Dimerization of the thyrotropin-releasing hormone receptor potentiates hormone-dependent receptor phosphorylation

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The G protein-coupled thyrotropin (TSH)-releasing hormone (TRH) receptor forms homodimers. Regulated receptor dimerization increases TRH-induced receptor endocytosis. These studies test whether dimerization increases receptor phosphorylation, which could potentiate internalization. Phosphorylation at residues 355–365, which is critical for internalization, was measured with a highly selective phospho-site-specific antibody. Two strategies were used to drive receptor dimerization. Dimerization of a TRH receptor-FK506-binding protein (FKBP) fusion protein was stimulated by a dimeric FKBP ligand. The chemical dimerizer caused a large increase in TRH-dependent phosphorylation within 1 min, whereas a monomeric FKBP ligand had no effect. The dimerizer did not alter phoshorylation of receptors lacking the FKBP domain. Dimerization of receptors containing an N-terminal HA epitope also was induced with anti-HA antibody. Anti-HA IgG strongly increased TRH-induced phosphorylation, whereas monomeric Fab fragments had no effect. Anti-HA antibody did not alter phosphorylation in receptors lacking an HA tag. Furthermore, two phosphorylation-defective TRH receptors functionally complemented one another and permitted phosphorylation. Receptors with a D71A mutation in the second transmembrane domain do not signal, whereas receptors with four Ala mutations in the 355–365 region signal normally but lack phosphorylation sites. When D71A- and 4Ala-TRH receptors were expressed alone, neither underwent TRH-dependent phosphorylation. When they were expressed together, D71A receptor was phosphorylated by G protein-coupled receptor kinases in response to TRH. These results suggest that the TRH receptor is phosphorylated preferentially when it is in dimers or when preexisting receptor dimers are driven into microaggregates. Increased receptor phosphorylation may amplify desensitization.

G protein | desensitization | G protein-coupled receptor | G protein-coupled receptor kinase | PKC

The thyrotropin (ISH)-releasing hormone (TRH) receptor belongs to the superfamily of seven transmembrane helix G protein-coupled receptors (GPCRs). TRH is a tripeptide that functions as a hormone regulating TSH and prolactin secretion. TRH receptors signal through G_q and G_{11}, leading to the production of inositol (1, 4, 5) triphosphate and the release of intracellular calcium. After TRH binds, the TRH receptor is rapidly desensitized (1). Desensitization of GPCRs is initiated when receptors are phosphorylated by G protein-coupled receptor kinases (GRKs) or second messenger-activated kinases. Phosphorylated receptors recruit β-arrestins and, as a result, become uncoupled from G proteins. Once docked on activated GPCRs, β-arrestins also bind to clathrin and adapter proteins, causing receptor internalization (2).

A growing body of evidence suggests that many GPCRs, including the TRH receptor, form dimers or oligomers (3–6). Atomic force microscopy reveals higher order oligomers of rhodopsin (7). Receptor heterodimerization also has been shown to modulate ligand binding, signaling, and trafficking. Ligand-binding properties are altered by heterodimerization of various opioid receptors (8, 9); adenosine A2A and dopamine D1 receptors (10); and somatostatin SSTR5 and dopamine D2 receptors (11). Heterodimerization between AT1 and bradykinin B receptors enhances signaling triggered by angiotensin II (12). Heterodimers of δ-opioid and β2-adrenergic receptors undergo endocytosis in response to either an opioid or adrenergic agonist (13). As shown by these examples, heterodimerization of GPCRs can generate diversity in cell signaling.

The function of homodimerization of GPCRs has been more difficult to define. Inhibitors of receptor homodimerization and dimerization-defective mutants are not available for most GPCRs, including the TRH receptor. Although dimerization of the TRH receptor has been demonstrated by several approaches and TRH has been found to increase the fraction of receptors behaving as dimers (14, 15), the physiological ratio of the monomer:dimer:higher oligomer in cells is unknown. Solubilized TRH receptors run at the size of monomers and dimers on SDS/PAGE, and receptors with different epitope tags coimmunoprecipitate when coexpressed (14). However, these approaches are limited by the possibility that receptors dissociate or aggregate in detergent solutions. TRH receptor dimerization has been demonstrated in living cells by BRET, which detects changes in the distance between reporters, but it is not known whether such changes result from formation of new dimer pairs or conformational changes of receptors that are already dimerized. Because of the limitations in our information about receptor oligomerization, we developed a regulated homodimerization system that exploits FKBP12 and its small molecular ligands (16). We showed that dimerization of the TRH receptor does not affect TRH signaling based on the increases in intracellular calcium and inositol phosphates, but does increase TRH-dependent receptor internalization.

In the study reported here, we tested the hypothesis that dimerization promotes receptor internalization by potentiating phosphorylation. We show that TRH-dependent receptor phosphorylation is dramatically increased when receptor dimerization is induced by either a synthetic dimerizer or an antibody, and that a mutant receptor that does not undergo phosphorylation when expressed alone becomes phosphorylated in response to TRH when expressed together with a different phosphorylation-defective TRH receptor. The data provide direct evidence that formation of TRH receptor multimers amplifies receptor phosphorylation and show that dimerization of GPCRs has important

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Abbreviations: BIM, bisindolylmaleimide; CDE, chlordiazepoxide; FKBP, FK506-binding protein; GPCR, G protein-coupled receptor; GRK, GPCR kinase; PMA, phorbol 12-myristate-13-acetate; TRH, thyrotropin-releasing hormone; TSH, thyrotropin.

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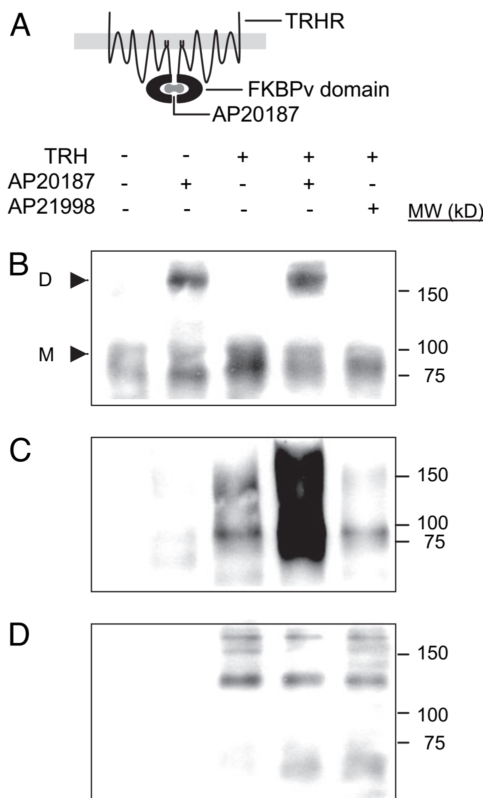

Fig. 1. Effect of AP20187-induced dimerization on TRH receptor phosphorylation. (*A*) Dimerization strategy. (*B–D*) CHO cells stably expressing TRHR-FKBP-HA (*B* and *C*) or 2HA-TRHR (*D*), which lacks an FKBP domain, were incubated with 100 nM dimeric (AP20187) or monomeric (AP21998) FKBP ligand or 100 nM TRH, as shown, for 5 min. Total receptors were precipitated with anti-HA antibody (*B*), and phosphorylated receptors were precipitated with anti-phospho-TRH receptor antibody 6959 (*C* and *D*). All blots were probed with anti-HA antibody. The figure is representative of three independent experiments. D, dimer; M, monomer.

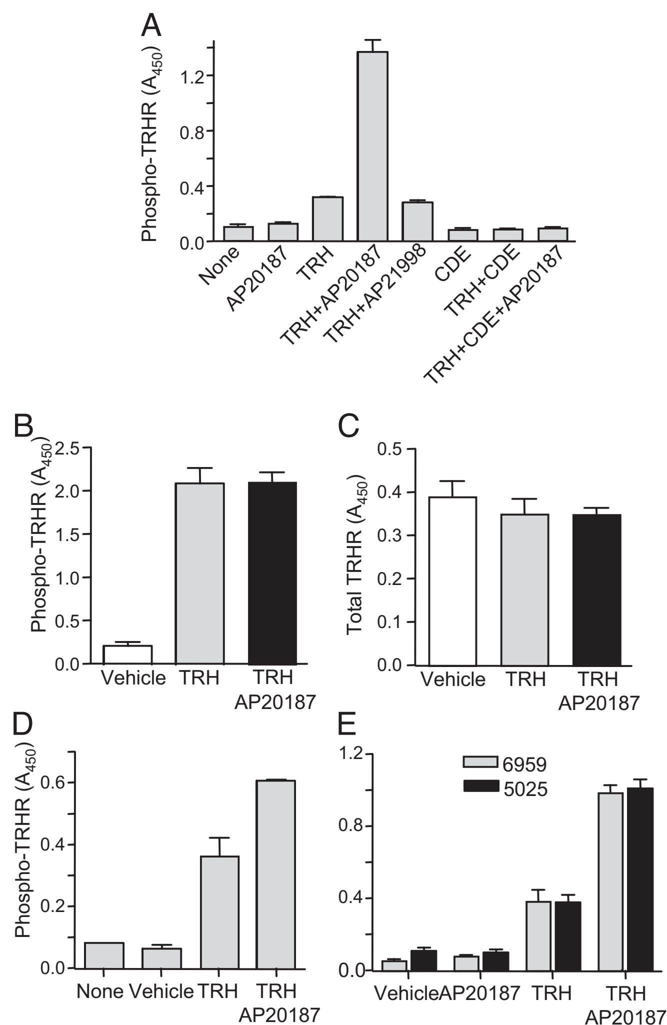

Fig. 2. Effect of regulated dimerization on TRH receptor phosphorylation measured by ELISA. CHO cells stably expressing TRHR-FKBP-HA (*A* and *C–E*) or 2HA-TRHR (*B*) were incubated with 100 nM TRH, 100 nM AP20187, 100 nM AP21998, or 10 μM CDE, alone or in combination, for 5 min. The inverse agonist CDE was not toxic as tested. ELISAs were carried out with anti-phospho-TRH receptor antibody 6959 (*A*, *B*, and *D*), polyclonal antibody 1135 against a nonphosphorylated peptide (residues 353–371) (*C*), or antibodies against fully phosphorylated peptides from residues 351–370 (Ab 6969, gray bars) and 366–385 (Ab 5025, black bars) of the receptor tail (*E*). Cells were fixed except in *D*, where lysates were used. TRH significantly increased phosphorylation in all cases ($P < 0.01$) but had no significant effect on total receptor. In *A* and *D*, TRH-stimulated phosphorylation was greater in the presence of dimerizer ($P < 0.01$), but dimerizer had no significant effect in *C*.

consequences for phosphorylation that will impact signal transduction, desensitization, and receptor trafficking.

Results

Effect of Regulated Dimerization on Receptor Phosphorylation. To control TRH receptor dimerization, we stably expressed a receptor fusion protein with an HA-tagged FKBP domain at the carboxyl terminus in CHO cells (Fig. 1*A*). The construct, TRHR-FKBP-HA, responds to TRH with normal inositol phosphate production, intracellular calcium signals, and mitogen-activated protein kinase activation (16). A synthetic dimeric ligand of FKBP, AP20187, induced receptor dimerization (Fig. 1*B*, second and fourth lanes), whereas a monomeric ligand, AP21998, did not (Fig. 1*B*, fifth lane). To study receptor phosphorylation, we used a recently developed polyclonal antibody against a multiply phosphorylated peptide representing residues 351–370 of the C-terminal tail of the TRH receptor, which recognizes phosphorylated TRH receptor specifically (17). Cells stably expressing the TRH receptor-FKBP fusion protein were treated with or without dimerizer and TRH. Phosphorylated receptors were immunoprecipitated with anti-phospho-TRH receptor antibody, run on SDS/PAGE, and immunoblotted with anti-HA antibody. No phosphorylated receptors were detected in lysates from untreated cells or from cells exposed to dimerizer alone (Fig. 1*C*, first and second lanes), whereas phosphorylated TRH receptors were clearly seen in TRH-treated cells (Fig. 1*C*, third lane). Receptor phosphorylation was markedly increased in cells incubated with dimerizer and TRH (Fig. 1*C*, fourth lane). The monomeric ligand AP21998 did not potentiate phosphorylation (Fig. 1*C*, fifth lane). In a control experiment, we tested cells expressing an HA-tagged TRH receptor that had no FKBP domain (Fig. 1*D*). TRH caused the expected increase in receptor phosphorylation, but dimerizer had no effect alone or with hormone.

We quantified phosphorylated receptors after incubation with or without AP20187 and TRH by using an ELISA developed to measure receptor phosphorylation in fixed cells. Cells expressing TRHR-FKBP-HA were incubated with agonist or the weak inverse agonist chlordiazepoxide (CDE) in the presence or absence of AP20187 and then fixed. Dimerizer did not cause phosphorylation by itself, but dramatically increased TRH-dependent receptor phosphorylation (Fig. 2*A*). Monomeric

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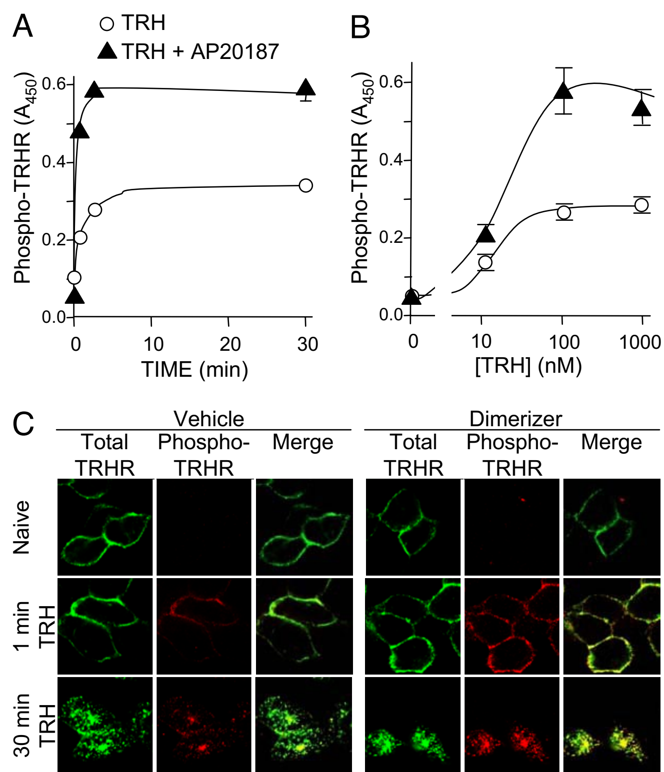

Fig. 3. Dose and time dependence of TRH receptor phosphorylation and receptor localization. CHO cells stably expressing TRHR-FKBP-HA were stimulated with TRH (circles) or TRH plus AP20187 (triangles). (*A* and *B*) Cells were incubated with 100 nM TRH for 0–30 min (*A*) or with 0–1 μM TRH for 5 min (*B*). Dimerizer did not change the EC_{50} for TRH-induced phosphorylation (EC_{50} = 35.5 ± 8.6 nM without and 15.9 ± 3.6 nM with AP20187; $n = 4$, $P > 0.1$). (*C*) Cells were incubated with or without 100 nM TRH and 100 nM AP20187 as indicated and were stained with anti-HA antibody for total receptor (green) or with antibody 6959 for phosphoreceptor (red). The overlay shows colocalization of total and phosphorylated TRH receptor (yellow). All images were processed identically.

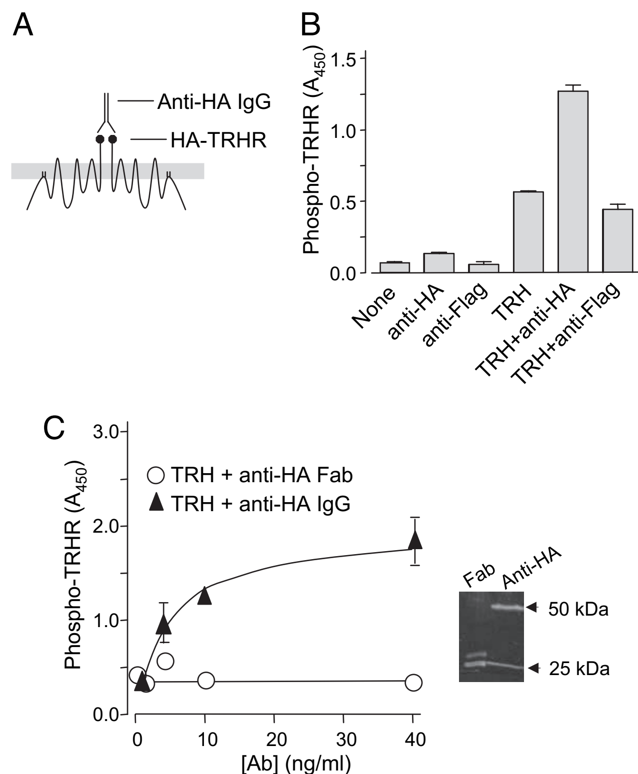

Fig. 4. Effect of antibody on receptor phosphorylation. (*A*) Dimerization strategy. (*B*) CHO cells stably expressing HA-TRH receptor were incubated with anti-HA antibody (1:250) or anti-Flag antibody (1:250) in the presence or absence of 1 μM TRH for 5 min. TRH significantly increased phosphorylation versus control ($P < 0.001$), and anti-HA but not anti-Flag antibody significantly increased the response to TRH ($P < 0.001$). (*C*) Cells were treated with TRH and indicated concentration of anti-HA IgG or Fab fragments for 5 min. Receptor phosphorylation was measured by ELISA by using antibody 6959. (*Inset*) Fab fragments of anti-HA antibody were prepared by papain digestion and analyzed on SDS/PAGE.

AP21998 did not increase phosphorylation alone or potentiate the effect of TRH. CDE completely blocked receptor phosphorylation. These data demonstrate that increased receptor phosphorylation requires an agonist and is markedly potentiated by dimerizer. In cells expressing a receptor without the FKBP domain, dimerizer did not potentiate receptor phosphorylation (Fig. 2*B*).

Additional control experiments ruled out the possibility that dimerizer altered reactivity with the phospho-site-specific antibody by changing receptor localization or conformation. Immunoreactivity with antibody against a nonphosphorylated peptide from the same region of the receptor was unaffected by incubation with TRH alone or with dimerizer (Fig. 2*C*). A dimerizer-dependent increase in receptor phosphorylation (Fig. 2*D*) was observed in detergent lysates, where any effect of receptor localization would be negated.

We also asked whether AP20187 stimulates phosphorylation at a more distal site on the TRH receptor cytoplasmic tail by using an antibody against a phosphorylated peptide from residues 367–386 (17). As shown in Fig. 2*E*, dimerizer increased TRH-dependent phosphorylation in a distal region of the receptor (black bars) to exactly the same extent as phosphorylation at the proximal site (gray bars). Receptor phosphorylation occurred rapidly when TRH was added, reached a maximum within 5 min (Fig. 3*A*), and was dose-dependent (Fig. 3*B*).

Subcellular Localization of Phosphorylated TRH Receptor. The subcellular distribution of phosphorylated and total TRH receptors was observed by immunofluorescence microscopy by using anti-phosphoreceptor or anti-HA antibodies, respectively (Fig. 3*C*). In unstimulated cells, the receptor was mainly localized at the cell surface, and phosphorylated TRH receptor was not detectable. Receptor localization was not affected by dimerizer alone, and no receptor phosphorylation was stimulated by dimerizer. The intensity of phosphoreceptor staining was greatly increased at all time points when cells were exposed to both dimerizer and TRH. After 1 min with TRH, the phosphorylated receptor was visible at the plasma membrane and colocalized with total receptor. After 30 min, the receptor was localized in large vesicles, and treatment with dimerizer caused more of these vesicles to be deeper inside the cell. Some internalized receptor that was not phosphorylated (green in overlays) was detectable after TRH addition, likely representing the receptor that was dephosphorylated after internalization.

Effect of Antibody-Induced Dimerization on Receptor Phosphorylation. In a second, distinct strategy to regulate dimerization of TRH receptors (Fig. 4*A*), we incubated cells expressing receptor with an amino-terminal HA tag with dimeric IgG against the HA epitope for 5 min with or without TRH and assessed receptor phosphorylation. Anti-HA antibody did not induce phosphorylation by itself, but clearly increased TRH-dependent phosphorylation (Fig. 4 *B* and *C*). Control anti-Flag IgG had no effect on

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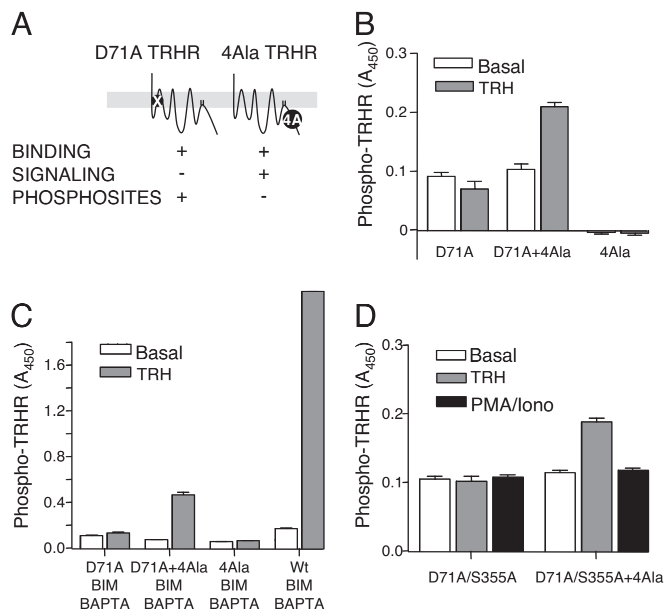

Fig. 5. Phosphorylation of coexpressed mutant TRH receptors. (*A*) Functional complementation strategy. (*B–D*) CHO cells were transfected with D71A and 4Ala-TRH receptors (*B* and *C*) or D71A/S355A and 4Ala-TRH receptors (*D*), alone or together. Cells were incubated with 1 μM TRH for 5 min, and receptor phosphorylation was measured by ELISA with antibody 6959. (*C*) Ten nanomolars BIM and 30 μM BAPTA/AM were added 15 min before and during TRH exposure. (*B–D*) TRH had no significant effect on phosphorylation in cells expressing D71A or 4Ala-TRH receptors but caused a significant increase ($P <$ 0.01) in cells expressing both receptors.

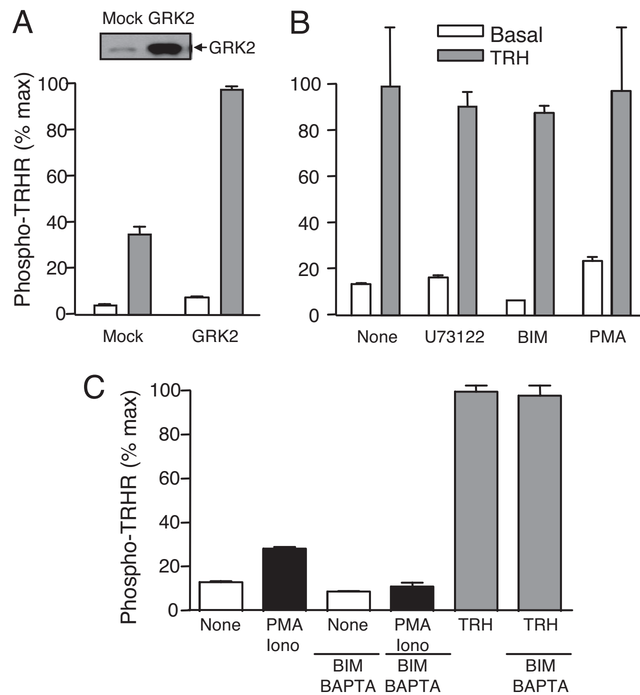

Fig. 6. Effects of GRK2 and second messenger-activated kinases on TRH receptor phosphorylation. (*A*) CHO cells were transfected with TRH receptor and either GRK2 or pcDNA3 vector and stimulated with or without 100 nM TRH for 5 min. (*Inset*) To confirm the overexpression of GRK2, cell lysates were separated on SDS/PAGE, and the blot was probed with anti-GRK2 antibody. (*B*) CHO cells stably expressing HA-TRH receptors were preincubated with 10 μM U73122, 10 nM BIM, or 100 nM PMA for 1 h and treated with or without TRH for 5 min. (*C*) Cells were preincubated with 10 nM BIM and 30 μM BAPTA/AM for 15 min when the indicated combinations of drugs (vehicle, 1 μM TRH or 100 nM PMA, and 1 μM ionomycin) were added for 5 min. Receptor phosphorylation was monitored by using ELISA with antibody 6959. TRH significantly increased phosphorylation in all groups ($P < 0.001$). (*A* and *B*) The response was significantly increased by GRK overexpression ($P < 0.001$), but not significantly altered by U73122, BIM, or PMA. (*C*) PMA plus ionomycin significantly increased phosphorylation alone ($P < 0.01$), but not when BIM and BAPTA/AM were present.

phosphorylation of the HA-tagged TRH receptor, and anti-HA antibody did not affect phosphorylation in cells expressing receptor with an amino-terminal Flag epitope (data not shown). The ability of anti-HA IgG to promote agonist-induced dimerization resulted from its ability to dimerize receptors because monovalent Fab anti-HA antibody did not affect phosphorylation alone or with TRH (Fig. 4*C*).

Complementary Phosphorylation of D71A TRH Receptor Mutants. We used a functional complementation approach to determine whether two different mutant receptors that cannot be phosphorylated by themselves can complement one another in dimers and rescue the phosphorylation defect. In one mutant, Asp-71 in TM 2 was mutated to Ala; 1 μM TRH did not increase inositol phosphate formation in cells expressing this mutant receptor (ref. 18 and data not shown). The D71A receptor has lower affinity for TRH ($K_d \approx 30$ nM) than the wild-type receptor, but would have been fully occupied at 1 μM TRH. We also generated a mutant with all four potential phosphorylation sites in the 355–365 region mutated to Ala, the 4Ala-TRH receptor. The 4Ala-TRH receptor binds TRH normally and signals strongly, but cannot undergo phosphorylation detectable by antibody 6959 (17), although it is phosphorylated normally at downstream residues.

Neither the 4Ala- nor the D71A TRH receptor was phosphorylated at all in response to 1 μM TRH (Fig. 5*B*). When the D71A mutant was coexpressed with the 4Ala mutant, however, the D71A receptor became phosphorylated in response to TRH. It is noteworthy that only half of the D71A receptor is predicted to be in a dimeric complex with 4Ala receptors if the two receptors are expressed at equal concentrations. We also demonstrated a direct interaction between the D71A and 4Ala receptor mutants by coimmunoprecipitating the HA-tagged 4Ala-TRH receptor with Flag-tagged D71A TRH receptor (data not shown).

It was important to rule out the possibility that the D71A mutant was phosphorylated by a second messenger-activated kinase activated by the agonist-occupied 4Ala-TRH receptor. GRKs are mammalian serine/threonine protein kinases that phosphorylate agonist-activated GPCRs as their primary substrates (19). When GRK2 was overexpressed, TRH-dependent phosphorylation was increased (Fig. 6*A*), confirming previous evidence that TRH receptor phosphorylation is primarily because of GRKs (14, 17, 20). Multiple lines of evidence show that the TRH-dependent phosphorylation observed with the coexpression of D71A and 4Ala-TRH receptors is not because of second messenger-activated kinases. Neither U73122, a phospholipase C inhibitor that should block all downstream signaling, nor bisindolylmaleimide (BIM), a protein kinase C inhibitor, prevented TRH-dependent receptor phosphorylation, and phosphorylation with TRH and PMA was no greater than with TRH alone (Fig. 6*B*). Maximal activation of downstream kinase pathways with a combination of phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, and the calcium ionophore ionomycin did cause some receptor phosphorylation, although much less than that stimulated by TRH (Fig. 6*C*) (17). This phosphorylation was prevented by the combination of BIM and BAPTA/AM, a calcium chelator, but phosphorylation in re-

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sponse to TRH was not prevented (Fig. 6*C*). However, BIM and BAPTA/AM did not prevent the phosphorylation seen when D71A and 4Ala-TRH receptors were coexpressed (Fig. 5*C*). We previously reported that phosphorylation by protein kinase C and ionomycin occurs at Ser 355 and is completely absent in a S355A TRH receptor mutant (17). We constructed a D71A/S355A double mutant of the TRH receptor and confirmed that, as expected, it was not phosphorylated in response to PMA and ionomycin (Fig. 5*D*). The D71A/S355A TRH receptor did become phosphorylated in response to TRH when the 4Ala-TRH receptor was coexpressed, consistent with phosphorylation by a GRK.

Discussion

To learn whether receptor dimerization alters receptor phosphorylation in live cells, we used two different strategies to regulate dimerization. A dimeric FKBP ligand increased the fraction of a receptor-FKBP fusion protein running as a dimer on SDS/PAGE and simultaneously increased TRH-induced receptor phosphorylation; a bivalent antibody directed against a small N-terminal epitope on the receptor likewise increased TRH-stimulated phosphorylation. Regulated dimerization potentiated receptor phosphorylation between 2.5- and 10-fold in different experiments, but normalization is inexact because it relies heavily on the small background signal seen without hormone. The fraction of receptors dimerized also is difficult to quantify, although most receptors ran as dimers on SDS/PAGE after addition of dimerizer. If TRH receptors are synthesized as dimers and driven into larger oligomers by regulated dimerization, the data suggest that phosphorylation takes place preferentially in receptor microaggregates.

The consequences of regulated dimerization need to be interpreted cautiously. It is not known whether the chemical dimerizer or the antibody drives the receptor into the same dimeric structure as hormone. It also is uncertain whether controlled dimerization causes dimerization of receptor monomers, stabilizes preexisting dimers, or forces preexisting multimers into higher order structures. Like FRET and BRET, the receptor-FKBP fusion required addition of a large polypeptide to the cytoplasmic tail of the receptor, but the use of an antibody against a small N-terminal epitope did not involve a major change in receptor structure. Despite these caveats, the effects of regulated dimerization were clear: Dimerization dramatically increased phosphorylation of TRH receptors at sites in the cytoplasmic tail critical for desensitization and internalization.

Phosphorylated and activated TRH receptors recruit β-arrestins, which target receptors to clathrin-coated pits for internalization. C-terminally truncated TRH receptors and receptors with Ala mutations for Ser and Thr residues at residues 355–365 in the cytoplasmic tail are impaired in β-arrestin binding and receptor internalization (17). Numerous other GPCRs fail to recruit β-arrestin or undergo endocytosis when phosphorylation sites are lacking. The increase in agonist-dependent phosphorylation caused by dimerization would be expected to increase β-arrestin binding to the TRH receptor and explain our previous observation that dimerization promotes receptor internalization. The importance of TRH receptor dimerization to the endocytic pathway is consistent with previous studies (5, 13, 21). For example, both the V1a and V2 receptors are internalized in a β-arrestin-dependent manner. The V1a receptor internalizes without β-arrestin and recycles to the plasma membrane rapidly, whereas the V2 receptor internalizes with β-arrestin and becomes degraded. When V1a and V2 receptors are coexpressed, V1a and V2 heterodimers internalize together and recycle slowly (22). V1a receptor phosphorylation is normally transient (23), whereas V2 receptor phosphorylation is sustained (24). Based on the present study, it seems possible that cross-phosphorylation in a dimer pair contributes additional sites that help determine receptor trafficking.

The conclusion that receptor phosphorylation takes place in dimeric complexes is supported by the ability of the 4Ala-TRH receptor lacking phosphorylation sites to rescue phosphorylation of the D71A mutant that does not signal or undergo phosphorylation alone. Two hypotheses can explain the phosphorylation of the inactive D71A mutant receptor coexpressed with the 4Ala-TRH mutant by GRKs. First, the D71A TRH receptor mutant may be converted to an active conformation by dimerization with agonist-activated 4Ala-TRH mutant receptor, causing GRKs to phosphorylate the D71A receptor. There is precedent for this model because the agonist induces cooperative conformational changes in the leukotriene B4 receptor-BLT1 dimer (25). This model is not likely to explain our findings, however, because regulated dimerization of the TRH receptor does not initiate signaling or potentiate TRH-dependent signaling (16). Second, GRKs recruited by an agonist-activated 4Ala-TRH mutant receptor may cross-phosphorylate the D71A partner in the dimeric complex because of physical proximity, although the D71A receptor is not in an active conformation. This hypothesis is supported by a recent finding showing that a ligand-binding mutant of chemokine receptor CCR5 undergoes GRK-mediated phosphorylation when coexpressed with a different mutant that recruits GRK to the plasma membrane (26). Likewise, recruitment of rhodopsin kinase is believed to account for the observation that bleaching a small number of rhodopsin molecules in the retina causes phosphorylation of many rhodopsin molecules (27). This model is consistent with results for the TRH receptor.

For some GPCRs, phosphorylation alone seems to be sufficient to desensitize signaling and induce receptor internalization. For others, the major role of phosphorylation is to promote β-arrestin binding, which causes uncoupling from G proteins and internalization. We have shown that dimerization potentiates TRH receptor phosphorylation and that cross-phosphorylation takes place in receptor pairs. By increasing receptor phosphorylation, the formation of receptor oligomers can be expected to amplify the desensitization process. In this manner, dimerization of GPCRs may exert a fundamental effect on the intensity and duration of signal transduction.

Materials and Methods

Plasmids and FKBP Ligands. Construction of the TRHR-FKBP-HA, 2HA-TRH receptor with double N-terminal HA epitopes and 2Flag-TRH receptor with prolactin signal sequence and double N-terminal Flag epitopes has been described (14, 16). D71A and D71A/S355A mutant receptors were made from 2Flag-TRH receptors and 4Ala (S355A/S360A/S364A/T365A) mutant receptors from 2HA-TRH receptor by using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). DNA sequences were confirmed by sequencing. Plasmid-encoding GRK2 cDNA was a gift from Jeffrey Benovic (Jefferson University, Philadelphia, PA). FKBP ligands were from Ariad Pharmaceuticals (Cambridge, MA).

Antibodies. Rabbit antibodies against phosphorylated peptides from the TRH receptor cytoplasmic tail were prepared and tested as described previously (17). Antibody 6959 against the peptide 351–370, ALNY(pS)VIKE(pS)DRF(pS)(pT)ELDDI, was used unless noted. Antibody 5025 against 366–385, ELDDI(pT)VTD(pT)YV(pS)TTKVSFD, also was used. These antibodies specifically recognize phosphorylated TRH receptors from TRH-treated cells. Fab fragments of anti-HA antibody were generated by digestion with papain conjugated to agarose beads (Pierce, Rockford, IL). Purified monoclonal anti-HA antibody (Covance, Berkeley, CA) was incubated with papain in digestion buffer [20 mM sodium phosphate, 10 mM EDTA, 20

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mM cysteine-HCl (pH 7.0)] for 16 h in a shaker at 37°C. After centrifugation, the supernatant containing Fab fragments was run on SDS/PAGE gel, and protein was stained with SYPRO Ruby (Bio-Rad, Hercules, CA) to confirm complete digestion. Control experiments confirmed that the Fab fragments bound to HA-tagged TRH receptors.

Cell Growth. Cells were maintained in DMEM/F12 medium supplemented with 5% FBS at 37°C in a humidified 5% CO_2 atmosphere and transfected with 1 μg of plasmid DNA and 3 μl of Lipofectamine per well in six-well plates. Cells were treated with hormones and drugs in serum-free medium.

Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting were performed as described (17) by using 1:5,000 anti-HA (Covance) or anti-FLAG (Sigma–Aldrich, St. Louis, MO) antibody or 1:100 anti-phosphoreceptor serum 6959 for precipitation and blotting, followed by 1:7,500 HRP-conjugated anti-mouse antibody.

ELISA and Immunofluorescence. Phosphoreceptor was quantified by ELISA according to Jones *et al.* (17). Receptor phosphorylation in lysates was monitored by a modified ELISA. Protein A/G-coated plates (Pierce) were coated with 1:100 anti-phospho-TRH receptor serum and then blocked, and cell lysates were added before washing and incubating with anti-HA antibody (1:5,000). HRP-conjugated anti-mouse antibody (1:5,000) was added for 45 min, followed by substrate.

To visualize the phosphorylated TRH receptor, cells on coverslips were treated, fixed, and blocked as described for ELISAs. The cells were then incubated with primary antibodies (1:500 mouse anti-HA and 1:100 rabbit anti-phospho-TRH receptor) for 2 h and secondary antibodies (1:1,000 anti-mouse Alexa 488 and 1:500 anti-rabbit Alexa 546) (Invitrogen, Carlsbad, CA) for 1 h, washed, and mounted. Cells were viewed on a Nikon (Tokyo, Japan) C1 visible light laser scanning confocal microscope with a 60× (1.4 NA) oil-immersion objective by using 488-nm argon and 543-nm He-Ne lasers and 543- and 585-nm bandpass emission filters, respectively. All images were processed identically by using Metamorph Imaging Software (Molecular Devices, Downingtown, PA).

Statistical Analysis. Experiments were performed a minimum of two times, and values shown represent the mean and range of duplicate or standard error of triplicate determinations. Significance of differences between multiple groups was analyzed by ANOVA with Tukey's post hoc analysis. Where not visible, error bars fell within symbol size.

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