**Gβγ Binds Histone Deacetylase 5 (HDAC5) and Inhibits Its Transcriptional Co-repression Activity**

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In a yeast two-hybrid screen designed to identify novel effectors of the Gβγ subunit of heterotrimeric G proteins, we found that Gβγ binds to histone deacetylase 5 (HDAC5), an enzyme involved in a pathway not previously recognized to be directly impacted by G proteins. Formation of the Gβγ-HDAC5 complex in mammalian cells can be blocked by overexpression of Gαq, and this inhibition is relieved by activation of α2A-adrenergic receptor, suggesting that the interaction occurs in a signal-dependent manner. The C-terminal domain of HDAC5 binds directly to Gβγ through multiple motifs, and overexpression of this domain mimics the C terminus of G protein-coupled receptor kinase 2, a known Gβγ scavenger, in its ability to inhibit the Gβγ-HDAC5 interaction. The C terminus of HDAC4 shares significant similarity with that of HDAC5, and accordingly, HDAC4 is also able to form complexes with Gβγ in cultured cells, suggesting that the C-terminal domain of class II HDACs is a general Gβγ binding motif. Activation of a Gαq-coupled receptor results in a time-dependent activation of MEF2C, an HDAC5-regulated transcription factor, whereas inhibition of the interaction with a Gβγ scavenger inhibits MEF2C activity, suggesting a reduced potency of HDAC5-mediated inhibition. Taken together, these data imply that HDAC5 and possibly other class II HDACs can be added to the growing list of Gβγ effectors.

Signaling through G protein-coupled receptors (GPCRs) is a ubiquitous mechanism mediating cellular responses to such diverse stimuli as photons of light, odorants, and hormones. Information from such stimuli is initially transduced through heterotrimeric G proteins; activated receptors catalyze the dissociation of the Gα and Gβγ subunits and, Thus, modulation of the subunits’ downstream effectors (1). In addition to regulating the activation state of a partner Gα subunit, the Gβγ functional monomer interacts with an increasingly apparent number of signaling proteins (2), including well known effectors such as phosphoinositide 3-kinases and phospholipases C and more recently discovered interacting partners such as the ubiquitin-related protein PLIC-1 (3) and the glucocorticoid receptor (4).

In a recent effort to expand our understanding of Gβγ function, we conducted a yeast two-hybrid screen to identify novel Gβγ-interacting proteins. Results from this screen led to the identification of an isomorph of the receptor for activated C kinase (RACK1) and several other WD40-repeat containing proteins as Gβγ binding partners (5). These results led to the hypothesis that interactions between WD40 domains may be a general phenomenon and that the WD40 domain may serve in part to increase the speed and specificity of signaling events by acting as a versatile scaffold in diverse signaling pathways (5, 6). Importantly, RACK1 was identified as an effector-specific modulator of Gβγ signaling in vivo (7).

An emerging paradigm in the field of GPCR function that is typified by the RACK1/Gβγ interaction is the role of cross-talk with other signaling paradigms. For example, in its ability to interact with molecules downstream of receptor tyrosine kinases and transforming growth factor receptors, RACK1 may serve as a nexus mediating the cross-talk between numerous types of pathways (8). Furthermore, both Gβγ and receptor tyrosine kinases modulate the activity of the β isomorph of phosphoinositide 3-kinase (for review, see Ref. 9), and GPCR activation can result in transactivation of receptor tyrosine kinases in the modulation of mitogen-activated protein kinase (MAPK) cascades (for review see 10).

One potential signaling partner for G protein pathways that has been largely unexplored is protein acetylation, a signaling mechanism of increasingly apparent importance (11). The most well studied acetylation events govern access to the information encoded in the genome. At appropriate times, the acetylation states of histones associated with specific genes are controlled by a plethora of histone acetyltransferases (HATs) and histone deacetylases (HDACs). By altering local chromatin structure and providing binding sites for numerous transcription factors, HATs typically act to increase transcription at a specific site, whereas HDACs often act as transcriptional co-repressors by opposing the actions of HAT enzymes (12).

Two types of enzyme catalyze histone deacetylation, namely the NAD+-dependent sirtuins and the Zn2+-dependent HDACs (13, 14). The latter family is subdivided into at least two classes. Class I HDACs (HDACs 1, 2, 3, and 8) are most similar to the yeast regulator of potassium dependence 3 (Rpd3p). These enzymes are ubiquitously expressed in mammalian tissues and cell lines. Homologous to the class I HDACs are class II HDACs, which are most similar to a yeast HDAC known as Hda1p. Class II HDACs (HDACs 4, 5, 6, 7, 9, 10, and 11) generally have more restricted distributions than those of class I; HDAC5, for example, is most highly expressed in cardiac smooth muscle, skeletal muscle, and brain (15, 16).

In addition to distinct tissue distributions, a subset of class II HDACs are characterized by N-terminal extensions with well studied roles in protein-protein interactions (17, 18) and subcellular distribution. In addition to their long (~400–600 amino acid) N-terminal domains, some class II HDACs also have a relatively short (~150 amino acid) and well conserved domain at their extreme C termini. In addition to a signal-responsive nuclear export signal (19), other functions of this domain are emerging (20).
Interaction between Gβγ and HDAC5

The current report investigates a role for the interaction of Gβγ with class II histone deacetylases, including HDAC5, which is the second novel Gβγ binding partner discovered in the previous yeast two-hybrid screen. Interestingly, the C terminus of a related class II HDAC, HDAC7, has been shown to interact with a GPCR, endothelin receptor A, and possibly to modulate an endothelin-1-stimulated MAPK pathway (20). Here, we show that Gβγ interacts with the C-terminal domain of HDAC5 and provide evidence that signaling through Gβγ has an inhibitory effect on HDAC5 function. Thus, HDAC5 may be added to the long and growing list of effectors of the signaling capacity of Gβγ.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-pan-Gβ (T20) and a control irrelevant antibody (rabbit anti-His probe (H115)) were from Santa Cruz Biotechnological. Mouse anti-FLAG (M2) and anti-HA antibodies and antibody-Sepharose conjugates were from Sigma. HEK293A cells (Invitrogen) were passaged at 37 °C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin. Spodoptera frugiperda (Sf9) (Invitrogen) was grown in SF-900 II SFM medium (Invitrogen) at 28 °C with gentle shaking. Trichostatin A (TSA) and other chemicals were of the highest grade available and were typically from Sigma.

Endogenous Immunoprecipitations and Deacetylation Assays—Heart tissue from two normal adult Sprague-Dawley rats was lysed with 15 strokes of a tight-fitting Dounce homogenizer in phosphate-buffered saline with 1% deoxycholate, 1% Igepal, 0.5% SDS, 5 mM dithiothreitol, and protease inhibitors (lysis buffer). The soluble portion of this lysate was pre-cleared by incubation with protein G-Sepharose beads. Four micrograms of purified rabbit polyclonal antibodies against Gβ or the His probe were added, and the mixtures were incubated on ice overnight. A 50-μl bed volume of protein G-Sepharose was added, and the tubes were incubated with gentle mixing at 4 °C for 1 h. Immunoprecipitates were collected via centrifugation and washed twice with 1 ml of lysis buffer and once with phosphate-buffered saline (PBS). Ten percent of the resin was removed for Western blot analysis. The remaining resin was assayed for deacetylation activity using a commercially available assay kit essentially as described (Upstate).

Plasmid Construction—Plasmids directing the expression of bovine Gγ2 and N-terminally HA-tagged bovine Gβ1 in mammalian cells under the control of the human cytomegalovirus promoter were created using conventional molecular biological techniques. Plasmids directing the expression of C-terminally FLAG-tagged human HDACs 4, 5, and 6 were generously provided by Dr. Ed Seto, University of South Florida. A plasmid directing the expression of Gaαx with an internal EE epitope tag was obtained from the Guthrie cDNA Resource Center. The αx-adrenergic receptor expression vector was obtained from Dr. Lee Limbird, Vanderbilt University.

The C termini of HDACs 4 and 5 (HDAC4ct and HDAC5ct) were amplified from the corresponding FLAG tag plasmids with the following primers: HDAC4 sense (5′-CACCATGGATTTGCCCGGATGTGGTG-3′); HDAC4 antisense (5′-AATCTCGAGTACAAGGGGCCGTCCTCTTT-3′); HDAC5 sense (5′-CACCATGGATTTGCTCAGTGTGGTCG-3′); and HDAC5 antisense (5′-AATCTCGAGCTTTTGTACAGGAGCTTGGTCG-3′). The PCR amplification products were ligated into pENTR/D/D-TOPO from the Gateway cloning system (Invitrogen). The genes were transferred to pDEST26 or pDEST27 (Invitrogen) for expression in mammalian cells with an N-terminal His, or glutathione S-transferase (GST) tag, respectively. Additionally, HDAC5ct was transferred to pDEST20 for creation of baculoviruses directing the expression of GST-HDAC5ct in S9 cells.

Viruses were created and amplified according to the Bac-to-Bac system (Invitrogen).

Protein Purification—S9 cells (1 × 10⁶) were infected at an approximate multiplicity of infection of one with baculovirus GST-HDAC5ct. Following a 60-h incubation at 28 °C, cells were collected by centrifugation. The cells were swollen for 20 min on ice in hypotonic buffer (20 mM Tris pH 8.0, 5 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride). The cells were lysed using a hand-held rotary homogenizer. NaCl was added to a final concentration of 150 mM, and the lysates were cleared by centrifugation. Glutathione-Sepharose purification proceeded as recommended (Amersham Biosciences), except that proteins were eluted overnight at 4 °C with 50 mM Tris, 20 mM glutathione, 250 mM NaCl, 5 mM diethiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The proteins were concentrated, and glutathione was removed by buffer exchange with PBS using centrifugal concentrators (Millipore). Gβγ1 was purified from bovine rod outer segments as described (21).

In Vitro Binding Assays—GST or GST-HDAC5ct were incubated at noted concentrations in a final volume of 100 μl with Gβγ1. Binding buffer consisted of PBS with 5 mM diethiothreitol, 5 mM ethylenediamine tetraacetic acid, and 0.2 mg/ml bovine serum albumin. After a 1-h incubation at 4 °C, 30 μl of a 50:50 slurry of glutathione-Sepharose resin was added to each reaction. The complexes were allowed to bind to the resin for 30 min at 4 °C with constant mixing. The resin was collected by brief centrifugation and then washed three times with 1 ml of PBS plus 0.2% Tween 20 and 5 mM diethiothreitol. The complexes were eluted from the resin with SDS-PAGE running buffer and separated by electrophoresis.

Cell Culture and Transfections—Cells were transfected using Lipofectamine 2000 (Invitrogen). Typically, for a 6-well plate at 80–90% confluence, 1 μg of total plasmid DNA was complexed with 5 μl of lipoid reagent in 500 μl of Opti-MEM I (Invitrogen). After a 20-min incubation at room temperature, the complexes were added to the cells with 2 ml of Dulbecco’s modified Eagle’s medium and 10% serum but no antibiotics. For some transfections, empty vector (pcDNA3.1) was added to keep total DNA at 1 μg.

Immunoprecipitations—Forty-eight hours after transient transfections, cells were washed with PBS, and 400 μl of PBS with 0.5% Igepal, 0.1% SDS, protease inhibitors, and 1 mM diethiothreitol (lysis buffer) was added. Following a 5-min incubation at 4 °C, the cells were recovered with a cell lifter. The cells were lysed further by 10 passages through a 23-gauge needle with a 1-cc syringe. Cellular debris was removed by centrifugation and a pre-clearing step using protein G-Sepharose (Amersham Biosciences). Complexes were immunoprecipitated for 1 h at 4 °C using either anti-FLAG (M2)-agarose or anti-HA agarose (Sigma). The pellets were washed three times with 1 ml of lysis buffer. Proteins were eluted with SDS-PAGE loading dye prior to electrophoresis. Typically, one-half of the supernatant was analyzed by electrophoresis and Western blot.

Western Blots—Samples separated with SDS-PAGE were transferred to polyvinylidene fluoride membranes (Millipore). Primary antibodies were detected with specific secondary antibodies covalently linked to Alexa Fluor 680 (Invitrogen) or IRDye800 (Rockland, Inc.). Fluorescent signal was imaged with an Odyssey infrared imaging system (LI-COR Inc.).

Assays for Reporter Gene Transfection—Plasmids encoding MEF2C and luciferase downstream of three MEF2C binding sites (3× MEF2C-luciferase) were kindly provided by Dr. Eric Olson (22). LacZ under control of a cytomegalovirus promoter (Invitrogen) was included as a transfection control. HEK293A cells on 12-well plates were transfected with 0.1 μg of MEF2C, 0.2 μg of 3× MEF2C-luciferase, 0.0125 μg of CMV-LacZ, and other plasmids as noted using Lipofectamine 2000.
transfection reagent as described above. Total DNA was adjusted to 0.5 μg with empty vector (pcDNA3.1). Two days after transfection, luciferase and β-galactosidase were harvested with 250 μl of passive lysis buffer as recommended (Promega). Luciferase and β-galactosidase activities were determined with reagents obtained from Promega. Luciferase activity was measured with a Wallac Victor2 V 1420 multi-label HTS counter equipped with a 700-nm infrared cutoff filter. Measurements were linear with respect to time and dose of extract.

RESULTS

Yeast Two-hybrid Screen—Bovine Gβ1 was used as bait to screen a mouse brain library in a previously described yeast two-hybrid assay (5). Two clones whose expression resulted in reporter gene transcription were found to encode the C-terminal ~160 amino acids of HDAC5 (data not shown). This result was confirmed by retransformation of the plasmids and co-immunoprecipitation from yeast lysates as described (5) (data not shown). In addition, the interaction was found to be independent of the co-expression of mammalian Gγ (data not shown), suggesting that Gβ1 formed a functional dimer with a yeast Gγ-like protein in situ and that binding was mediated by Gβ rather than Gγ.

Investigating an in Vivo Interaction—Because of an apparent insensitivity of several anti-HDAC5 antibodies in immunoblotting and immunoprecipitation experiments, the HDAC content of immunoprecipitated Gβ complexes was assessed with in vitro deacetylase assays to confirm the association of Gβ with HDACs in situ. Lysates of heart tissue from normal adult rats were subjected to immunoprecipitation with a rabbit polyclonal anti-Gβ antibody or a control irrelevant rabbit polyclonal antibody. The anti-Gβ antibody specifically precipitated Gβ, as shown in Fig. 1A. As displayed in Fig. 1B, the anti-Gβ antibody precipitates displayed significantly more deacetylase activity than did the control precipitates.

To functionally characterize the activity present in the complex, in vitro assays were performed in the presence of TSA, which is a selective inhibitor of the Zn2+-dependent HDAC family of deacetylases. No activity was detected in control or anti-Gβ immunoprecipitates (Fig. 1B), suggesting that HDAC enzymes exist in stable complexes with Gβ in vivo.

Interaction with HDAC5 in Particular—Comunoprecipitation of epitope-tagged proteins expressed in cultured cells was employed to confirm that Gβ could interact with HDAC5. First, empty vector or N-terminally HA-tagged Gβ1 and untagged Gγ2 were transiently transfected into HEK293A cells with C-terminally FLAG-tagged HDAC5. Two days after transfection, cell lysates were subjected to immunoprecipitation with an anti-HA antibody. As shown in Fig. 2A, HA-Gβ1 was specifically purified when HDAC5-FLAG was immunoprecipitated, indicating that the proteins can exist in an identical complex in mammalian cells. In the converse experiment, immunoprecipitation of HA-Gβ1 and HA-Gγ2 also specifically co-purified HDAC5-FLAG, further supporting the ability of HDAC5 and Gβγ to form a complex in mammalian cells.

Direct Gβγ/HDAC5 Interaction—The interaction between HDAC5 and Gβγ has thus been shown in both yeast and mammalian systems, suggesting that the interaction is likely direct. However, to further demonstrate that the interaction is not mediated through other molecules, purified proteins were assayed for binding. The C-terminal domain of HDAC5, HDAC5ct, was expressed and purified as a GST fusion protein from a baculovirus/SP9 system. This protein was assayed for interaction with Gβγ γ, highly purified from bovine rod outer segments. Equimolar amounts (50 nM) of GST or GST-HDAC5ct were incubated with increasing concentrations of Gβγ γ, followed by purification with glutathione-Sepharose. As displayed in Fig. 3, Gβ1 specifically co-purified...
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**FIGURE 3. The HDAC5ct/Gβγ interaction is direct.** Purified GST or GST-HDAC5ct (50 ng) was incubated with the indicated concentrations of Gβγ purified from bovine retinal rod outer segments. A, Western blot analysis. The pellets from glutathione-Sepharose purification were analyzed by Western blot with anti-GST (top) and anti-Gβγ (bottom) antibodies. Bands are identified on the left, whereas mobility of standards are indicated on the left (kDa). B, quantification. The anti-Gβγ signal from three similar experiments was quantified, and the results are displayed as a dose dependence curve. Background binding of Gβγ to GST was negligible, whereas Gβγ associated with GST-HDAC5 with an EC50 of ~330 nM.

with GST-HDAC5ct, confirming that the proteins interact directly. In addition, to estimate the HDAC5ct/Gβγ affinity, an apparent EC50 was calculated; at 50 nM GST-HDAC5ct, 50% binding is achieved at 330 ± 120 nM Gβγ. Thus, the apparent affinity is similar to that observed for the interaction of Gβγ with RACK1 (5).

**The Gβγ/HDAC5 Interaction Is Controlled by Heterotrimeric G Protein Activation**—In regard to the regulation of effector molecules, heterotrimeric G proteins containing Ga-GDP are thought to be inactive, with effector regulation occurring upon the receptor-catalyzed exchange of GTP for GDP on Ga and resultant dissociation of the heterotrimer. This mechanism allows activation of receptors to be involved in the temporal regulation of downstream pathways. Accordingly, we sought to determine whether coexpression of Ga could block the ability of overexpressed Gβγ to form a complex with HDAC5. HEK293A cells transfected with HDAC5-FLAG, HA-Gβγ1γ2, EE-tagged GaαoA, and the Gαo-coupled receptor α2A-adrenergic receptor were serum-starved overnight. In subsequent anti-FLAG immunoprecipitation assays we observed that complex formation was greatly reduced by Gaαo expression (Fig. 4), and anti-EE reactivity was never observed in the immunoprecipitates (data not shown). Together, these data suggest that HDAC5 binds only to free Gβγ, allowing the possibility that the interaction is controlled by activated receptors.

To test the hypothesis that Gβγ interacts with HDAC5 upon G protein activation, the ability of stimulation of a Gaαo-coupled receptor to relieve inhibition of complex formation by Gaαo was tested. Following serum starvation as described above, the cells were incubated with a β-adrenergic receptor antagonist, 10 μM propanolol, for 10 min at 37 °C. The cells were then exposed to 10 μM (-)-epinephrine to stimulate the α2A-adrenergic receptor for various times, whereupon complex formation was assayed by anti-FLAG immunoprecipitation. As shown in Fig. 4, receptor stimulation relieved the Gaαo-mediated inhibition of Gβγ/HDAC5 complex formation, suggesting that the interaction is signal-responsive in vivo.

**FIGURE 4. The Gβγ/HDAC5 interaction is controlled by GPCR activation.** HEK293A cells were transfected with plasmids encoding α2A-adrenergic receptor and HA-Gβγ1γ2, as well as HDAC5-FLAG and EE-GαoA, plasmids as indicated. Following serum starvation overnight, the cells were treated for the indicated times with 10 μM (-)-epinephrine (epi) after a 10 min pre-incubation with 10 μM propanolol. Detergent lysates were subjected to immunoprecipitation (IP) with anti-FLAG agarose. Fifty percent of the immunoprecipitate and 2.5% of the lysates were analyzed by Western blot. A, Western blot representative of three independent experiments. IB, immunoblot. B, quantification of Gβγ in immunoprecipitates. The anti-HA signal from three experiments were quantified with an Odyssey infrared imaging system as described under “Experimental Procedures.” Results are displayed relative to signal from cells without ectopic Gaαo. The immunoprecipitation was significantly inhibited by overexpression of Gaαo (compare the column marked 0 with column marked 0 +; *, p < 0.001; two-tailed Student’s t test). The interaction was rescued by stimulation of α2A-adrenergic receptor (compare column marked 0 + with columns marked 5, 10, 20, 40, and 60 min, *p < 0.001; **p < 0.005).

The C Terminus of HDAC5 Mediates Gβγ Binding—The yeast two-hybrid assay identified the C terminus of HDAC5 as sufficient for binding to Gβγ. To demonstrate that this domain is the major binding site for Gβγ, we determined the ability of the C-terminal domain to block the interaction between Gβγ and full-length HDAC5. A construct encompassing the C-terminal 172 amino acids of HDAC5 (residues 951-1122 of human HDAC5) was created by PCR amplification. HEK293A cells were transfected with plasmids encoding HDAC5-FLAG and HA-Gβγ1γ2 along with increasing amounts of DNA directing the expression of the C-terminal fragment with an N-terminal His6 tag. As shown in Fig. 5A, subsequent co-immunoprecipitation assays indicated that the C-terminal fragment was capable of inhibiting the interaction between HDAC5 and Gβγ.

Identification of the C terminus of HDAC5 as a major Gβγ binding site suggested that it might comprise a novel Gβγ interaction domain. Scanning protein sequence data bases with this minimal domain did not reveal conservation among multiple protein families, but it did identify the C-terminal 164 amino acids of HDAC4 as containing significant similarity (~68% identity and 80% similarity). The C termini of both HDAC4 and HDAC5 were subcloned into a mammalian expression vector directing the expression of N-terminally GST-tagged proteins. Precipitates with glutathione-Sepharose from lysates of HEK293A cells expressing the GST fusions and HA-tagged Gβγ1γ2 demonstrated that Gβγ interacts with both proteins (Fig. 5B).
In addition to the C-terminal domain, full-length HDAC4 was tested for Gβγ binding by immunoprecpitation C-terminally FLAG-tagged full-length HDAC4. As shown in Fig. 4C, full-length HDAC4 and HDAC5 formed complexes with HA-Gβγγ, demonstrating that HDAC4 is another novel Gβγ-binding protein. Furthermore, HDAC6, which has two catalytic domains that are homologous to those of HDAC4 and HDAC5 but lacks the corresponding C-terminal domain, was not observed to form a complex with Gβγγ. Lysates from HEK293A cells expressing empty vector (pcDNA3) or HDAC4-FLAG, HA-Gβγγ and Gβγγ; and increasing amounts of His-tagged C-terminal domain were subjected to anti-FLAG immunoprecipitation (IPs). The amount of HA-Gβγγ co-precipitated decreased with increasing expression of the C-terminal domain (middle section). IB, immunoblot. B, the C terminus of HDAC4 also interacts with Gβγγ. Lysates from HEK293A cells expressing empty vector (pcDNA3), GST, or GST-tagged HDAC4 or HDAC5 C termini (HDACct and HD5ct) and HA-Gβγγ and Gβγγ were subjected to precipitation with glutathione-Sepharose. Both C termini precipitated HA-Gβγγ, with similar potency (middle section). IB, immunoblot. C, Gβγγ binding is specific for HDACs 4 and 5. HEK293A cells co-transfected with HDAC 4, 5, or 6 or empty vector (CTRL) and HA-Gβγγ and Gβγγ were lysed and subjected to anti-FLAG immunoprecipitation (IPs). Both HDAC4 and HDAC5, which have similar C termini, co-immunoprecipitated HA-Gβγγ, whereas no binding to HDAC6 was detected. Approximately 2.5% of lysates and 50% of immunoprecipitates were analyzed by Western blot. IB, immunoblot.

**The Gβγ Binding Motif Comprises Multiple Non-colinear Sequences**—To further distinguish the Gβγ-binding element in the C terminus of HDAC5, the domain was dissected via PCR into several fragments (Fig. 6A). These fragments were expressed with HA-Gβγγ in HEK293A cells and were tested for Gβγγ binding by gluthathione-Sepharose-mediated precipitation from cell lysates. As displayed in Fig. 6B, fragments consisting of the first 58 (fragment 4) or 88 (fragment 5) amino acids bound Gβγγ, as did a fragment consisting of the last 113 amino acids (fragment 6). Because fragments 4 and 6 do not overlap, these results suggest that the domain contains multiple sequences required for full Gβγγ binding. Of additional significance, fragments consisting of the extreme C-terminal 55 (fragment 2) or 28 (fragment 3) amino acids did not pull down a significant amount of Gβγγ under these conditions, suggesting that Gβγγ does not bind to the signals-responsive nuclear export signal located within amino acids 1081 to 1122 (19).

**Signaling through Gβγ Influences Transcription through an HDACS-Represible Factor**—One well studied role of HDAC5 and other class II HDACs is co-repression of the transcriptional activity of the muscle differentiation factor MEF2C. When HDAC5 is associated with MEF2C, the deacetylase creates a repressive environment, inhibiting the ability of the transcription factor to activate gene transcription. The repressive effect of HDAC5 is controlled in several ways, for instance by prevention of the interaction with MEF2C and by sequestration from the nucleus, which is enhanced by phosphorylation and by binding to 14-3-3 proteins (19, 23–25).

To test the hypothesis that Gβγ impacts HDAC5 function, we employed a quantitative assay of MEF2C transcriptional activity in cultured cells using a procedure originally described by Zhang et al. (22). In this system, ectopic expression of MEF2C activates transcription of a reporter gene (luciferase) that is downstream of multiple MEF2C binding sites. In HEK293A cells, transcriptional activation is repressed in a dose-dependent manner by co-expression of HDACs 4 and 5, as expected (data not shown). In addition, in cells grown in normal serum the activation is potentiated by the cell-permeable HDAC inhibitor TSA (EC50 = 396 nM) (Fig. 7A), suggesting that the activity of MEF2C is limited by endogenous HDACs in resting cells.

The interaction between Gβγ and HDAC5 is enhanced through the activation of a G1-coupled receptor (Fig. 4). To determine whether interaction with Gβγ correlates with a modulation of HDAC5 activity, we assayed MEF2C activity upon activation of the α1A-adrenergic receptor.
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Overnight serum starvation of transfected HEK293A cells resulted in an almost complete inhibition of the MEF2C activity (data not shown). However, stimulation of the receptor with 10 μM (-)-epinephrine caused a time-dependent activation of MEF2C-mediated transcription (Fig. 7B). Note that because this assay relies on transcription and translation of the reporter gene, the observed time dependence is significantly different from the rate of formation of the protein-protein interaction upon receptor stimulation (Fig. 4). To demonstrate the specificity of this response, the cells were treated overnight with pertussis toxin. In these cells, (-)-epinephrine stimulation failed to induce MEF2C activation (Fig. 7B), consistent with a role of G12α heterotrimers in the response.

To determine whether modulation of MEF2C output is dependent on Gβγ or is downstream of G12α, we utilized a Gβγ scavenger, the C-terminal 132 amino acids of GPCR kinase 2 (GRKct2) (26, 27). Importantly, we found that expression of GRKct2 inhibits complex formation between Gβγ and HDAC5. Cells were transfected with HDAC5-FLAG, HA-G12α, and His6-GRKct2 or empty vector. Subsequent immunoprecipitation with an anti-FLAG antibody showed that less G12α was co-precipitated with HDAC5 when GRKct2 was expressed (Fig. 8A).

In addition to disrupting complex formation, we found that expression of GRKct2 inhibited MEF2C-mediated gene transcription (Fig. 8B) in cells grown in normal serum, suggesting that endogenous Gβγ influences MEF2C activity. To show that the inhibition was due to modulation of HDAC activity, we determined the sensitivity of the inhibition to treatment with a cell-permeable HDAC inhibitor. A reduction of the ability of GRKct2 to inhibit MEF2C activity by an HDAC inhibitor would suggest that the effect is mediated through an alteration of HDAC activity (28). At 30 nM TSA, neither MEF2C-independent nor MEF2C-dependent transcription was altered significantly (Fig. 8B). This at this concentration, however, the GRKct2-mediated inhibition was abated (Fig. 8B), suggesting that scavenging Gβγ causes an increase in endogenous HDAC activity.

DISCUSSION

Gβγ-mediated signaling occurs through interactions with a variety of molecules, including enzymes, ion channels, and small G proteins (2). One major signaling role for Gβγ is the management of MAPK cascades, which allows communication between Gβγ and the nucleus (for review, see Ref. 29). Here, we have shown that Gβγ interacts with another nuclear protein, HDAC5, and that this interaction correlates with inhibition of its nuclear function. Our results therefore suggest that inhibition of the activity of transcriptional co-repressors is another mechanism by which Gβγ influences gene expression.

Adding to the List of Gβγ Effectors—The abundance of pathways impacted by Gβγ is evident by the number of biological roles ascribed to Gβγ signaling, such as mediation of chemokine-stimulated chemotaxis (30) and alteration of neurotransmitter release from presynaptic vesicles (31). The finding that Gβγ directly interacts with and affects the function of enzymes involved in protein deacetylation expands the signaling capacity and physiological responsibilities of G proteins.

A New Level of Control of Transcriptional Co-repressors—In addition to expanding the cellular role of Gβγ, the current report also adds to the understanding of HDAC regulation. The regulation of HDAC5 and other class II HDACs is of clear interest. For example, there exists a
strong link between these enzymes and the physiological differentiation of muscle cells and the pathological development of cardiac hypertrophy. For example, Zhang et al. (32) demonstrated that ectopic expression of a hyperactive HDAC5 mutant inhibited the stimulated expression of genes marking pathologic hypertrophy. In addition, mice lacking a related HDAC, HDAC9, displayed increased susceptibility to hypotrophic stimuli (32). Moreover, histone deacetylase inhibitors have been demonstrated to be effective at limiting pathology in a model of hypertrophy (33).

Accordingly, the regulation of class II HDACs has been well studied (34). Phosphorylation by kinases, including calmodulin-dependent protein kinase I, leads to the dissociation of HDAC5 from MEF2C, relieving its inhibitory effect (35). Phosphorylation induces export of HDAC5 from the nucleus into the cytosol, where it is sequestered by members of the 14-3-3 protein family (19, 23–25). Direct interaction with Gβγ provides an additional level through which signaling from cell surface receptors regulates HDAC activity.

An effect of Gβγ on transcription is not without precedent. For instance, Gβγ was found to play a role in the induction of intercellular adhesion molecule 1 (ICAM-1) transcription by thrombin stimulation of protease-activated receptor 1 in endothelial cells (36). In addition, Reusch et al. (37) showed that serum stimulation of a smooth muscle-specific promoter in vascular smooth muscle cells was up-regulated by Gβγ activation and inhibited by expression of a Gβγ scavenger. Accordingly, Gβγ is thought to play an important role in cell proliferation (38–40), and a Gβγ scavenger has been shown recently to inhibit growth in a cancer cell model (41). Several mechanisms are likely responsible for the effect of Gβγ on gene transcription and cell proliferation, including activation of phosphoinositide 3-kinase (36) and the MAPK cascades (42). We propose that modulation of HDAC activity is yet another mechanism influencing nuclear effects of G protein signaling.

Gβγ signaling may be responsible for fine tuning the activity of HDAC5 and MEF2C. Whereas activation of Gβγ in serum starved-cells resulted in a maximal induction of a MEF2C responsive reporter gene of ~2-fold (Fig. 6B), and a Gβγ scavenger had a significant but modest effect on transcription in cells in normal serum (Fig. 7B), treatment with a small molecule inhibitor of HDAC catalytic activity induced an increase of ~10-fold (Fig. 6A). This disparity suggests that Gβγ influences a relatively small portion of cellular HDAC. Such a modulation may be critical under physiological conditions, however.

**Mechanism of HDAC5 Inhibition by Gβγ**—We are currently studying the mechanism by which interaction with Gβγ inhibits HDAC5 cellular activity. Gβγ might collaborate with other proteins in altering subcellular distribution of HDAC5. Alternatively, interaction with Gβγ may reduplicate a fraction of the cellular HDAC pool to an alternate job. The impact of the Gβγ/HDAC5 interaction on signaling through MEF2C may be secondary to a yet to be discovered role for the interaction near the plasma membrane. It is abundantly clear that histones are not the only proteins impacted by the activity of HDACs (11). Numerous transcription factors, including STAT3 (signal transducers and activators of transcription 3) (43) and p53 (44), are acetylated; autoacetylation of HATs can impact catalysis and protein-protein interactions (11, 45), and the roles of acetylation of proteins such as importin-α have yet to be determined (46). Association with HATs and HDACs has been reported to affect the biological roles of extranuclear proteins such as endothelin receptor A (20) and cytosolic phospholipase A2 (47), but the acetylated substrates mediating these effects have not been identified. In mediating the interaction of HDAC5 with other factors, Gβγ thus may be relieving MEF2C of a small portion of its complement of class II HDACs, leading to our observations.

On the other hand, it is interesting to note that the Gβγ dimer may play a direct role in transcriptional regulation. Park et al. (48) showed that Gβγ directly binds another transcriptional repressor, the adipocyte enhancer-binding protein 1 (AEBP1). Levels of Gγγ are regulated during adipogenesis and correlate inversely with AEBP1 transcriptional repression, thus potentially implicating Gβγ in a direct regulation of transcription (48). It is possible, therefore, that Gβγ activates MEF2C-dependent transcriptional activity by shunting class II HDACs to other nuclear complexes.

Additional recent data have identified another direct nuclear function for Gβγ. Kino et al. (40) discovered through a yeast two-hybrid screen that Gβγ is a binding partner of the glucocorticoid receptor. This association correlates with nuclear import of Gβγ upon glucocorticoid stimulation and plasma membrane association of the glucocorticoid receptor upon activation of the somatostatin GPCR (4). In contrast to our findings, however, where Gβγ signaling enhanced the activity of a transcription factor, Gβγ was found by Kino et al. (4) to suppress glucocorticoid-dependent transcriptional activity. The effect of Gβγ could thus depend on the identity of the transcription factor or the contents of the transcriptionally active complex. On the other hand, it is possible that the impact of Gβγ on MEF2C activity in vivo is secondary to recruitment of HDACs from MEF2C to nuclear hormone receptors. Interestingly, Kino et al. (4) found no effect of GAL4-fused Gβγ expression on basal transcription from a GAL4 promoter, indicating that Gβγ was not recruiting co-activators or co-repressors to DNA. The effects of HDACs on transcription are not restricted to modulating the acetylation state of histones, however. The Gβγ-mediated recruitment of class II HDACs could modulate the acetylation state of the transcription factor itself or some other protein; this effect would be predicted to be apparent only in the context of an appropriate transcription factor.

**Conclusion**—The discovery of a direct link between G proteins and histone deacetylases was a novel and unexpected finding. HDACs are emerging as a valuable drug target in the treatment of cancer and other disorders. Therefore, the newly described association may facilitate drug discovery, because signaling pathways involving G proteins have long been known as a rich source of validated drug targets.

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