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Activation of Ras-Dependent Signaling Pathways by G_{14}-Coupled Receptors Requires the Adaptor Protein TPR1

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ABSTRACT

Many G_q-coupled receptors mediate mitogenic signals by stimulating extracellular signal-regulated protein kinases (ERKs) that are typically regulated by the small GTPase Ras. Recent studies have revealed that members of the $G\alpha_q$ family may possess the ability to activate Ras/ERK by interacting with the adaptor protein tetratricopeptide repeat 1 (TPR1). Within the $G\alpha_q$ family, the highly promiscuous $G\alpha_{14}$ can relay signals from numerous receptors. Here, we examined if $G\alpha_{14}$ interacts with TPR1 to stimulate Ras signaling pathways. Expression of the constitutively active $G\alpha_{14}$QL mutant in HEK293 cells led to the formation of GTP-bound Ras as well as increased phosphorylations of downstream signaling molecules including ERK and IκB kinase. Stimulation of endogenous G_{14}-coupled somatostatin type 2 and α_2-adrenergic receptors produced similar responses in human hepatocellular HepG2 carcinoma cells. Co-immunoprecipitation assays using HEK293 cells demonstrated a stronger association of TPR1 for $G\alpha_{14}$QL than $G\alpha_{14}$, suggesting that TPR1 preferentially binds to the GTP-bound form of $G\alpha_{14}$. Activated $G\alpha_{14}$ also interacted with the Ras guanine nucleotide exchange factors SOS1 and SOS2. Expression of a dominant negative mutant of TPR1 or siRNA-mediated knockdown of TPR1 effectively abolished the ability of $G\alpha_{14}$ to induce Ras signaling in native HepG2 or transfected HEK293 cells. Although expression of the dominant negative mutant of TPR1 suppressed $G\alpha_{14}$QL-induced phosphorylations of ERK and IκB kinase, it did not affect $G\alpha_{14}$QL-induced stimulation of phospholipase Cβ or c-Jun N-terminal kinase. Our results suggest that TPR1 is required for $G\alpha_{14}$ to stimulate Ras-dependent signaling pathways, but not for the propagation of signals along Ras-independent pathways. J. Cell. Biochem. 113: 3486–3497, 2012.

KEY WORDS: ERK; $G\alpha_{14}$; IKK; PLCβ; Ras; TPR1

G protein-coupled receptors (GPCRs) regulate a broad array of cellular functions through heterotrimeric G proteins that belong to one of the four families, namely G_s, G_i, G_q, and G_{12}. Members of the $G\alpha_q$ family are renowned for their role in linking cell surface receptors to the stimulation of phospholipase Cβ (PLCβ). Of the four family members, $G\alpha_q$ and $G\alpha_{11}$ are ubiquitously expressed whereas $G\alpha_{14}$ and $G\alpha_{16}$ are mostly found in hematopoietic cells and other peripheral tissues [Wilkie et al., 1991]. Considerable functional redundancy presumably exists among the $G\alpha_q$ members since transgenic mice with single [Offermanns et al., 1997] and even certain double knockouts [Davignon et al., 2000] did not exhibit lethality or gross abnormalities. Since all $G\alpha_q$ members can activate PLCβ isozymes [Smrcka et al., 1991; Lee et al., 1992; Offermanns and Simon, 1995] and be deactivated by the same panel of regulator of G protein signaling (RGS) proteins [Ross and Wilkie, 2000], it is conceivable that they can subserve each other's role. However, there is also evidence to suggest functional specificity. For instance, GRK2 binds to all $G\alpha_q$ members except $G\alpha_{16}$ [Day et al., 2003] which has

Abbreviations used: ERK, extracellular signal-regulated kinase; GPCRs, G protein-coupled receptors; HEK293, human embryonic kidney 293; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; PLCβ, phospholipase Cβ; SOS, Son of Sevenless; SSTR2, somatostatin type 2 receptor; TPR1, tetratricopeptide repeat 1.

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the lowest sequence homology with the others [Wilkie et al., 1991; Hubbard and Hepler, 2006]. Recent studies have identified several novel signaling partners for the $G\alpha_q$ members. These include Ric8A [Tall et al., 2003] and p63RhoGEF [Lutz et al., 2005] for $G\alpha_q$ as well as tetratricopeptide repeat 1 (TPR1) for $G\alpha_{16}$ [Marty et al., 2003].

TPR1 is a ubiquitous protein with three TPR motifs [Murthy et al., 1996]. Although the biological role of TPR1 has not been clearly established, it appears to function as a scaffold for protein–protein interaction [Das et al., 1998]. Our recent discovery that TPR1 can link $G\alpha_{16}$ activation to Ras signaling [Marty et al., 2003; Liu et al., 2010] is both interesting and tantalizing, especially in view of the critical roles of Ras in cell proliferation and differentiation [Macara et al., 1996]. The unique ability of $G\alpha_{16}$ to recognize a broad range of GPCRs [Offermanns and Simon, 1995] further allows numerous extracellular signals to tap into the Ras signaling pathway. Ras signaling is critical for $G\alpha_{16}$-mediated regulation of transcription factors such as the signal transducer and activator of transcription 3 (STAT3) [Lo et al., 2003] and nuclear factor κB (NFκB) [Liu and Wong, 2004], and disruption of $G\alpha_{16}$/TPR1 interaction can severely compromise their transcriptional activities in response to the stimulation of the $G\alpha_{16}$-coupled adenosine A_1 receptor [Liu et al., 2010]. Interaction with TPR1 requires the β3 region of $G\alpha_{16}$ which is distinct from the PLCβ-interacting domain [Liu et al., 2011], indicating that dual Ras and PLCβ signaling may actually occur.

As compared to $G\alpha_{16}$, $G\alpha_q$ is a poor binding partner for TPR1 [Marty et al., 2003]. This is not entirely surprising since the two Gα subunits only share 57% sequence homology [Wilkie et al., 1991; Hubbard and Hepler, 2006]. $G\alpha_{14}$ has the highest homology to $G\alpha_{16}$ and it closely resembles $G\alpha_{16}$ in many aspects. Like $G\alpha_{16}$, $G\alpha_{14}$ has a restrictive expression pattern in peripheral tissues and cells [Wilkie et al., 1991] and it can regulate STAT3 and NFκB through complex signaling networks involving Ras [Lo and Wong, 2004; Liu and Wong, 2005]. $G\alpha_{14}$ is perhaps the most promiscuous Gα subunit second only to $G\alpha_{16}$ [Ho et al., 2001]. The ability of $G\alpha_{14}$ to link G_i-, G_s-, and G_q-coupled receptors to the stimulation of PLCβ can be extended to the Ras pathway indirectly through the actions of other signaling molecules such as protein kinase C (PKC). However, if $G\alpha_{14}$ can interact with TPR1, it can then modulate Ras signaling independent of PLCβ activities and provide alternative modes of transcriptional control for many GPCRs. In the present study, we examined the ability of $G\alpha_{14}$ to interact with TPR1 and assessed the role of TPR1 in $G\alpha_{14}$QL as well as in receptor-mediated signaling. We demonstrated that TPR1 is indeed required for $G\alpha_{14}$-mediated Ras signaling.

MATERIALS AND METHODS

REAGENTS

The human cDNAs of $G\alpha_{14}$ and $G\alpha_{14}$QL were obtained from Guthrie Research Institute (Sayre, PA). Cell culture reagents, including LipofectAMINE PLUS reagents, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Anti-$G\alpha_{14}$ targeting the N-terminal was obtained from Gramsch Laboratories (Schwabhausen, Germany). Anti-FLAG antibody and anti-FLAG affinity gel were from Sigma–Aldrich (St. Louis, MO). Anti-TPR1 antibody, siRNA against TPR1 and control siRNA used in HepG2 cells were purchased from Abnova (Taiwan). Other antibodies were purchased from Cell Signaling Technology (Danvers, MA). siRNA against TPR1 and control siRNA with high GC content used in HEK293 cells were purchased from Invitrogen. Protein G-agarose and dithiobis[succinimidylpropionate] (DSP) cross-linker were from Pierce Biotechnology (IL). Osmonics nitrocellulose membrane and ECL kit were from Westborough (MA) and GE Medical (Piscataway, NJ), respectively. Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA), and octreotide (OCT) was from Sigma–Aldrich.

CELL CULTURE AND CO-IMMUNOPRECIPITATION

HEK293 cells were obtained from the American Type Culture Collection (CRL-1573, Rockville, MD). They were maintained in Eagle's minimum essential medium at 5% CO_2, 37°C with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin. HepG2 cells were grown in Eagle's minimum essential medium adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and supplemented with 10% (v/v) FBS, 50 U/ml penicillin and 50 μg/ml streptomycin.

For co-immunoprecipitation experiments, HEK293 cells were grown to 80% confluency in 100 mm tissue culture plates and then co-transfected with 200 ng Gα and 200 ng FLAG-TPR1 cDNAs using 15 μl PLUS and LipofectAMINE reagents in Opti-MEM. Serum was replenished 3 h after transfection. Cross-linking was performed 1 day after transfection; transfected HEK293 cells were washed with PBS twice and then treated with 0.5 mM DSP in PBS for 15 min at room temperature. Cells were then washed again with PBS and maintained in quenching solution (50 mM glycine in PBS, pH 7.4) for 5 min. Subsequently, cells were lysed in ice-cold RIPA buffer (25 mM HEPES at pH 7.4, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 200 μM Na_3VO_4, 4 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin). Cell lysates were gently rocked with an anti-$G\alpha_{14}$ antiserum at 4°C overnight, and then incubated in 30 μl protein G-agarose (50% slurry) at 4°C for 2 h. Alternatively, the cell lysates were incubated in 30 μl anti-FLAG affinity agarose gel (50% slurry) at 4°C overnight. Immunoprecipitates were washed with ice-cold RIPA buffer (400 μl) for four times, resuspended in 50 μl RIPA buffer and 10 μl 6× sample buffer, and then boiled for 5 min. $G\alpha_{14}$ and FLAG-TPR1 proteins in the immunoprecipitates were analyzed by Western blots.

Protein samples were resolved on 12% SDS–PAGE and transferred to nitrocellulose membrane. Resolved proteins were detected by their specific primary antibodies and horseradish peroxidase-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the ECL kit from Amersham, and the images detected in X-ray films were quantified by densitometric scanning using the Eagle Eye II still video system (Stratagene, La Jolla, CA).

RAS ACTIVATION ASSAY

HEK293 cells were co-transfected with 200 ng Gα, 200 ng FLAG-TPR1 or FLAG-TPR1ΔC and 100 ng Ras cDNAs. After 1 day, transfectants were serum starved for 4 h. Cells were then washed

twice with ice-cold PBS and lysed with the Mg^{2+} lysis buffer (MLB; 125 mM HEPES at pH 7.5, 750 mM NaCl, 5% Nonidet P-40, 50 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, and appropriate protease inhibitors). Clarified cell lysates were immunoprecipitated with 20 μl Raf-1 RBD agarose for 45 min and subsequently washed three times with 400 μl ice-cold MLB. Eluted protein samples in 50 μl MLB and 10 μl 6× sampling dye were then resolved in SDS gels and analyzed using specific anti-Ras antibody.

INOSITOL PHOSPHATES (IP) ACCUMULATION ASSAY

HEK293 cells were seeded on a 12-well plate at 2×10^5 cells/well 1 day prior to transfection. Cells were then transfected with 200 ng Gα and 200 ng FLAG-TPR1 or FLAG-TPR1ΔC cDNAs using 2 μl PLUS and Lipofectamine reagents in Opti-MEM. On the next day, cells were labeled with inositol-free Dubecco's modified Eagle's medium (DMEM; 750 μl) containing 5% FBS and 2.5 μCi/ml *myo*-[^{3}H]inositol overnight. Labeled cells were washed twice with the inositol phosphates (IP) assay medium (DMEM buffered with 20 mM HEPES, pH 7.5 and 5 mM LiCl) and were incubated for 1 h at 37°C. Reactions were stopped by replacing the assay medium with 750 μl ice-cold 20 mM formic acid and the lysates were kept in 4°C for 30 min before the separation of [^{3}H]IP from other labeled species by sequential ion-exchange chromatography as described previously [Tsu et al., 1995].

3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DEPHENYL-TETRAZOLIUM BROMIDE (MTT) COLORIMETRIC ASSAY

The day after transfection, HEK293 cells were seeded into 96-well plates (10,000 cells/well) and cultured for 4 days under standard culture conditions. Following replacement of the medium with 0.5 mg/ml MTT in MEM, cells were returned into the incubator for 4 h. Cells and MTT formazan crystals were then solubilized by trituration in a solubilization buffer (10% SDS in 0.01 M HCl) and the survival profile of the cells were quantified by spectrophotometric determination at 570 nm.

ASSAYS FOR PHOSPHORYLATED ERK, JNK, AND IKK

HEK293 cells were seeded on a six-well plate at 4.5×10^5 cells/well 1 day prior to transfection. Transfection was performed with 200 ng Gα and 200 ng FLAG-TPR1 cDNAs using 4 μl PLUS and Lipofectamine reagents in Opti-MEM. The tranfectants were serum starved overnight 1 day after transfection. Cells were harvested with 150 μl lysis buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 40 mM NaP_2O_7, 1 mM dithiothreitol, 200 μM Na_3VO_4, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin) and then gently shaken on ice for 30 min. Supernatants were collected by centrifugation at 16,000*g* for 5 min. For HepG2 cells, the cells were seeded on a six-well plate at 4×10^5 cells/well 1 day before overnight PTX treatment (100 ng/ml) in serum-free medium. Before cell lysis, cells were challenged with 100 nM OCT for the indicated time. Clarified lysates were resolved and analyzed using specific antibodies against phospho-ERK-Thr^{202}/Tyr^{204}, phospho-IKKα-Ser^{174}/β-Ser^{180}, phospho-JNK-Thr^{183}/Thr^{185}, and their unphosphorylated forms.

KNOCKDOWN OF TPR1

Per 35 mm plate, 4×10^5 HEK293 cells were transfected with 40 or 60 nM of mixture of siRNA containing three different siRNAs targeting different regions of TPR1 from Invitrogen, 60 nM of high GC content negative control and 200 ng $G\alpha_{14}$ using 4 μl Lipofectamine 2000 reagent. After 2 days, transfectants were serum starved for 4 h prior to semi-quantitative reverse transcriptase-PCR (RT-PCR) or Western blotting analysis. For HepG2 cells, cells were seeded at 3×10^5 per well in six-well plate. Cells were then transfected with different concentrations of siRNA using 5 μl Lipofectamine 2000 reagents. One day after transfection, cells are serum starved overnight prior to semi-quantitative RT-PCR and Western blotting analysis.

SEMI-QUANTITATIVE REVERSE TRANSCRIPTASE-PCR (RT-PCR)

Two days after siRNA transfection, HEK293 or HEP G2 cells were collected and their total RNA extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA from each sample was used to make first-strand cDNA by reverse transcription at 50°C for 50 min. Reactions were terminated at 85°C for 5 min. Afterwards, cDNA was denatured at 95°C for 5 min. The primer sequence targeting TPR1 are as follows; TPR1-sense: 5′-AGAGCC CAA AAG GTC GGA GAA C-3′ and TPR1-antisense: 5′-TGC CCA TCC TGC TTC CAA A-3′ (403 bp). PCR (30 cycles each with 95°C for 60 s, 60°C for 60 s, and 72°C for 60 s) was carried out using AccuPrime PCR Supermix. Amplification of β-actin was achieved with 30 cycles of 95°C (1 min), 56°C (1 min), and 72°C (1 min), and a final extension for 10 min at 72°C. The primers used were 5′-CAA CAC TGA TCT GGG TCA TCT TCT C-3′ and 5′-GCT CGT CGT CGA CAA CGG CTC-3′ (353 bp). RT-PCR products were electrophoresed on 1% agarose gels and visualized with ethidium bromide staining.

RESULTS

G_{14}-COUPLED RECEPTORS STIMULATE RAS SIGNALING IN HepG2 CELLS

We began our study by addressing whether G_{14}-coupled receptors can indeed activate Ras in a PTX-insensitive manner. As the expression of $G\alpha_{14}$ is restricted to several tissues such as the lung and liver [Wilkie et al., 1991], we screened a number of available cell lines for the presence of $G\alpha_{14}$ by semi-quantitative RT-PCR with $G\alpha_{14}$-specific primers. Among the cell lines examined, human hepatocellular HepG2 carcinoma cells were found to express $G\alpha_{14}$ (Fig. 1A). The other cell lines including human hepatocellular carcinoma Hep3B cells and human lung adenocarcinoma epithelial A549 cells, as well as the negative control murine NIH-3T3 fibroblasts, did not express $G\alpha_{14}$ (Fig. 1A). The expression of $G\alpha_{14}$ in HepG2 cells was also confirmed by Western blot analysis (Fig. 1A, lower panel). Based on the known receptor coupling specificity of $G\alpha_{14}$ [Ho et al., 2001], we selected the G_i-coupled somatostatin type 2 receptor (SSTR2) and α_2-adrenergic receptor for further study. Both SSTR2 and α_2-adrenergic receptors are known to be expressed in HepG2 cells [Schaak et al., 1997; Raderer et al., 2000] and they are incapable of signaling through $G_{q/11}$ proteins [Ho et al., 2001]. Hence, by eliminating possible interference from $G\alpha_i$ proteins by PTX treatment (100 nM, 16 h), agonist-induced responses would be channeled through $G\alpha_{14}$. Application of the SSTR2-selective

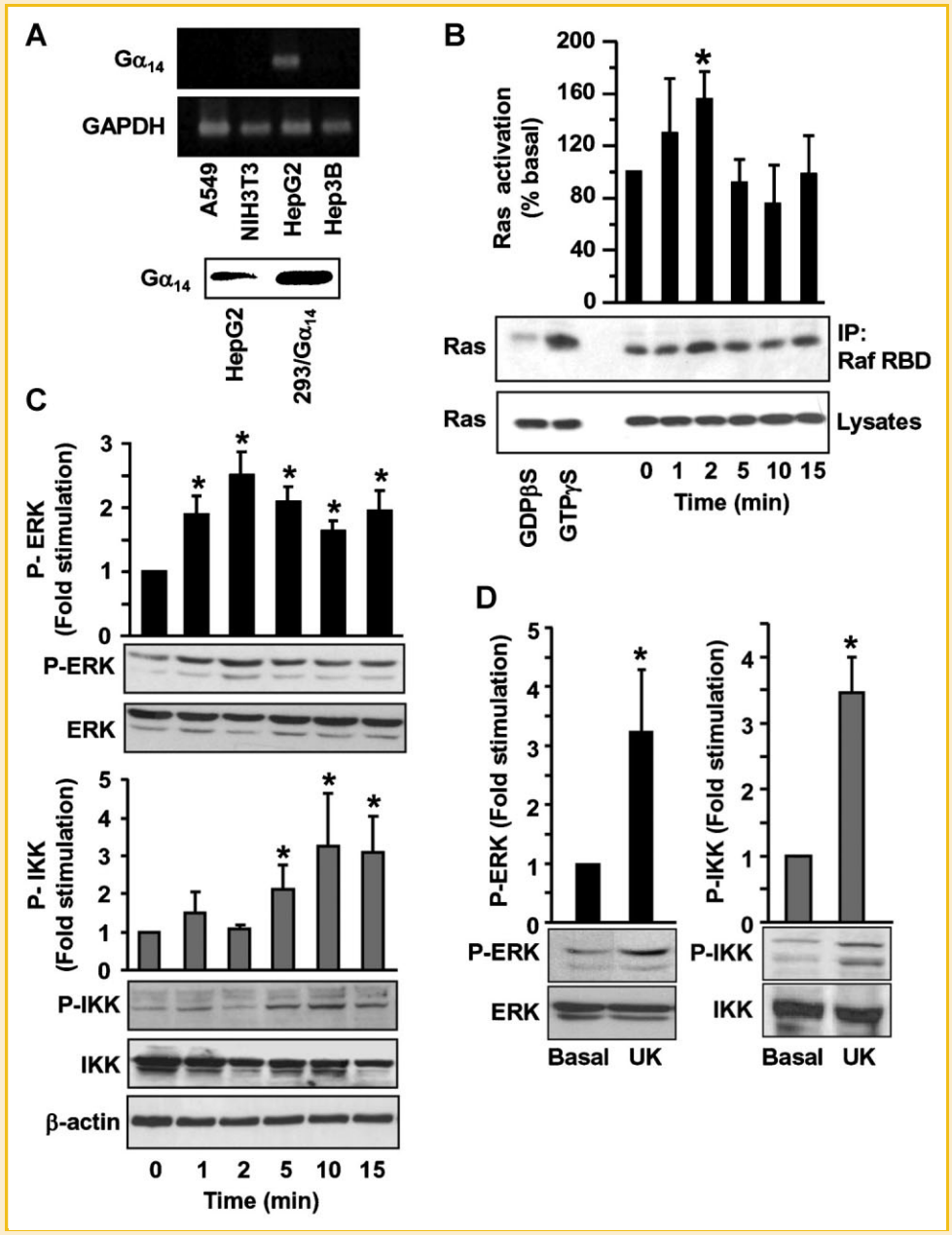

Fig. 1. G_{14}-coupled receptors stimulate Ras signaling in HepG2 cells. A: Total RNA was extracted from A549, NIH 3T3, HepG2, and Hep3B cells, and 1 μg of the total RNA was subjected to RT-PCR using specific primers for $G\alpha_{14}$ and GAPDH. Detection of $G\alpha_{14}$ transcript (200 bp) in HepG2 cells (*upper panel*) was confirmed by Western blot analysis; HEK293 cells transiently expressing $G\alpha_{14}$ was used as a positive control (*lower panel*). B: PTX-treated (100 ng/ml, 16 h) HepG2 cells were stimulated with octreotide (100 nM) for various durations and then subjected to Ras activation assay using Raf-1 RBD agarose beads. Octreotide-induced Ras activation was expressed as a percentage of the basal activity (time 0). GDPβS and GTPγS (100 μM each) was used as negative and positive controls, respectively. *Ras activity was significantly enhanced. C: HepG2 cells were treated as in (B) but the cell lysates were analyzed for ERK or IKK phosphorylation using phospho-specific antisera. Immunodetection of β-actin was used as loading control. Octreotide-induced ERK and IKK phosphorylations were normalized against basal activities (time 0). *Octreotide significantly stimulated ERK/IKK phosphorylation. D: PTX-treated HepG2 cells were stimulated with UK14,304 (1 μM, 10 min) and then analyzed for ERK or IKK phosphorylation as in (C). *UK14,304 significantly stimulated ERK/IKK phosphorylation. Immunoblots shown represent one of three sets; two other sets yielded similar results. Data represent mean ± SEM (n = 3) and were analyzed by one-way ANOVA with Dunnett's post-tests, $P < 0.05$.

agonist, OCT (100 nM), resulted in the activation of Ras in HepG2 cells (Fig. 1B); active Ras was detected with a pull-down assay using beads containing the Ras-binding domain (RBD) of Raf-1 that only recognizes GTP-bound Ras. Significantly more active Ras was detected by 2 min of agonist treatment. Since it has been previously reported that OCT can stimulate NFκB via the Ras/Raf-1/ERK pathway [Liu and Wong, 2005], we further determined if activation of SSTR2 can indeed lead to the phosphorylation of ERK and IκB kinase (IKK; an upstream regulator of NFκB) in HepG2 cells using phospho-specific antisera against the target molecules. As shown in

Figure 1C, OCT induced the phosphorylation of both ERK and IKK, with the ERK response occurring earlier than the IKK response, presumably because ERK is upstream of IKK [Liu and Wong, 2005]. Similarly, stimulation of α_2-adrenergic receptor by its selective agonist (UK14,304; 1 μM, 10 min) resulted in Ras activation (data not shown) as well as ERK and IKK phosphorylation in HepG2 cells (Fig. 1D). These results indicate that receptor-induced $G\alpha_{14}$ activation can lead to the stimulation of Ras signaling pathways in a native cellular environment.

$G\alpha_{14}$ INTERACTS WITH TPR1 IN AN ACTIVITY-DEPENDENT MANNER

- To test if the activation of Ras by G_{14}-coupled receptors was mediated via TPR1, we examined the ability of $G\alpha_{14}$ to interact with TPR1 by co-immunoprecipitation assays. Because of the lack of an antibody which can efficiently pull down endogenous TPR1, co-immunoprecipitation assays were performed with HEK293 cells expressing an N-terminal FLAG-tagged TPR1 (FLAG-TPR1) as described previously [Marty et al., 2003]. HEK293 cells were co-transfected with FLAG-TPR1 in combination with vector, $G\alpha_{14}$ or constitutively active $G\alpha_{14}$QL [Lo and Wong, 2004]. Co-immunoprecipitation was performed with an anti-FLAG affinity gel or anti-$G\alpha_{14}$ antiserum and protein G sepharose. The immunoprecipitates and cell lysates were then examined by Western blot analysis using anti-$G\alpha_{14}$ and anti-FLAG antisera. Both $G\alpha_{14}$ and $G\alpha_{14}$QL co-immunoprecipitated with FLAG-TPR1 but more $G\alpha_{14}$QL was apparently associated with FLAG-TPR1 (Fig. 2A, upper panels). In contrast, no $G\alpha_{14}$ protein was detected in the anti-FLAG immunoprecipitates of vector-transfected cells even though similar levels of FLAG-TPR1 were detected with anti-FLAG antiserum (Fig. 2A lane 1 on the left). Reciprocal co-immunoprecipitation assays were performed using a $G\alpha_{14}$-specific antiserum (Fig. 2A, middle panels). Although comparable levels of $G\alpha_{14}$ and $G\alpha_{14}$QL were detected in the immunoprecipitates (Fig. 2A, middle panels), more FLAG-TPR1 co-immunoprecipitated with $G\alpha_{14}$QL. Western blot analysis of the cell lysates revealed that FLAG-TPR1 was expressed at similar levels and that both $G\alpha_{14}$ and $G\alpha_{14}$QL were expressed to similar degrees (Fig. 2A, lower panels). Collectively, these results suggest that TPR1 can interact with $G\alpha_{14}$ and it prefers to bind to the active form of $G\alpha_{14}$.

The C-terminus of TPR1 has previously been shown to be required for the binding of $G\alpha_{16}$ [Marty et al., 2003]. If $G\alpha_{14}$ binds to the same site as $G\alpha_{16}$, then it would not be able to co-immunprecipitate along with FLAG-TPR1ΔC, a C-terminal truncated version of TPR1 which only contained the first 190 amino acids. As shown in Figure 2A, neither $G\alpha_{14}$ nor $G\alpha_{14}$QL co-immunoprecipitated with FLAG-TPR1ΔC, confirming that the C-terminus of TPR1 is essential for the interaction between $G\alpha_{14}$ and FLAG-TPR1.

$G\alpha_{14}$QL/TPR1 INDUCES THE ACTIVATION OF Ras

The association of TPR1 with activated $G\alpha_{16}$ has been shown to result in Ras activation [Liu et al., 2010]. To determine if the $G\alpha_{14}$/TPR1 complex can similarly regulate Ras activity, HEK293 cells were transfected with FLAG-TPR1, Ras, and $G\alpha_{14}$ or $G\alpha_{14}$QL, and then assayed for Ras activation. In transfectants co-expressing FLAG-TPR1 and Ras, the presence of $G\alpha_{14}$QL significantly increased the amount of GTP-bound Ras as assessed by immunoprecipitation using RBD-Raf-1 (Fig. 2B, lane 6). In contrast, co-expression of wild-type $G\alpha_{14}$ did not affect the Ras activity (Fig. 2B, lane 5). $G\alpha_{14}$QL-induced Ras activation was significantly reduced when FLAG-TPR1 was replaced by FLAG-TPR1ΔC, indicating that functional interaction between $G\alpha_{14}$QL and TPR1 was required for Ras activation (Fig. 2B). It should be noted that due to the presence of endogenously expressed TPR1, expression of FLAG-TPR1ΔC could not completely abolish the $G\alpha_{14}$QL-induced Ras activation. Complete blockade of Ras activation can be achieved by doubling the expression of FLAG-TPR1ΔC [Liu et al., 2010].

The ability of $G\alpha_{14}$QL to stimulate Ras activity via an association with TPR1 suggested that these signaling components may form a macromolecular complex. Since TPR1 has a much lower affinity for inactive Ras than for GTP-bound Ras [Marty et al., 2003], activation of $G\alpha_{14}$ is expected to promote the formation of a TPR1/Ras complex. Indeed, both TPR1 and $G\alpha_{14}$QL were pulled down by HA-tagged Ras in HEK293 cells co-expressing these constructs (Fig. 2C, lane 4). In contrast, HA-tagged Ras failed to interact with $G\alpha_{14}$/TPR1 or $G\alpha_{14}$QL/TPR1ΔC (Fig. 2C, lanes 3 and 8), indicating that activation of $G\alpha_{14}$ and its subsequent association with TPR1 is required for Ras interaction. Moreover, both activated $G\alpha_{14}$ and TPR1 could be pulled down by RBD-Raf-1 in Ras activation assay (Supplementary Fig. S1). These results implied that Ras can recognize the complex composed of activated $G\alpha_{14}$ and TPR1.

Since other small GTPases including Rac1/2, Cdc42, and RhoA cannot bind to TPR1 [Marty et al., 2003], $G\alpha_{14}$QL is therefore expected to selectively stimulate Ras through TPR1. Indeed, $G\alpha_{14}$QL failed to elicit any stimulatory activity in Rac1, Cdc42, and RhoA activation assays (data not shown). Given that TPR1 does not act as a RasGEF to facilitate GDP/GTP exchange [Marty et al., 2003], the formation of a $G\alpha_{14}$QL/TPR1/Ras complex cannot fully explain the mechanism of $G\alpha_{14}$-induced Ras activation. The missing link may be provided by Son of Sevenless (SOS) 2 which has recently been shown to form a macromolecular complex with TPR1 and $G\alpha_{16}$ [Liu et al., 2010]. Both isoforms of SOS, SOS1 and SOS2, are ubiquitously expressed [Bowtell et al., 1992]. We thus co-expressed $G\alpha_{14}$ or $G\alpha_{14}$QL with HA-tagged SOS1 or SOS2 in HEK293 cells and then performed co-immunoprecipitation with anti-$G\alpha_{14}$ or anti-HA antibody. Unlike $G\alpha_{16}$QL [Liu et al., 2010], $G\alpha_{14}$QL interacted with both SOS1 and SOS2 to similar extents, whereas wild-type $G\alpha_{14}$ interacted weakly with SOS1/2 (Fig. 2D).

SELECTIVE INVOLVEMENT OF TPR1 IN $G\alpha_{14}$QL-INDUCED PHOSPHORYLATION OF IKK

The TPR1-interacting domain on $G\alpha_{16}$ has been mapped to the β3 region [Liu et al., 2011] which is distinct from the putative PLCβ-interacting domain (α2-β4-α3-β5 regions) [Venkatakrishnan and Exton, 1996; Yu et al., 2008; Waldo et al., 2010]. The segregation of these domains allows $G\alpha_{16}$ to independently regulate the activities of Ras and PLCβ. This notion is supported by the ability of several $G\alpha_{16/z}$ chimeras to activate Ras despite a complete lack of stimulatory effect on PLCβ [Liu et al., 2011]. If $G\alpha_{14}$ behaves like $G\alpha_{16}$, then its ability to stimulate PLCβ should not be affected by the

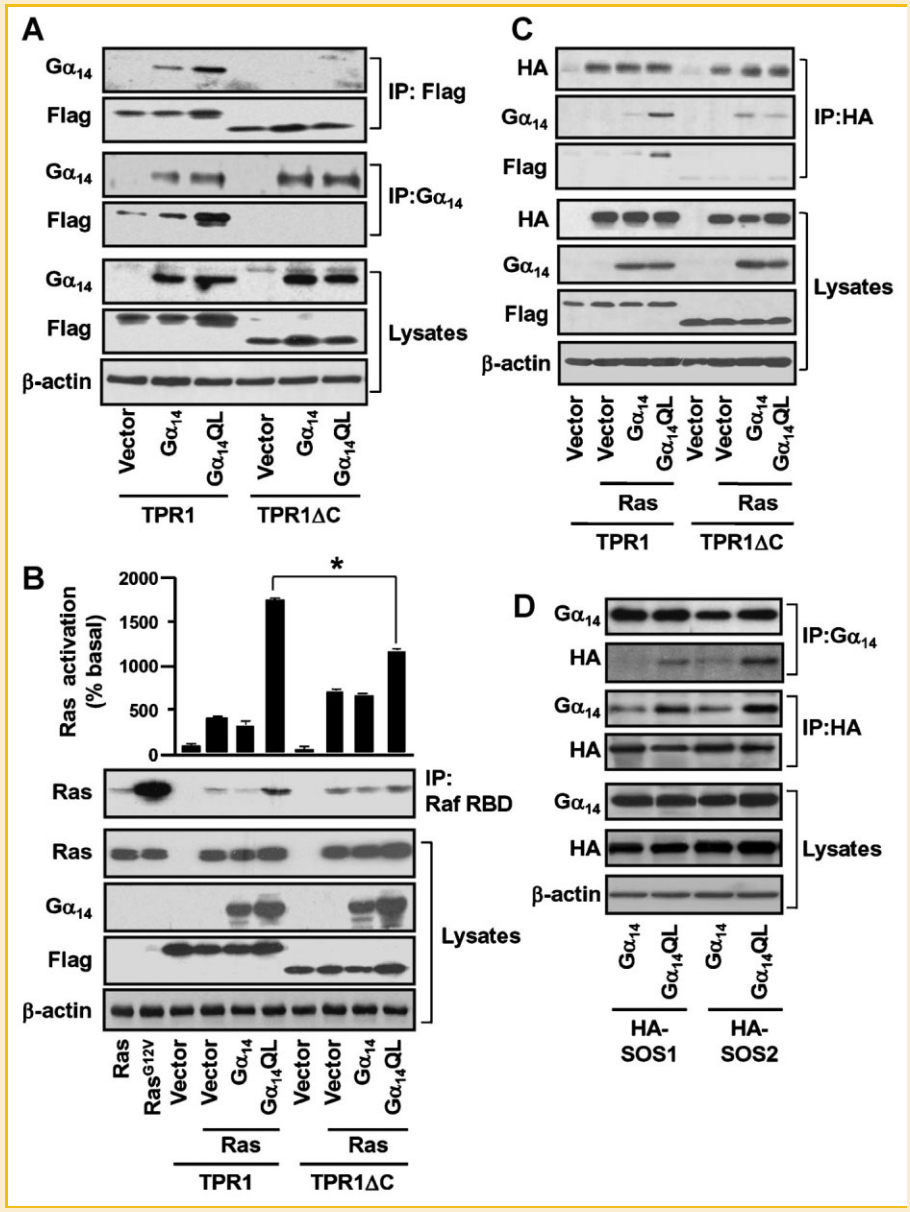

Fig. 2. $G\alpha_{14}QL$ interacts with TPR1 and activates Ras in HEK293 cells. A: HEK293 cells were transiently co-transfected with FLAG-TPR1 or FLAG-TPR1ΔC in combination with pcDNA1 (vector), $G\alpha_{14}$ or $G\alpha_{14}QL$. Cell lysates were immunoprecipitated (IP) by anti-$G\alpha_{14}$ antiserum or anti-FLAG affinity agarose gel. The immunoprecipitates were immunoblotted with anti-$G\alpha_{14}$ or anti-FLAG antiserum. Cell lysates were also assessed for the expression of $G\alpha_{14}$, FLAG-TPR1, or FLAG-TPR1ΔC. B: HEK293 cells were transiently co-transfected as in (A) with or without Ras. Transfectants were subjected to Ras activation assay as in Figure 1B. *TPR1ΔC significantly inhibited $G\alpha_{14}QL$-induced Ras activation. C: HEK293 cells were transiently co-transfected as in (B). Cell lysates were subjected to immunoprecipitation of Ras using an anti-HA affinity agarose gel. The immunoprecipitates were immunoblotted with anti-HA, anti-$G\alpha_{14}$, or anti-FLAG antiserum. D: HEK293 cells were transiently co-transfected with HA-SOS1/2 in combination with $G\alpha_{14}$ or $G\alpha_{14}QL$. Cell lysates were immunoprecipitated by anti-$G\alpha_{14}$ or anti-HA antisera. Immunoblots shown represent one of three sets; two other sets yielded similar results. Data represent mean ± SEM ($n = 3$) and were analyzed by one-way ANOVA with Dunnett's post-tests, $P < 0.05$.

disruption of TPR1/Ras signaling. To test this prediction, we used FLAG-TPR1ΔC as a dominant negative mutant to determine the role TPR1 in $G\alpha_{14}$-mediated PLCβ signaling; FLAG-TPR1ΔC can act as a dominant negative mutant of TPR1 because it remains capable of binding Ras [Marty et al., 2003] despite of its loss of interaction with $G\alpha_{14}$. HEK293 cells were co-transfected with pcDNA1, $G\alpha_{14}$ or $G\alpha_{14}QL$ in combination with either FLAG-TPR1 or FLAG-TPR1ΔC, and the transfectants were analyzed for IP_3 production. Expression of $G\alpha_{14}QL$, but not $G\alpha_{14}$, significantly stimulated IP_3 production in the transfectants (Fig. 3A). The $G\alpha_{14}QL$-induced IP_3 formation was unaffected by the co-expression of FLAG-TPR1 or FLAG-TPR1ΔC, confirming that the regulation of PLCβ by $G\alpha_{14}$ was independent of TPR1/Ras signaling.

Since $G\alpha_{14}$ is capable of stimulating the phosphorylation of ERK and IKK via Ras activation [Lo and Wong, 2004; Liu and Wong, 2005], these activities should be sensitive to blockade by

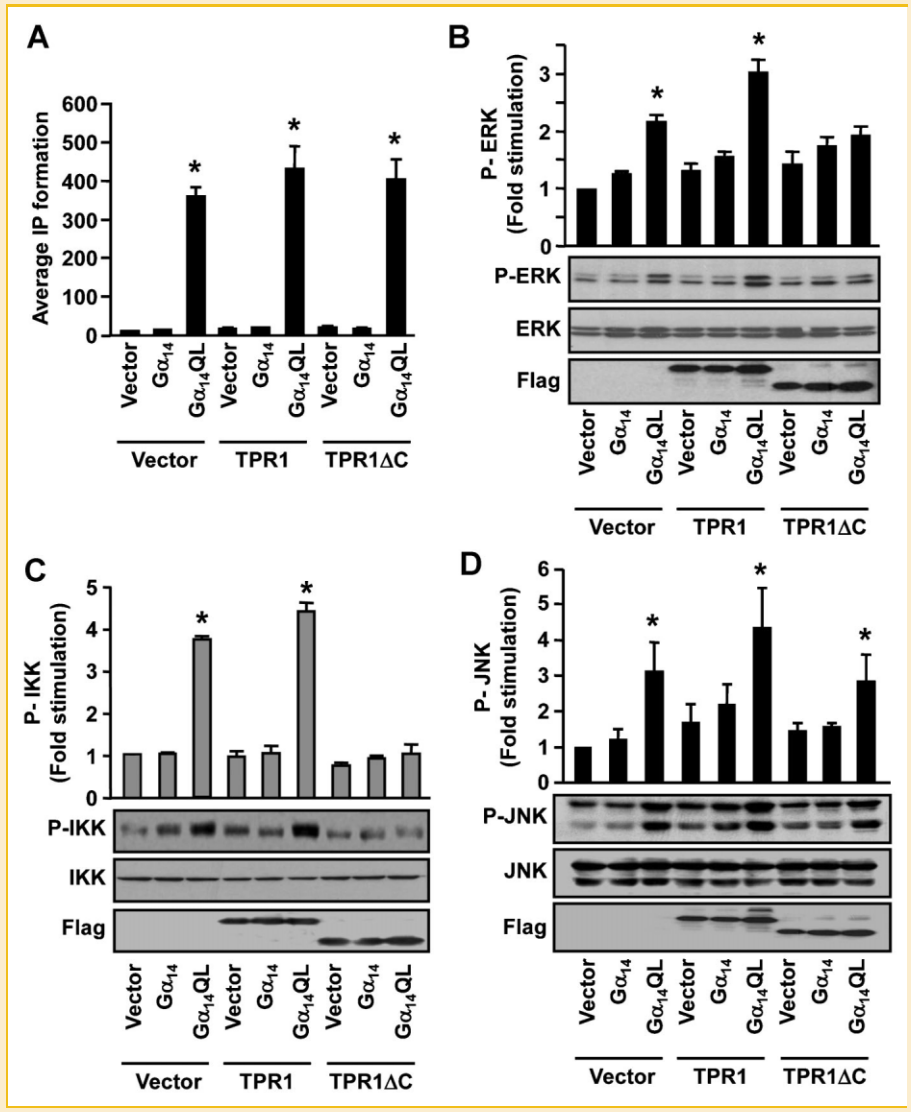

Fig. 3. TPR1ΔC inhibits $G\alpha_{14}$QL-induced Ras signaling in HEK293 cells. HEK293 cells were co-transfected with pcDNA1 (vector), $G\alpha_{14}$ or $G\alpha_{14}$QL in combination with TPR1 or TPR1ΔC. A: Transfectants were labeled with [^{3}H]inositol (2.5 μCi/ml) in DMEM containing 5% FBS overnight. IP formations were examined in the presence of 5 μM LiCl for 60 min. Data represent the mean ± SD of triplicate determinations of a single representative experiment, n = 3. B–D: Transfectants were analyzed for the expression levels of FLAG-proteins as well as the phosphorylation levels of ERK (B), IKK (C), and JNK (D) using anti-phospho-ERK, anti-ERK, anti-phospho-IKK, anti-IKK, anti-phospho-JNK, and anti-JNK antibodies. *$G\alpha_{14}$QL-induced IP formation and ERK/IKK/JNK phosphorylations were significantly higher than the corresponding vector controls (one-way ANOVA with Dunnett's post-tests, $P < 0.05$). Data shown are the mean ± SEM, and the immunoblots shown are representatives of three individual sets of experiments.

FLAG-TPR1ΔC. HEK293 cells were co-transfected with $G\alpha_{14}$ or $G\alpha_{14}$QL with or without FLAG-TPR1 or FLAG-TPR1ΔC, and the transfectants were analyzed for the phosphorylation of ERK and IKK. In agreement with our previous findings [Lo and Wong, 2004; Liu and Wong, 2005], expression of $G\alpha_{14}$QL induced the phosphorylations of ERK and IKK as compared to the vector and $G\alpha_{14}$ controls (Fig. 3B,C). $G\alpha_{14}$QL-induced ERK and IKK phosphorylations were significantly inhibited to levels close to their basal values (cells transfected with vector alone) in transfectants co-expressing FLAG-TPR1ΔC, but not in cells co-expressing FLAG-TPR1 (Fig. 3B,C). Similar expression levels of total ERK, IKK, and FLAG-tagged proteins were observed in the different transfectants (Fig. 3B,C).

$G\alpha_{14}$ is also capable of stimulating the phosphorylation of c-Jun N-terminal kinase (JNK) in a Ras-independent manner [Yung et al., 1999] and thus $G\alpha_{14}$QL-induced phosphorylation of JNK should not be affected by FLAG-TPR1ΔC. Indeed, co-expression of Flag-TPR1 or Flag-TPR1ΔC had no effect on the $G\alpha_{14}$QL-induced phosphorylation of JNK (Fig. 3D). Collectively, these results suggest that disruption of $G\alpha_{14}$/TPR1 interaction might selectively interfere with Ras signaling pathways.

KNOCKDOWN OF TPR1 ABOLISHED $G\alpha_{14}$-MEDIATED IKK PHOSPHORYLAYION IN HEK293 AND HepG2 CELLS

The preceding experiments suggest that $G\alpha_{14}$QL-induced Ras signaling requires TPR1. Thus, siRNA-mediated knockdown of

TPR1 is expected to inhibit Ras-dependent responses. Validated TPR1-specific siRNA (siTPR1) [Liu et al., 2010] was tested in HEK293 cells for its ability to inhibit $G\alpha_{14}$QL-induced IKK phosphorylation. At 60 nM of siTPR1, the level of TPR1 mRNA in HEK293 transfectants was significantly suppressed by ~80%, while the TPR1 transcript level was unaffected in cells transfected with a negative control siRNA (siCon) having minimal sequence homology to any known vertebrate transcript and a GC content similar to siTPR1 (data not shown). Hence, we co-transfected HEK293 cells with $G\alpha_{14}$ or $G\alpha_{14}$QL in the absence or presence of siTPR1 or siCon (60 nM each), and the transfectants were examined for IKK phosphorylation. $G\alpha_{14}$QL-induced IKK phosphorylation was unaffected by siCon but was completely inhibited in the presence of siTPR1 (Fig. 4A). The siRNA-mediated knockdown of TPR1 was selective because siTPR1 neither affected the expression of total IKK nor β-actin (Fig. 4A).

Using the G_{14}-coupled α_2-adrenergic receptor in HepG2 cells as a model system, we ascertained whether the knockdown of TPR1 can suppress receptor-mediated simulation of Ras-dependent signaling pathways by first confirming the ability of siTPR1 to knockdown TPR1 in HepG2 cells. As shown in Figure 4B, the level of TPR1 mRNA was reduced by ~80% in cells transfected with 100 nM of siTPR1, whereas the negative control (200 nM siCon) had no effect. Again, the transcript level of β-actin was not affected by siTPR1 or siCon. The knockdown of TPR1 by siTPR1 in HepG2 cells was further confirmed using an anti-TPR1 antibody; the expression of endogenous TPR1 was almost abolished in HepG2 cells transfected with 100 nM siTPR1 (Fig. 4B, bottom panel). In agreement with our previous observation (Fig. 1D), mock-transfected (control) and siCon-treated HepG2 cells responded to UK14,304 challenge (1 μM, 10 min) with increased ERK phosphorylation; these responses were PTX-resistant and presumably mediated via $G\alpha_{14}$ (Fig. 4C). The knockdown of TPR1 in siTPR1-treated HepG2 cells resulted in the loss of UK14,304-induced ERK phosphorylation (Fig. 4C) and Ras activation (Fig. 4D), indicating a critical role of TPR1 in mediating α_2-adrenergic/$G\alpha_{14}$ signaling along the Ras/ERK axis.

The loss of UK14,304-induced Ras activation in TPR1-depleted cells (Fig. 4D) suggest that TPR1 is required for linking activated $G\alpha_{14}$ to the stimulation of the Ras GEF, SOS1/2. To test the critical role of TPR1, HEK293 cells co-expressing $G\alpha_{14}$ and HA-tagged SOS1 were subjected to siRNA-mediated knockdown of TPR1, followed by the immunoprecipitation of SOS1 with an anti-HA antibody. In line with our expectation, the knockdown of TPR1 resulted in the corresponding loss of interaction between $G\alpha_{14}$ and SOS1 (Fig. 4E), suggesting that TPR1 plays an essential role in linking $G\alpha_{14}$ activation to SOS1.

Since many G_q-linked receptors are known to stimulate mitogenesis [Rozengurt, 2007], the functional significance of $G\alpha_{14}$-induced Ras activation was explored by examining the effect of siTPR1 on $G\alpha_{14}$QL-induced cell proliferation. HEK293 transfectants expressing $G\alpha_{14}$ or $G\alpha_{14}$QL were analyzed by means of the MTT assay for their ability to proliferate in culture. As shown in Figure 4E, HEK293 cells expressing $G\alpha_{14}$QL attained a significantly higher density than those of $G\alpha_{14}$ after 4 days in culture. This growth advantage of $G\alpha_{14}$QL-expressing cells was abolished upon siRNA-mediated knockdown of TPR1, whereas siCon did not affect the enhanced proliferation of $G\alpha_{14}$QL-expressing cells (Fig. 4F). These results suggest that TPR1 is required for mediating $G\alpha_{14}$QL-induced cell proliferation.

DISCUSSION

Activation of transcription factors such as NFκB [Liu and Wong, 2005] and STAT3 [Lo and Wong, 2004] by $G\alpha_{14}$ allows a variety of GPCRs to regulate different cellular processes that range from inflammatory response to cell proliferation and differentiation. Although it has been established that $G\alpha_{14}$QL-induced activation of IKK/NFκB is mediated via Ras and ERK [Liu and Wong, 2005], the mechanism by which $G\alpha_{14}$ activates Ras remains unknown. Due to the complexity of signaling networks operated by GPCRs, activation of Ras by $G\alpha_{14}$ may be relayed through the PLCβ/PKC pathway [Marais et al., 1998; Bivona et al., 2006], transactivation of epidermal growth factor receptor [Schäfer et al., 2004], or interaction with the TPR1 adaptor protein [Liu et al., 2010]. The present study has demonstrated that the latter mechanism is primarily responsible for mediating Ras activation by $G\alpha_{14}$ in native HepG2 and transfected HEK293 cells. Several lines of evidence support this notion. Firstly, TPR1 interacted preferentially with the activated form of $G\alpha_{14}$ in co-immunoprecipitation assays (Fig. 2A), thereby providing a linkage as well as a regulatory switch for the pathway. Secondly, disruption of $G\alpha_{14}$/Ras signaling by scavenging Ras with TPR1ΔC resulted in the inhibition of Ras-dependent signals but not the PLCβ or JNK signals; presumably because TPR1ΔC can bind Ras [Marty et al., 2003] and prevent it from being recruited to the activated $G\alpha_{14}$. Lastly, siRNA-mediated knockdown of TPR1 successfully suppressed $G\alpha_{14}$-induced Ras signaling in native HepG2 and transfected HEK293 cellular models. Collectively, these results indicate that TPR1 is a key molecule in supporting G_{14}-coupled receptors to stimulate the Ras signaling pathway.

TPR1 was initially identified as a $G\alpha_{16}$-interacting protein, but it can also interact with other Gα subunits including $G\alpha_q$ [Marty et al., 2003]. Since $G\alpha_{14}$ closely resembles $G\alpha_{16}$ in terms of sequence homology [Simon et al., 1991], restrictive tissue distribution [Wilkie et al., 1991], and receptor coupling promiscuity [Ho et al., 2001], it is not entirely surprising that it can interact with TPR1 and activate Ras signaling in a manner similar to $G\alpha_{16}$ [Liu et al., 2010]. However, unlike $G\alpha_{16}$ which activates Ras by interacting with TPR1 and SOS2, $G\alpha_{14}$ appeared to associate with both SOS1 and SOS2. The significance of this difference is not immediately apparent since the SOS proteins are functionally identical and ubiquitously expressed [Bowtell et al., 1992]. Nevertheless, it should be noted that SOS2, but not SOS1, can be degraded rapidly by an ubiquitination-dependent pathway [Nielsen et al., 1997]. In growth factor signaling, activation of receptor tyrosine kinases results in the recruitment of SOS proteins to the plasma membrane where they act as GEFs for Ras. Recruitment of SOS proteins to the plasma membrane is achieved by growth factor receptor-bound protein 2 (Grb2), an adaptor protein which interacts with both the activated receptor tyrosine kinase and SOS [Lowenstein et al., 1992; Rozakis-Adcock et al., 1993]. The role of Grb2 is probably taken up by TPR1 in GPCR-mediated Ras activation. TPR1 may interact with GTP-bound $G\alpha_{14}$, SOS proteins

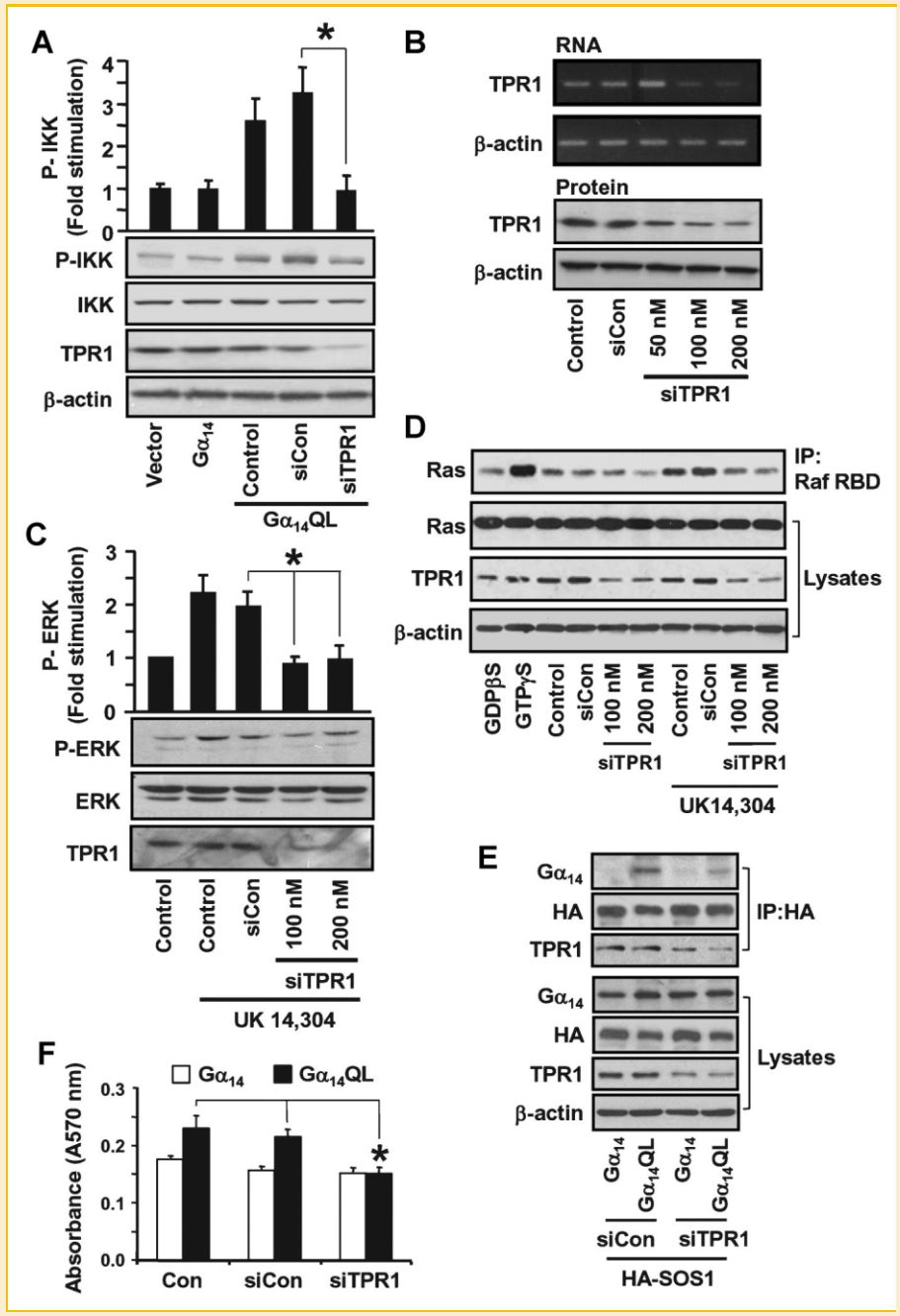

Fig. 4. siRNA-mediated knockdown of TPR1 disrupts $G\alpha_{14}$-induced Ras signaling. A: HEK293 cells were co-transfected with $G\alpha_{14}$ or $G\alpha_{14}$QL in the absence or presence of 60 pM control (siCon) or TPR1-targeting siRNA (siTPR1). Transfectants were assayed for IKK phosphorylation as in Figure 1C. Knockdown of TPR1 was confirmed by Western blot analysis using an anti-TPR1 antiserum. B: HepG2 cells were co-transfected with or without siCon or siTPR1 (50–200 nM) as indicated. Total RNA was extracted 2 days after transfection and subjected to semi-quantitative RT-PCR for the detection of endogenous RNA levels of TPR1 and β-actin. PCR products were resolved on a 1.2% agarose gel. The TPR1 transcript level was normalized against the control (set as 1.0). Knockdown of TPR1 was confirmed by Western blot analysis using an anti-TPR1 antiserum (*lower panel*). C: HepG2 cells were transfected as in (B) and the transfectants were assayed for UK14,304-induced ERK phosphorylation as in Figure 1D. D: HepG2 cells were transfected as in (C) and the transfectants were assayed for UK14,304-induced Ras activation as in Figure 1B. E: HEK293 cells were transfected the cDNA of HA-tagged SOS1 and $G\alpha_{14}$ or $G\alpha_{14}$QL in the absence or presence of siCon or siTPR1. Lysates from the transfectants were subjected to immunoprecipitation by an anti-HA affinity agarose gel and subsequently immunoblotted with anti-HA, anti-$G\alpha_{14}$, or anti-TPR1 antisera. F: HEK293 cells were transfected as in (E) except with the omission of SOS1. Transfectants were seeded into 96-well plates the following day and cultured for another 4 days prior to the MTT assay. *siTPR1 significantly suppressed the TPR1 transcript level and inhibited $G\alpha_{14}$-mediated cell proliferation or IKK and ERK phosphorylation (one-way ANOVA with Dunnett's post-tests, $P < 0.05$). Data shown represent one of three sets of immunoblots; two other sets yielded similar results. The result of the densitometric analysis is shown above the immunoblots and the numerical values represent relative intensities expressed as a ratio of the control (set as 1.0).

and Ras to form a signaling complex for Ras activation (Fig. 5); the existence of a $G\alpha_{16}$/TPR1/SOS2 complex has indeed been demonstrated [Liu et al., 2010]. However, it is not known if TPR1 or $G\alpha_{14}$ interacts directly with SOS or if other adaptor proteins are involved. It is worth noting that $G\alpha_q$ and $G\alpha_{16}$ can interact with the C-terminal PH domain of p63RhoGEF (a GEF for another small GTPase RhoA) [Lutz et al., 2005; Yeung and Wong, 2009], and that the SOS proteins also contain a PH-domain for localization to the plasma membrane [Chen et al., 1997]. It will be interesting to determine whether the PH domain of SOS proteins is involved in the interaction with $G\alpha_{14}$ and $G\alpha_{16}$.

Like most Gα subunits, $G\alpha_{14}$ possesses the capacity to regulate a multitude of pathways. For instance, upon activation by an agonist-bound receptor, $G\alpha_{14}$ can stimulate PLCβ and TPR1/Ras, while the released Gβγ can modulate a host of effectors such as adenylyl cyclases and ion channels (Fig. 5). Each of these signals may drive a different or identical cellular response (e.g., mitogenesis) independently or collectively. The selective disruption of Ras-dependent signals by TPR1ΔC and siTPR1 has multiple ramifications on $G\alpha_{14}$ signaling. The lack of effect of TPR1 or TPR1ΔC expression on $G\alpha_{14}$-induced PLCβ activity (Fig. 3A) suggests that TPR1 and PLCβ do not compete for the same site on $G\alpha_{14}$, and that both pathways

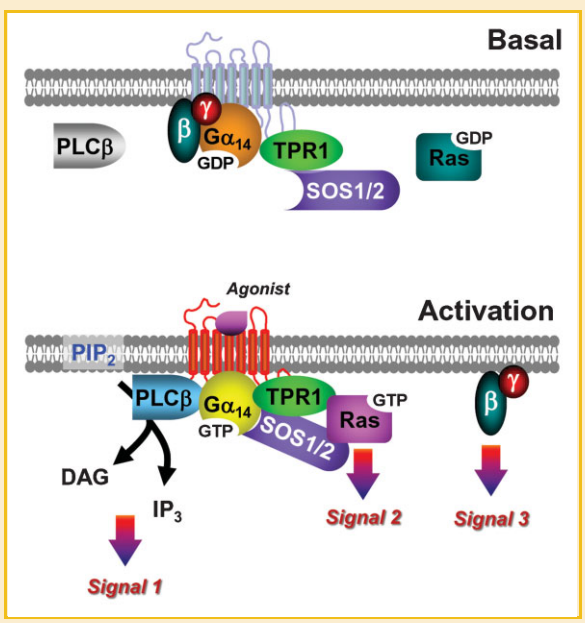

Fig. 5. Schematic representation of signal processing by G_{14}-coupled receptors. In the GDP-bound basal state, $G\alpha_{14}$ binds to Gβγ and forms a complex with the receptor. Both TPR1 and SOS1/2 can weakly associate with $G\alpha_{14} \times$ GDP, but unstimulated PLCβ and Ras are not part of the complex. Upon agonist binding to the GPCR, $G\alpha_{14}$ exchanges GDP for GTP and becomes activated and dissociates from the Gβγ dimer. The conformation of $G\alpha_{16} \times$ GTP is recognized by PLCβ which results in the conversion of PIP_2 into DAG and IP_3 (*Signal 1*). $G\alpha_{16} \times$ GTP also interacts with TPR1 and SOS1/2, leading to the recruitment and activation of Ras (*Signal 2*). The GTP-bound active Ras then regulates downstream signaling such as the phosphorylation of ERK and IKK. The released Gβγ can interact with a panel of effectors (*Signal 3*) that may or may not overlap with those regulated by $G\alpha_{14}$. Integration of the various signals will modulate the final outcome of the biological response.

can be stimulated simultaneously. Since both PLCβ/PKC and TPR1/Ras/ERK signals can contribute to mitogenesis, their simultaneous activation may ensure a robust mitogenic response. When the TPR1/Ras/ERK signal becomes limiting (e.g., lack of TPR1 expression or competition for SOS1/2 by Grb2), the PLCβ/PKC signal should remain unaltered but it alone may not be sufficient to drive mitogenesis. One analogy of this signaling mechanism is the regulation of STAT3 by $G\alpha_{14/16}$ where both PLCβ and Ras signals are required [Lo et al., 2003; Lo and Wong, 2004]; the absence of one signal input would result in the loss of a response. Hence, the ability of a cell to integrate multiple signals provides it with a means to select a final outcome as well as to fine-tune a response.

Given that $G\alpha_{14}$-mediated stimulation of the TPR1/Ras signaling has the capacity to stimulate cell proliferation in HEK293 (Fig. 4F) and NIH3T3 cells (unpublished data), activation of G_{14}-coupled receptors is expected to enhance tumorigenesis in tissues where the Ras pathway constitutes a strong mitogenic signal. In this regard, elevated SSTR2 expression is often found to associate with human neuroendocrine tumors such as corticotroph adenomas [Hofland, 2008] and somatostatin receptor scintigraphy is being developed as a diagnostic tool [Nguyen-Khac et al., 2009]. Yet, recent studies have revealed that somatostatin analogs are effective in suppressing tumor growth in several types of cancer [Barbieri et al., 2009; Martinez-Alonso et al., 2009; Zou et al., 2009; Mariniello et al., 2011]. Moreover, SSTR2 expression is apparently correlated to a better survival of patients with well-differentiated endocrine carcinomas [Corleto et al., 2009]. The mechanism by which somatostatin induces antiproliferative activity has not been fully elucidated but there is evidence to suggest that SSTR2 signaling interferes with growth factor receptor-mediated pathways [He et al., 2009]. Our observation that $G\alpha_{14}$ utilizes TPR1 and SOS1/2 for Ras signaling may thus provide a linkage to the growth factor signaling pathways. It is conceivable that TPR1 can compete with Grb2 for SOS proteins, thereby limiting the growth factor-mediated responses. Although the recruitment of SOS proteins by $G\alpha_{14}$/TPR1 will similarly lead to the activation of Ras/ERK, GPCR-elicited ERK signaling is generally weaker and less sustained than those stimulated by growth factor receptors [Wu and Wong, 2005]. The promiscuous nature of $G\alpha_{14}$ [Ho et al., 2001] allows it to couple a wide range of GPCRs to the TPR1/Ras pathway and thus it is pertinent to establish a thorough understanding on how their intracellular signals are processed. Such information may provide additional handles for therapeutic intervention of diseases like Cushing's disease that involve G_{14}-coupled receptors.

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