibodies generated against synthetic peptides corresponding to β1 residues 127–136 (KT) or residues 256–265 (RA) (36). Chimeras with a heterologous sequence replacing a portion of β1 corresponding to an immunogenic peptide were necessarily evaluated by the other antisera. Secondary detection employed 125I-Protein A followed by autoradiography on film or an enhanced chemiluminescence using goat anti-rabbit antibody coupled to horseradish peroxidase (Boehringer Mannheim). Limited tryptic digestion of cytosolic fractions was performed as described previously (28).

Phosphoinositol Phospholipase C Activity Assay—The PI-PLC activity of transfected cells was estimated by a modification of the procedure of Berridge et al. (37) as described previously (9). Subconfluent COS cells were transfected with combinations of 1.5 μg of human PLC-β2 cDNA (in vector pMT2) (24), 2–20 μg of wild-type β1, or one of the chimeras, and 5 μg of wild-type γ2 per 75-cm2 flask as indicated in the figure legends. In experiments like that shown in Fig. 2, 3 μg of m2 mAChR cDNA (25) was included, and γ-cDNA was omitted to assess the impact of wild-type and mutant G protein β and γ cDNAs on receptor-mediated activation of PLC-β2 via heterotrimeric G proteins composed of endogenous α and γ subunits. On the second day of transfection, the cells were trypsinized and replated in 12-well plates (4.5 cm2/well) at a cell density of ~4.0 × 104 cells per well, and the cells were allowed to reattach. Approximately 20 h before the assay, the medium was replaced with DMEM without insulin (Life Technologies, Inc.) supplemented with 10% dialyzed fetal bovine serum and 10 mM LiCl in DMEM without inositol for 25 min at 37°C. For experiments involving pertussis toxin, 100 ng/ml holotoxin (List) was included in this medium for overnight incubation. For the assay, the cells were incubated in 400 μl of 10 mM LiCl in DMEM without inositol for 25 min at 37°C. For experiments involving m2 muscarinic receptor transfectants, this medium was supplemented with 50 μM carbacid, or not, as indicated in the figure legends. Isolation of inositol phosphates was by the method of Berridge et al. (37) as modified by Katz et al. (9).

MAPK Activity Assay—The assay for MAPK activity was essentially as described by Crespo et al. (11). Approximately 1.6 × 106 COS-7 cells were plated on 100-mm plates and then incubated at 37°C overnight. On the following day, the cells were transfected by the DEAE-dextran method using a total of 12 μg of DNA per cotransfection, including 2 μg of pMT2-EGF (21) epidermal growth factor-tagged human PLC-β2 and 10 μg of human PLC-β1 cDNAs produced a nearly 10-fold enhancement of PLC activity consistent with previous reports (9). In transfection experiments employing increasing amounts of wild-type β1 cDNA with constant amounts of γ2 and PLC-β2 cDNA, it was found that 10 μg of plasmid gave a near-saturating signal in this assay (Fig. 2A, inset). Thus, for screening of chimeric β constructs, 10 μg of plasmid was used per transfection to make the assay most sensitive for detection of loss of function.

RESULTS

Chimera Construction—In an attempt to identify residues of the G protein β subunit critical for activation of phospholipase C-β, we introduced segments of phylogenetically remote β sequence into a background of mammalian β1 by homolog-scanning mutagenesis with the aim of creating loss of function mutants. After unsuccessful trials employing segments of STE4, the Gβ homolog from S. cerevisiae, we turned to Dicyostelium β, which is 67% identical to mammalian β1 (39), as the source of heterologous β sequence. In the slime mold Dicyostelium, there is a single form of phospholipase C which is most homologous to the mammalian PLC-β1 isoform (40, 41). Furthermore, Dicyostelium PLC appears to be under the control of a G protein α and not β (42, 43).

An initial series of 15 Dicyostelium β/mammalian β1 chimeras was constructed as shown in Fig. 1. The series of chimeras incorporates all the residues which are nonconserved between the two parental subunits, in clusters of 2 to 27 per chimera arranged according to primary sequence. The boundaries between the Dicyostelium β and β1 sequences in chimeras A through O were chosen prior to the elucidation of the three-dimensional structure of the βγ complex (44–46).

Ability of β Chimeras to Activate PLC-β—Screening for possible loss of function mutations among the 15 chimeras shown in Fig. 1 involved two stages: (i) an initial activity screen to evaluate βγ-dependent PLC activation following cotransfection of wild-type or chimeric β in combination with wild-type γ2 and human PLC-β2, and (ii) assessment of γ-dependent protein expression by cotransfection of chimeric β constructs with nonphosphorylated γ (27, 28). Cotransfection of G protein β and γ cDNAs has been used by others to evaluate the βγ-dependent activation of PLC-β (9, 47). As shown in Fig. 2A, the transfection of COS cells with PLC-β2 and either γ2 or β1 alone had little effect on PLC activity, whereas cotransfection of both γ2 and β2 cDNAs produced a nearly 10-fold enhancement of PLC activity consistent with previous reports (9). In transfection experiments employing increasing amounts of wild-type β1 cDNA with constant amounts of γ2 and PLC-β2 cDNA, it was found that 10 μg of plasmid gave a near-saturating signal in this assay (Fig. 2A, inset). Thus, for screening of chimeric β constructs, 10 μg of plasmid was used per transfection to make the assay most sensitive for detection of loss of function.

The results with the 15 Dicyostelium β/mammalian β1 chimeras are shown in Fig. 2A. A wide range of activities is evident among the 15 chimeras with the lowest PLC activity demonstrated by chimera O. The level of PLC activity seen with chimera O transfection was most often at or below the level of activity seen with control (Fig. 2A). Chimeras A also demonstrated consistently low activity, but was always more active than chimera O. The levels of PLC activity seen with chimeras B, E, F, G, I, J, and L approached or exceeded that of wild-type β2, while chimeras C, D, H, K, M, and N demonstrated intermediate activities. Whereas the ability of chimeric β constructs such as B, E, F, G, I, J, and L to activate PLC strongly implies that they are expressed and assembled with γ, the lower activity of the other constructs may be due, at least in part, to low levels of expression. Thus, the second stage of screening the chimeras involved an estimation of the activity of their γ-dependent expression in cotransfected COS cells. For this purpose, a nonphosphorylated point mutant of γ2 (γ2 C685→γ2

Fig. 1. Sequence comparison of mammalian β1 and Dicyostelium β with boundaries of the "sliding window" of substituted sequence in chimeras A through O. Sequence alignment was performed by the GAP algorithm of the Genetics Computer Group of the University of Wisconsin (39). For each of chimeras A through O, only the sequence within the box was from Dicyostelium β (23) with any sequence preceding or following the box derived from β1 (21).
(\(\gamma^+\)) was employed (27). The observation that nonisoprenylated \(\gamma\) subunits direct expression of cotransfected \(\beta\) subunits to the cytosol has been used extensively to estimate the ability of wild-type and mutant subunits to assemble (27, 28, 48).

Estimation of the \(\gamma\)-dependent Expression Level of the \(\beta\) Chimeras A and O—The results of experiments in which wild-type \(\beta\) or chimeras A or O were cotransfected with \(\gamma^+\) in COS cells are shown as immunoblots of the cytosolic fractions in Fig. 2B. Transfection of vector or \(\beta_1\) alone produced no significant \(\beta\) immunoreactivity in the cytosol, whereas transfection of \(\gamma^+\) alone resulted in faint \(\beta\) immunoreactivity representing translocation of endogenous \(\beta\) subunits, as described previously (27, 28, 48) (Fig. 2B). In contrast, cotransfection of both wild-type \(\beta_1\) and \(\gamma^+\) resulted in a large increase in cytosolic \(\beta\) immunoreactivity as seen previously (27, 28, 48) (Fig. 2B). Cotransfection of both chimera A with \(\gamma^+\) and chimera O with \(\gamma^+\) produced weak \(\beta\)-immunoreactive signals (Fig. 2B). Chimera O was consistently expressed to a higher level than A, however (Fig. 2B), even though its PLC-\(\beta\) stimulatory activity was lower (Fig. 2A).

Chimera O was therefore used as a starting point to look for a more highly expressed variant with the same loss of function phenotype. Construction and testing of 14 additional variants of chimera O (data not shown) identified chimera O\(_{2,3,4}\), containing only four heterologous Dictyostelium residues at codons 327, 328, 335, and 340 of \(\beta_1\), which was expressed to a higher level than chimera O (Fig. 2B). Like chimera O, chimera O\(_{2,3,4}\) was severely impaired in its ability to activate PLC-\(\beta\), exhibiting <10% of wild-type activity at 20 \(\mu\)g of transfected plasmid (Fig. 2D). The corresponding maximal expression of chimera O\(_{2,3,4}\) was ~40% of wild-type \(\beta\), as estimated by quantitation of immunoblots of CHAPS extracts of crude particulate fractions (data not shown). Limited tryptic digestion of COS cell cytosolic fractions containing \(\beta_1/\gamma^+\) and chimera O\(_{2,3,4}/\gamma^+\) produced stable ~26-kDa C-terminal \(\beta\) fragments indicative of properly folded \(\beta\) polypeptide chains (49–51) (Fig. 2C). Chimeras B through N also demonstrated variable levels of cytosolic \(\beta\) immunoreactivity when cotransfected with \(\gamma^+\), in a rank order roughly parallelizing their relative activity in the PLC assays of Fig. 2A (data not shown).

Dominant Negative Effects of Chimeras O and O\(_{2,3,4}\) on Receptor-stimulated PLC Activation—G protein \(\beta\)-mediated stimulation of PLC by a pertussis toxin-sensitive pathway is seen upon activation of a variety of \(G\)-coupled receptors, including the m2 muscarinic receptor (9). We reasoned that if chimeras O and O\(_{2,3,4}\) were deficient in their ability to activate PLC-\(\beta\), retained their ability to interact with endogenous elements of this receptor-driven pathway they might exhibit dominant inhibitory effects on agonist signaling. Experiments were therefore performed in which carbachol-induced PLC activation was assessed in COS cells cotransfected with cDNAs for PLC-\(\beta_2\) and m2 mAChR without or with wild-type \(\beta_1\) or chimeras O or O\(_{2,3,4}\). As seen in Fig. 3, carbachol induces an activation of PLC activity in m2 mAChR-transfected cells which is largely abolished by pretreatment with pertussis toxin. Co-

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**Fig. 2.** Identification and characterization of C-terminal chimeras O and O\(_{2,3,4}\) deficient in PLC-\(\beta\) stimulation. A, PI-PLC activity of wild-type \(\beta_1\) and chimeras A through O determined in a cotransfection assay. COS cells in 75-cm² flasks were transfected with PLC-\(\beta_2\) (1.5 \(\mu\)g) either alone (Control) or in combination with \(\gamma_2^+\) (5 \(\mu\)g) alone, \(\beta_1\) (10 \(\mu\)g) alone, or \(\gamma_2^+\) in combination with \(\beta_1\) or one of the chimeras A through O (10 \(\mu\)g) as indicated, and PI-PLC activity was assayed as described under "Experimental Procedures." Inset, PI-PLC activity in response to increasing amounts of wild-type \(\beta_1\) cDNA (0–20 \(\mu\)g as indicated) cotransfected with constant PLC-\(\beta_2\) (1.5 \(\mu\)g) and \(\gamma_2^+\) (5 \(\mu\)g) cDNA. B, \(\gamma_2^+\)-dependent cytosolic expression of wild-type \(\beta_1\) and chimeras A, O, and O\(_{2,3,4}\). RA antibody immunoblot of the soluble fraction (10 \(\mu\)g of protein/lane) of COS cells transfected with the indicated constructs and either maintained on ice or incubated with trypsin (1 \(\mu\)g for 30 min at 37°C prior to SDS-PAGE on 11% polyacrylamide gel as described previously (28). Antibody detection was by 125I-Protein A chemiluminescence as under "Experimental Procedures." Arrow indicates molecular mass of bovine brain G\(\beta\)i standard (not shown). C, limited tryptic digestion of cytosolic fractions of COS cells cotransfected with G\(\gamma_2^+\) and \(\beta_1\) or chimeras A, O, or O\(_{2,3,4}\). RA antibody immunoblot of the soluble fraction (25 \(\mu\)g of protein per lane) of COS cells transfected with the indicated constructs and either maintained on ice or incubated with trypsin (1 \(\mu\)g for 30 min at 37°C prior to SDS-PAGE on 11% polyacrylamide gel as described previously (28). Antibody detection was by 125I-Protein A as described under "Experimental Procedures." *Indicated to the left are the molecular masses of intact G\(\beta_1\) subunit (36 kDa) or its major C-terminal tryptic fragment (26 kDa). D, PI-PLC activity as a function of transfected wild-type \(\beta_1\) or chimera O\(_{2,3,4}\) cDNA. COS cells were transfected with a fixed amount of PLC-\(\beta_1\) (1.5 \(\mu\)g) and \(\gamma_2^+\) (5 \(\mu\)g) cDNA, without or with increasing amounts of plasmid cDNA for wild-type \(\beta_1\) (open squares) or chimera O\(_{2,3,4}\) (solid circles) (0–20 \(\mu\)g as indicated), and PI-PLC activity was measured as described under "Experimental Procedures."
transfection of wild-type \( \beta_1 \) enhances this effect, perhaps by promoting the formation of \( m2 \)-coupled wild-type G protein heterotrimers. It should be noted that the transfected \( \beta \) subunit must assemble with endogenous COS cell G protein \( \alpha \) and \( \gamma \) subunits for its effect since neither cDNA is being supplied exogenously. In contrast to the effect of wild-type \( \beta_1 \), cotransfection of chimeras O and O₂,₃,₄ abolished the carbachol-induced activation of PLC, producing an effect resembling that of pertussis toxin (Fig. 3). These two mutant \( \beta \) subunits function as dominant negative inhibitors of the \( m2 \) muscarinic-PLC stimulatory pathway in this system.

Effects of Chimera O₂,₃,₄ on Mitogen-activated Protein Kinase—It has recently been demonstrated that, as in budding yeast, the mammalian MAPK pathway can be activated by the \( \beta \gamma \) complex of heterotrimeric G proteins (11–13). We therefore questioned whether the loss of function of chimeras O and O₂,₃,₄ with respect to PLC-\( \beta_2 \) stimulation would extend to the MAPK pathway. To this end, COS cells were cotransfected with cDNAs encoding HA epitope-tagged ERK2, wild-type \( \gamma_2 \), and either wild-type \( \beta \) or one of chimeras A, O, or O₂,₃,₄, and ERK2 kinase activity was measured in the HA immunoprecipitates as described (11). As found previously for wild-type \( \beta_1 \) (11), both chimeras O and chimera O₂,₃,₄ were capable of MAPK activation and required \( \gamma \) cotransfection for this activity (Fig. 4A). Chimera A, however, was inactive, as in the PLC assay (Fig. 2A), suggesting a broader deficiency of function in this mutant. In contrast to its inactivity over a wide range of transfected cDNA in the PLC assay (Fig. 2D), chimera O₂,₃,₄ was comparable to wild-type \( \beta_1 \) in its ability to activate the kinase activity of HA epitope-tagged ERK2 (Fig. 4B).

**DISCUSSION**

Homolog-scanning mutagenesis has been used to generate loss-of-function mutants of the Gβ₁ subunit to investigate domain critical for the activation of PLC-\( \beta_2 \). Dictyostelium \( \beta \) was used as the source of heterologous sequence because the single Dictyostelium PLC isoform (homologous to the mammalian PLC-\( \delta \) subclass) appears to be under the control of a G protein \( \alpha \) and not \( \beta \gamma \) (42, 43). Of the 15 initial Gβ chimeras outlined in

![Fig. 3. Dominant inhibitory activity of chimeras O and O₂,₃,₄ on receptor-mediated PLC activation. COS cells were transfected with vector alone, or PLC-\( \beta_2 \) (5 \( \mu \)g) and m2 muscarinic receptor (3 \( \mu \)g) alone or in combination with \( \beta_1 \), chimera O, or chimera O₂,₃,₄ as indicated. PLC activity was determined as described under “Experimental Procedures” without (open bars) or with (solid bars) 50 \( \mu \)m carbachol. Data are expressed as fold stimulation relative to the activity of PLC-\( \beta_2 \) transfected alone (not shown). In samples treated with pertussis toxin (PTX), 100 ng/ml holotoxin (List) was included in the cell culture medium overnight prior to the assay.

![Fig. 4. MAPK activation induced by overexpression of wild-type or mutant \( \beta \gamma \) complexes. A, COS cells were transfected with HA-ERK2 (2 \( \mu \)g) and vector alone (Control) or the indicated constructs (5 \( \mu \)g each) and MAPK activity measured as described previously (11) by quantification of \(^{32}P\)-phosphorylated myelin basic protein substrate in dried SDS-PAGE gels by PhosphorImager analysis. Data are expressed as fold stimulation relative to the activity of \( \gamma_2 \) alone. The activity induced in untransfected control cells assayed with 100 ng/ml epidermal growth factor (EGF) is shown. B, MAPK activity in COS cells cotransfected with HA-ERK2 (2 \( \mu \)g) and \( \gamma_2 \) (5 \( \mu \)g) cDNA alone or with increasing amounts of wild-type \( \beta_1 \) (open symbols) or chimera O₂,₃,₄ (solid symbols) plasmid cDNA as indicated. Data are expressed as fold stimulation relative to the activity of \( \gamma_2 \) alone.

Fig. 1, it is remarkable that so many were capable of PLC-\( \beta_2 \) stimulation at or near wild-type levels (Fig. 2A). This suggests that the interaction of \( \beta_2 \) with PLC-\( \beta_2 \) may involve a limited domain or domains and is therefore insensitive to mutations in many parts of Gβ.

Our strategy succeeded in the identification of a segment in the extreme C terminus of Gβ₁, which when substituted with a minimum of four homologous residues from Dictyostelium Gβ, produced mutant \( \beta \gamma \) complexes deficient in their ability to stimulate phospholipase C-\( \beta_2 \) in a transient transfection model system. Because our strategy relied on differences in the primary sequences of Gβ₁ and Dictyostelium Gβ, it would be expected to miss any residues critical for PLC-\( \beta \) activation which happened to be conserved between the divergent \( \beta \) subunits. The C-terminal chimera O₂,₃,₄ identified in this study was nevertheless competent to assemble with \( \gamma \) as evidenced by its ability to stimulate the MAPK pathway in a \( \gamma \)-dependent manner, translocate to the cytosol upon cotransfection with nonisoprenylated \( \gamma \), and resist tryptic proteolysis with generation of a stable 26-kDa C-terminal fragment. These latter findings exclude the possibility that chimera O₂,₃,₄ is globally misfolded and stand in contrast to results with another chi-
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...maling to stimulate PLC activity in the cotransfection assay, chimera A. Chimera A has multiple substitutions in the coiled-coil domain of Gβ (44–46), a region where point mutations blocking βγ assembly have been demonstrated previously (52), and its generalized lack of function appears to result from failure to form a stable βγ complex. In light of our findings with chimera O2,3,4 it is interesting to note that a six-amino acid deletion in the extreme C-terminal region of the STE4 is reported to block βγ-mediated effector signaling in yeast (19).

In addition to the loss-of-function exhibited by chimeras O and O2,3,4 with respect to PLC-β stimulation, these two mutants demonstrated a dominant inhibitory activity when coexpressed with wild-type signaling elements. Chimeras O and O2,3,4 functioned as dominant inhibitors of carbachol-stimulated PLC-β2 activation when cotransfected with the m2 muscarinic receptor (Fig. 3). These inhibitory effects of chimeras O and O2,3,4 may result from competition with wild-type β complexes at the level of the effector (PLC-β), the receptor, or the Gα subunit. Definitive analysis of these possibilities awaits reconstitution of purified wild-type and mutant Gβ2 by PLC-β, Gα, and receptor.

The molecular basis for the phenotype exhibited by chimera O2,3,4 remains unclear. One possibility would be that the C-terminal β1 residues mutated in chimera O2,3,4 (Val327, Ala328, Phe329, and Asn330) comprise all or part of a signaling domain with PLC-β2. Alternatively, the effect of the mutations in chimera O2,3,4 may result from indirect alteration of a single effector interface, may present different facets to different M- and O2,3,4 may result from competition with wild-type O2,3,4 may result from indirect alteration of a...