Ribozyme-mediated Suppression of the G Protein γ7 Subunit Suggests a Role in Hormone Regulation of Adenylcyclase Activity*

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Human HEK 293 cells present a simple and tractable system to directly test the hypothesis that the G protein γ subunits contribute to the specificity of receptor signaling pathways in vivo. To begin to elucidate the functions of the individual γ subunits in these cells, a ribozyme strategy was used to specifically inactivate the mRNA encoding the γ7 subunit. A phosphorothioated DNA-RNA chimeric hammerhead ribozyme was constructed and analyzed for specificity toward the targeted γ7 subunit. In vitro cleavage analysis of this ribozyme revealed a highly efficient cleavage activity directed exclusively toward the γ7 RNA transcript. In particular, this ribozyme did not result in cleavage of the γ12 RNA transcript, which is 75% identical to the γ7 RNA transcript. Using a transient transfection assay, in vivo analysis of this ribozyme showed a specific reduction in both the mRNA and protein expression of the γ7 subunit in HEK 293 cells. Coincident with this loss in γ7 subunit, there was a specific reduction in the protein expression of the β7 subunit, suggesting that the β7 and γ7 subunits may functionally interact to form a βγ dimer in vivo. Functional analysis of the consequences of ribozyme-mediated suppression of the γ7 subunit expression indicated that it was associated with significant attenuation of isoproterenol-, but not prostaglandin E1-stimulated adenyl cyclase activity. Suppression of the γ7 subunit expression had no effect on carbachol- and ATP-mediated stimulation of phosphatidylinositol turnover. Taken together, these results not only indicate the feasibility of using the ribozyme technology to determine the roles of individual γ subunits in receptor-G protein-effector pathways in vivo, but they point to a specific role of the γ7 subunit in the regulation of adenylcyclase activity in response to isoproterenol.

While the role of heterotrimeric G proteins in signal transduction is well established, a central question that remains to be resolved is how the specificity of signal transduction from receptor to G protein to effector is encoded in the protein-protein interactions between an expanding number of signaling partners. Perhaps the simplest way to encode the specificity of signal transduction would be for each type of receptor to interact with a specific G protein α and βγ subunit combination to converge on one or more types of effectors. In this scenario, the specific combination of G protein α and βγ subunits would provide the level of selectivity that is needed to interact with the different types of receptors. That βγ subunits, as well as α subunits, contribute to the selectivity of interaction with receptor is supported by several studies. Reconstitution of receptors with G proteins consisting of common α subunits but distinct βγ subunits shows a range of differences in coupling (1–3). Moreover, the differences in coupling are attributable to the γ component, with both the prenyl group and the structure of the γ subunit tail being specific determinants of receptor-G protein interaction (4, 5). While these reconstitution studies have begun to provide invaluable information on the ability of specific G protein α or βγ subunits to interact with particular receptors, the results indicate and probably overestimate the potential interactions that actually occur in native membranes or cellular systems. In fact, it appears that the βγ subunits do not readily substitute for one another in vivo. Antisense oligonucleotide blockage of expression of different βγ subunits in GH3 pituitary cells has been shown to selectively disrupt signaling through either the muscarinic or somatostatin receptor pathways, both of which couple to inhibition of the same Ca2⁺ current. These results clearly suggest that the muscarinic and somatostatin receptor pathways involve distinct βγ dimers (6, 7). Presumably, this requirement for particular βγ subunits reflects a specificity at the receptor-G protein interface that does not allow their substitution. Taken together, these and other findings strongly support a major role for the G-protein βγ subunits in receptor recognition and, hence, a role in the assembly of physiologically appropriate receptor signaling pathways.

Since the βγ subunits exist as a tightly associated complex under physiological conditions and function as a single entity, the relative contributions of the individual β and γ components in receptor recognition have been difficult to investigate. The complexity of this task is reflected by the diversity and variety of potential interactions of the β and γ subunits that have been described (8, 9). Both the β and γ subunits belong to large multigene families. The known existence of at least 6 β subunits (10, 11) and 11 γ subunits (12, 13) provides the potential to generate more than 60 possible combinations of βγ dimers. To begin to assign specific βγ subunits to various receptor-G protein-effector pathways in vivo, we have been developing a reverse genetics approach to specifically inactivate G protein γ subunits at the mRNA level. In this study, we have described the use of the ribozyme technology to specifically inactivate the mRNA encoding the γ7 subunit in HEK 293 cells to directly test the hypothesis that the particular subtype of γ subunit present in a heterotrimeric G protein is a determinant of the specificity of receptor signaling pathways. The results presented in this paper demonstrate for the first time the feasibility of using the newly described ribozyme technology to determine the roles of individual γ subunits in receptor-G protein-effector pathways in vivo.

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Ribozyme-mediated Suppression of G Protein \( \gamma \) Expression

**EXPERIMENTAL PROCEDURES**

**Ribozyme Synthesis**—A chimeric DNA-RNA hammerhead ribozyme targeted against the G protein \( \gamma \) subunit mRNA was chemically synthesized in 1-\( \mu \)mol scale on a model 394 DNA/RNA synthesizer (Applied Biosystems Division of Perkin Elmer, Foster City, CA). Phosphorothioate linkages were introduced by sulfurization with 3H,12-benzodithiol-3-one-1,1-dioxide. The ribozyme was autoclaved on the synthesizer using sodium hydroxide/ethanol (3:1) for 2 h, and excess hydroxide protecting groups were removed by heating at 65 °C for 3 h. The ribozyme was desilylated with neat triethylamine trihydrofluoride and desalted by precipitation in n-butyl alcohol according to a published procedure (14). The sequence of this ribozyme is shown, with the ribonucleotides designated by lowercase letters and the deoxyribonucleotides by capital letters (Fig. 1).

**In Vitro Ribozyme Cleavage**—To generate an *in vitro* \( \gamma \) RNA transcript, a pBSK plasmid containing the \( \gamma \) subunit cDNA was linearized as template, and the transcription reaction was carried out with T3 RNA polymerase as recommended by the supplier (Promega, Madison, WI). The \( \gamma \) RNA transcript was labeled by including 50 \( \mu \)Ci of \( [\alpha-\text{32P}]\)CTP (10 mCi/ml, 3000 Ci/mmol, NEN Life Science Products) in the reaction. To perform the *in vitro* cleavage experiment, the ribozyme and RNA transcript were denatured in separate tubes in 50 mM Tris-HCl, pH 8.5, at 85 °C for 1 min. The contents of the two tubes were then mixed together in the presence of 20 mM MgCl\(_2\) and incubated at 42 °C for 1 h. The ratio of ribozyme to target RNA (50 nM) was varied over a 32-fold range. The specific activity of ribozyme was determined by exposure to Biomax MS film (Eastman Kodak Co.), and the intensities of the immunodetectable bands were quantified using the Molecular Dynamics PhosphorImager SI.

**Northern Slot Blotting for RNA Analysis**—The HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Cells were plated and grown in either 100-mm dishes (for analysis of protein suppression) or six-well plates (for analysis of mRNA suppression) and transfected at approximately 80% confluency. Prior to transfection, the cells were preincubated with serum-free Dulbecco’s modified Eagle’s medium for 1 h. Cells were then transfected for 5 h at 37 °C with fresh serum-free media containing premixed ribozyme (2 \( \mu \)M) and the cationic lipid, LipofectAMINE (15 \( \mu \)g/ml, Life Technologies, Inc.). At 5 h post-transfection, heat-inactivated fetal calf serum was added to a final concentration of 6%. Subsequently, at 5, 24, 30, and 48 h after transfection, the dishes were supplemented with additional 0.5 \( \mu \)M ribozyme such that the total concentration of added ribozyme at 48 h was equivalent to 4 \( \mu \)M. Control cells were treated identically but without added ribozyme.

**Northern Slot Blotting for RNA Analysis**—To determine the effect of ribozyme exposure on \( \gamma \) mRNA levels, a 3-\( \mu \)g aliquot of total RNA was slot blot hybridized with \( \gamma \) subunit RNA probes. Ribozyme RNA was prepared from the transiently transfected cultured cells using the RNeasy kit from Qiagen Inc., as recommended by the manufacturer. The specificity of \( \gamma \) subunit probes was tested by Southern blot analysis against different \( \gamma \) cDNAs, including \( \gamma_2 \), \( \gamma_5 \), \( \gamma_7 \), \( \gamma_9 \), \( \gamma_{11} \), and \( \gamma_{12} \). The sequences of the probes that were used in this study were as follows: \( \gamma_2 \), 5’-TGGCTATCTGGTTAGGCGC-3’; \( \gamma_5 \), 5’-CTGGTAGCAGATCGGATGCCC-3’; elongation factor 1a (EF1a), 5’-CGTTGAACCGCTCATTGCGCC-3’; the probes were 3’-end-labeled with terminal deoxynucleotidyl transferase (Promega) and [\( \alpha-\text{32P}]\)dATP (10 mCi/ml, 3000 Ci/mmole, NEN Life Science Products). The membrane was prehydrated with QuikHyb hybridization solution from Stratagene for 15 min at 30 °C and then hybridized with labeled probe for another 2 h at 30 °C. After hybridization, the membrane was washed twice with 2 \( \times \) SSC, 0.1% SDS at room temperature and then washed once with 0.5 \( \times \) SSC, 0.1% SDS for 30 min at 38 °C. The hybridization signal was analyzed and quantitated using a Molecular Dynamics PhosphorImager SI. Values shown throughout this study are expressed as the mean \( \pm \) S.E., unless otherwise noted.

**Cyclic AMP Accumulation**—Approximately 1.4 \( \times \) 10⁶ HEK 293 cells were plated into six-well plates and transfected with \( \gamma \), ribozyme as described above. At 72 h post-transfection, the cells were stimulated with 10 nM forskolin in serum-free Dulbecco’s modified Eagle’s medium. The cells were subsequently stimulated with a variety of hormonal stimuli in the presence of 10 mM LiCl for 30 min and stopped by the addition of 1 M of ice-cold 6% trichloroacetic acid. The precipitated cellular proteins were removed by centrifugation, and the supernatants were extracted three times with 2 volumes of diethylther. The total cyclic AMP was determined by retention and elution from Dowex AG1-X8 (100–200 mesh, formate form) columns using a standard procedure (21) and quantitated by liquid scintillation counting.

**RESULTS**

Human HEK 293 cells are an excellent model cell system for studying the role of heterotrimeric G protein subunits in signal transduction. They contain a variety of endogenous receptor-G protein-effector signaling pathways. Of particular interest, we have found that they express multiple receptors that regulate either adenylylcyclase or phospholipase C activity, thereby allowing us to test the hypothesis that receptors that converge on a common effector may utilize G proteins composed of the same \( \alpha \) subunit but distinct \( \beta \gamma \) subunits. Furthermore, consistent with the variety of endogenous receptor-G protein-effector signal pathways, we have identified a number of G protein \( \gamma \) subunits that are expressed at both the mRNA and protein levels in these cells. As shown in Table I, 7 of the 11 known \( \gamma \) subunits have been detected at this RNA level, with 5 of the \( \gamma \) subunits also being detected at the protein level. This varied complement of \( \gamma \) subunits allows us to directly test the hypothesis that the \( \gamma \) subunits contribute to the specificity of signaling pathways in *vivo*. To begin to elucidate the functions of the individual \( \gamma \) subunits in these cells, we have initially focused on the \( \gamma_7 \) subunit for two reasons. First, the \( \gamma_7 \) subunit is widely expressed in a number of tissues, thereby suggesting a possible role of this protein in a common receptor-effector pathway.
Second, the γ subunit is readily detected at both the RNA and protein levels in these cells, thereby allowing its fate to be easily followed.

Design and Construction of the γ Ribozyme—To determine the functional role of the γ subunit, we designed and utilized a hammerhead ribozyme that was targeted to the GUC sequence at positions +3 to +5 in relation to the transcription start site of the mRNA encoding the γ subunit. Since this study was ultimately aimed at using the ribozyme in vivo, the ribozyme was chemically synthesized as a chimeric DNA-RNA construct to improve resistance to endonucleases. As shown in Fig. 1, the constructed chimeric DNA-RNA ribozyme consisted of a catalytic core containing ribonucleotides (in lowercase letters) and flanking sequences containing deoxyribonucleotides (in capital letters), which hybridize to the γ mRNA transcript by Watson-Crick base pairing. In addition, we examined the effect of adding two phosphorothioate linkages to the 3'-end of the chimeric DNA-RNA ribozyme on improving the stability and cellular delivery of the ribozyme in vivo.

Catalytic Activity of γ Ribozyme in Vitro—To confirm that the ribozyme was capable of cleaving the targeted γ RNA, the catalytic activity of the γ ribozyme was examined using an in vitro synthesized γ RNA transcript. As shown in Fig. 2A, the RNA transcript synthesized from the γ cDNA generated a product of 418 bases that includes 21 bases of the multicloning site, 172 bases of 5'-untranslated sequence, 203 bases of translated sequence, and 22 bases of 3'-untranslated sequence. Upon addition of the ribozyme to the γ RNA transcript, two fragments of 220 and 198 bases were produced, corresponding to the predicted fragment sizes for cleavage at the targeted GUC site at positions +3 to +5 (Fig. 2A). This result indicated that ribozyme-mediated cleavage of the γ RNA transcript was site-specific. Since the ratio of ribozyme to template has previously been shown to influence the efficiency of cleavage (22–24), we next varied the ratio of ribozyme to template over a 1000-fold range to optimize conditions for cleavage by the γ ribozyme. As shown in Fig. 2A, over 95% of the template was converted to cleavage products with a 10-fold or higher excess of ribozyme to template. Accordingly, the time course of cleavage was examined at a ribozyme:template ratio of 20:1. As shown in Fig. 2B, the majority of the template was cleaved within 15 min of exposure to ribozyme, with essentially complete cleavage within 1 h. Taken together, these results confirm that the designed γ ribozyme elicited the expected catalytic activity.

Specificity of γ Ribozyme in Vitro—Since the γ subunit is a member of a large, multigene family, it was necessary to validate that the γ ribozyme cleaved the γ RNA transcript in a gene-specific manner. Therefore, we evaluated the specificity of the γ ribozyme cleavage against in vitro synthesized γ, γ, γ, γ, γ, and γ RNA transcripts. (The in vitro synthesized γ, γ, and γ RNA transcripts were not examined, since their corresponding mRNAs are not expressed in HEK 293 cells.) As shown in Fig. 3, the sizes of the full-length RNA transcripts varied from 418 to 1632 bases. No cleavage of the RNA transcripts occurred in the absence of the γ ribozyme (Fig. 3, Control panel). Upon addition of the γ ribozyme, cleavage of the γ RNA transcript into fragments of the expected sizes was observed (indicated by arrows in the right panel labeled γ). Of particular importance, no cleavage of the γ, γ, γ, γ, γ, and γ RNA transcripts occurred in the presence of the γ ribozyme. These data demonstrate that the γ ribozyme specifically cleaved its own template in a gene-specific manner.

Effect of γ Ribozyme on the Level of γ mRNA in Vivo—To examine its activity in vivo, the γ ribozyme was introduced into HEK 293 cells by transient transfection with LipofectAMINE. At 24, 48, or 72 h after transfection, total RNA was extracted from cells, and the level of γ RNA was determined by Northern slot blotting analysis. Since ribozymes have been shown to be rapidly degraded in vivo (25), two modifications were made to try to improve the stability of the γ ribozyme. The first modification was to design the ribozyme as a chimeric DNA-RNA molecule (26), while the second modification was to
add two phosphorothioate linkages at the 3'-end of the chimeric DNA-RNA ribozyme (27). Fig. 4 shows that the degree of RNA suppression achieved by the γ7 ribozyme was highly dependent on this latter modification. As shown in panel A, the phosphorothioate-modified γ7 ribozyme (Thio RZ) was much more efficacious in suppressing the level of γ7 RNA than the γ7 ribozyme alone (RZ). The greatest reduction with the phosphorothioate-modified γ7 ribozyme was observed at 48 h after transfection, where the level of the γ7 RNA was reduced to 40 ± 9% (n = 3) of its control level. Taken together, these results demonstrate the superior efficacy of the phosphorothioate-modified γ7 ribozyme over the γ7 ribozyme alone in suppressing the level of the γ7 RNA in vivo. Accordingly, the phosphorothioate-modified γ7 ribozyme was used in all of the subsequent experiments.

To establish that the specificity of RNA cleavage was maintained upon in vivo delivery of the phosphorothioate-modified γ7 ribozyme, we examined the effect of transfection with the phosphorothioate-modified γ7 ribozyme on the levels of other RNAs that do not contain the predicted cleavage site. EF1α is expressed in a wide variety of cells at a relatively constant level. As shown in Fig. 4B, transfection of cells with the γ7 ribozyme did not reduce the level of the EF1α RNA (112 ± 18%). As a more important control for the specificity of the γ7 ribozyme, we also examined the effect of the γ7 ribozyme on the expression of the γ12 subunit RNA level. Of the 11 γ subunits cloned to date, the γ7 and γ12 subunits show the greatest homology, exhibiting 75% identity within the coding region of the nucleotide level.2 As shown in Fig. 4B, transfection of cells with the phosphorothioate-modified γ7 ribozyme had no effect on the level of the γ12 RNA (115 ± 16%, n = 3), although in these same cells the level of the γ7 RNA was reduced to 40% of its control value. Taken together, these data clearly indicate that the effect of the phosphorothioate-modified γ7 ribozyme was highly specific for the targeted γ7 RNA when delivered in vivo.

Effect of γ7 Ribozyme on the Level of γ7 Subunit Protein in Vivo—Next, we investigated whether the suppression in γ7 RNA was paralleled by a reduction in the amount of γ7 protein in cells transfected with the γ7 ribozyme. For this purpose, cells were transfected with either no ribozyme (Control) or phosphorothioate-modified γ7 ribozyme (Thio RZ). At 48 or 72 h after transfection, membranes were prepared from these cells and evaluated by immunoblotting with various antibodies. Because the greatest suppression of γ7 protein was seen at 72 h after transfection (data not shown), cells were routinely harvested at 72 h after transfection in the experiments described below. Fig. 5A shows representative immunoblots where the nitrocellulose was cut along the 30-kDa marker, where the lower molecular weight portion of the blot was probed with an antibody (A-67) previously shown to specifically recognize the γ7 subunit (16), and where the higher molecular portion of the blot was probed with antibodies (A-54 and A-56) previously shown to detect the γ4, γ12, or γ13 subunits (17). Of particular interest, the γ7 antibody

2 J. D. Robishaw, unpublished data.
detected a distinct band of 6.5 kDa in control cells that was noticeably reduced in the cells transfected with the phosphorothioate-modified γ7 ribozyme. Identification of this band as the γ7 subunit is based on the findings that it has the same electrophoretic mobility as the recombinantly expressed γ7 subunit and is specifically recognized by an antibody shown previously to detect the γ7 subunit, but not the γ1, γ2, γ6, γ7-like, γ10, or γ11 subunits (16). Fig. 5B shows the quantitation of these results in which the relative amounts of the γ7 protein were quantitated and then expressed as a percentage of the control level. Based on this analysis, the level of the γ7 protein was suppressed to 15 ± 5% (n = 3) in ribozyme-treated cells compared with control cells.

The specific action of the phosphorothioate-modified γ7 ribozyme to suppress the level of the γ7 protein was further demonstrated by examining the effect of ribozyme on the levels of other G protein γ and α subunits. Recently, we identified a second band of approximately 7 kDa that is detected by the γ7 antibody (A-67) in HEK 293 cells. The identity of this cross-reacting band is not known, but by process of elimination, it is likely to represent an alternatively spliced form of the human γ7 subunit, the newly identified human γ7 subunit, or a novel γ subunit that has not been previously cloned. Of particular interest, this band of 7 kDa, designated γ7-like, was only marginally reduced in γ7 ribozyme-treated cells to 73 ± 14% (n = 3) of its control value (Fig. 5, A and B). Likewise, bands of 41 kDa that were detected with antibodies previously shown to be specific for the α1β1γ1 subunits were present at roughly similar levels (97 ± 7%, n = 3) in cells transfected with or without the γ7 ribozyme (Fig. 5, A and B). Taken together, these results attest to both the efficacy and specificity of the γ7 ribozyme in suppressing the expression of γ7 subunit protein in vivo.

Effect of γ7 Ribozyme on the Level of β1 Subunit Protein in Vivo—Under physiological conditions, the G protein β1 subunits exist in a tightly associated complex, with some studies suggesting that the expression of the β and γ subunits may be linked (28–31). Therefore, to determine whether the ribozyme-mediated reduction in the expression of the γ7 subunit affected the expression of one or more of the β subunits, membranes prepared from HEK 293 cells treated in the presence or absence of the phosphorothioate-modified γ7 ribozyme were immunoblotted with various antibodies against the β subunits. Fig. 6 shows the results of three sets of blots, where the nitrocellulose was cut along the 30-kDa marker, where the higher molecular portion of the blots was probed with antibodies against the β subunits, and where the lower molecular weight portion of the blots was probed with the γ7-specific antibody (A-67). The relative amounts of these proteins were quantitated in γ7 ribozyme-treated and control cells and then expressed as a percentage of their levels in control cells. Based on this analysis, the level of the β1 protein in ribozyme-treated cells was reduced to 23 ± 3% (n = 3) of its level in control cells. These results indicate that the loss of γ7 protein resulted in a similar reduction of the β1 protein. The levels of other β proteins in these cells have not been determined due to the lack of specific, high titer antibodies. However, the effect of the γ7 ribozyme treatment to suppress the level of the β1 subunit

FIG. 5. Selective suppression of the endogenous level of γ7 subunit protein expression in γ7 ribozyme-transfected HEK 293 cells. Membrane proteins from 72-h transfected control and Thio RZ-treated cells were extracted, resolved on 15% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted as described under "Experimental Procedures." Following transfer, the nitrocellulose was cut along the 30-kDa marker, the lower molecular weight blot was probed with a γ7-specific antibody (A-67), and the higher molecular blot was probed with γ-specific antibodies (A-56 and A-54). The γ7-specific antibody (A-67), which, of the 10 γ subunits tested, only detects γ7, detected two bands in HEK 293 cells. These bands corresponded to a 6.5-kDa protein, which is γ7, since this is the observed molecular mass of the γ7 standard (baculovirus-expressed recombinant γ7 subunit) and a novel protein of 7 kDa. This novel 7-kDa protein is referred to as γ7-like. Panel A shows representative immunoblots demonstrating the selective loss of γ7 protein in γ7 ribozyme-treated cells (Thio RZ). Expression of the γ7-like protein and α1 subunit were only slightly attenuated in the ribozyme-treated cells. Panel B shows the quantitation of the loss of γ7 protein expression in the γ7 ribozyme-treated cells. The intensities of the bands from three separate experimental sets of immunoblots were determined by PhosphorImager analysis. The relative amounts of proteins in ribozyme-treated cells (shaded bars) were expressed as a percentage of their control levels (white bars). Data shown are mean ± S.E.

FIG. 6. Suppression of β1 subunit expression in γ7 ribozyme-transfected HEK 293 cells. The higher molecular nitrocellulose blot probed with α1-specific antibodies presented in Fig. 5 was stripped and reprobed with a β1-specific antibody (B-69) and a β common antibody (SW/1 from NEN Life Science Products) as described under "Experimental Procedures." The data shown are the quantitation of expression of the β1 subunit and the total β expression in control (white bars) and γ7 ribozyme-transfected cells (shaded bars). Expression of β1 subunit was observed to be suppressed in a similar fashion to that of the γ7 subunit in the γ7 ribozyme-transfected cells. The relatively modest suppression of the total β subunit pool suggests that it is lowered primarily by the very large attenuation of the β1 subunit expression. The data shown are from three separate experimental sets of immunoblots in which the intensities of the bands were determined by PhosphorImager analysis. Data shown are mean ± S.E.
appeared to be relatively specific, since the level of total β proteins, as detected by an antibody (SW1; NEN Life Science Products) that recognizes an epitope common to all of the known β subunits, was only modestly suppressed to 76 ± 1% of its level in control cells.

**Effect of Ribozyme-mediated Loss of γ7 Subunit Expression on Receptor Signaling**—The heterotrimeric G proteins transmit signals from receptors to effectors. Therefore, we tested how the ribozyme-mediated loss of the γ7 subunit affects receptor-effector coupling. There are a number of hormone agonists capable of activating receptors that couple to heterotrimeric G proteins to regulate the activity of common effectors, such as phospholipase C or adenylylcyclase, in HEK 293 cells. Initially, we studied the effect of ribozyme-mediated loss of the γ7 subunit on regulation of phospholipase C activity through activation of the muscarinic or P2 purinergic receptors. Fig. 7 shows the results in which agonist-induced phospholipase C activity was quantitated in control and ribozyme-treated cells and expressed as a percentage of the control value. In control cells, both carbachol, a muscarinic receptor agonist, and ATP, a P2-purinergic receptor agonist, resulted in an approximately 2-fold stimulation of inositol phosphate accumulation. Interestingly, cells transfected with the phosphorothioate-modified γ7 ribozyme showed a similar inositol phosphate accumulation in response to carbachol and ATP as did control cells (112 ± 4% (n = 6) and 89 ± 8% (n = 6) of the control-transfected levels, respectively). These results demonstrate that the transfection procedure itself did not alter hormonal responsiveness of cells. More importantly, they indicate that the γ7 subunit does not appear to take part in signaling between the muscarinic or P2 purinergic receptors and phospholipase C in these cells.

Next, we examined the effect of ribozyme-mediated loss of the γ7 subunit on regulation of adenylylcyclase activity through activation of prostaglandin or β-adrenergic receptors. Fig. 8 shows the results in which agonist-induced adenylylcyclase activity was quantitated in control and ribozyme-treated cells and expressed as a percentage of the control value. In control HEK 293 cells, both isoproterenol, a β-adrenergic receptor agonist, and PGE1, a prostaglandin receptor agonist, resulted in marked stimulation of cAMP formation. Cells transfected with the phosphorothioate-modified γ7 ribozyme responded to PGE1 in the same manner as did control cells (96 ± 7%, n = 3). Intriguingly, however, cells transfected with the phosphorothioate-modified γ7 ribozyme showed a reduction in cAMP accumulation in response to isoproterenol to 70 ± 2% (n = 5) of its level in control cells (Fig. 8A). From the time course, the effect of the γ7 ribozyme to suppress cAMP accumulation appeared to result from a reduced rate of cAMP formation in response to isoproterenol (Fig. 8B). Taken together, these data indicate a role for the γ7 subunit in transducing the signal from the β-adrenergic receptor to adenylylcyclase. Furthermore, these data suggest that the γ subunit composition of the Gs protein that couples the β-adrenergic receptor to adenylylcyclase differs from that of the Gs protein that couples the prostaglandin receptor to the same effector.

**DISCUSSION**

In this study, we explore the use of the ribozyme technology to specifically suppress the expression of the γ7 subunit of the heterotrimeric G proteins in HEK 293 cells. The creation of a cell line deficient in the γ7 subunit will be a useful tool in dissecting its role in receptor-G protein-effector pathways. Three major conclusions can be derived from this study: 1) ribozymes can be designed to suppress the expression of the γ7 subunit at both the RNA and protein levels in a selective fashion; 2) the ribozyme-mediated suppression of γ7 expression is associated with a similar reduction in the expression of the β1 subunit at the protein level, suggesting they are coordinately expressed; and 3) the combined reduction in the expression of the γ7 and β1 subunits at the protein level is associated with an impaired responsiveness of the cells to stimulation of adenylylcyclase by isoproterenol, thereby suggesting a specific role for these proteins in transducing the signal from the β-adrenergic receptor to adenylylcyclase.

In evaluating reverse genetic approaches to suppress endogenous G protein subunit expression, ribozymes have the marked advantage of operating as site-specific ribonucleases, resulting in the degradation of the target RNA. The in vivo results showed that the ribozyme directed against the γ7 subunit produced the expected cleavage products of the γ7 RNA transcript but had no effect on the γ2, γ10, γ11, and γ12 RNA transcripts, demonstrating its ability to cleave the RNA transcript in a site-specific fashion at physiologic temperature (Figs. 2 and 3). However, a potential problem of ribozymes has been their rapid degradation in vivo (25). To circumvent this problem, we found that two modifications suggested by other
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![Graph A](image)

**Panel A** shows the effect of PGE1 on cAMP accumulation in control-transfected and $\gamma_7$ ribozyme-transfected HEK 293 cells. Data shown are mean $\pm$ S.E. for 3–6 separate experiments.

**Panel B** shows the time course of cAMP accumulation. In control-transfected cells (shaded bars), stimulation with PGE1 resulted in a 245- and 111-fold increase in cAMP concentration relative to control-transfected cells. With isoproterenol stimulation, however, the increase in cAMP was significantly attenuated relative to control-transfected cells.

In control-transfected cells, stimulation with PGE1 resulted in a 245- and 111-fold increase in cAMP concentration relative to control-transfected cells. With isoproterenol stimulation, however, the increase in cAMP was significantly attenuated relative to control-transfected cells.

In view of the transient transfection nature of these experiments, the partial reduction of the $\gamma_7$ subunit at both the RNA and protein levels could be interpreted in two possible ways. On the one hand, the finding that the $\gamma_7$ RNA and protein was reduced to 40 and 15% of their control levels, respectively, could be interpreted to indicate a partial loss of the $\gamma_7$ subunit in all of the cells. On the other hand, a large population of the cells could have a complete loss of the $\gamma_7$ subunit, with a smaller population of the cells showing no loss of the $\gamma_7$ subunit, due to variation of the transfection efficiency. To try to distinguish between these two possibilities, pCMV LacZ plasmid DNA was transfected into HEK 293 cells by the same transient transfection technique. By counting the $\beta$-galactosidase-stained cells, we found that about 70% of the cells were effectively transfected by the LipofectAMINE technique. Thus, in those cells transfected with the $\gamma_7$ phosphorothioate-modified ribozyme, it is likely that the suppression of the $\gamma_7$ subunit at the RNA and protein levels was even greater than the measured values. Finally, the finding that the reduction of the $\gamma_7$ subunit at the protein level was even greater than its reduction at the RNA level may suggest that the ribozyme was both cleaving the $\gamma_7$ RNA as well as restricting synthesis of the $\gamma_7$ protein through the formation of RNA duplexes that arrest the translation process.

The specificity of ribozyme suppression is crucial to deducing the functional consequences of such suppression. Specificity can be achieved by complementarity between the ribozyme and the sequences flanking the targeted GUC sequence in the RNA template. In particular, the $\gamma_7$ ribozyme must be able to discriminate among the eight known $\gamma$ subunits that have been identified to date in HEK 293 cells. Based on the fact that the amino-terminal regions of the $\gamma$ subunits are the most structurally diverse (12), and in line with the assumption that the region surrounding the transcription start site is likely to be the most accessible, we selected the GUC sequence at positions +3 to +5 in relation to the transcription start site as the target for the $\gamma_7$ ribozyme. A striking finding of the present study was the degree of specificity that could be achieved with the phosphorothioate-modified $\gamma_7$ ribozyme in the *in vivo* studies. When the $\gamma_7$ ribozyme suppressed the $\gamma_7$ RNA to 40% of its level in control cells, the EF1a and $\gamma_{12}$ RNAs were maintained at 112 and 115% of their levels in control cells, respectively. Likewise, when the $\gamma_7$ ribozyme attenuated synthesis of $\gamma_7$ protein to 15% of its level in control cells, the $\gamma_{12}$ and $\alpha_{1,2,3}$ proteins were only marginally suppressed to 73 and 89% of their levels in control cells, respectively. Taken together, the present results show the ability of ribozyme to recognize its target in a natural context and to discriminate the target sequence in $\gamma_7$ from similar sequences in related $\gamma$ RNAs as well as other unrelated RNAs. This provides the first evidence that the ribozyme technology can be used to inhibit the expression of specific subunits of the heterotrimeric G proteins, which is likely to have important implications for these and other multigene families. Furthermore, the present study shows the ability of ribozyme to target a naturally occurring G protein $\gamma$ subunit in cells, demonstrating the specificity that can be achieved in a normal cellular context.

The Ribozyme-mediated Reduction in $\gamma_7$ Expression Can Result in Similar Suppression of $\beta$ Expression—Unlike the expression of EF1a, the G protein $\alpha_{1,2,3}$ subunits, and the other G protein $\gamma_{12}$ subunit, which were at most only marginally affected by introduction of the $\gamma_7$ ribozyme, the expression of the $\beta_i$ subunit was found to be reduced to 23% of its control value. This was remarkably similar to the magnitude of suppression observed for the targeted $\gamma_7$ subunit (15% of its control value). While the levels of the other $\beta$ subunit subtypes in these cells have not been determined due to the lack of specific antibodies of the requisite titer, analysis of total $\beta$ subunit protein expression indicated that it was suppressed only mod-
Ribozyme-mediated Suppression of G Protein $\gamma_7$ Expression

The Ribozyme-mediated Reduction in $\gamma_7$ and $\beta_1$ Expression Is Associated with Impaired Responsiveness of Cells to Isoproterenol but Not PGE$_1$—How the specificity of signaling from receptor to G protein to effector is encoded in the protein-protein interactions between an expanding number of signaling partners is not clear. The possibility that the specificity of signal transmission is determined in part by the G protein $\beta$ and $\gamma$ subunits is supported by the present study. HEK 293 cells treated with the phosphorothioate-modified $\gamma_7$ ribozyme showed a 30% attenuation in cAMP accumulation in response to a $\beta$-adrenergic receptor agonist, isoproterenol. That the attenuated response was due to the specific loss of the $\gamma_7$ subunit rather than a nonspecific effect of the $\gamma_7$ ribozyme is supported by the following lines of evidence. First, hormone responsiveness of cells to carbachol, ATP, and PGE$_1$ was not affected by treatment with the $\gamma_7$ ribozyme (Figs. 7 and 8), demonstrating that receptor signaling pathways were not altered in a general way. Second, $\beta$-adrenergic receptor content was not affected by treatment with $\gamma_7$ ribozyme (data not shown), indicating that there was no defect in the receptor upstream of the G protein. Finally, cAMP accumulation in response to a prostaglandin receptor agonist, PGE$_1$, was not altered by treatment with the $\gamma_7$ ribozyme (Fig. 8), indicating that there was no defect in the effector downstream of the G protein. Taken together, these results indicate that the attenuation of cAMP response to isoproterenol was due to a specific loss of the G protein $\gamma_7$ subunit. By extension, these results further suggest that the $\gamma_7$ subunit participates in signal transduction between the $\beta$-adrenergic receptor and the adenylyl cyclase. Moreover, because loss of the $\gamma_7$ subunit was associated with a similar reduction of the $\beta_1$ subunit, a participatory role of the $\beta_1$ subunit in signal transduction between the $\beta$-adrenergic receptor and the same effector is also suggested. Taken together, these results provide strong evidence that the $\gamma_7$ and $\beta_1$ subunits play a role in signal transduction between the $\beta$-adrenergic receptor and adenylyl cyclase. Intriguingly, a previous study has proposed a functional interaction between the G protein $\gamma_7$ subunit and adenylyl cyclase based on the finding that the $\gamma_7$ subunit is selectively enriched in the striatum of the brain in a pattern highly reminiscent of that of adenylyl cyclase (35). While the present study did not directly address the role of the $\gamma_7$ subunit in the $\beta$-adrenergic receptor signaling pathway, two possible mechanisms are suggested. On the one hand, it is known from previous studies that the $\beta\gamma$ subunits themselves are capable of regulating certain types of adenylyl cyclases (35). In this scenario, the reduced assembly of the required $\beta_1\gamma_7$ dimer would compromise the necessary G protein-effector interaction, thereby accounting for the attenuated response. On the other hand, it is known from previous studies that the $\beta\gamma$ subunits are required for efficient interaction of the $\alpha$ subunit with the receptor (1–3). In this scenario, the reduced synthesis of the $\gamma_7$ subunit would lead to reduced assembly of the required $\beta_1\gamma_7$ dimer and, by extension, the required $\alpha_\beta_1\gamma_7$ heterotrimer. This in turn would compromise the necessary receptor-G protein interaction, thereby accounting for the attenuated response. At the present time, we favor the latter mechanism, since a $\beta\gamma$-regulated form of adenylyl cyclase has not been detected in HEK 293 cells (36). If we assume the latter scenario is correct, in which the interaction between the $\beta$-adrenergic receptor and the G protein is compromised, the question arises as to why the suppression of $\gamma_7$ and $\beta_1$ subunits did not result in greater attenuation of adenylyl cyclase in response to isoproterenol. There are several possible explanations. First, an obvious explanation lies with the efficiency of the transient transfection procedure. Only 70% of the cells are effectively transfected with the $\gamma_7$ ribozyme by the LipofectAMINE procedure. Therefore, the remaining 30% of cells that are not transfected would be expected to show a normal response to isoproterenol. Second, it is well established that there is not necessarily a linear relationship between the receptor-G protein content and the functional response in cells (37), with the receptor-G protein content often exceeding the number needed to give a maximal response in vivo. Therefore, a partial reduction in the levels of the G protein $\gamma_7$ and $\beta_1$ subunits would not necessarily be expected to result in a similar degree of attenuation of the adenylyl cyclase response. Finally, the $\beta$-adrenergic receptor may couple to multiple G proteins (38), each of which may contain a different $\beta\gamma$ subunit composition. Hence, a reduction in only the $\beta_1\gamma_7$ subunits would be expected to result in only a partial attenuation of the adenylyl cyclase response. Future experiments will be necessary to distinguish between these and other possibilities.

Implications for $\alpha\beta\gamma$ Composition of G—Given the known existence of at least 6 $\beta$ and 11 $\gamma$ subunits, it is clear that G proteins should be named on the basis of their specific combinations of $\alpha\beta\gamma$ subunits rather than solely on the basis of their $\alpha$ subunits as they were in the past. However, this nomenclature has been difficult to put into practice, since most cell types express multiple $\alpha\beta\gamma$ complexes, and in the course of detergent extraction from the membrane, the various $\alpha$ and $\beta\gamma$ subunits making up these complexes undergo an appreciable rate of dissociation and exchange. This results in homogeneously pure $\alpha$ subunits co-purifying with multiple $\beta\gamma$ subunits (39). As a consequence, it has not been possible to determine the $\alpha\beta\gamma$ subunit compositions of many of the physiologically important G proteins, such as Gs, Gi, and Gq. In the present study, we used the ribozyme technology to provide evidence for the $\beta\gamma$ subunit composition of Gs in HEK 293 cells. A particularly striking finding of the present study was that cells treated with the $\gamma_7$ ribozyme showed a 30% attenuation in cAMP accumulation in response to isoproterenol. This result strongly suggests that the $\beta$-adrenergic receptor interacts with a form of Gs containing the $\beta_1$ and $\gamma_7$ subunits. In contrast, cells treated with $\gamma_7$ ribozyme showed no change in cAMP accumulation in response to PGE$_1$ (Fig. 8). This result indicates that the prostaglandin receptor interacts with a form of Gs that does not contain the $\beta_1$ and $\gamma_7$ subunits. Taken together, these data strongly support the hypothesis that different receptors interact with distinct G proteins, defined by their unique $\alpha\beta\gamma$ subunit compositions. These findings underscore the importance of naming G proteins on the basis of their $\alpha\beta\gamma$ subunit compositions. Moreover, they establish the contribution of the $\beta\gamma$ subunits in determining the selectivity of signal transduction from...
receptor to G protein to effector, as first suggested in studies by Kleuss and colleagues (6, 7).

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