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siRNA Screen Identifies the Phosphatase Acting on the G Protein-Coupled Thyrotropin-Releasing Hormone Receptor

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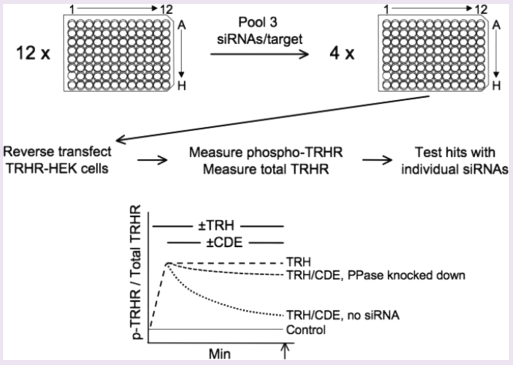
Supporting Information

ABSTRACT: G protein-coupled receptors (GPCRs) are an ubiquitously expressed class of transmembrane proteins involved in the signal transduction of neurotransmitters, hormones and various other ligands. Their signaling output is desensitized by mechanisms involving phosphorylation, internalization, and dissociation from G proteins and resensitized by mechanisms involving dephosphorylation, but details about the phosphatases responsible are generally lacking. We describe here the use of an siRNA-based library to knock down expression of specific phosphatase subunits to identify protein phosphatase 1-α (PP1α) as important for the thyrotropin-releasing hormone (TRH) receptor. Inhibition of PP1α synthesis and overexpression of dominant negative PP1α preserved receptor phosphorylation under conditions favoring dephosphorylation, whereas overexpression of PP1α accelerated dephosphorylation. Knockdown of all three PP1 catalytic subunits inhibited TRH receptor phosphorylation much more powerfully than knockdown of PP1α alone, suggesting that different PP1 isoforms function redundantly. Knockdown of a structural subunit of PP2A, a second potential hit in the library screen, was ineffective. Calyculin A, a potent inhibitor of PP1 family phosphatases, strongly inhibited dephosphorylation of transfected TRH receptors and endogenous receptors in pituitary cells, but fostriecin, which is selective for PP2A family phosphatases, did not. We conclude that the PP1 class of phosphatases is essential for TRH receptor dephosphorylation.



G protein-coupled receptors (GPCRs), the largest class of transmembrane proteins, regulate a multitude of cellular responses. Desensitization of GPCRs, which often follows phosphorylation in their intracellular loops and C-terminal domains, has been extensively characterized.[1,2] Resensitization is equally important but remains more obscure. Resensitization of GPCRs is usually initiated by receptor dephosphorylation, and identifying the phosphatases involved is an important objective.

The majority of GPCR phosphorylation sites are Ser and Thr residues. Protein phosphatases are classified into several families based on sequence homology.[3,4] Protein phosphatases 1 (PP1) and 2A (PP2A) account for approximately 90% of the total Ser/Thr phosphatase activity in eukaryotic cells. They are not selective about the amino acid sequence surrounding the phosphorylated residue but achieve substrate specificity through association with various regulatory subunits.[3−6] PP1s are heterodimers composed of catalytic and regulatory subunits, whereas PP2As are heterotrimers with a large structural subunit that docks both a catalytic and a regulatory subunit.[4,6] PP1 and PP2A have been implicated in the dephosphorylation of numerous GPCRs,[7−10] primarily based on the effects of phosphatase inhibitors such as calyculin A and okadaic acid. These inhibitors target the catalytic subunits of PP1 and PP2A.[11] They are quite toxic since they block dephosphorylation globally and severely disrupt kinase/phosphatase balance. Phosphatase inhibitors can identify the phosphatase family responsible for activity but provide no information about the targeting or catalytic isoforms involved.

The thyrotropin-releasing hormone (TRH) receptor, a GPCR expressed highly in anterior pituitary, binds TRH released from the hypothalamus. Activation of the TRH receptor leads to secretion of thyrotropin, which in turn stimulates thyroid hormone biosynthesis. Since circulating levels of thyroid hormone must be tightly regulated for metabolic homeostasis, control of TRH receptors is essential. When the TRH receptor is activated, its C-terminal tail is rapidly phosphorylated at multiple sites.[12] When extracellular TRH is removed, the receptor undergoes dephosphorylation.[12−14]

Typically, GPCR dephosphorylation has been measured by time-consuming procedures such as immunoblotting. To circumvent this bottleneck, we quantified TRH receptor phosphorylation with a modified ELISA[12] using an affinity-

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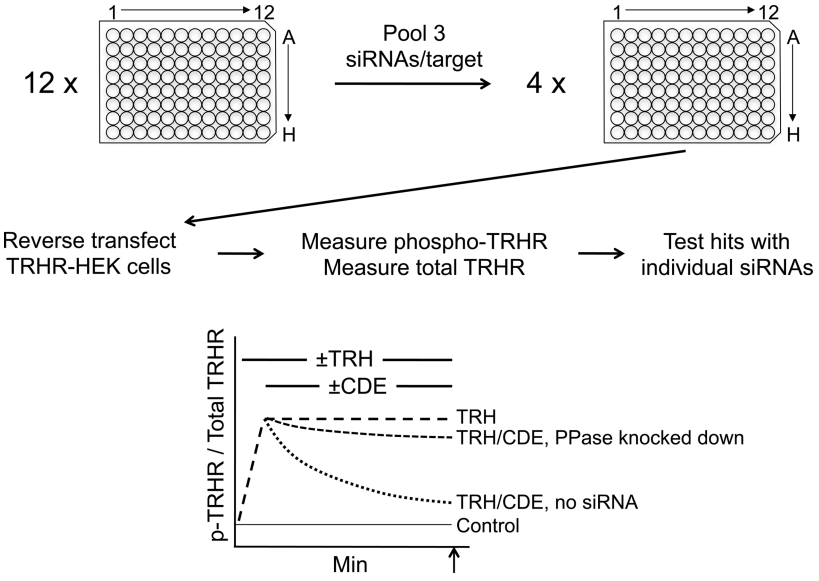

Figure 1. TRH receptor (TRHR) dephosphorylation assay design. Three different siRNAs from the Human *Silencer* Phosphatase siRNA library targeting 267 unique phosphatase genes were arrayed per well. To screen for effects on phosphatases, TRHR phosphorylation was stimulated with TRH for 5 min, and CDE was added to promote dephosphorylation for 30 additional min, when phosphorylated (p-TRHR) and total TRHR were quantified (arrow).

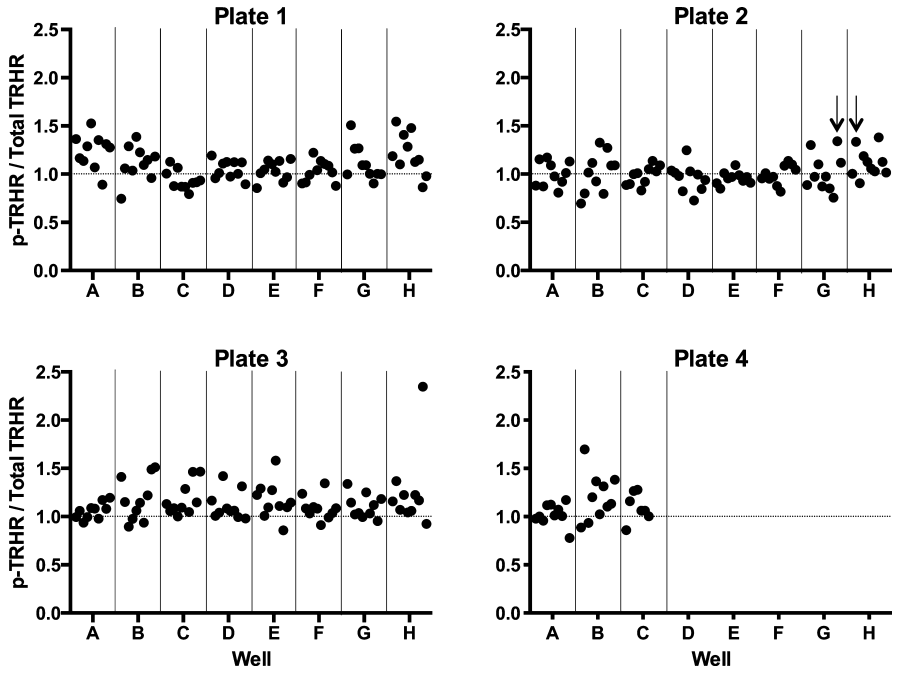

Figure 2. Inhibition of TRHR dephosphorylation by siRNA duplexes. TRHR-HEK cells were reverse transfected with 40 nM pooled siRNAs. p-TRHR was measured in cells incubated with 50 nM TRH for 5 min followed by 100 μM CDE for 30 min. Total TRHR was measured in parallel dishes incubated in buffer alone. Points show the mean p-TRHR divided by the mean total TRHR, each determined in two independent experiments. p-TRHR/total TRHR ratios were normalized to ratios from control wells incubated without any siRNA but otherwise treated exactly as experimental wells (horizontal line). Control wells incubated with no siRNA gave p-TRHR/total TRH ratios of 0.4−0.45 (buffer alone) and 2.25−2.5 (TRH alone). Arrows denote wells containing PP1α and PP2A-Aα siRNAs. Supplementary Table S1 shows the gene targeted for each well number.

purified phosphosite-specific antibody raised against a peptide containing four phoshorylated residues between amino acids 355 and 365 of the receptor tail. The antibody recognizes a peptide phosphorylated at Thr365 but not other phosphopeptides. Phosphorylation in this region represents approximately half of all receptor phosphorylation, based on ^{32}P incorporation, and Ala substitution for Thr365 prevents receptor internalization. Phosphorylation in this region is also required for desensitization. Additional phosphorylation occurs at more distal sites in the tail but apparently not in intracellular loops. The rates of phosphorylation and dephosphorylation at two different distal regions are the same as those reported for sites in the 355−365 region, yet deletion of distal sites has no effect on internalization or desensitization.[12] In this study, we used a

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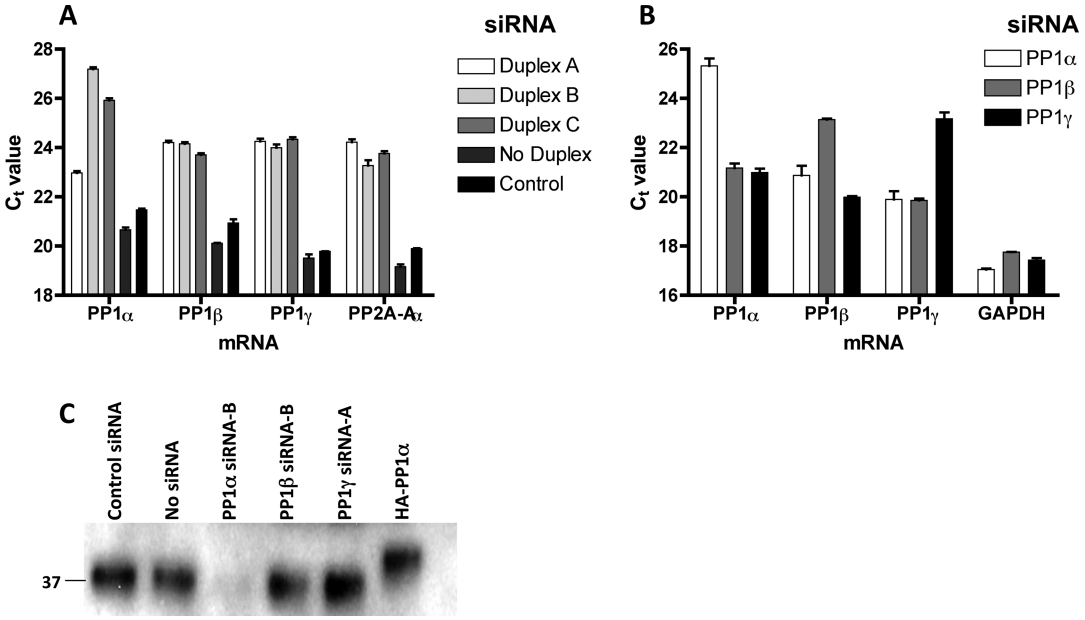

Figure 3. Analysis of protein phosphatase knockdown by individual siRNAs. TRHR-HEK cells were reverse transfected with 40 nM of the indicated siRNAs. (A) mRNA levels for the indicated genes were measured in cells treated with individual targeting siRNA duplexes, without siRNA (no duplex), or with nontargeting siRNA (control). The small decrease in mRNA caused by control siRNA was also observed for GAPDH mRNA and probably results from a small decrease in cell number (not shown). (B) Specificity of single siRNAs (duplex B for PP1α and PP1β; duplex A for PP1γ). Shown are results from one of two independent experiments performed in duplicate. (C) TRHR-HEK cells were reverse transfected with individual siRNAs as above or transfected with plasmid encoding HA-PP1α. Lysates were analyzed by immunoblot using pan-PP1 antibody.

commercially available siRNA library targeting human phosphatase proteins to identify those involved in TRH receptor dephosphorylation. An antibody against phosphosites in the 355−365 region was used to quantify receptor phosphorylation. We report that PP1 is a major regulator of TRH receptor dephosphorylation.

RESULTS AND DISCUSSION

Effects of siRNAs Targeting Phosphatase Genes. To identify phosphatase(s) regulating TRH receptors, we assayed TRH receptor dephosphorylation in cells incubated with siRNAs from a library directed against 267 unique human phosphatase targets (Supplementary Table S1). The library targets catalytic and regulatory subunits of numerous protein phosphatases and phosphatases that dephosphorylate lipids and metabolic intermediates. Human HEK293 cells stably expressing HA-tagged TRH receptors (TRHR-HEK) provided the model system.

The library contained three different siRNAs for each target plus spare wells for controls in 12 96-well plates. For the initial screen, the three siRNAs were pooled at a final concentration of 40 nM and reverse transfected into TRHR-HEK cells for 2 days (Figure 1). Because of the rapid rates of receptor phosphorylation and dephosphorylation, TRH was added to stimulate phosphorylation and the inverse agonist chlordiazepoxide (CDE) was added without a wash step to initiate dephosphorylation. Typical rates of receptor phosphorylation and dephosphorylation following the addition of TRH and then CDE are depicted in Supplementary Figure S1. Phosphorylation reached a stable maximum within 5 min of TRH addition.

In the library screen, phosphoreceptor levels were quantified 30 min after the addition of CDE when most receptor is normally dephosphorylated. If an siRNA knocked down an important phosphatase, we predicted an increased phosphoreceptor signal. Parallel plates were used to quantify HA-epitope as a measure of total receptor concentration. When the assay was evaluated on scattered wells (n = 8), phosphoreceptor absorbance averaged 0.167 ± 0.020 for cells incubated with buffer, 0.768 ± 0.083 for cells incubated with TRH for 5 min, and 0.423 ± 0.020 for cells incubated with TRH for 5 min followed by chlordiazepoxide for 30 min. Total receptor absorbance was 0.554 ± 0.014 in control cells and 0.537 ± 0.025 in cells exposed to agonist.

siRNA screening has the potential to identify unanticipated catalytic or targeting subunits, but the consequence of knocking down an important phosphatase component, delayed dephosphorylation, is subtle and sensitive to cell loss. Potential hits were identified based on the ability of pooled siRNAs to inhibit dephosphorylation reproducibly in initial screens and further evaluated to determine whether: (1) individual siRNAs exerted the same effect as pooled siRNAs; (2) mRNA encoding the target protein was present and reduced by the siRNAs; and (3) transfection with DNA encoding the target protein but resistant to siRNA reversed the effect.

The ratio of phosphorylated to total TRH receptor was used to identify potential hits. The ratios with all siRNAs are shown for cells stimulated with TRH for 5 min (Figure 2) or 30 min (Supplementary Figure S2). The dotted lines in Figure 2 show the control baseline (no siRNA, TRH 5 min, and CDE for 30 min). Raw data for phospho-TRH receptor are provided in Supplementary Figure S3 and for total TRH receptor in Supplementary Figure S4 and Table S2, where particularly toxic siRNAs are noted in red and pink. A fold-increase in phospho-/total receptor of ≥1.2 was scored as a potential hit. siRNAs that caused >40% cell loss were not pursued.

Using these criteria, 8 siRNA targets were identified as possible hits (green in Supplementary Table S1). No siRNA blocked dephosphorylation completely. Potential hits (legend

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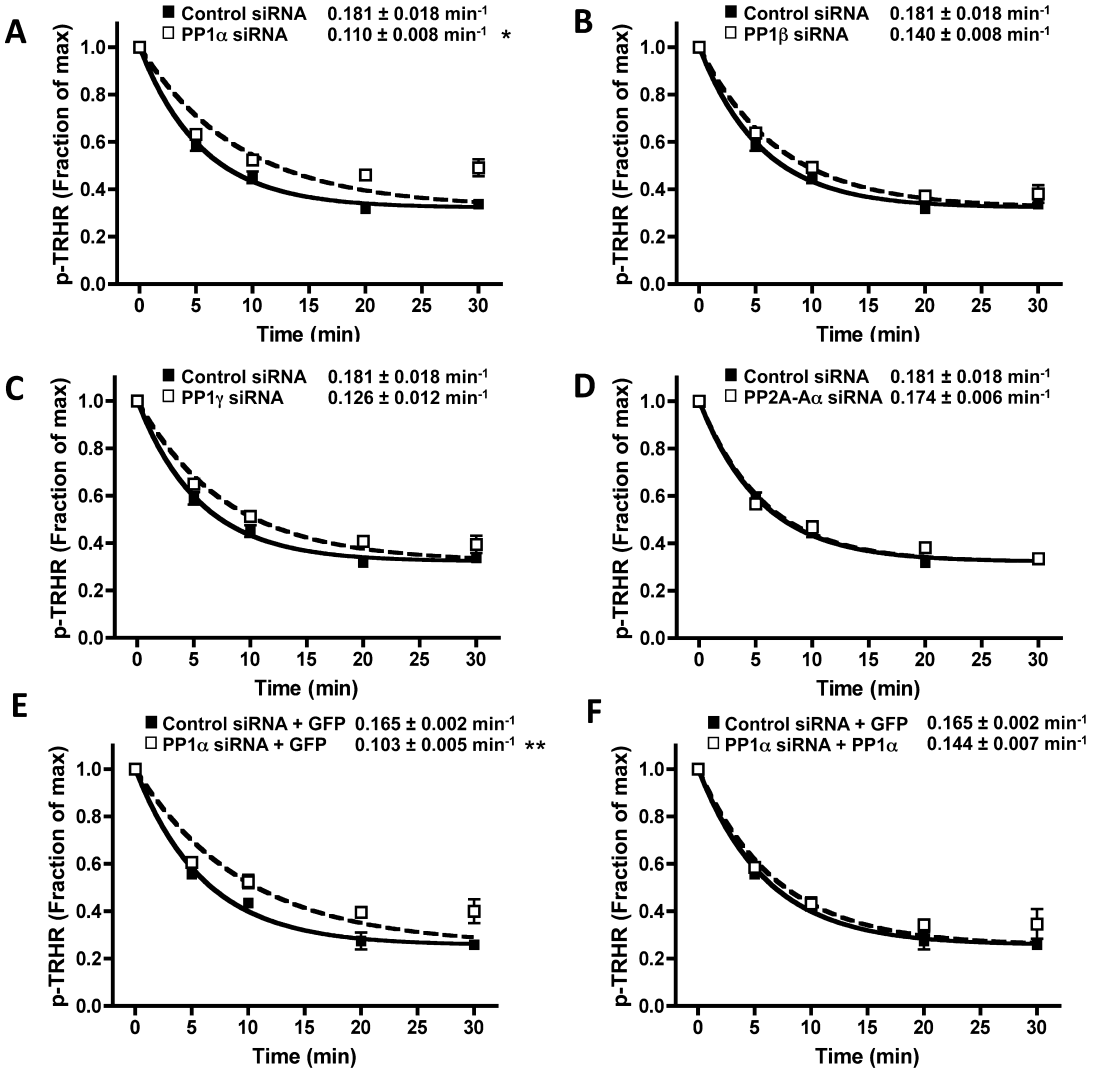

Figure 4. Inhibition of TRHR dephosphorylation by individual siRNA duplexes. (A–D) TRHR-HEK cells were reverse transfected with 40 nM siRNAs targeting (A) PP1α, (B) PP1β, (C) PP1γ, or (D) PP2A-Aα or (A–D) negative control siRNA. Cells were stimulated with 50 nM TRH for 5 min when 100 μM CDE was added (time zero). Shown are mean ± SEM of three experiments performed in duplicate. (E,F) Cells were reverse transfected with 40 nM siRNA and 40 ng of siRNA-resistant plasmid encoding PP1α or GFP. Shown are mean ± range of two experiments performed in duplicate. *$p < 0.05$; **$p < 0.01$.

to Supplementary Table S1) included targets that seem unlikely: enzymes not thought to act on proteins, a tyrosine phosphatase, and a mitochondrially localized phosphatase. Two hits were essential subunits of ubiquitously expressed Ser/Thr phosphatases: protein phosphatase 1, catalytic subunit alpha isoform (PPP1CA or PP1α) and protein phosphatase 2A, regulatory A subunit alpha isoform (PPP2R1A or PP2A-Aα). PP1α is one of three PP1 catalytic subunits, while PP2A-Aα is one of two structural subunit isoforms that scaffolds various PP2A catalytic and targeting subunits. PP1α and PP2A-Aα were identified in screens carried out following both 5 and 30 min exposure to TRH. siRNAs targeting PP1α and PP2A-Aα increased phosphoreceptor signal without decreasing total receptor appreciably, and they did not alter phosphoreceptor signal in control screens using HEK293 cells without receptor and TRHR-HEK cells not stimulated with TRH (data not shown).

Effects of Individual siRNA Duplexes. To rule out the possibility that off-target effects of pooled siRNAs affected receptor dephosphorylation, we measured PP1α, β, and γ and PP2A-Aα transcript levels in the presence of each siRNA duplex using quantitative polymerase chain reaction (qPCR) (Figure 3A). With the exception of one siRNA for PP1α, every siRNA effectively reduced target mRNA. On the basis of the qPCR data, we selected the most efficacious siRNA for further studies; each was specific and reduced mRNA levels by ≥90% (Figure 3B). PP1 protein levels were estimated by immunoblotting with a pan-PP1 antibody that recognizes PP1α, β, and γ (Figure 3C). The results indicate that PP1α is the dominant PP1 in the TRHR-HEK cells but may underestimate the concentration of PP1β and PP1γ because these isoforms were detected poorly by the antibody. The single PP1α siRNA used in subsequent studies reduced PP1α protein by 91.9 ± 2.9% based on densitometry in 5 experiments. PP1β and PP1γ siRNAs had no significant effect and none of the siRNAs significantly altered α-actin.

We measured the effects of individual siRNAs on TRH receptor dephosphorylation. Reduction of PP1α significantly decreased the rate of dephosphorylation (Figure 4A), whereas reduction of PP1β or PP1γ had no significant effect (Figure

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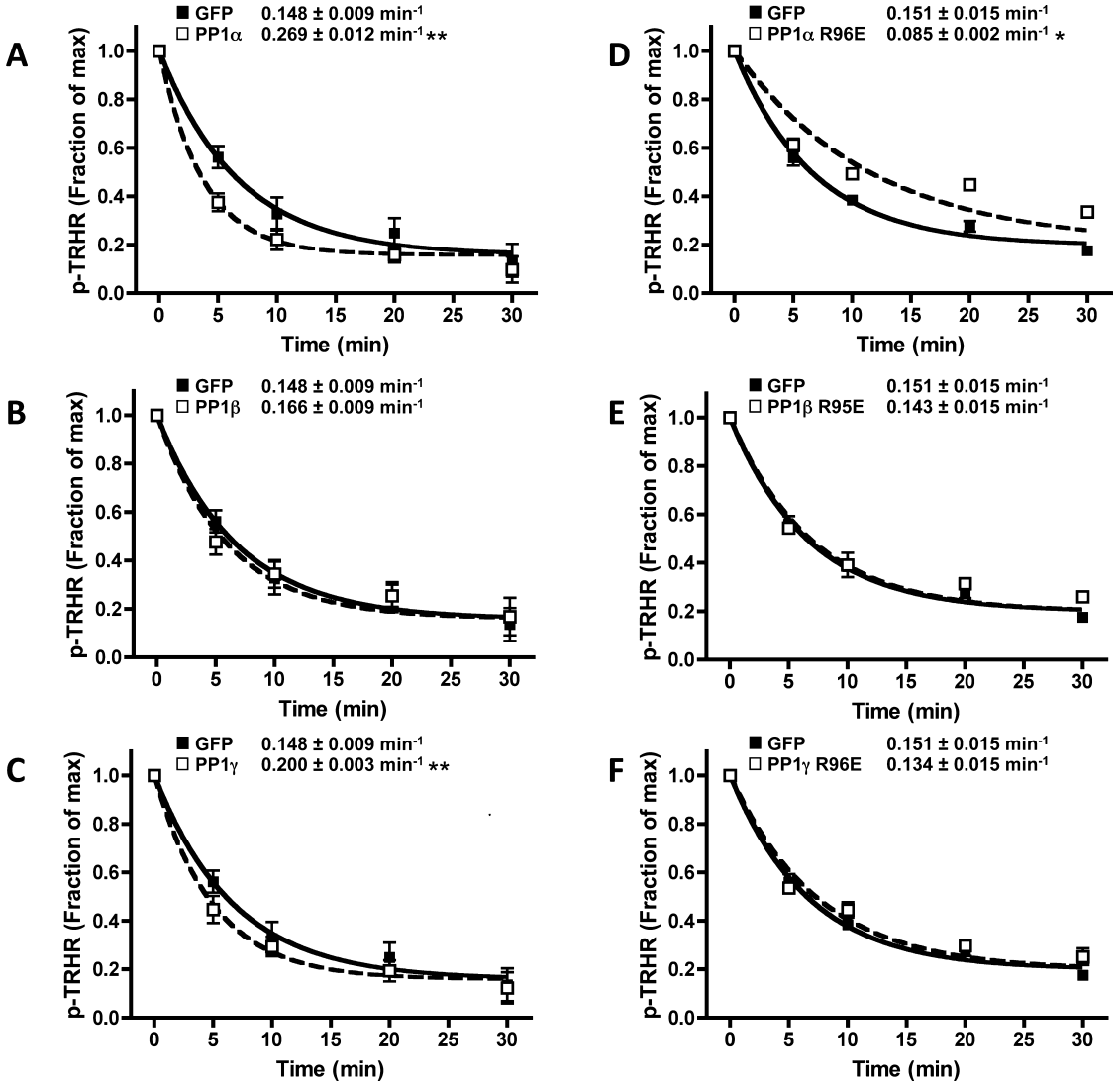

Figure 5. Overexpression of wild-type and dominant negative PP1 isoforms. HEK293 cells were transiently transfected with a 1:4 DNA ratio of V5-tagged TRHR and HA-tagged (A–C) wild-type or (D–F) dominant negative mutant catalytic subunits of PP1. Cells were stimulated with 50 nM TRH for 5 min when 100 μM CDE was added (time zero). Results are the mean ± SEM of three experiments performed in duplicate. *$p < 0.05$; **$p < 0.01$.

4B,C). Knockdown of PP2A-Aα also had no discernible effect (Figure 4D). To show that the decrease in TRH receptor dephosphorylation was directly attributable to a reduction in cellular PP1α, we measured TRH receptor dephosphorylation in the presence of both the siRNA and a plasmid encoding siRNA-insensitive PP1α. Expression of siRNA-insensitive PP1α restored the rate of TRH receptor dephosphorylation (Figure 4E,F).

Overexpression of Protein Phosphatases Supports a Role for PP1α. All three PP1 catalytic subunits were cloned with HA-tags and expressed in cells with V5-tagged TRH receptor. PP1α and PP1γ overexpression increased the rate of TRH receptor dephosphorylation (Figure 5A–C). Mutation of active site Arg residues in PP1s (R96 for α and γ; R95 for β) disrupts catalytic efficiency but not substrate binding, generating dominant negative proteins.[15−17] Overexpression of dominant negative (R96E)PP1α significantly decreased TRH receptor dephosphorylation (Figure 5D). Inhibition was not complete, probably because the R96E enzyme was competing with endogenous PP1α. Dominant negative mutants of PP1β and PP1γ did not alter TRH receptor dephosphorylation (Figures 5E,F). Expression of each phosphatase was confirmed by immunoprecipitation and immunoblotting (Figure 6D). Both wild-type and dominant negative PP1α expressed well, PP1γ expressed better than its dominant negative mutant, and both PP1βs expressed poorly. It is possible that the β and γ isoforms of PP1 failed to affect dephosphorylation because they were not expressed as well as PP1α (Figures 5 and 6D).

The siRNA library targeted 15 different regulatory subunits of PP2A, including the two structural subunits (PP2A-Aα and PP2A-Aβ). Knockdown of PP2A-Aα reduced TRH receptor dephosphorylation in the screen, but its overexpression also inhibited dephosphorylation (Figure 6A), suggesting these effects may be indirectly related to PP2A-Aα expression. PP2A-Aβ was not tested because it is expressed at much lower levels than PP2A-Aα.[18] The library did not contain siRNAs targeting the two catalytic subunits of PP2A (PP2A-Cα and PP2A-Cβ). Nevertheless, we overexpressed both isoforms and found that

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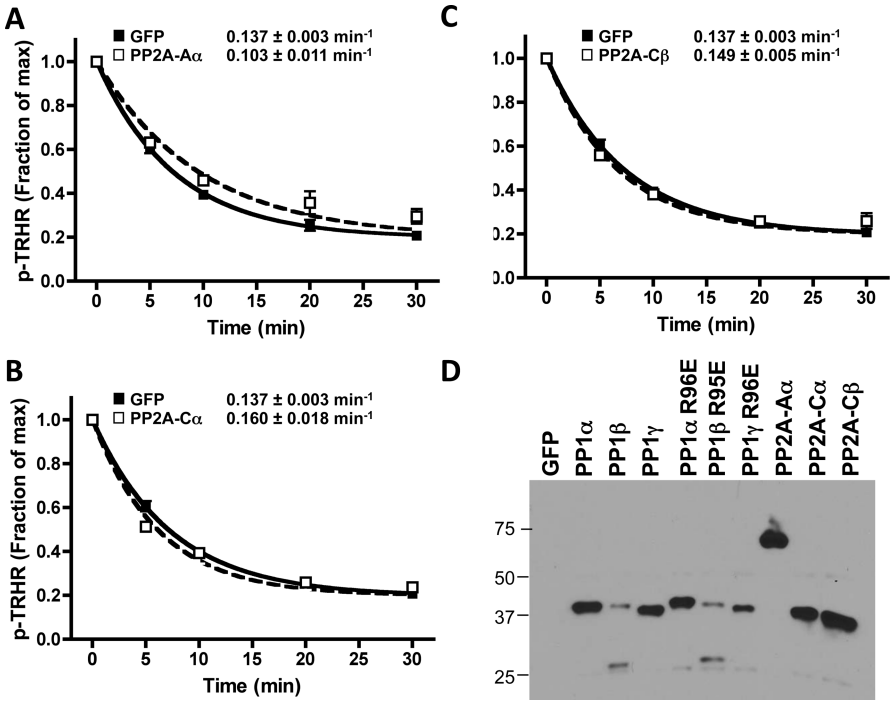

Figure 6. Overexpression of PP2A catalytic and regulatory subunits. HEK293 cells were transiently transfected with a 1:4 DNA ratio of V5-tagged TRHR and HA-tagged subunits of PP2A. Cells overexpressing (A) PP2A-Aα or (B) PP2A catalytic subunits α or (C) β were stimulated with 50 nM TRH for 5 min when 100 μM CDE was added (time zero). Results are the mean ± range of two experiments performed in duplicate. (D) Lysates were prepared from transfected HEK293 cells, and HA-tagged proteins were immunoprecipitated and visualized on immunoblots.

neither (Figure 6B,C) altered receptor dephosphorylation despite robust expression (Figure 6D).

Effect of Combinations of PP1 siRNAs. To better assess whether all three isoforms could regulate the TRH receptor, we tested for additive effects of PP1 siRNAs, while maintaining the same total concentration of siRNA (40 nM) as before. Knockdown of all three PP1 isoforms dramatically inhibited TRH receptor dephosphorylation (Figure 7A). siRNA targeting PP1α inhibited receptor dephosphorylation by itself, but simultaneous knockdown of PP1β and PP1γ amplified the inhibition caused by PP1α knockdown (Figure 7B).

We attempted to rescue the inhibition resulting from knockdown of all three PP1 isoforms by overexpressing siRNA-resistant plasmids encoding each isoform individually. When all endogenous PP1s were depleted, the rate of receptor dephosphorylation was partially restored by each isoform of PP1, and PP1α was again the most effective (Figure 7C).

To determine whether receptor trafficking was altered by inhibition of dephosphorylation, we knocked down all three PP1 isoforms and measured the density of receptors on the plasma membrane of cells exposed to vehicle only, TRH for 5 or 35 min, or TRH for 5 min followed by CDE for 30 min. When cells were treated with PP1$\alpha/\beta/\gamma$ siRNAs, the fraction of receptors internalized was increased, and recovery of receptors on the cell surface following CDE treatment was decreased (Figure 7D). These data are consistent with previous results showing that receptor phosphorylation is required for internalization and that dephosphorylation is required for recycling.[19] They also raise the possibility that altered trafficking contributes to the effects of PP1 levels on dephosphorylation.

Effects of Phosphatase Inhibitors. siRNA library screening and follow-up studies implicated PP1 but not PP2A in TRH receptor dephosphorylation. To confirm this conclusion by an independent method, we tested the effects of okadaic acid, calyculin A, and fostriecin, widely used cell-permeant Ser/Thr phosphatase inhibitors that target the catalytic subunits.[20−24] Fostriecin demonstrates approximately 10 000-fold and okadaic acid 100-fold greater potency toward PP2A than PP1, whereas calyculin A is a potent inhibitor of both enzymes.[20,21,23] TRHR-HEK cells were preincubated with high concentrations of the phosphatase inhibitors. Cultures were then incubated with TRH for 5 min when the inverse agonist CDE was added to initiate dephosphorylation, and phosphorylated TRH receptor was quantified after 30 min. Dephosphorylation was strongly inhibited by calyculin A, moderately inhibited by okadaic acid and insensitive to fostriecin (Figure 8A).

All experiments described above were performed on TRH receptors expressed in HEK293 cells. To validate that our findings were relevant to *in vivo* regulation, we investigated endogenous TRH receptor dephosphorylation in rat pituitary GH3 cells. Because libraries of siRNAs directed against rat phosphatase subunits are not available, we assessed the effects of phosphatase inhibitors. Dephosphorylation was strongly inhibited by calyculin A but not by fostriecin or okadaic acid (Figure 8B), consistent with results obtained with transfected receptors in human TRHR-HEK cells. Calyculin A caused some apparent phosphorylation of TRH receptors in the absence of agonist, but little signal was seen in cells that did not express TRH receptors (data not shown), implying that phosphatase inhibitors amplify a low level of constitutive activity.[25]

The TRH receptor undergoes endocytosis following hormone binding,[26] and the rate of dephosphosphorylation is lower once receptors have internalized.[12,13] This is intriguing because it raises the possibility that the receptor is a substrate for different phosphatases during intracellular trafficking, perhaps resulting in altered phosphatase inhibitor sensitivity. To test this possibility, we incubated cells with inhibitors and stimulated with TRH for 5 or 30 min before adding CDE.

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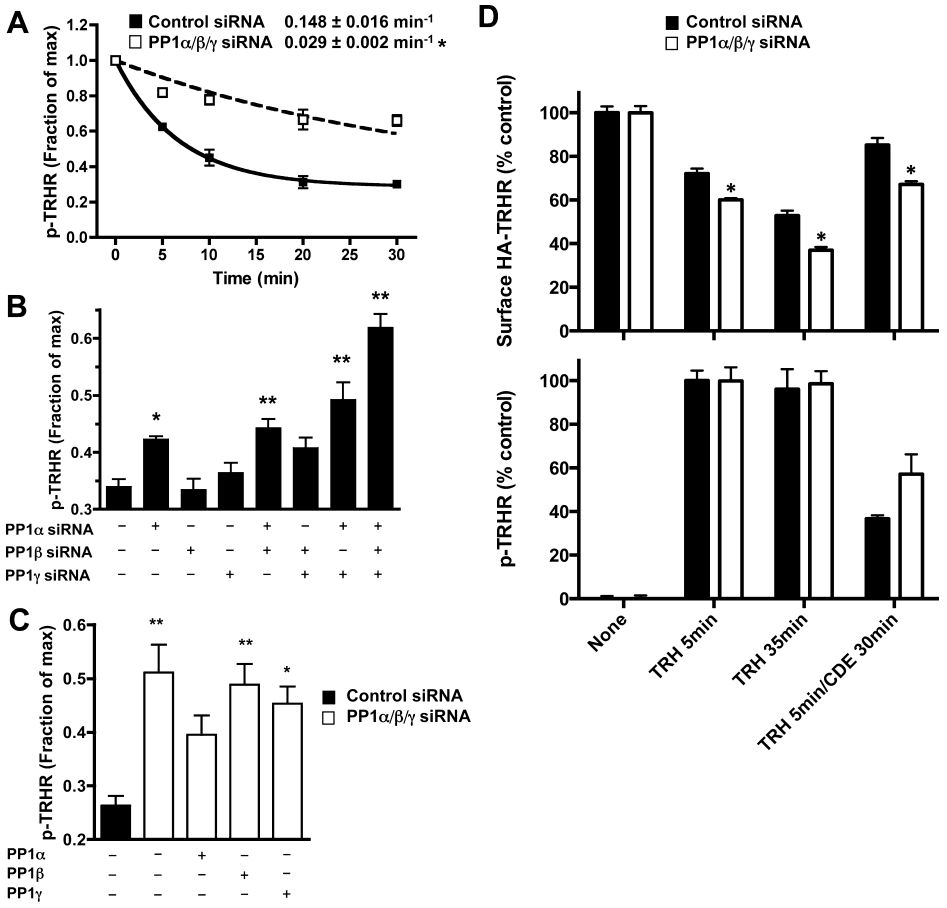

Figure 7. Additive effects of PP1 siRNAs on TRHR dephosphorylation. (A,B,D) TRHR-HEK cells were reverse transfected with the indicated combinations of siRNAs (40 nM total, each siRNA at 13.3 nM). (C) Cells were cotransfected with plasmids shown (80 ng) and 40 nM control siRNA or 13.3 nM each of PP1α, PP1β, and PP1γ siRNAs. Cells were stimulated with 50 nM TRH for 5 min when 100 μM CDE was added for (A) times shown or (B,C) 30 min. Shown are mean ± range or SEM of (A) two, (B) five, or (C) three experiments performed in duplicate. (D) Cells reverse transfected with control or combined PP1 siRNAs were incubated with buffer alone or 50 nM TRH for 5 or 35 min or with TRH for 5 min and CDE for 30 min when surface TRHR or p-TRHR were quantified. HA-TRHR levels are expressed relative to no treatment and p-TRHR levels to 5 min TRH. One of three independent experiments is shown. *$p < 0.05$; **$p < 0.01$ vs siRNA control.

Dephosphorylation was more sensitive to calyculin A than to okadaic acid with IC_{50}s of ~30 and 160 nM, respectively (Figure 8C). In the experiments shown in Figure 8, preincubation was carried out for 30 min because cells tended to detach with longer exposures to calyculin.

Sensitivity to the inhibitors did not change whether receptors were activated for 5 min, when they remained on the surface, or for 30 min, when they had moved to endosomes. Fostriecin was inactive at concentrations as high as 1 μM at 5 and 30 min (data not shown). Receptor dephosphorylation was insensitive to the PP2B (calcineurin) inhibitor cyclosporin A at concentrations up to 1 μM and to the tyrosine phosphatase inhibitor sodium orthovanadate at 1 mM (data not shown). These results indicate that PP1, or a phosphatase with similar drug sensitivity, dephosphorylates TRH receptors regardless of its intracellular location.

The major finding of this article is that PP1 plays an essential role in dephosphorylation of the TRH receptor. This conclusion is based on (1) inhibition of dephosphorylation by pooled and individual siRNAs targeting PP1α; (2) inhibition of dephosphorylation by dominant negative PP1α; (3) acceleration of dephosphorylation by catalytically active PP1α and PP1γ; (4) strong inhibition of dephosphorylation by calyculin A, relative resistance to okadaic acid, and complete resistance to fostriecin, as expected for the PP1 phosphatase family. Inhibitor data also support the involvement of PP1 in dephosphorylation of endogenous receptors in pituitary cells.

PP1 catalytic subunits bind to their regulatory subunits and some substrates in a mutually exclusive manner through a conserved RVxF motif.[6,27] Several class C metabotropic glutamate receptors bind PP1 via RVxF motifs in the receptor.[9] The three isoforms of the PP1 catalytic subunit share greater than 90% sequence identity, including the regions that interact with the RVxF sequence.[28] The TRH receptor has a potential PP1-binding motif, KVSF, in a conserved region of its C-terminal tail. We tested whether this motif was important for dephosphorylation by mutating the Val and Phe residues to Ala, changes known to disrupt the interaction with PP1.[29] These mutations did not affect the rate of TRH receptor dephosphorylation nor did truncation of the C-terminus including the KVSF sequence (data not shown), proving that the TRH receptor does not bind to PP1 exclusively by the canonical RVxF motif. Instead, association of PP1 may occur directly through a noncanonical interaction or multiple weak interactions or indirectly via one or more regulatory subunits of PP1. Targeting subunits[4,27] are prime candidates to bring phosphatases in proximity to phosphorylated GPCRs, and it was somewhat surprising that no targeting subunits were

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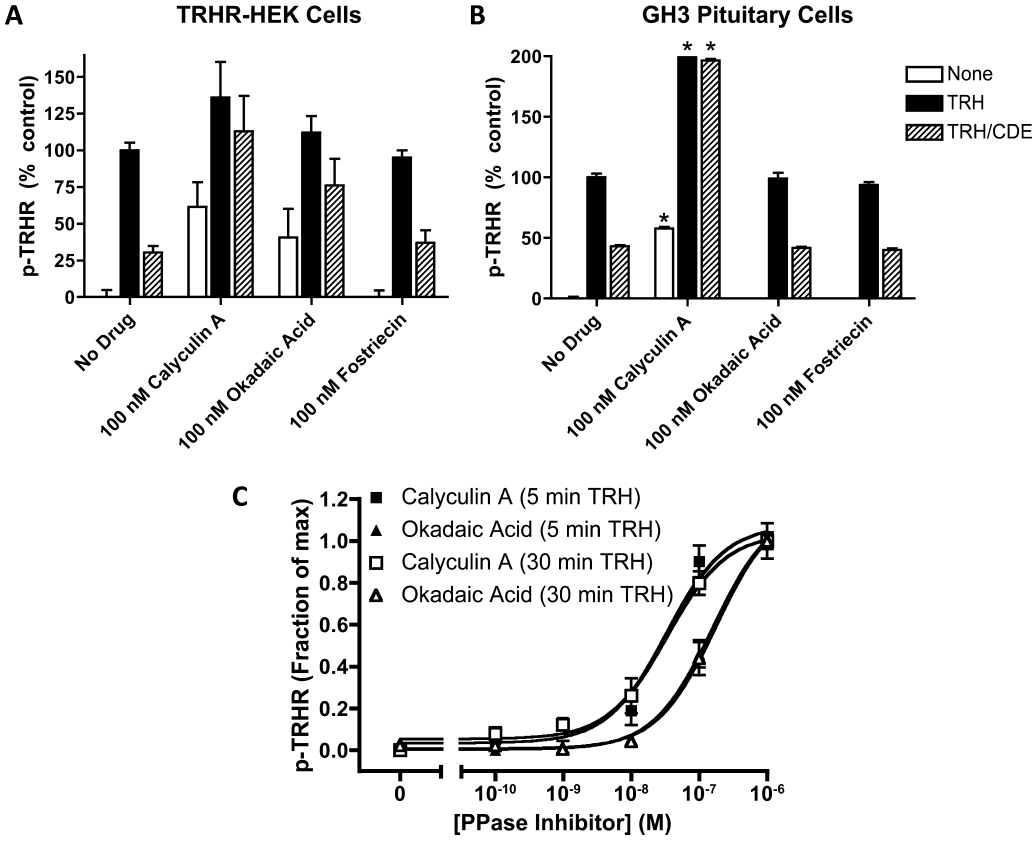

Figure 8. Author: Please verify that the changes made to improve the English still retain your original meaning.Inhibition of TRHR dephosphorylation by protein phosphatase inhibitors. (A) TRHR-HEK cells or (B) pituitary GH3 cells were preincubated with drugs for 30 min at 37 °C and then incubated with vehicle (none), TRH for 5 min (TRH), or TRH for 5 min followed by 100 μM CDE for 30 min (TRH/CDE). Signal obtained in vehicle-treated cells exposed to no drug has been subtracted. (C) TRHR-HEK cells incubated with the indicated concentrations of calyculin A or okadaic acid as above when 50 nM TRH was added for 5 min and 100 μM CDE was added for 30 additional min; p-TRHR levels normalized to (A,B) p-TRHR in TRH-stimulated control cells or (C) the highest levels of p-TRHR in each treatment group. Shown are the mean ± range or SEM of 2−12 experiments performed in duplicate or triplicate. *$p < 0.05$ vs no drug.

uncovered in this screen, but more than half of 26 PP1 regulatory subunits in the library were highly toxic. Interestingly, some of proteins targeted by siRNAs that caused cell loss in the present screen were targets of 73 phosphatase-related siRNAs reported to promote apoptosis in Hela cells.[30]

There are a number of caveats in interpreting the results of our screen. PP1 has been reported to interact with more than 180 different proteins;[6] hence, many potential regulatory partners were not represented in the siRNA library. Important roles for phosphatase subunits cannot be ruled out simply because they were required for cell viability. Knockdown of targeting subunits may have been insufficient at the two day time point selected for screening. Finally, legitimate targeting subunits in the library would not have been detected if they were functionally redundant.

Several other mechanisms are known to localize phosphatases near GPCRs. As discussed above, PP1 binds some GPCRs directly, and PP2C binds directly to mGluR1.[7] PP2A can also bind to certain GPCRs directly, associate with arrestin, and colocalize via an A kinase-anchoring protein.[31] Activation of β2-adrenergic receptors simulates phosphoinositide 3-kinase γ (PI3Kγ), best known as a lipid kinase. In this case, PI3Kγ phosphorylates the inhibitory I2 protein of PP2A, prolonging receptor desensitization.[32] Early work suggested that the β2-adrenergic receptor must undergo endocytosis before being dephosphorylated by an endosomally localized PP2A,[33] but it is now known that both PKA and GRK sites can be dephosphorylated at the plasma membrane.[36] Endocytosis is not a prerequisite for dephosphorylation of the TRH receptor.[12,14] In fact, dephosphorylation is faster while the TRH receptor remains at the plasma membrane. Different phosphatases, including PP1, PP2C, and PP2B (calcineurin), have been found to dephosphorylate GPCRs.[7,10,31,34,35] As information about individual phosphosites has become available, it has become apparent that phosphorylation and dephosphorylation of different sites on receptors can require different enzymes and result in different downstream consequences. For example, in CHO cells, certain phosphosites in the somatostatin receptor are dephosphorylated regardless of subcellular localization, while others are dephosphorylated only after internalization and when different enzymes act on different sites.[34] This level of complexity has not been detected for the TRH receptor, which is phosphorylated and dephosphorylated at the same rate at several sites in the cytoplasmic tail in a nonhierarchical manner,[12] but the situation may differ at phosphosites that have not been analyzed.

There are 500−1000 distinct GPCRs in mammalian species but only 7 GRKs. Knockdown of one of them, GRK2, with siRNA or overexpression of dominant negative GRK2, reduces but does not completely prevent TRH receptor phosphorylation.[12] The situation is similar for phosphatases, where a small number of phosphatases acts on a huge variety of GPCRs.

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As shown here, loss of a single phosphatase (PP1α) causes reproducible but incomplete inhibition of TRH receptor dephosphorylation. Knockdown of all three PP1 catalytic subunits inhibits dephosphorylation quite strongly, suggesting functional redundancy among isoforms. Our data implicating PP1α in TRH receptor dephosphorylation are of particular interest in light of a recent report that siRNA knockdown of PP1β and calyculin A inhibit dephosphorylation of a group of Thr residues in the cytoplasmic tail of the rat somatostatin (SST-2A) receptor expressed in HEK293 cells.[10] Because expression of the three PP1 subunits may differ even among HEK293 cell lines, caution should be observed in ascribing specific roles to individual subunits.

siRNA screens have been used previously to investigate phosphatases involved in cell growth and apoptosis [30] and signaling via NF-kB,[37,38] Foxo,[39] and Hedgehog [40] pathways. Published studies measured downstream outputs affected by multiple signaling molecules and tended to identify large numbers of targets. Hits in our screen were much rarer. The siRNA phosphatase screen reported here uncovered the previously unknown involvement of PP1α in TRH receptor regulation. This information could not have been obtained with currently available phosphatase inhibitors. The bias-free screen can be applied to other GPCRs for which phosphosite-specific antibodies are available. As the mechanisms underlying phosphatase specificity become clearer, regulation of GPCR dephosphorylation and resensitization may prove to be a fertile area for therapeutic intervention.

METHODS

Materials. The Human *Silencer* Phosphatase siRNA Library V3, individual *Silencer* Predesigned siRNAs (siRNA ID#s 2069, 41840, 104508, 105829), and *Silencer* Negative Control #1 siRNA were purchased from Applied Biosystems/Ambion. Calyculin A and okadaic acid were from LC Laboratories, fostriecin from Enzo and Santa Cruz, TRH from Bachem, and chlordizepoxide from Sigma. Protein phosphatases were amplified from cDNA by PCR using oligos that attached two N-terminal HA-tags (oligo sequences available upon request). PCR products were digested with *Hin*dIII and XbaI and ligated into pcDNA3. Plasmids encoding PP1α and PP1β should be resistant to library siRNAs that target 3′ UTRs not present in the constructs. A PP1γ plasmid was designed to create two silent mutations rendering it resistant to its Predesigned siRNA. Immunoblotting confirmed that PP1γ siRNA strongly reduced expression of wild-type PP1γ but not expression of PP1γ containing silent mutations. Protein phosphatase 2 catalytic subunits α and β (PP2α and PP2β) were also amplified using primers to append two N-terminal HA-tags, but those PCR products were first cloned into pCR2.1-TOPO (Invitrogen) and then subcloned into pcDNA3 using *Hin*dIII and XbaI. Mutants were generated using QuikChange Lightning from Stratagene and confirmed by sequencing. All oligos were created by Integrated DNA Technologies or Invitrogen.

Cell Growth and Transfection. Rat pituitary GH3 cells, adherent HEK293 cells, and HEK293 cells stably expressing a rat TRH receptor with two N terminal HA-tags were grown in DMEM/F12 media supplemented with 5% fetal bovine serum on BD Falcon multiwell plates coated with poly(L)lysine. DNA was transfected with Lipofectamine (Invitrogen) 24 h before use. Individual or pooled siRNAs were aliquoted into wells of 48 or 96 well plates, and TRHR-HEK cells were simultaneously plated and transfected (reverse-transfected) using RNAiMAX (Invitrogen). Experiments were performed 48 h later. siRNA and DNA were cotransfected with RNAiMAX for 24 or 48 h. Cells were rinsed once and then equilibrated at RT in HBSS containing 10 mM HEPES, pH 7.4, and treated in the same buffer as described.

Quantification of Phosphorylated and Total TRH Receptor. Phosphorylated TRH receptor was measured in a fixed cell ELISA using Ab6959, a previously described affinity-purified rabbit phosphosite-specific antibody.[12] Total TRH receptor was quantified similarly using HA-11 antibody to the HA-epitope (Covance). Antibodies were added in RIPA/milk buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Triton-X100, 0.1% SDS, 0.5% sodium deoxycholate, pH 8.0, and 5% nonfat dried milk). Antiphospho-TRH receptor was used at 1:500 in 96 well plates and 1:1000 in 48 or 24 well plates and anti-HA antibody at 1:5000. HRP-conjugated antirabbit and antimouse antibodies (Bio-Rad) were used at 1:5000 and 1:10 000, respectively.

Immunoprecipitation and Immunoblotting. Protein phosphatases 1 and 2A with HA-epitopes were detected by immunoprecipitation followed by immunoblotting. Cells were lysed as described[41] and the postnuclear supernatant incubated with 1:5000 anti-HA antibody, and immunoprecipitates collected on Protein A/G PLUS-Agarose (Santa Cruz). Samples were run on 10% or 12% gels and HA-labeled proteins detected via immunoblot using 1:5000 anti-HA and 1:10000 HRP-conjugated goat antimouse antibody. In some experiments, lysates were run on SDS-PAGE directly and immunoblotted with a mouse monoclonal antibody against the catalytic subunit of PP1 (pan-PP1 antibody E-9 from Santa Cruz) at 1:500, rabbit antibody against alpha-actin at 1:250, or anti-HA at 1:5000.

Quantitative PCR. mRNA levels were quantified using an ABI Prism 7000 Sequence Detection System. Samples were generated using the Power SYBR Green Cells-to-Ct Kit (Ambion). Cells were lysed in 96 well format. Primers were designed using Primer3Plus, a web interface primer design program.[42] Primers for qPCR were selected to amplify regions containing intron(s) using the web-based program BLAT.[43] C_t values were calculated as using ABI Prism 7000 software.

TRH Receptor Internalization. Following treatment, live cells were incubated on ice for 20 min with monoclonal antibody to the HA epitope diluted 1:1000 in PBS with 5% milk, washed, fixed for 15 min in 3% paraformaldehyde in PBS, and then processed as described for total TRH receptor quantification.

Data Analysis. All data were analyzed using GraphPad Prism 4.0. Dephosphorylation data were fitted for one-phase exponential decay and constrained to the plateau attained under control conditions; rate constants are shown on figures. Inhibitor data were fitted for sigmoidal dose−response. Data were statistically analyzed using Student's two-tailed t test or one-way ANOVA using Dunnett's post hoc test.

ASSOCIATED CONTENT

Supporting Information

TRHR phosphorylation and dephosphorylation; effects of siRNAs targeting human phosphatase subunits on TRHR dephosphorylation after 30 min TRH stimulation; effect of siRNAs targeting human phosphatase subunits on TRHR dephosphorylation; effect of siRNAs targeting human phosphatase subunits on total TRHR; potential hits in screens with Human *Silencer* Phosphatase siRNA Library V3; toxic siRNAs from Human *Silencer* Phosphatase Library. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Gurevich, V. V., and Gurevich, E. V. (2006) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol. Ther. 110*, 465−502.

(2) Krupnick, J. G., and Benovic, J. L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol. 38*, 289−319.

(3) Barford, D., Das, A. K., and Egloff, M. P. (1998) The structure and mechanism of protein phosphatases: insights into catalysis and regulation. *Annu. Rev. Biophys. Biomol. Struct. 27*, 133−164.

(4) Virshup, D. M., and Shenolikar, S. (2009) From promiscuity to precision: protein phosphatases get a makeover. *Mol. Cell 33*, 537−545.

(5) Shenolikar, S. (1994) Protein serine/threonine phosphatases: new avenues for cell regulation. *Annu. Rev. Cell Biol. 10*, 55−86.

(6) Bollen, M., Peti, W., Ragusa, M. J., and Beullens, M. (2010) The extended PP1 toolkit: designed to create specificity. *Trends Biochem. Sci. 35*, 450−458.

(7) Flajolet, M., Rakhilin, S., Wang, H., Starkova, N., Nuangchamnong, N., Nairn, A. C., and Greengard, P. (2003) Protein phosphatase 2C binds selectively to and dephosphorylates metabotropic glutamate receptor 3. *Proc. Natl. Acad. Sci. U.S.A. 100*, 16006−16011.

(8) Mao, L., Yang, L., Arora, A., Choe, E. S., Zhang, G., Liu, Z., Fibuch, E. E., and Wang, J. Q. (2005) Role of protein phosphatase 2A in mGluR5-regulated MEK/ERK phosphorylation in neurons. *J. Biol. Chem. 280*, 12602−12610.

(9) Croci, C., Sticht, H., Brandstatter, J. H., and Enz, R. (2003) Group I metabotropic glutamate receptors bind to protein phosphatase 1C. Mapping and modeling of interacting sequences. *J. Biol. Chem. 278*, 50682−50690.

(10) Poll, F., Doll, C., and Schulz, S. (2011) Rapid dephosphorylation of G protein-coupled receptors by protein phosphatase 1{beta} is required for termination of {beta}-arrestin-dependent signaling. *J. Biol. Chem. 286*, 32931−32936.

(11) McCluskey, A., Sim, A. T., and Sakoff, J. A. (2002) Serine-threonine protein phosphatase inhibitors: development of potential therapeutic strategies. *J. Med. Chem. 45*, 1151−1175.

(12) Jones, B. W., Song, G. J., Greuber, E. K., and Hinkle, P. M. (2007) Phosphorylation of the endogenous thyrotropin-releasing hormone receptor in pituitary GH3 cells and pituitary tissue revealed by phosphosite-specific antibodies. *J. Biol. Chem. 282*, 12893−12906.

(13) Gehret, A. U., and Hinkle, P. M. (2010) Importance of regions outside the cytoplasmic tail of G-protein-coupled receptors for phosphorylation and dephosphorylation. *Biochem. J. 428*, 235−245.

(14) Jones, B. W., and Hinkle, P. M. (2005) Beta-arrestin mediates desensitization and internalization but does not affect dephosphorylation of the thyrotropin-releasing hormone receptor. *J. Biol. Chem. 280*, 38346−38354.

(15) Huang, H. B., Horiuchi, A., Goldberg, J., Greengard, P., and Nairn, A. C. (1997) Site-directed mutagenesis of amino acid residues of protein phosphatase 1 involved in catalysis and inhibitor binding. *Proc. Natl. Acad. Sci. U.S.A. 94*, 3530−3535.

(16) Zhang, J., Zhang, Z., Brew, K., and Lee, E. Y. (1996) Mutational analysis of the catalytic subunit of muscle protein phosphatase-1. *Biochemistry 35*, 6276−6282.

(17) Zhuo, S., Clemens, J. C., Stone, R. L., and Dixon, J. E. (1994) Mutational analysis of a Ser/Thr phosphatase. Identification of residues important in phosphoesterase substrate binding and catalysis. *J. Biol. Chem. 269*, 26234−26238.

(18) Zhou, J., Pham, H. T., Ruediger, R., and Walter, G. (2003) Characterization of the A-alpha and A-beta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. *Biochem. J. 369*, 387−398.

(19) Jones, B. W., and Hinkle, P. M. (2009) Subcellular trafficking of the TRH receptor: effect of phosphorylation. *Mol. Endocrinol. 23*, 1466−1478.

(20) Swingle, M., Ni, L., and Honkanen, R. E. (2007) Small-molecule inhibitors of Ser/Thr protein phosphatases: specificity, use and common forms of abuse. *Methods Mol. Biol. 365*, 23−38.

(21) Bialojan, C., and Takai, A. (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem. J. 256*, 283−290.

(22) Fagerholm, A. E., Habrant, D., and Koskinen, A. M. (2010) Calyculins and related marine natural products as serine-threonine protein phosphatase PP1 and PP2A inhibitors and total syntheses of calyculin A, B, and C. *Mar. Drugs 8*, 122−172.

(23) Ishihara, H., Martin, B. L., Brautigan, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., and Uemura, D. (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun. 159*, 871−877.

(24) Sheppeck, J. E., Gauss, C. M., and Chamberlin, A. R. (1997) Inhibition of the Ser−Thr phosphatases PP1 and PP2A by naturally occurring toxins. *Bioorg. Med. Chem. 5*, 1739−1750.

(25) Jinsi-Parimoo, A., and Gershengorn, M. C. (1997) Constitutive activity of native thyrotropin-releasing hormone receptors revealed using a protein kinase C-responsive reporter gene. *Endocrinology 138*, 1471−1475.

(26) Ashworth, R., Yu, R., Nelson, E. J., Dermer, S., Gershengorn, M. C., and Hinkle, P. M. (1995) Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling. *Proc. Natl. Acad. Sci. U.S.A. 92*, 512−516.

(27) Cohen, P. T. (2002) Protein phosphatase 1: targeted in many directions. *J. Cell Sci. 115*, 241−256.

(28) Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T., Cohen, P., and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J. 16*, 1876−1887.

(29) Trinkle-Mulcahy, L., Ajuh, P., Prescott, A., Claverie-Martin, F., Cohen, S., Lamond, A. I., and Cohen, P. (1999) Nuclear organisation of NIPP1, a regulatory subunit of protein phosphatase 1 that associates with pre-mRNA splicing factors. *J. Cell Sci. 112* (Pt 2), 157−168.

(30) MacKeigan, J. P., Murphy, L. O., and Blenis, J. (2005) Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nat. Cell Biol. 7*, 591−600.

(31) Shih, M., Lin, F., Scott, J. D., Wang, H. Y., and Malbon, C. C. (1999) Dynamic complexes of beta2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. *J. Biol. Chem. 274*, 1588−1595.

(32) Vasudevan, N. T., Mohan, M. L., Gupta, M. K., Hussain, A. K., and Naga Prasad, S. V. (2011) Inhibition of protein phosphatase 2A activity by PI3Kgamma regulates beta-adrenergic receptor function. *Mol. Cell 41*, 636−648.

(33) Pitcher, J. A., Payne, E. S., Csortos, C., DePaoli-Roach, A. A., and Lefkowitz, R. J. (1995) The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc. Natl. Acad. Sci. U.S.A. 92*, 8343−8347.

(34) Ghosh, M., and Schonbrunn, A. (2011) Differential temporal and spatial regulation of somatostatin receptor phosphorylation and dephosphorylation. *J. Biol. Chem. 286*, 13561−13573.

(35) Spurney, R. F. (2001) Regulation of thromboxane receptor (TP) phosphorylation by protein phosphatase 1 (PP1) and PP2A. *J. Pharmacol. Exp. Ther. 296*, 592−599.

(36) Iyer, V., Tran, T. M., Foster, E., Dai, W., Clark, R. B., and Knoll, B. J. (2006) Differential phosphorylation and dephosphorylation of beta2-adrenoceptor sites Ser262 and Ser355,356. *Br. J. Pharmacol. 147*, 249−259.

(37) Gewurz, B. E., Towfic, F., Mar, J. C., Shinners, N. P., Takasaki, K., Zhao, B., Cahir-McFarland, E. D., Quackenbush, J., Xavier, R. J., and Kieff, E. (2012) Genome-wide siRNA screen for mediators of NF-kappaB activation. *Proc. Natl. Acad. Sci. U.S.A. 109*, 2467−2472.

(38) Li, S., Wang, L., Berman, M. A., Zhang, Y., and Dorf, M. E. (2006) RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-kappaB signaling. *Mol. Cell 24*, 497−509.

(39) Mattila, J., Kallijarvi, J., and Puig, O. (2008) RNAi screening for kinases and phosphatases identifies FoxO regulators. *Proc. Natl. Acad. Sci. U.S.A. 105*, 14873−14878.
(40) Jia, H., Liu, Y., Yan, W., and Jia, J. (2009) PP4 and PP2A regulate Hedgehog signaling by controlling Smo and Ci phosphorylation. *Development 136*, 307−316.
(41) Zhu, C. C., Cook, L. B., and Hinkle, P. M. (2002) Dimerization and phosphorylation of thyrotropin-releasing hormone receptors are modulated by agonist stimulation. *J. Biol. Chem. 277*, 28228−28237.
(42) Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J. A. (2007) Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res. 35*, W71−74.
(43) Kent, W. J. (2002) BLAT: the BLAST-like alignment tool. *Genome Res. 12*, 656−664.