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# A Novel Nuclear Signaling Pathway for Thromboxane $A_2$ Receptors in Oligodendrocytes: Evidence for Signaling Compartmentalization during Differentiation[▽]

Fozia Mir and Guy C. Le Breton*

*Department of Cellular and Molecular Pharmacology, University of Illinois at Chicago, College of Medicine, 835 South Wolcott Avenue (MC 868), Chicago, Illinois 60612-7343*

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**The present study investigated G protein expression, localization, and functional coupling to thromboxane $A_2$ receptors (TPRs) during oligodendrocyte (OLG) development. It was found that as OLGs mature, the expression levels of $G_q$ increase while those of $G_{13}$ decrease. In contrast, the expression levels of $G_s$, $G_o$, and $G_i$ do not change significantly. Localization studies revealed that $G_q$, $G_{13}$, and $G_i$ are present only in the extranuclear compartment, whereas $G_s$ and $G_o$ are found in both the extranuclear and the nuclear compartments. Purification of TPR-G protein complexes demonstrated that TPRs couple to both $G_q$ and $G_{13}$ in the extranuclear compartment but only to $G_s$ in the nuclear compartment. Furthermore, functional analysis revealed that stimulation of nuclear TPR in OLGs stimulates CREB phosphorylation and myelin basic protein transcription and increases survival. Collectively, these results demonstrate that (i) OLGs selectively modulate the expression of certain G proteins during development, (ii) G proteins are differentially localized in OLGs leading to subcellular compartmentalization, (iii) TPRs couple to $G_q$ and $G_{13}$ in the extranuclear compartment and to $G_s$ only in the nucleus, (iv) mature OLGs have a functional nuclear TPR-$G_s$ signaling pathway, and (v) nuclear TPR signaling can stimulate CREB phosphorylation and myelin gene transcription and increase cell survival. These findings represent a novel paradigm for selective modulation of G protein-coupled receptor–G protein signaling during cell development.**

G protein-coupled receptors (GPCRs) are a large family of heptahelical cell membrane receptors (50) that interact with a limited number of heterotrimeric G proteins for signal transduction through downstream effectors (6, 39). These Gα subunits can be divided into four subfamilies based on sequence homology, i.e., $G_q$, $G_{13}$, $G_s$, and $G_i$. Using different cell expression systems, it has become apparent that most GPCRs can couple to multiple Gα subunits. For example, many GPCRs, including thyroid-stimulating hormone, α2-adrenergic receptors, protease-activating receptors, and thromboxane $A_2$ ($TXA_1$) receptors (TPRs), have been reported to signal through several Gα subunits (24; reviewed in reference 16). Therefore, it appears that a single GPCR has the potential to activate a number of intracellular signaling processes. However, such GPCR-G protein promiscuity would seem to be inconsistent with the regulation of specific cellular events initiated when a particular GPCR is activated. This in turn indicates that cells in some way regulate the coupling of specific GPCRs to specific Gα subunits. In this connection, we previously provided evidence that changes in GPCR/Gα subunit expression can result in alterations in their coupling profiles (17). Additionally, the effective GPCR/G protein levels may also be modified within the cell by compartmentalization of the different GPCR/Gα subunits.

Historically, GPCR signaling pathways have for the most part been restricted to the plasma membrane compartment. However, this concept has recently been challenged by various reports describing GPCRs and G proteins in other subcellular locations. For instance, functional GPCRs have been shown to be associated with certain cellular organelles, such as internal calcium storage sites, the endoplasmic reticulum (ER)/Golgi apparatus, and more recently the nucleus of different cell types. Specifically, β-adrenergic receptors localize to the sarcolemma and T-tubular network in the cardiac myocytes (4), whereas group I metabotropic glutamate receptors selectively localize to the ER (37). Among the GPCRs localized to the nucleus are the prostaglandin (EP3) receptors (14), endothelin receptors/β-adrenergic receptors (4), platelet-activating factor receptors (26), and metabotropic glutamate receptors (21). In addition, Gα subunits have been reported to exist at various subcellular locations, including the Golgi apparatus (46), ER (2), cytoskeleton (7), and nucleus (9). While not well characterized, such localization may be regulated by numerous factors, including cell development, ligand activation, cellular disease/stress, etc. Thus, GPCRs and G proteins localize within selective subcellular compartments, and it appears that this compartmentalization not only limits certain GPCR-G protein interactions but also facilitates other interactions. A separate feature of this process is the potential for the dynamic distribution of GPCRs/G proteins within subcellular compartments at different time points during cellular maturation. Such an effect would result in the appearance of new signaling pathways for a given GPCR as the cells develop or respond to external stimuli.

The present study investigated the changes in G protein signaling interactions for a particular GPCR, i.e., TPR known to be involved in the development and function of oligodendrocytes (OLGs) (25, 41). To this end, we determined the expression and localization of TPR-associated heterotrimeric Gα subunits and purified TPR-Gα complexes formed during OLG differentiation.

* Corresponding author. Mailing address: Department of Cellular and Molecular Pharmacology, University of Illinois at Chicago, College of Medicine, 835 South Wolcott Avenue (MC 868), Chicago, IL 60612-7343. Phone: (312) 996-4929. Fax: (312)-996-1225. E-mail: gcl@uic.edu.

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Our results demonstrate that changes in both TPR/G protein expression and cellular localization allow the dynamic modulation of TPR-G protein signaling during OLG development.

## MATERIALS AND METHODS

**Materials.** The stable TPR mimetic U46619, TPR antagonist SQ29548, and the thromboxane $B_2$ ($TXB_2$) enzyme immunoassay kit were purchased from Cayman Chemical (Ann Arbor, MI). The stable TPR antagonist BM13.505 was from Boehringer Mannheim (Mannheim, Germany). The A2B5 hybridoma cell line was obtained from ATCC (Manassas, VA). Platelet-derived growth factor and fibroblast growth factor 2 were obtained from Pepro Tech (Rocky Hill, NJ). Prolong Antifade mounting medium and DAPI (4′,6-diamidino-2-phenylindole) were from Molecular Probes (Eugene, OR). Unlabeled cyclic AMP (cAMP) and [$^3$H]cAMP were purchased from Assay Designs (Ann Arbor, MI) and Amersham Biosciences (Piscataway, NJ), respectively. Adenylyl cyclase inhibitor, SQ22536, was purchased from Biomol (Plymouth, PA). Affi-Gel HZ hydrazide gel matrix was from Bio-Rad Laboratories (CA). $G_s$ and control Accell small interfering RNAs (siRNAs) were purchased from Thermo Scientific Dharmacon. Dibutyryl cAMP (db-cAMP) was purchased from Sigma-Aldrich (St. Louis, MO).

**Antibodies.** Mouse anti-2′,3′-cyclic nucleotide phosphodiesterase (CNPase) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-histone H1, anti-actin, anti-G protein ($G_q$/$G_{13}$/$G_s$/$G_o$/$G_i$) antibodies, anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP) conjugate, anti-mouse IgG HRP conjugate, and anti-goat IgG HRP conjugate were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CREB and anti-phospho-CREB were from Cell Signaling (Beverly, MA). The secondary Alexa Fluor-conjugated antibodies were from Molecular Probes (Eugene, OR).

**Cell culture.** Primary rat OLGs were obtained from mixed glial cultures prepared from 1- to 2-day-old Sprague-Dawley rat pups as reported previously (41), using a modification of the method of McCarthy and de Vellis (28). Oligodendrocyte progenitor cells (OPCs) were allowed to differentiate into mature OLGs by removal of the B104 conditioned growth medium and were cultured in differentiation medium for 16 days. The progenitor CG-4 cell line was cultured and passaged in N1 medium containing 35% B104 conditioned medium and induced to differentiate into OLGs by being subcultured in the differentiation medium as described previously (25, 41).

**Immunocytochemistry.** Cells grown on glass coverslips were fixed with 4% paraformaldehyde and washed with 100 mM glycine and Hanks' balanced salt solution-HEPES as reported previously (41). Cells were incubated overnight with primary antibodies in permeabilization buffer (containing 5% goat serum, 0.1% Triton X-100, 0.2% bovine serum albumin) at 4°C. The following dilutions were used: TPR (1:400), G protein ($G_q$/$G_{13}$/$G_s$/$G_o$/$G_i$) (1:200), A2B5 (1:250), and CNPase (1:250). The coverslips were washed and incubated with Alexa Fluor-conjugated secondary antibodies at a 1:1,000 dilution for 1 hour at room temperature. Cells were then stained with DAPI (2 μg/ml) for 5 min before coverslips were mounted. Each experiment was repeated on at least three individual culture preparations. All images were acquired using a Zeiss LSM510 META laser scanning confocal microscope.

**Immunostaining in rat brain sections.** Cryosections (12 μm) from rat brains were used for immunostaining as described previously (41). Briefly, rat brain sections were incubated with primary antibodies to $G_s$ and CNPase at a 1:150 dilution, followed by incubation with Alexa Fluor-conjugated secondary antibodies at a 1:300 dilution. Nuclei were visualized by staining with DAPI. The sections were mounted in Antifade mounting medium with coverslips. All the images were acquired using a Zeiss LSM510 META laser scanning confocal microscope.

**Immunoblotting.** Purified day 0 OPCs and day 16 OLGs were harvested on ice with a modified radioimmunoprecipitation assay buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.25% sodium deoxycholate,1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM $Na_3VO_4$, 1 mM NaF, 1 mM β-glycerolphosphate, and protease inhibitor cocktail III (Calbiochem, La Jolla, CA). Cell lysates were sonicated and centrifuged, and the supernatant was used for biochemical analysis. Protein concentrations were estimated using a bicinchoninic acid assay kit (Pierce Biochemicals, Rockford, IL), and samples (5 to 20 μg) were resolved on 10% or 15% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to Immobilon polyvinylidene difluoride (Millipore) membranes. The blots were blocked for 30 min and incubated with primary antibody at 4°C overnight at the following dilutions: histone H1 (1:400), G protein (1:250), TPR (1:400), actin (1:400), and myelin basic protein (MBP) (1:500). The blots were washed and incubated with the appropriate HRP-conjugated secondary antibodies (1:4,000) for 1 h at room temperature. Bands were visualized using the Pierce Supersignal kit (Pierce Biochemicals, Rockford, IL). The resulting bands were scanned, and the relative density of each band was calculated using the Scion Image software. Each experiment was repeated at least three times using different primary cultures. To demonstrate equal protein loading, blots were stripped with stripping buffer (containing 0.2 M glycine [pH 2.2], 1% Tween-20, and 0.1% SDS) for 1 h at room temperature and subsequently reprobed with antibodies as indicated above.

**Nuclear fractionation.** For cell fractionation, nuclear and extranuclear fractions were separated by sucrose density gradient centrifugation performed using previously published methods (41, 15). For immunoblotting, nuclei were solubilized in buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 2% Triton X-100 and were briefly sonicated on ice. Protein concentrations were measured using a bicinchoninic acid assay kit. Efficiency of the fractionation was confirmed by probing the fractions with antibodies for compartment-specific markers, i.e., histone H1 for nuclear fraction and MBP for the nonnuclear fraction.

**Immunoaffinity purification of the TPR-G protein complex.** TPR-G protein complexes were purified by the method described previously (3, 5, 10). Briefly, 5 mg of anti-TPR antibody was coupled to the Affi-Gel HZ hydrazide gel matrix overnight at 4°C. The gel matrix was then washed with a phosphate-buffered saline. Next, the isolated extranuclear and nuclear samples were resuspended in the buffer containing 50 mM KCl, 0.5 mg/ml azolectin, 20% glycerol, 0.2 mM EGTA, 2 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The samples (4 mg of protein) were incubated with the immunoaffinity matrix overnight at 4°C. The unbound proteins were removed by washing the matrix with 15 ml of buffer (20 mM Tris-HCl, 10 mM CHAPS, 20% glycerol, 500 mM KCl, 0.2 mM EGTA, 0.5 mg/ml azolectin, pH 7.4). The specifically coupled proteins were then eluted with a 100 mM glycine buffer, pH 2.0. Fractions of 200 μl were collected and immunoblotted. A normal rabbit IgG–Affi-Gel HZ matrix was used as a control.

**Knockdown of $G_s$ using siRNA.** These experiments were carried out in CG4 cells. Briefly, Accell siRNAs to $G_s$ (CCUACAUGUUAAUGGGUUU) or control (nontargeting) were purchased from Dharmacon and used as per the manufacturer's instructions. Briefly, 24 h after plating, CG4 cells were transfected with the siRNA (0.75 μM) and subsequently cultured in the differentiation medium. The differentiated OLGs were analyzed 72 h after transfection for knockdown efficiency.

**cAMP assay.** Freshly isolated nuclei and the extranuclear fraction were resuspended in cytosolic comparable buffer (25 mM HEPES, 125 mM KCl, 2 mM $K_2HPO_4$, 200 nM $CaCl_2$, 4 mM $MgCl_2$, and 0.5 mM ATP, pH 7.0). Samples were incubated with agonist U46619 (5 μM) or forskolin (5 μM) after pretreatment with adenylyl cyclase inhibitor, SQ22536, and/or the TPR antagonist, BM13.505, for 20 min. Control samples were treated with the vehicle. The phosphodiesterase inhibitor RO20-1724 (100 μM) was then added to the samples, which were flash frozen. The pellet was then resuspended, sonicated, boiled for 4 min and centrifuged, and the supernatant was used for the cAMP assay. The concentration of cAMP in the samples was measured as previously described (12, 18). The standard curve was generated by adding known concentrations of unlabeled cAMP to the buffer.

**CREB phosphorylation.** Freshly isolated nuclei were resuspended in cytosolic comparable buffer. Samples were incubated with agonist U46619 (5 μM) or BM13.505 (10 μM) after pretreatment with SQ22536 (300 μM) or the vehicle for 20 min. In $G_s$ knockdown cells, db-cAMP (0.5 mM) was added to the isolated nuclei. The nuclei were sonicated, followed by boiling with the SDS-PAGE loading buffer. Samples were subjected to SDS-PAGE (10% gels), blotted, and probed with phospho-CREB antibody (1:1,000 dilution). Total CREB (antibody dilution, 1:1,000) was used as a loading control.

**MBP gene expression in a cell-free nuclear system.** Freshly isolated OLG nuclei ($10^6$ nuclei/assay) were placed in the cytosolic buffer containing ATP, UTP, GTP, and CTP (0.5 mM) along with RNase inhibitor and incubated at 37°C for 1 hour with agonist U46619 (5 μM) or the vehicle in the presence or absence of the TPR antagonist, BM13.505. Total nuclear RNA was isolated by the standard guanidine isothiocyanate method. One microgram of total nuclear RNA was transcribed using the ImProm-II reverse transcription system from Promega. Nascent MBP mRNA was quantified using semiquantitative PCR. Initially, the PCR cycle was run for 32 cycles to get the 548-bp product. In order to quantify the newly synthesized MBP mRNA, cycles 15 to 32 of the PCR were broken to achieve the best time point for quantification before the curve plateaus. Cycle 18 was determined to be the optimal cycle and was used for further experiments. Primers for MBP were as follows: forward, 5′CGGACCCAAGAT GAAAACCC-3′, and reverse, 5′-AAAGGAAGCCTGGACCACACAG-3′. Primers for TPR were as follows: forward, 5′ TGCTGCAGACGCTACCTGT C-3′, and reverse, 5′-GATTGGCACCGTCCTTCAGG-3′ (31). As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified under the same conditions using the sense and reverse primers 5′-CC

ACTCACGGCAAATTCAACGGCA-3′ and 5′-TCCAGGCGGCACGTCAGA TCCACG-3′, respectively. PCR products were run on a 1.5% agarose gel and imaged, and the individual bands were quantified. Data are represented as means ± standard errors of the means (SEMs) of results obtained from three different experiments.

**Cell survival assay.** To study OLG cell survival, CG4 cells were plated at a density of 5,000 cells/well in 96-well poly-L-lysine-coated plates. After 24 h, the cells were transfected with either $G_s$ or control siRNA and subsequently cultured in differentiation medium. The cells were treated with the TPR agonist U46619 or the vehicle for 6 days. Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega) as per the manufacturer's instructions.

**$TXB_2$ measurement.** Nuclei isolated from OPCs and mature OLGs were washed twice in Hanks' balanced salt solution and suspended in cytosolic buffer. The vehicle or 50 μM of indomethacin was added for 15 min before treatment with 100 μM arachidonic acid (AA) for 10 min. At the end of all time points, supernatants were collected and a 100-μl aliquot was used to measure the levels of the stable metabolite of $TXA_2$, i.e., $TXB_2$ using a colorimetric enzyme-linked immunosorbent assay.

**Statistical analysis.** Data were analyzed using GraphPad Prism 4 statistical software (San Diego, CA) and expressed as means ± SEMs, and statistical significance was assessed by using one-way analysis of variance. Differences were considered statistically significant when values were $P < 0.05$.

## RESULTS

**G protein distribution and expression during OLG maturation.** We first investigated the presence of different Gα subunits in OLGs during development by immunocytochemistry. Primary rat OPCs were plated on poly-L-lysine-coated coverslips and maintained in the OPC growth medium or differentiated using OLG differentiation medium as previously reported (28, 41). For immunocytochemistry, day 0 OPCs and day 16 mature OLGs were used. A2B5 was employed as a stage-specific marker for the bipolar proliferating OPCs (Fig. 1A, row I), CNPase was used as a marker for mature myelinating OLGs (Fig. 1B, row II), and DAPI was used to stain the nuclei (Fig. 1A and B, row III). The OPCs and OLGs were coimmunostained for the different Gα proteins, i.e., $G_q$, $G_{13}$, $G_o$, $G_s$, and $G_i$. As can be seen in Fig. 1A and B, $G_q$ (column a), $G_{13}$ (column b), $G_o$ (column c), $G_s$ (column d), and $G_i$ (column e) are expressed in both OPCs and mature OLGs during development. The specificity of the antibodies was confirmed using normal IgG control (data not shown) as well as secondary antibody control (data not shown). Furthermore, the coimmunostaining revealed that there were differences in the distribution of these G proteins within OPCs and OLGs. Specifically, it was found that while $G_s$ (Fig. 1A and B, column d) and $G_o$ (Fig. 1A and B, column c) were present in the extranuclear compartment (plasma membrane and cytosolic region) as well as the nuclear compartment of both OPCs and OLGs, $G_q$ (Fig. 1A and B, column a), $G_{13}$ (Fig. 1A and B, column b), and $G_i$ (Fig. 1A and B, column e) were present only in the extranuclear compartment. This differential localization was next investigated by subcellular fractionation and biochemical analysis using methods reported previously (41, 15). Furthermore, since our previous results demonstrated that TPRs are associated with the nuclear compartment of mature OLGs and not OPCs, these studies used only mature rat OLGs.

In these experiments, primary rat OLGs (day 16) were lysed and fractionated by sucrose gradient centrifugation to separate the extranuclear fractions (plasma membrane, cytosol, and ER/Golgi apparatus) and the nuclear fractions. In order to establish the purity of these fractions, subcellular markers were employed. In this regard, it is known that MBP is not present in the nucleus of mature OLGs and that histone H1 is restricted to the nuclear compartment. Consequently, these proteins can serve as indicators of cross-contamination between the two fractions in mature OLGs. As can be seen in Fig. 2, no MBP immunoreactivity was detected in the nuclear fraction and no histone H1 was detected in the extranuclear fraction. Regarding Gα subunit distribution, $G_q$, $G_{13}$, and $G_i$ were detected only in the OLG extranuclear fraction, whereas $G_s$ and $G_o$ were present in both fractions (Fig. 2). Thus, these results demonstrate that the distribution of G proteins within subcellular compartments is not uniform.

The next studies measured the expression levels of the G proteins in OPCs and OLGs. As can be seen in Fig. 3, the expression level of $G_q$ increased as the OPCs differentiated into myelin-producing OLGs. In contrast, $G_{13}$ levels were downregulated and the expression levels of $G_i$, $G_s$, and $G_o$ (Fig. 3) did not change significantly as OPCs differentiated. Therefore, it appears that as OLGs mature, they selectively modify only certain G protein expression levels.

**Functional coupling of TPRs to G proteins in OLGs.** The next experiments identified the different G proteins that couple to TPRs. Specifically, the TPR-G protein complexes from OPCs and OLGs were isolated by immunoaffinity chromatography (3, 5, 10), and the eluates from the immunoaffinity columns were probed for TPR and Gα subunits. Since our previous studies (41) had established that TPRs are not present in the nuclei of OPCs, the present experiments evaluated only TPR-G protein complex formation in the OPC extranuclear fraction. It can be seen (Fig. 4A, lane 2) that passage of the extranuclear fraction of OPCs through the TPR-immunoaffinity column resulted in purification of TPRs in the eluate. It can also be seen that immunoblotting the affinity column eluate with various G protein antibodies revealed TPR complex formation with $G_q$ and $G_{13}$ but not with $G_s$, $G_o$ or $G_i$ (Fig. 4A, lane 2). The passage of the nuclear fraction of OPCs through the TPR-immunoaffinity column did not reveal any detectable purification of TPRs in the eluate (data not shown).

In the case of mature OLGs, TPR was detected in the unpurified lysates from both the extranuclear and nuclear fractions (Fig. 4B), which is a distribution pattern consistent with our previous reports (5, 41). In separate G protein immunoblotting studies, it was found that $G_q$, $G_{13}$, $G_s$, $G_o$, and $G_i$ were all present in the unpurified lysate of the extranuclear compartment (Fig. 4B, lane 1). Furthermore, affinity purification of this fraction revealed coelution of TPRs with $G_q$ and to a smaller extent $G_{13}$ (Fig. 4B, lane 2). On the other hand, $G_s$, $G_o$, and $G_i$ were not detected in the eluate from this extranuclear compartment (Fig. 4B, lane 2). Collectively, these findings are comparable to those observed for OPCs and indicate that TPRs couple to both $G_q$ and $G_{13}$, but not to $G_i$, $G_o$, or $G_s$, in the extranuclear compartment.

In the OLG nuclear fraction, it can also be seen that $G_q$, $G_{13}$, and $G_i$ were not detected in either the unpurified lysate or the eluate following affinity purification (Fig. 4C, lanes 1 and 2). While both $G_s$ and $G_o$ were present in the unpurified nuclear lysate, interestingly, only $G_s$ copurified with TPRs from the nuclear lysate (Fig. 4C, lanes 1 and 2). In control experiments, passage of the lysates through a normal rabbit IgG immunoaffinity matrix did not reveal the purification of either

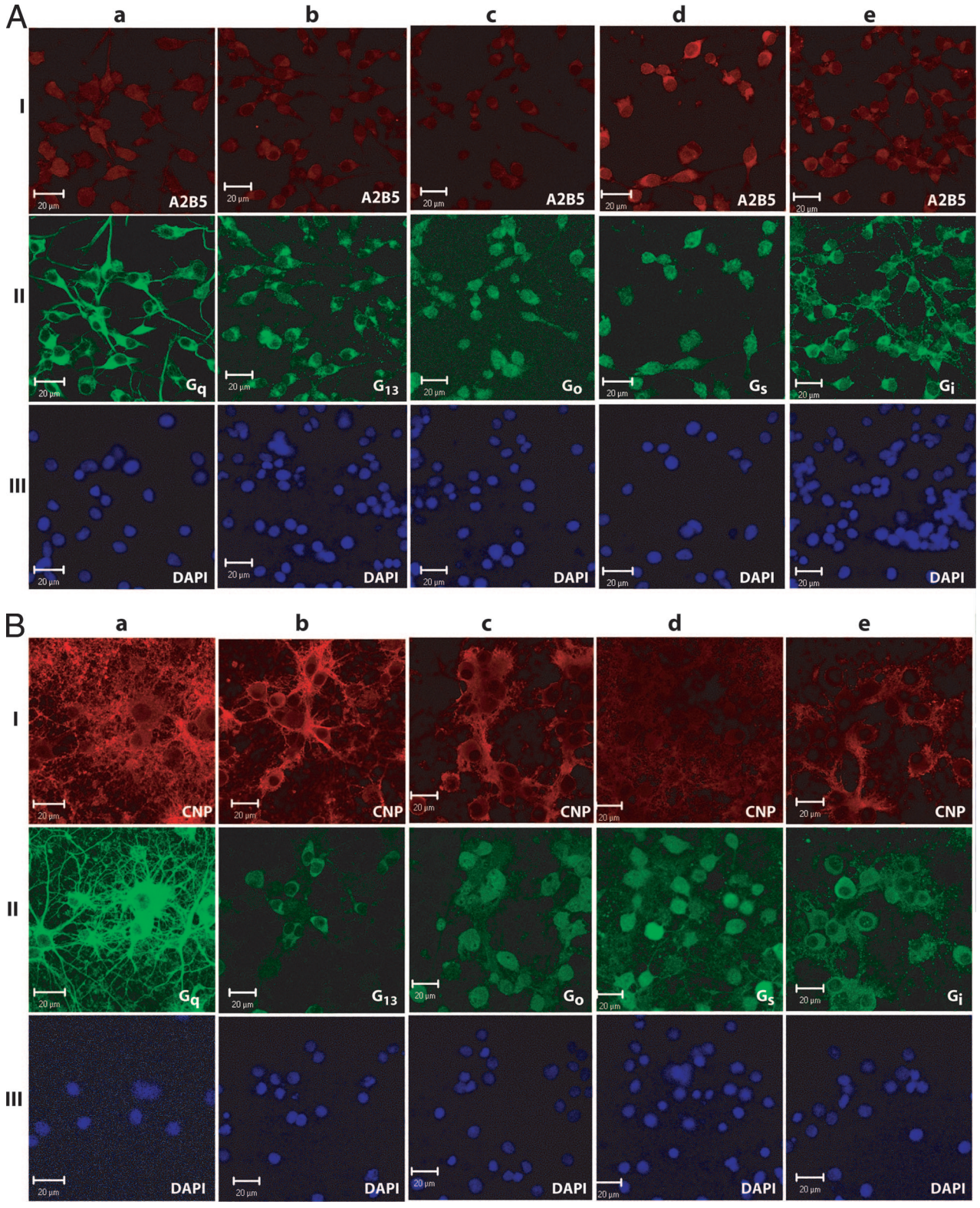



FIG. 1. Expression and distribution of G proteins in OPCs and OLGs. Primary rat OPCs were plated onto poly-L-lysine-coated coverslips, induced to differentiate, fixed (days 0 and 16 of differentiation), stained, and analyzed by confocal microscopy. The antibodies to the OLG lineage-specific markers A2B5 (red staining for OPCs) (A) and CNPase (red staining for OLGs) (B) were used to identify cells at each time point (row I, columns a to e). The second row (row II, columns a to e) shows the coimmunostaining for G proteins $G_q$ (column a), $G_{13}$ (column b), $G_o$ (column c), $G_s$ (column d), and $G_i$ (column e) for the same field of cells. The nucleus is indicated by DAPI (blue staining) (A and B, row III). The IgG isotype control for the primary antibodies and the secondary antibody control (in the absence of primary antibodies) were done to confirm the specificity of the antibody labeling (data not shown). Scale bars in all cases are 20 μm. Images shown are representative of at least three separate experiments done on different primary cultures.

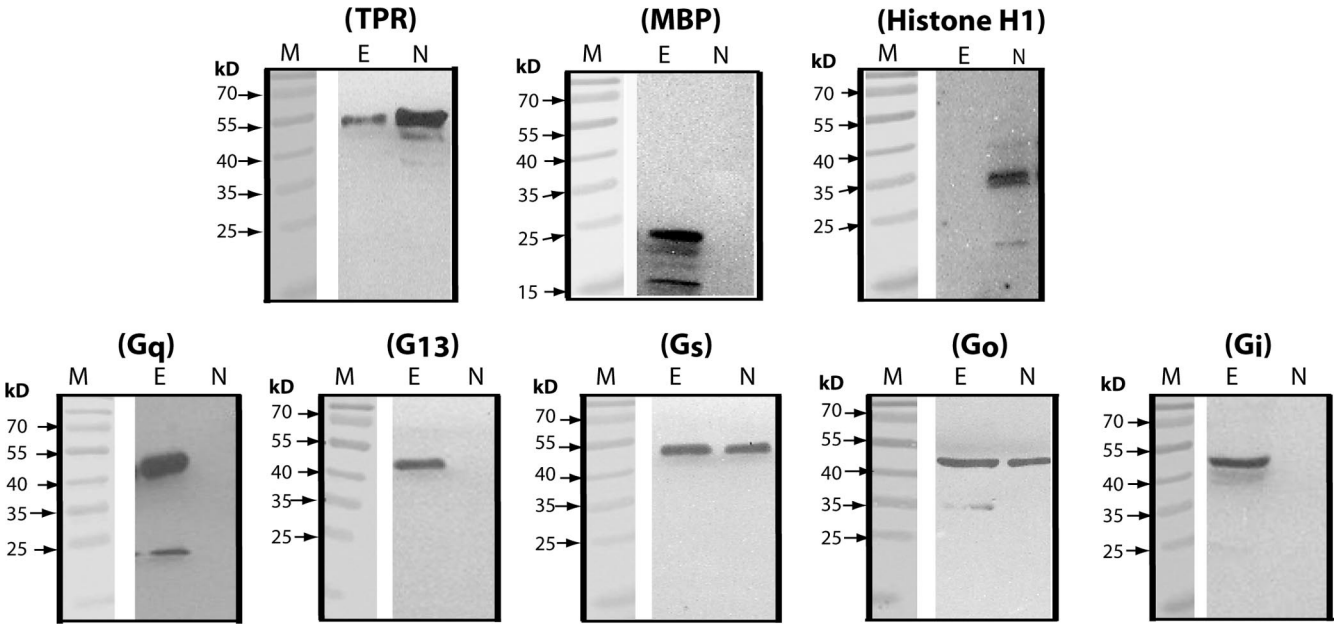



FIG. 2. Intracellular localization of G proteins. OLGs (day 16) were separated into extranuclear (E) and nuclear (N) fractions. Twenty micrograms of protein from each fraction was subjected to SDS-PAGE and then immunoblotting. These fractions were also probed for TPR and G protein α subunits using antibodies to $G_q$, $G_{13}$, $G_o$, $G_s$, and $G_i$. The molecular mass markers (M) for each representative blot are shown in the first lane. Each experiment was repeated at least three times ($n = 3$).

TPR or any of the associated G proteins (Fig. 4A to C, lane 3), demonstrating that the observed elution profiles were indeed TPR specific.

Previous immunocytochemical studies determined that TPRs localize to the nucleus of OLGs in vivo (41), and the results described above suggest a coupling of TPRs to $G_s$ in the nuclear compartment. On this basis, we next determined whether $G_s$ also localizes to OLG nuclei in whole brain. It was found that $G_s$ immunoreactivity was uniformly distributed among the cytoplasmic and nuclear compartments in OLGs in the rat cerebellum (Fig. 4D) and spinal cord (not shown).

Taken together, the results described above provide the first documentation of differential G protein expression, distribution, and coupling to TPRs during OLG development. Furthermore, these results demonstrate the novel coupling of TPRs to $G_s$ which is restricted to the nucleus of mature OLGs.

**TPR activation elevates nuclear cAMP levels in OLGs.** The next experiments investigated the functional activity of the nuclear TPR-$G_s$ complex in OLGs. Briefly, the nuclei and the extranuclear fractions isolated from mature OLGs were treated with the specific TPR agonist U46619 or the vehicle alone. These experiments were also conducted in the presence/absence of the adenylyl cyclase inhibitor, SQ22536, or the TPR antagonist, BM13.505. It was found that stimulation with forskolin substantially raised cAMP levels in both the extranuclear and nuclear fractions (Fig. 5) and that this elevation in cAMP was inhibited by preincubation with SQ22536 (data not shown). These results establish the presence of functional adenylyl cyclase in each of these subcellular fractions, consistent with earlier reports (4). In the next experiments, nuclear TPRs were stimulated with various doses of U46619 (0.25 to 5 μM) and the effect on cAMP levels was determined. It was found that 5 μM of U46619 produced a nearly 100% increase in nuclear cAMP but had no effect on cAMP levels in the extranuclear fraction (Fig. 5A).

Furthermore, this increase in nuclear cAMP was inhibited by both SQ22536 (300 μM) and BM13.505 (10 μM). Finally, stimulation of nuclei isolated from OPCs with U46619 did not produce any significant change in cAMP (data not shown), as would be expected since these nuclei do not contain TPRs (41).

To further establish the role of $G_s$ in TPR-mediated elevation of nuclear cAMP, $G_s$ knockdown experiments were conducted. Briefly, CG4 cells were transfected with $G_s$ or control siRNA and analyzed after OLG differentiation. As can be seen in Fig. 5B, treatment of CG4 cells with siRNA substantially decreased the $G_s$ expression levels (71% knockdown; $n = 3$). The next experiments analyzed the TPR-stimulated nuclear cAMP production in these $G_s$ knockdown cells. It was found that the U46619-induced increase in cAMP production was not only blocked by the TPR antagonist BM13.505 but also by $G_s$ knockdown (Fig. 5C). Furthermore, the ability of $G_s$ knockdown to lower cAMP production was rescued by the addition of db-cAMP.

**Effect of nuclear TPR signaling on phosphorylation of CREB.** Separate experiments next investigated the functional consequences of signaling through the TPR nuclear pathway. In this connection, cAMP has been reported to influence OLG development (38, 40), in part through activation of the transcription factor CREB (43). Consistent with this notion, our results demonstrated that stimulation of OLG nuclei with the TPR agonist U46619 (5 μM) did indeed lead to activation of CREB, as indicated by an increase in phospho-CREB over basal levels (Fig. 6A). As expected, this activation was inhibited by the antagonist BM13.505 (10 μM) as well as by the adenylyl cyclase inhibitor, SQ22536 (300 μM) (Fig. 6A).

Additional studies used $G_s$ knockdown OLGs (derived from CG4 cells treated with $G_s$ siRNA) to examine CREB phosphorylation in response to TPR stimulation. It was found (Fig. 6B) that knockdown of $G_s$ also led to a reduction in TPR-

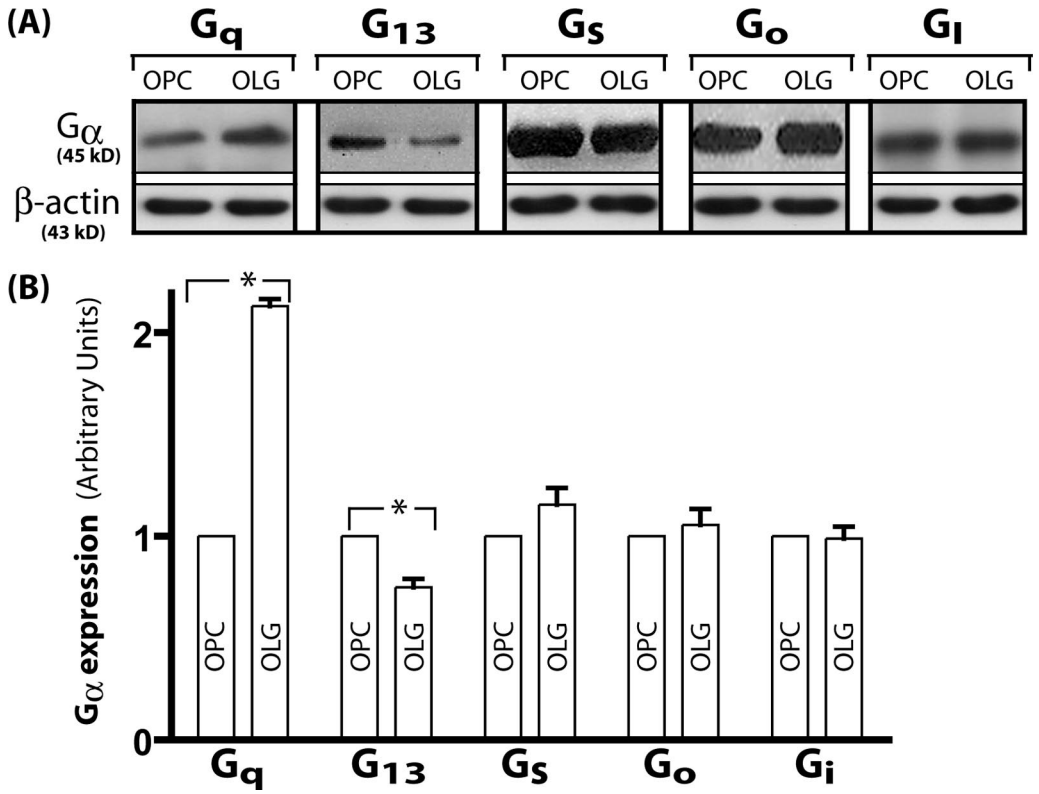



FIG. 3. Expression levels of G proteins during OLG development. (A) Primary rat OPCs (day 0) and OLGs (day 16) were harvested, and 20-μg protein samples were run on SDS-PAGE. After being transferred, the proteins were immunoblotted using antibodies against $G_q$, $G_{13}$, $G_s$, $G_o$, and $G_i$. The blots were stripped and reprobed for β-actin, which was used as a loading control. Representative blots from three separate experiments are shown. (B) The resulting bands were scanned and quantified and are represented as means ± SEMs ($n = 3$). Differences were statistically significant (*) at a $P$ value of $<0.05$.

mediated CREB phosphorylation and that this reduction was reversed by the addition of db-cAMP.

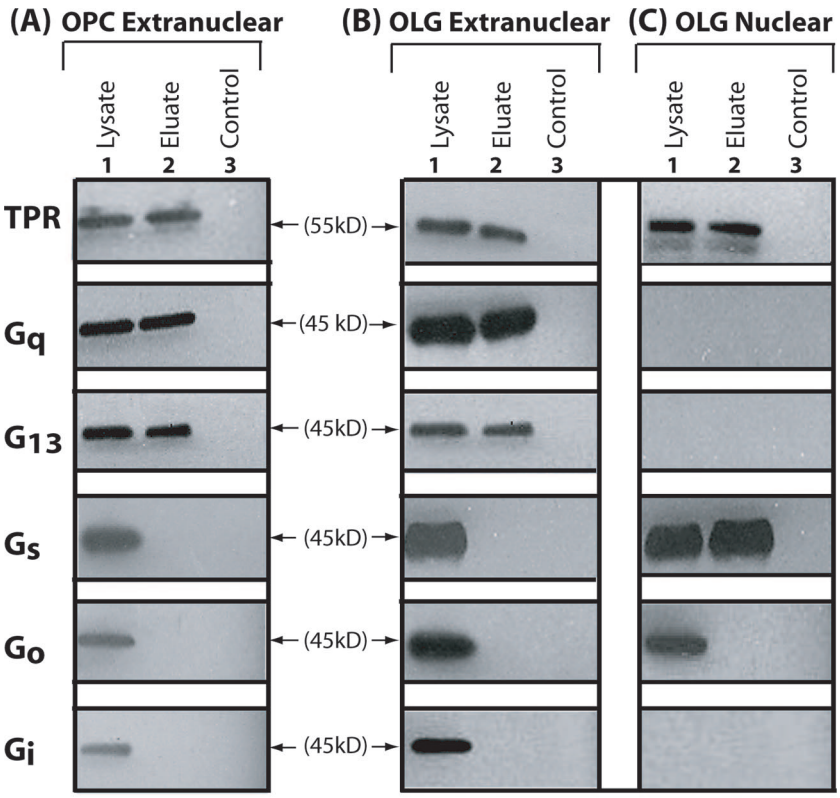
**Nuclear TPR activation increases MBP gene expression.** While TPR stimulation in OLGs is known to elevate MBP expression (41), the mechanism is not known. In the present experiments, we sought to determine if nuclear TPR signaling is involved in this simulation of the MBP promoter. Briefly, freshly isolated OLG nuclei ($10^6$ nuclei/assay) were incubated (37°C) in cytosolic buffer containing RNase inhibitor and U46619 (1 μM) in the presence or absence of BM13.505 (5 μM). The newly synthesized MBP mRNA in the isolated nuclei was measured using semiquantitative PCR. As can be seen in Fig. 7A and B, stimulation of TPRs with U46619 resulted in a significant increase in the MBP mRNA levels compared to the vehicle-treated control nuclei. This increase in MBP levels was blocked by the TPR antagonist BM13.505 (Fig. 7A and B). On the other hand, it can also be seen that TPR stimulation in the isolated OLG nuclei did not produce any significant change in the levels of mRNA for TPR itself or for GAPDH (Fig. 7A). These results demonstrate that the TPR nuclear signaling pathway can specifically activate MBP transcription in OLGs and that this pathway is sufficient to stimulate MBP transcription independently of extranuclear signaling events.

In separate experiments, the effect of TPR-$G_s$ coupling on OLG survival was examined. As can be seen in Fig. 7C, stimulation of CG4 cells with the TPR agonist U46619 (1 μM) increases cell viability. This increased survival was blocked by both the TPR antagonist BM13.505 (5 μM) and by knockdown of $G_s$. Finally, the addition of db-cAMP increased cell survival even in the in the $G_s$ knockdown cells (Fig. 7C), indicating that cAMP is the modulator of TPR-mediated cell survival in OLGs.

**$TXA_2$ is produced in OLG nuclei.** The findings described above establish that OLG nuclei contain TPRs that signal through cAMP and CREB. In order to determine whether these nuclei also contain the functional enzymes necessary for elaboration of $TXA_2$, synthesis of $TXA_2$ was measured in the nuclear compartment. In these experiments, isolated nuclei from OPCs or mature OLGs were evaluated for their abilities to metabolize exogenously added AA (100 μM). The synthesis of $TXA_2$ was assayed by quantifying the production of its stable metabolite, $TXB_2$. It can be seen in Fig. 8 that in contrast to nuclei isolated from OPCs, the nuclei from mature OLGs produced significant amounts of $TXA_2$ in response to added AA, and this production was blocked by preincubation with the cyclooxygenase inhibitor indomethacin (50 μM).

## DISCUSSION

The eicosanoid $TXA_2$ has previously been shown to signal through the seven-transmembrane GPCR TPR. TPR signaling is known to play an important role in hemostasis, renal physiology, immune function, inflammation, parturition, fertility, etc. (32, 51). Furthermore, TPR activation is also known to



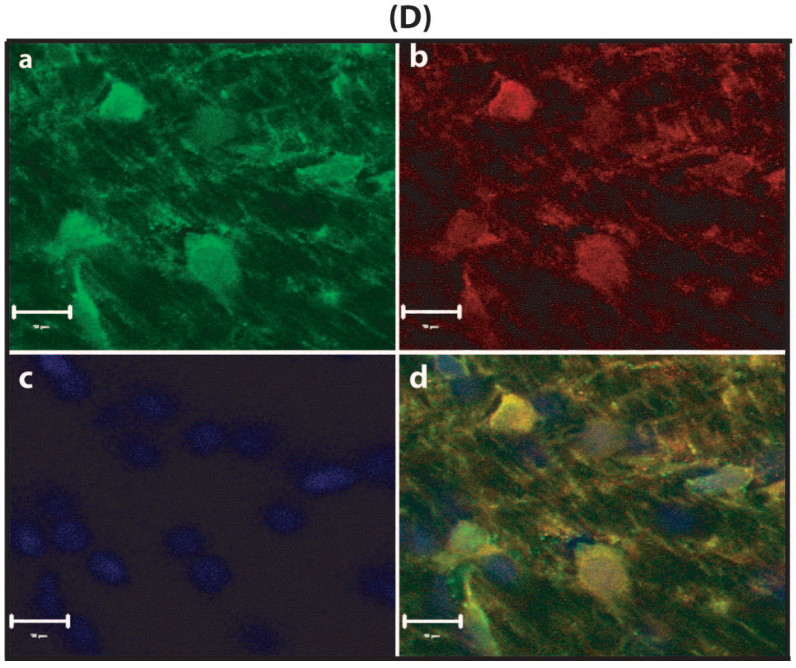




FIG. 4. (A to C) Immunoaffinity purification of TPR-G protein complex from OLGs. Four milligrams of protein of isolated extranuclear (E) and nuclear (N) fractions from rat OPCs/OLGs was incubated with the TPR-immunoaffinity matrix. After washing, the bound proteins were eluted, subjected to SDS-PAGE, and probed using antibodies against TPR and various Gα subunits: $G_q$, $G_{13}$, $G_s$, $G_o$, and $G_i$. Panel A represents the OPC extranuclear fraction, panel B represents the OLG extranuclear fraction, and panel C represents the OLG nuclear fraction. Representative immunoblots are shown. The lanes in each panel correspond to unpurified lysate (lane 1), eluate from the TPR-immunoaffinity matrix (lane 2), and eluate from the control rabbit IgG matrix (lane 3). Each experiment was repeated at least three times. (D) Localization of $G_s$ in OLGs in vivo. Brain cryostat sections from adult rat cerebellum were coimmunostained for $G_s$ (a), CNPase (b), and DAPI (c). Panel d shows the overlay, showing colocalization of $G_s$ (green) with DAPI (blue) in the nuclear compartment of CNPase-positive OLGs. Scale bars = 10 μm.

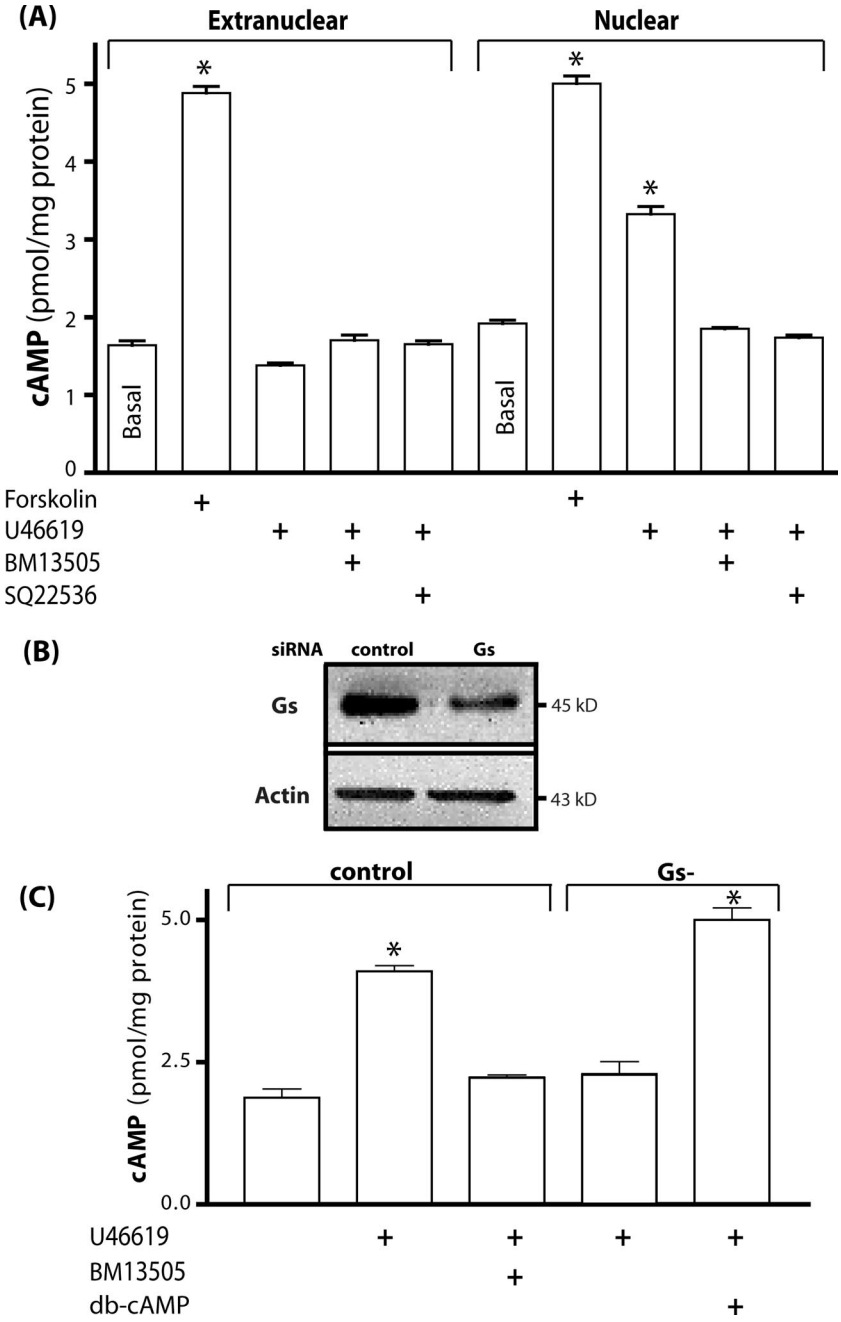



FIG. 5. Effect of TPR activation on nuclear cAMP levels in OLGs. (A) Isolated extranuclear and nuclear samples from mature OLGs in cytosolic buffer (containing 0.5 mM ATP) were treated with the TPR agonist, U46619 (5 μM), in the presence of the vehicle, the adenylyl cyclase inhibitor, SQ22536 (300 μM), or the TPR antagonist BM13.505 (10 μM). In order to establish intact adenylyl cyclase activity in this assay, forskolin was used. cAMP levels in the samples were then assayed as described in Materials and Methods. Results represent means ± SEMs of three separate experiments. A $P$ value of $<0.05$ was statistically significant (*) compared to basal cAMP levels. (B) Knockdown of $G_s$ in CG4 cells was achieved by transfection of CG4 cells with siRNA to $G_s$ or control. The cells were lysed 72 h after transfection and immunoblotted for $G_s$ expression. A representative blot is shown. The resulting bands from three separate experiments were scanned and quantified. A 71 ± 6.4% (means ± SEMs) decrease in $G_s$ expression was observed after treatment with the $G_s$ siRNA. (C) CG4 cells were transfected with $G_s$ siRNA, differentiated into OLGs for 6 days, and stimulated with the TPR agonist, U46619 (5 μM), and 0.5 mM db-cAMP was added to the $G_s$ knockdown cells (Gs-). cAMP levels in the nuclear samples were then assayed as described in Materials and Methods. Results represent means ± SEMs of three separate experiments. A $P$ value of $<0.05$ was statistically significant (*) compared to basal cAMP levels.

have multiple effects on development in different cell types. For instance, $TXA_2$ inhibits vascular endothelial growth factor-induced endothelial cell differentiation, promotes megakaryocytopoiesis, enhances mitogenesis of coronary artery smooth muscle cells, stimulates tumor cell proliferation (29, 34, 47), and is thought to be involved in apoptosis (11, 49). As is the case with other GPCRs, TPRs have been shown to activate multiple G proteins, in particular $G_q$ and $G_{13}$ (10, 23, 35, 45).

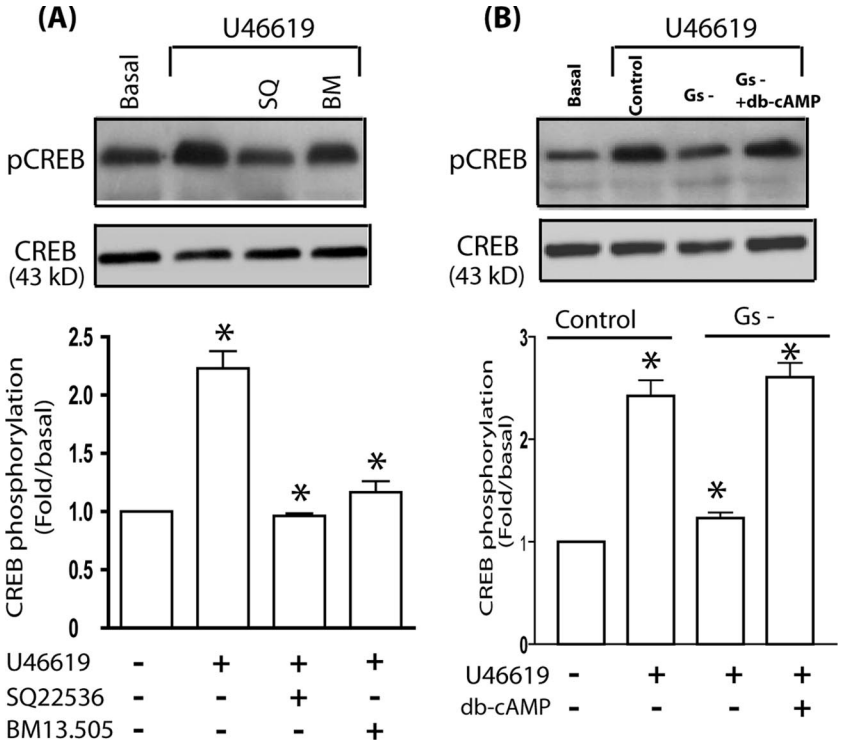



FIG. 6. Effect of nuclear TPR stimulation on CREB phosphorylation. (A) Nuclei isolated from mature OLGs were stimulated with the TPR agonist U46619 (5 μM) (or the vehicle) in the presence or absence of the antagonist BM13.505 (10 μM) and the adenylyl cyclase inhibitor, SQ22536 (300 μM). The nuclei were then sonicated and boiled. Twenty micrograms of protein was then subjected to SDS-PAGE and probed for phospho-CREB (upper blot). Total CREB antibody was used as a loading control (lower blot). A representative immunoblot is shown. Results were quantified after scanning the bands and are represented as means ± SEMs of measurements from three separate experiments. A $P$ value of $<0.05$ was statistically significant (*) compared to basal levels. (B) CREB phosphorylation in $G_s$ knockdown OLGs (Gs-) (cells derived from CG4 cells) was analyzed. Isolated nuclei from control or $G_s$ knockdown cells were stimulated with the TPR agonist U46619 (5 μM) in the presence of the antagonist BM13.505 (10 μM) or db-cAMP (0.5 mM). CREB phosphorylation was then analyzed as described above. Results from three separate experiments are represented in the bar graph. A $P$ value of $<0.05$ was statistically significant (*) compared to basal CREB phosphorylation levels.

While TPR signaling in certain organ systems, e.g., blood platelets, has been extensively documented, its role in brain development and function is poorly understood.

Studies from our laboratory have previously characterized the localization and functional effects of TPR activation in both OLGs and Schwann cells (5, 3, 25, 30, 41). These results demonstrated that stimulation of TPRs promotes proliferation of OPCs and prolongs survival of mature OLGs (25). In addition, it was found that TPR signaling in OLGs activates multiple downstream kinases, e.g., mitogen-activated protein kinase/extracellular signal-regulated kinase, as well as certain transcription factors, including CREB and c-*fos* (25). Recently, we reported that the distribution of TPRs shifts during OLG differentiation from an extranuclear to a predominately nuclear localization pattern (41). This nuclear localization, along with the capacity of TPRs to modulate both extranuclear and nuclear signaling pathways, led us to hypothesize that there is a functional TPR signaling mechanism in the OLG nucleus. The present study extended these findings by addressing several important questions concerning TPR signaling in OLG development and function. Firstly, do TPR signaling pathways change during OLG differentiation? If this is indeed the case, one would expect to see a similar change in G protein expression and distribution. Secondly, are nuclear TPRs functionally coupled to G proteins? If this occurs, it should be possible to demonstrate physical association of nuclear TPRs with specific Gα subunits. And lastly, what is the functional significance of the observed shift in TPR localization to the OLG nucleus?

The first experiments in this study mapped the expression and distribution of various G proteins during OLG development. It was found that there was a selective change in the expression levels of several G proteins as OLGs differentiate, suggesting a dynamic mechanism by which G protein signaling is regulated. Furthermore, analysis of the G protein distribution profile revealed a differential localization of these G proteins within OLGs. Specifically, $G_q$, $G_{13}$, and $G_i$ were found to be present only in the extranuclear region of OLGs, whereas $G_o$ and $G_s$ were present in both compartments. In order to further investigate these findings, immunoaffinity chromatography was used to copurify TPR-G protein complexes from each compartment. It was found that extranuclear TPRs specifically couple to $G_q$ and $G_{13}$ but not to $G_i$, $G_s$ or $G_o$. This apparent preference for $G_q$ and $G_{13}$ is consistent with the TPR-G protein coupling profile previously reported in blood platelets (10, 23). Interestingly, a completely different coupling profile was observed with the nuclear G proteins. In this case, nuclear TPRs of differentiated OLGs were found to couple to $G_s$ (but not to $G_o$). This latter result represents the first dem-

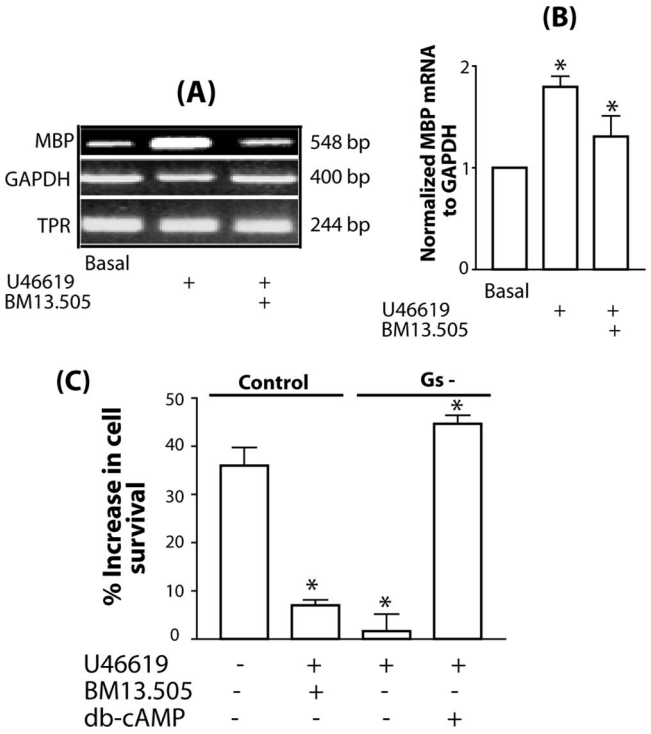



FIG. 7. (A, B) Activation of nuclear TPR and MBP gene expression. Freshly isolated OLG nuclei ($10^6$ nuclei/assay) were incubated (37°C) in cytosolic buffer (containing RNase inhibitor and 0.5 mM deoxynucleoside triphosphates) and U46619 (1 μM) in the presence or absence of BM13.505 (5 μM). Total RNA was isolated and transcribed using the ImProm-II reverse transcription system from Promega. The newly synthesized MBP mRNA in the isolated nuclei was measured using semiquantitative PCR. TPR and GAPDH mRNA were quantified as controls. (B) TPR mRNA levels were normalized to those for GAPDH. Results represent means ± SEMs of three separate experiments. A $P$ value of $<0.05$ was statistically significant (*) compared to basal mRNA levels. (C) TPR-$G_s$-mediated survival of mature OLGs. Control or $G_s$ knockdown OLGs ($G_s$-) were treated with the TPR agonist U46619 (1 μM), the presence of BM13.505 (5 μM), or db-cAMP (0.5 mM) for 6 days in differentiation medium. Cell viability (survival) was assessed by the CellTiter-Glo luminescent cell viability assay (Promega). Results are represented as means ± SEMs of measurements from three separate experiments. A $P$ value of $<0.05$ was statistically significant (*) compared to basal.

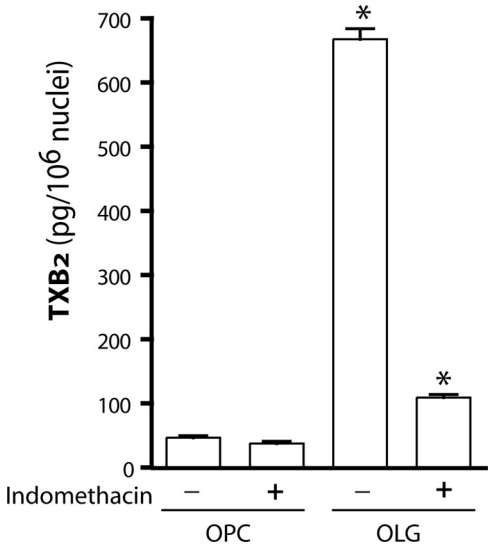



FIG. 8. $TXA_2$ production in OLG nuclei. Nuclei ($10^6$) isolated from OPCs and mature OLGs were treated with AA (100 μM) after pretreatment with cyclooxygenase inhibitor indomethacin (50 μM) or the vehicle. The synthesis of $TXA_2$ was assayed by quantifying the production of its stable metabolite, $TXB_2$. Results are represented as means ± the SEMs of measurements from three separate experiments. A $P$ value of $<0.05$ was statistically significant (*) compared to basal $TXB_2$ levels.

onstration that TPRs can physically form a complex with $G_s$ under endogenous levels of TPR/$G_s$ expression, as well as the first evidence that GPCRs interact with any Gα subunits in the cell nucleus.

Even though a number of recent reports have documented GPCR localization in the nuclei of various cell types, the functional consequences of this phenomenon have not been elucidated. On this basis, we next investigated TPR-$G_s$ signaling in OLG nuclei. It was found that stimulation of the nuclear TPR pool caused an elevation in nuclear cAMP levels. Furthermore, this elevated cAMP was found to activate CREB, which is known to promote OLG survival (20, 42) and stimulate MBP expression (1, 43). The direct association of TPRs with MBP expression was confirmed in subsequent experiments, revealing nuclear TPR-mediated increases in MBP mRNA levels. Further experiments revealed that the TPR-$G_s$ signaling increases OLG cell survival. Collectively, these results therefore provide evidence that OLGs possess self-contained nuclear GPCR signaling mechanisms, which are independent of the GPCR pathways normally associated with the plasma membrane compartment (as schematically represented in Fig. 9). The existence of such pathways would, in turn, dramatically expand the potential involvement of GPCR signaling in a host of nuclear events linked to cellular development, gene/protein expression, and cellular death.

From a mechanistic point of view, the abilities of TPRs to couple to different G proteins in the nuclear and extranuclear compartments presumably derive from TPR/Gα subunit compartmentalization that restricts the availability of each component for complex formation. For example, even though $G_s$ is present in both the extranuclear and nuclear compartments, TPRs preferentially couple to $G_q$ and $G_{13}$ (in the extranuclear regions) because they have a higher coupling affinity for TPRs than $G_s$ (17). On the other hand, in the nucleus, neither $G_q$ nor $G_{13}$ is available to form a complex with TPRs, whereas $G_s$ is abundantly available. Thus, the presence of nuclear $G_s$, in the absence of $G_q$ and $G_{13}$, facilitates TPR coupling to $G_s$ in this specific subcellular compartment. The compartmentalization of these GPCR-G protein complexes therefore has the potential to cause altered G protein signaling capacities within and between these subcellular regions. This process would in turn contribute to the manifestation of different GPCR effector profiles during stress or disease.

In addition, these differences in TPR-G protein coupling patterns would theoretically determine the consequences of TPR signaling at a particular stage of OLG maturation. Specifically, TPR coupling to $G_{13}$ in early OLG development would presumably mediate Rho-dependent mitogenesis, cytoskeletal alterations, and cell motility (44). Furthermore, as

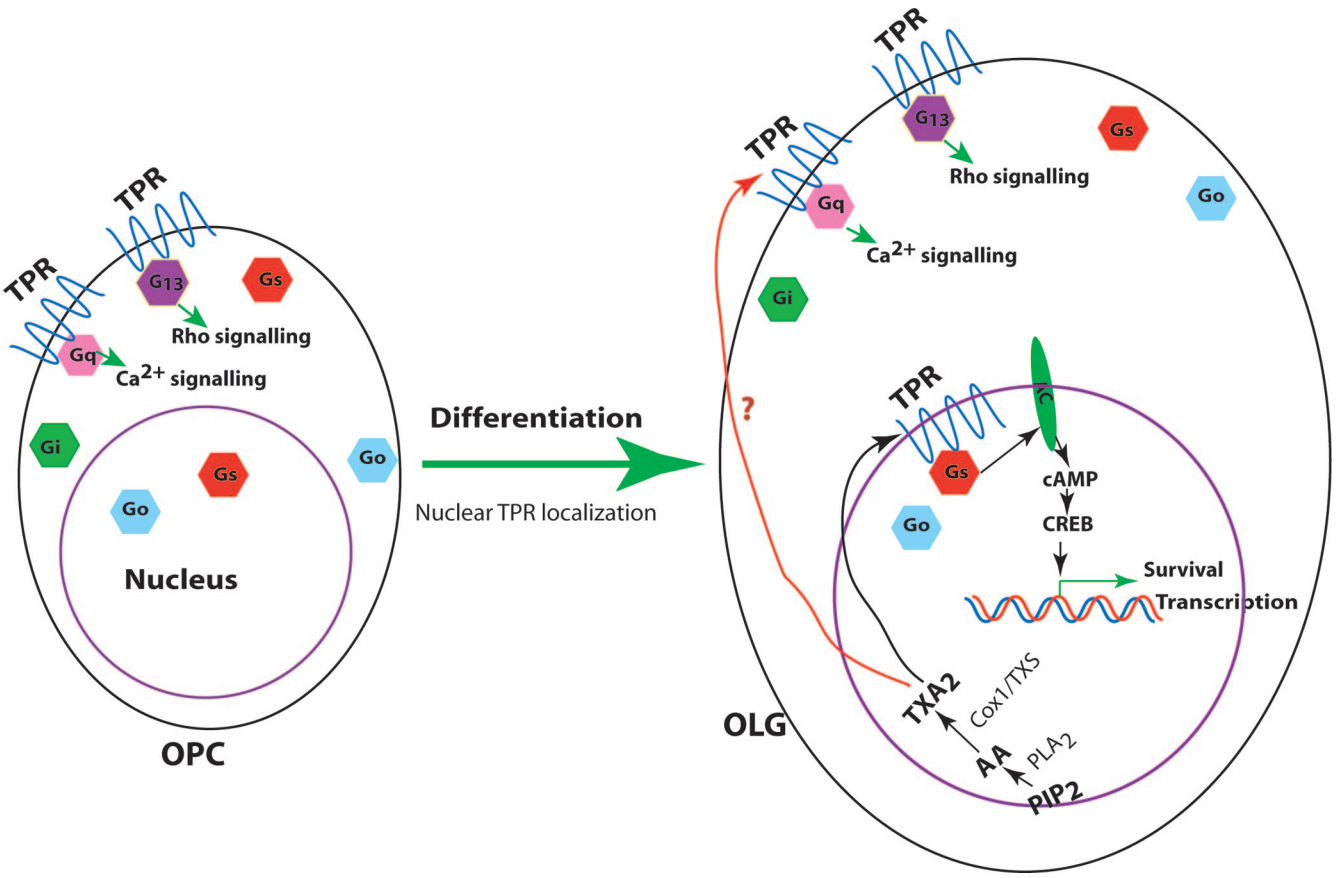



FIG. 9. Model depicting signaling pathways for TPR-mediated modulation of OLG development and function. See Discussion for details. Cox1/TXS, cyclooxygenase 1/thromboxane synthase; $PLA_2$, phospholipase $A_2$; $PIP_2$, phosphatidylinositol 4,5-biphosphate.

OLGs mature, TPR coupling with $G_q$ would lead to activation of the calcium-dependent signaling pathways involved in the modulation of different kinases (mitogen-activated protein kinase/extracellular signal-regulated kinase) affecting OLG differentiation (36). Finally, as nuclear TPR-$G_s$ complexes are formed, TPR stimulation would lead to cAMP-mediated activation of CREB, which is involved in MBP expression and OLG survival (38, 40).

While the results described above provide evidence that TPR activation can modulate nuclear signaling events, they do not establish the source of $TXA_2$ in this cellular compartment. In this regard, previous studies have shown that phospholipase $A_2$ activity increases as cells mature (33) and that nuclei of different mature cells possess the enzymes necessary to synthesize $TXA_2$ (48). Furthermore, differentiation-related shifts have been reported in the metabolism of AA in cell nuclei (13, 27). Consistent with this notion, our results demonstrate that treatment of isolated OLG nuclei with AA resulted in $TXA_2$ production. Furthermore, since $TXA_2$ synthesis was not detected in OPC nuclei, it appears nuclear $TXA_2$ can be produced only at specific stages of OLG differentiation. While the reason for this temporally regulated synthesis is not known, it presumably coincides with the shift toward nuclear TPR localization and signaling during OLG development.

Thus, it appears that GPCRs reside in the cellular nuclear compartments, that their levels are not constant during development, and that at least in the case of TPRs, these receptors have a functional signaling capacity through heterotrimeric G proteins. However, in spite of this knowledge, no information has been thus far provided to elucidate the mechanism by which these signaling components enter the nucleus. The "passive diffusion" model has been suggested to be involved in the transport of some GPCRs to the nucleus. In other cases, modified nuclear localization signals (NLS) have been proposed to facilitate the transport of large proteins to the nucleus via the Ran-GTP-importin mechanism (19) or putative karyopherins (22). In addition to the classical NLS, there are a large variety of heterogeneous sequence motifs that have no homology to the basic NLS but can still promote the import of proteins into the nucleus (8). While the precise mechanism of TPR localization to the mature OLG nucleus is not known, it does seem to be dependent on the stage of development of the OLGs as reported previously (41). Furthermore, ligand activation does not affect the receptor localization to the nucleus (unpublished data), which indicates that this redistribution process is associated with developmental factors and not ligand-induced receptor internalization.

The present results therefore demonstrate a novel signaling pathway for nuclear TPRs, which is schematically represented in Fig. 9. In this model, upon activation of the nuclear phospholipase $A_2$, AA is released from the nuclear phospholipid pool (i.e., phosphatidylinositol 4,5-biphosphate) and metabo-

lized through nuclear cyclooxygenase-1/thromboxane synthase to $TXA_2$ (13, 48). Once produced, the $TXA_2$ interacts with its receptor located on nuclear membrane structures, causing cAMP production, CREB phosphorylation, and pro-survival signaling. Whether this nuclear $TXA_2$ can also act as a ligand for the extranuclear TPRs is presently unknown. However, if this does indeed occur, it would require a complex translocation process involving movement across the nuclear membrane, the cytosol, and the plasma membrane in order to access surface TPRs. Alternatively, the function of nuclear-generated $TXA_2$ may be restricted to the nuclear compartment, with the surface TPRs serving only as a pathway for intercellular signaling. Therefore, taken together, the two TPR pools may each function in different capacities, i.e., autocrine for the nuclear pool and paracrine for the plasma membrane pool. Clearly, additional studies will be necessary to resolve these interesting possibilities.

In summary, the present study demonstrates a temporally dependent mechanism of GPCR/G protein compartmentalization. This process constitutes a novel mechanism by which GPCRs serve diverse cellular functions at different periods during cellular differentiation and development.

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