Identification of an Interaction between the TPα and TPβ Isoforms of the Human Thromboxane A2 Receptor with Protein Kinase C-related Kinase (PRK) 1

IMPLICATIONS FOR PROSTATE CANCER

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Elizabeth C. Turner¹, David J. Kavanagh¹, Eamon P. Mulvaney, Caitriona McLean, Katarina Wikström, Helen M. Reid, and B. Therese Kinsella²

From the School of Biomolecular and Biomedical Sciences, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

In humans, thromboxane (TX) A₂ signals through the TPα and TPβ isoforms of the TXA₂ receptor or TP. Here, the RhoA effector protein kinase C-related kinase (PRK) 1 was identified as an interactor of both TPα and TPβ involving common and unique sequences within their respective C-terminal (C)-tail domains and the kinase domain of PRK1 (PRK1640–942). Although the interaction with PRK1 is constitutive, agonist activation of TPα enhanced PRK1 phosphorylation and general survival of prostate cancer cells. Expression of PRK1 augmented TPα-mediated signaling in androgen-responsive prostate LNCaP and PC-3 cell lines but not in primary vascular neoplasms. Agonist-dependent phosphorylation of histone H3 at Thr11 (H3 Thr11), a previously recognized specificity determinant, is widely implicated in a number of cardiovascular disorders, including thrombosis, systemic- and pregnancy-induced hypertension, vessel remodeling, and atherosclerotic progression (10, 11). In recent evidence suggests aberrant TXA₂ signaling, and TXA₂ receptor (TP) expression is associated with certain cancers, in particular prostate cancer with protein-coupled receptor; H3 Thr11, histone H3 threonine 11; PG, prostaglandin; TP, thromboxane receptor; Y2H, yeast two-hybrid; βAR, β₂-adrenergic receptor; TX, thromboxane; PKN, protein kinase novel; ANOVA, analysis of variance; DDO, double dropout; QDO, quadruple drop-out; SDM, site-directed mutagenesis; HUVEC, human umbilical vein endothelial cell; AR, androgen receptor; IC, intracellular.

This article has been withdrawn by the authors. After reviewing the data, the corresponding author learned that there was duplication of data in Figs. 1A, 2D, 4A, 5, 6, 8C, 8D, S4F, and S4G. The authors wish to withdraw the article in the interests of maintaining their publication standards, while also respecting the highest standards of transparency and reliability of their research and of the JBC. Replica data sets for each of the figures in question that the authors state fully validate the findings and conclusions of the published article are available. A revised version of the manuscript with those replica data sets can be obtained by contacting the corresponding author.

WITHDRAWN

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The local control of hemostasis and vascular tone is controlled by a complex process involving platelets, the endothelium and vascular smooth muscle (VSM), various soluble coagulation factors, vasoconstrictor autacoids, and other diverse mediators (1). Agents such as thromboxane (TX) A₂, that signal through G protein-coupled receptors (GPCRs) to promote platelet aggregation or VSM contraction can typically co-couple to Gα, phospholipase Cγ, and G12/RhoA (2). These responses to promote myosin light chain phosphorylation and cell contraction is typically co-coupled with increased intracellular calcium and other physiological responses to direct vascular tone (3). Although the interaction with PRK1 is constitutive, agonist activation of TPα augmented TPα-mediated signaling in androgen-responsive prostate LNCaP and PC-3 cell lines but not in primary vascular neoplasms. Agonist-dependent phosphorylation of histone H3 at Thr11 (H3 Thr11), a previously recognized specificity determinant, is widely implicated in a number of cardiovascular disorders, including thrombosis, systemic- and pregnancy-induced hypertension, vessel remodeling, and atherosclerotic progression (10, 11). In recent evidence suggests aberrant TXA₂ signaling, and TXA₂ receptor (TP) expression is associated with certain cancers, in particular prostate cancer with protein-coupled receptor; H3 Thr11, histone H3 threonine 11; PG, prostaglandin; TP, thromboxane receptor; Y2H, yeast two-hybrid; βAR, β₂-adrenergic receptor; TX, thromboxane; PKN, protein kinase novel; ANOVA, analysis of variance; DDO, double dropout; QDO, quadruple drop-out; SDM, site-directed mutagenesis; HUVEC, human umbilical vein endothelial cell; AR, androgen receptor; IC, intracellular.

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1 To whom correspondence should be addressed. Tel.: 353-1-7166727; Fax: 353-1-7166456; E-mail: Therese.Kinsella@ucd.ie.

2 The abbreviations used are: VSM, vascular smooth muscle; AA, arachidonic acid; ACC, antiparallel coiled-coil fold; α-H8, α-helical 8 domain; AR, androgen receptor; C-tail, C-terminal tail; DHT, dihydrotestosterone; GPCR, G protein receptor; H3 Thr11, histone H3 threonine 11; PG, prostaglandin; TP, thromboxane receptor; Y2H, yeast two-hybrid; βAR, β₂-adrenergic receptor; TX, thromboxane; PKN, protein kinase novel; ANOVA, analysis of variance; DDO, double dropout; QDO, quadruple drop-out; SDM, site-directed mutagenesis; HUVEC, human umbilical vein endothelial cell; AR, androgen receptor; IC, intracellular.

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EXPERIMENTAL PROCEDURES

Materials—The MATCHMAKER™ human kidney cDNA library, HY4043AH, was obtained from Clontech; pGEX-5X-1 and glutathione-Sepharose 4B were from GE Healthcare; pCMVTag2a/b/c vectors and the QuickChange™ site-directed mutagenesis system were from Stratagene. The rabbit reticulocyte lysate in vitro transcription and translation system (TnT™) was from Promega. Anti-FLAG® M2 monoclonal antibody (F3165), mouse monoclonal anti-FLAG horseradish peroxidase (HRP) conjugate, histone H1, protein A-Sepharose CL-4B, protein G-Sepharose, and dihydrotestosterone (DHT) were from Sigma; anti-GST (B-14, sc-138), anti-RhoA (26C4, sc-418), goat anti-PRK1 (C-19, sc-1842), rabbit anti-histone H3 (FL-136, sc-10809) were from Santa Cruz Biotechnology; anti-phospho-histone H3 Thr11 (anti-phospho-H3 Thr11 antibody was from Active Motif; HRP-conjugated mouse anti-goat, HRP-conjugated goat anti-rabbit antibodies were from Santa Cruz Biotechnology; rat monoclonal 3F10 anti-HA-HRP-conjugated antibody was from Roche Applied Science; mouse monoclonal anti-hemagglutinin (HA)-101R antibody was from Eurogentec; U46619 and 17-phenyl trinor prostaglandin (PG) E2 was from Cayman Chemical Co.; [γ-32P]ATP (6000 Ci/mmol; 10 mCi/ml) was from PerkinElmer Life Sciences; Escherichia coli Rosetta 2 (DE3) from Merck Biosciences. All oligonucleotides were synthesized by Genosys Biotechnologies and all small interfering RNAs (siRNA) were from Dharmacon. Cicaprost was kindly donated by Schering AG (Berlin, Germany).

Expression Plasmids—The plasmids pGBK7T:TPC312–434 and pGBK7T:TPC312–407 were generated by subcloning the cDNA subfragments encoding the C-terminal domains of TPα (amino acid 312–434) and TPβ (amino acid 312–407) into the EcoRI/BamHI sites of the yeast bait vector pGBK7T (Clontech) such that inserts were c-Myc epitope-tagged and in-frame with the DNA-binding domain of the yeast GAL4 transcriptional regulator. Similarly, pGBK7T:TPβ312–328, pGBK7T:TPβ312–343, pGBK7T:TPβ312–353, pGBK7T:TPβ312–366, pGBK7T:TPβ312–392, pGBK7T:TPβ312–392, pGBK7T:TPβ329–392, pGBK7T:TPβ353–392 and pGBK7T:TPβ366–392, respectively, were generated by subcloning the respective PCR-amplified fragments into pGEX-5X-1, such that fragments were frame with glutathione S-transferase (GST). The plasmids pCMVTag2b:PRK1, pCMVTag2b:PRK11–357, pCMVTag2b:PRK11–343, pCMVTag2b:PRK1 561–942, and pCMVTag2b:PRK1K644E, encoding a dominant negative kinase-dead form of PRK1 (9), were generated by either subcloning of the respective PCR-amplified subfragments into pCMVTag2b such that they were in-frame with the N-terminal FLAG™ epitope tag or by QuikChange™ SDM cloning. The plasmids pCMVTag2b:PRK1 and pCMVTag2b:PRK1 561–942 were validated by DNA sequence analysis. Ala-scanning SDM of residues 221–246 expressed in the yeast bait plasmids pGBK7T:TPα312–434 and pGBK7T:TPβ312–407, respectively, was carried out using QuikChange™ SDM cloning. All in cases, sequences of the specific primers used are listed in supplemental Table 1. All plasmids were validated by DNA sequence analysis.

Yeast Two-hybrid Screening and Yeast Matings—The human kidney cDNA library (3.5 × 10⁸ independent clones; HY4043AH), cloned in-frame with the activation domain of the yeast GAL4 transcriptional activator in the yeast prey plasmid pACT2 and pre-transformed into Saccharomyces cerevisiae YI87, was obtained from Clontech. The MATa bait strains S. cerevisiae AH109 (pGBK7T:TPα312–434/pGBK7T:TPβ312–407) were mated with the MATa S. cerevisiae Y187 (pACT2; human kidney cDNA library) at a density of 2 × 10⁶ cells/ml and a ratio of 30:1 bait/prey cells, where 1 × 10⁶ individual diploids were screened. After 24 h of growth in SD/TRp⁺, Leu (DDO, double
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dropout) at 30 °C, diploids were plated onto SD/Trp−, Leu−, Ade−, His− medium (quadruple drop-out (QDO) medium) and maintained at 30 °C for 15 days. Following selection on QDO media, recombinant pACT2 plasmid DNA was extracted from putative interactants and transformed into supercompetent E. coli XL-1Blue cells. Following retransformation of the pACT2-based plasmid from putative interactants into S. cerevisiae Y187 and re-mating with an expanded range of bait strains, including S. cerevisiae AH109 (pGBK7:TPα312–343/pGBK7:TPβ329–343), pGBK7:TPβ329–407/pGBK7:TPα312–343 and pGBK7:TPβ312–407 (supplemental Table 1B), diploids were selected on DDO media, and positive interactants were on the basis of expression of HIS3, ADE2, and MELI reporter genes by growth on QDO medium and cleavage of 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside. cDNA inserts from positive interactants were identified by DNA sequence analysis.

Cell Culture and Transfections—Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (ATCC) and grown in minimal essential medium, 0.2% (v/v) l-glutamine, 10% (v/v) fetal bovine serum (FBS). Routinely, ~48 h prior to transfection, cells were plated at a density of 2 × 10^6 cells/10-cm dish in 8 ml of media. Thereafter, cells were transiently transfected with 400 ng of pCMV-based vectors using Effectene (Qiagen) and routinely harvested 48 h post-transfection. HEK, TPα, HEK, TPβ, and HEK, β2AR cells, stably expressing HA-tagged forms of TPα, TPβ, and the human thromboxane receptor (β2AR), respectively, have been described previously (27). HEK, β-galactosidase (HEK.β-gal), expressing the HA-tagged β-Galactosidase described previously (29). LNCaP cell lines were cultured in 90% RPMI 1640 medium and 10% FBS. PC-3 and LNCaP cells were cultured in DMEM (10% FBS, 1× Pen/Strep, 0.2% (v/v) L-glutamine). Thereafter, cells were transiently transfected with 2 μg of pCMV-based vectors using either Effectene (Qiagen) or Lipofectamine LTX (Invitrogen), respectively, and harvested 48 h post-transfection. For experiments using DHT treatments, both PC-3 and LNCaP cells were cultured in smooth muscle cell growth medium supplemented with 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 5% (v/v) FBS (Promocell GMBH, C-22062). 1° human umbilical vein endothelial cells (HUVECs), from Lonza (IRT9-048-0904D), were routinely cultured in M199 media (Sigma) supplemented with 0.4% (v/v) Endothelial Cell Growth Supplement/Heparin (ECGS/H; Lonza), 20% (v/v) FBS, and 0.2% (v/v) l-glutamine. 1° h.CoASMC and 1° HUVECs were routinely used between passages 2 and 8. All mammalian cells were grown at 37 °C in a humid environment with 5% CO2 and confirmed to be mycoplasma free.

Glutathione S-Transferase Pulldown Assays—E. coli Rosetta 2 (DE3) transformants of pGEX-5X-1:TPα312–343, pGEX-5X-1:TPβ329–343, and pGEX-5X-1:TPβ329–407, and pGEX-5X-1:TPβ329–407 were grown at 37 °C in LB selection medium, until an A600 of 0.8, and glutathione S-transferase (GST) proteins were induced at 4 °C for 1 h with 0.25 mM isopropyl β-D-thiogalactopyranoside. Cell pellets from 1-liter cultures were resuspended in Lysis Buffer (150 mM NaCl, 5 mM MgCl2, 1% (v/v) Triton X-100, 50 mM Tris-Cl, pH 7.5, 1 mM DTT, 10 μg/ml apro- tinin, 10 μg/ml leupeptin, 1 mM PMSF; 10 ml) and proteins purified on 1-ml (50% slurry) glutathione-Sepharose 4B beads pre-equilibrated in Wash Buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 0.5% (v/v) Triton X-100, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM PMSF), according to standard procedures. HA.PRK1640–942 was translated from the PCR-amplified product encoding the T7 promoter sequence, optimal translation initiation site (GAG GG ACC ATG), HA epitope and amino acids 640–942 of PRK1 using the TNT coupled T7 in vitro transcription/translation system (Promega). Per GST pulldown assay, 10 μg of final reaction product, and GST bound to glutathione-Sepharose 4B beads were pre-equilibrated at room temperature for 1 h, washed three successive changes of PBS, and beads containing 5 μg of each protein were combined with the in vitro translated product (Supplement 1A) (5 μg/ml aprotinin (v/v) Triton X-100) supplemented with 1% (w/v) Triton X-100 for 30–60 min. Thereafter, beads were incubated in IP Sample Buffer (10% (v/v) β-mercaptoethanol, 2% (v/v) SDS, 30% (v/v) glycerol, 0.025% (w/v) bromphenol blue). The input TNT* in vitro translated product HA.PRK1640–942 (1 μl) and protein-bound glutathione-Sepharose 4B beads were washed 10 min, followed by SDS-PAGE on 12.5% acrylamide gels, and transferred to polyvinylidene fluoride (PVDF). Membranes were immunoblotted versus anti-HA (3F10) (1:1000 dilution in TBS, 5% (w/v) skinned milk powder) or anti-GST antisera (1:1000 dilution in TBS, 5% (w/v) skinned milk powder). For GST pulldown assays using mammalian cell extracts, 48 h prior to pulldown experiments, HEK 293 cells were transfected with pCMVTag2b-PRK1. Thereafter, cells were lysed in Buffer B (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100 (v/v), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM PMSF). Cell debris was removed by centrifugation (14,000 rpm) at 4 °C for 20 min, and cellular lysates (500 μg) were incubated at 4 °C for 2 h with glutathione-Sepharose beads pre-loaded with 10 μg of GST. TPα312–343, GST. TPβ329–407, or as a control GST.Ip299–386. Beads were washed in three changes of Buffer B and finally resuspended in 30 μl of Immunoprecipitation (IP) Sample Buffer (10% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 30% (v/v) glycerol, 0.025% (w/v) bromphenol blue). The input TNT in vitro translated product HA.PRK1640–942 (1 μl) and protein-bound glutathione-Sepharose 4B beads were washed 10 min, followed by SDS-PAGE on 12.5% acrylamide gels, and transferred to polyvinylidene fluoride (PVDF). Membranes were immunoblotted versus anti-HA (3F10) (1:1000 dilution in TBS, 5% (w/v) skinned milk powder) or anti-GST antisera (1:1000 dilution in TBS, 5% (w/v) skinned milk powder).
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Disruption of PRK1 Expression by Small Interfering (si)RNA—For siRNA experiments, PC-3 and LNCaP cells were plated at ~2.5 × 10^5 cells/35-mm plate 24 h prior to transfection to achieve ~50–60% confluency. Thereafter, cells were transfected with 30 nM siRNA PRK1 (5′-CCUCCGAAGAUUUCAAGG-3′) or siRNAControl (5′-AATTCTCCG-AACG-3′) using DharmaFECT (14.4 μl per 500 l per well; Dharmacon) transfection reagent in PC-3 cells and DharmaFECT (2 μl per 500 l per well) in LNCaP cells, and harvested 48 h post-transfection. Membranes were blocked using 5% non-fat dry milk in TBS and incubated with 1:1000 dilution of either anti-PRK1 (101R; 1:300) or anti-PRK1 (1:1000), anti-TPα/TPβ (No. 168; 1:25, 20 μg per 500 l), and anti-RhoA, and anti-HA-POD (3F10) antibodies in Blocking Buffer (5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20 in PBS). Subsequently, membranes were washed four times with PBS and incubated with either 1:1000 diluted anti-HA HRP (1:1000 dilution) or anti-HA-POD (1:1000 dilution) antibodies in TBS and incubated for 1 h with 0.01% DMSO or the PRK1 inhibitor RO-31-8220 (10 μM) for 30 min, followed by four changes of PBS. Immunoreactive proteins were resolved by SDS-PAGE on 12.5% acrylamide gels, and transferred to PVDF membrane. The phosphorylated substrate(s) were visualized by autoradiography, and the PVDF membrane was subsequently stained with Ponceau S or screened by Western blot analysis using anti-GST antibody. HEK.TPa, HEK.TPB, HEKβ-Gal, LNCaP, PC-3, EA.hy926 cells, 1° h.CoASMCs or 1° HUVECs were grown in 10-cm dishes, as described previously, to achieve ~80% confluency. Prior to immunoprecipitation, cells were stimulated at 37 °C either with vehicle or U46619 (1 μM) for 0–60 min. Thereafter, PRK1 was precipitated from cell lysates either directly using anti-PRK1 (1:1000 dilution) or because of interaction with HA.TPa or HA.TPB using anti-HA (101R) (1:300 dilution). Precipitates were washed four times with RIP Buffer and four times with kinase buffer (20 mM Tris-Cl, pH 7.5, 4 mM MgCl2, 154 μM ATP, 18.5 KBq, 0.5 μCi of [γ-32P]ATP (3000 Ci/mmol, 10 μCi/ml) in a 50-μl final reaction volume. The reaction mixture (50 μl) was then subjected to SDS-PAGE (12.5% acrylamide gels) and transferred to PVDF membrane. Phosphorylated histone H1 was detected by autoradiography, and all membranes were subsequently immunoblotted with anti-PRK1 or anti-HA 101R to verify precipitations.

Investigation of H3 Thr11 Phosphorylation—To examine the effect of U46619 on H3 Thr11 phosphorylation, 1° HUVECs, 1° h.CoASMCs, PC-3, and LNCaP cells were routinely plated 48 h previously at 2 × 10^5 cells/10-cm dish in 8 ml of growth media where the 10% FBS in the respective growth media was replaced with 10% charcoal-stripped FBS (Pan Biotech). Cells were then either transfected with pCMVTag2b:PRK1 (2 μg), pCMVTag2b:PRK1K644E (2 μg), siRNAPRK1 (30 nM), or siRNAControl (30 nM) or incubated with vehicle (0.01% EtOH) or DHT (1 μM) for 24 h, followed by incubation with either vehicle (0.01% DMSO) or the PRK1 inhibitor RO-31-8220 (10 μM) for 1 h at 37 °C as indicated. The concentration of RO-31-8220 (10 μM) used is that routinely used to inhibit PRK1 in cells in culture (9). Cells were stimulated with U46619 (1 μM; 0–60 min), 17 phenyl trinor PGE2 (10 μM; 30 min), or appropriate vehicle at 37 °C. To examine the effect of U46619 on H3 Thr11 phosphorylation, mitotic cells were obtained by treatment with colcemid (50 ng/ml) for 3 h prior to harvesting. The cells were harvested by sequential extraction to remove soluble cytoplasmic and nucleoplasmic proteins to obtain nuclear extracts (containing histones) as described previously (30). Briefly, cells were washed with ice-cold PBS and lysed in hypotonic buffer (20 mM Tris-Cl, pH 8.0, 1 mM KCl, 1.5 mM MgCl2, 1 mM PMSF, 50 μM leupeptin and 1 mM DTT) for 30 min at 4 °C with rotation. The lysate and nuclei were separated by cen-
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fugation (10,000 × g), and the nuclei were resuspended in 0.4 M H2SO4 and incubated overnight at 4 °C with rotation. Nuclear debris was removed by centrifugation (16,000 × g), and the histone-containing supernatant was trichloroacetic acid-precipitated. The resulting pellets were washed with ice-cold acetone three times to remove acid, air-dried, resuspended in double distilled H2O, and histone concentrations analyzed by the Bradford assay. For Western blot analysis, nuclear extracts were resolved on 15% SDS-polyacrylamide gels, and electrophoretically transferred onto nitrocellulose membranes according to standard protocols. Membranes were screened using anti-phospho-H3 Thr11 sera (Active Motif) in 5% nonfat dried milk in TBS (0.01 M Tris-HCl, 0.1 M NaCl, pH 7.4) for 1 h at room temperature followed by washing and screening using goat anti-rabbit horseradish peroxidase followed by chemiluminescence detection. Membranes were also screened using anti-histone H3 antisera to confirm uniform histone H3 loading.

Investigation of PC-3 and LNCaP Cell Migration—Boyden chamber assays were used to assess the effects of TP activation on prostate cancer cell migration. Briefly, prior to migration assays, PC-3 and LNCaP cells were plated in 10-cm dishes in growth media containing charcoal-stripped FBS either in the presence or absence of DHT (1 nM) and containing either the presence or absence of DHT (1 nM) such that they were ≥90% confluent 24–36 h post-seeding. Alternatively, cells were seeded such that they were ≥50% confluent after 24–36 h and transfected with siRNA (siRNAPRK1 or siRNAControl) for 48 h prior to transfection.

RESULTS

As relevant, single, double, triple, and quadruple symbols signify p ≤ 0.05, p ≤ 0.01, p ≤ 0.001, and ≥ 0.0001, respectively, for post hoc Dunnett’s multiple comparison t test analyses.

Data Analyses—Statistical analyses of differences were carried out using the unpaired Student’s t test, two-way or one-way ANOVA followed by post hoc Dunnett’s multiple comparison tests, as indicated, throughout employing GraphPad Prism, version 4.00 package. p values of less than or equal to 0.05 were considered to indicate a statistically significant difference. To investigate overall differences between time-dependent, U46619-induced H3 Thr11 phosphorylation in the absence or presence of DHT, nonlinear regression (R2 and F-test analyses were carried out, where p values of less than or equal to 0.05 were considered to indicate a statistically significant difference. As relevant, single, double, triple, and quadruple symbols signify p ≤ 0.05, p ≤ 0.01, p ≤ 0.001, and ≥ 0.0001, respectively, for post hoc Dunnett’s multiple comparison t test analyses.

Concentration dependence of interaction of TPα/TPβ in human kidney—Here, a yeast-two-hybrid (Y2H) screen of a human kidney cDNA library was utilized to identify proteins by incubation for a further 48 h. Cells were routinely serum-starved for 24 h before harvesting and QCMTM 24-well colorimetric cell migration assay (Millipore) was performed as per the manufacturer’s instructions. Membranes were screened using antibody (1:2,000) against phospho-H3 Thr11 sera (Active Motif) in 5% nonfat milk in TBS for 1 h, followed by staining with 4-

Confocal Microscopy—LNCaP and PC-3 cells were seeded on 300 μl of serum-free growth media containing charcoal-stripped FBS either in the presence or absence of 1 nM DHT (1 μM). After the cells settled (1 h), 500 μl of serum-free growth media containing either vehicle (0.01% DMSO) or U46619 (1 μM) was added to the bottom chamber. Cell migration was assessed after 4 h at 37 °C.

The cells remaining in the top chamber were removed by cotton swabs, and the migrated cells were stained with a crystal violet dye (Millipore, catalog no. 90144) and washed with H2O. The cells were then extracted using an extraction buffer (Millipore, catalog no. 90145), and the A560 nm was measured. Migration was expressed as percentage of basal cell migration. All experiments were performed at least in duplicate, and each experiment was repeated at least two times.

Yeast Two-hybrid—A yeast-two-hybrid (Y2H) screen of a human kidney cDNA library was utilized to identify proteins by incubation for a further 48 h. Cells were routinely serum-starved for 24 h before harvesting and QCMTM 24-well colorimetric cell migration assay (Millipore) was performed as per the manufacturer’s instructions. Membranes were screened using antibody (1:2,000) against phospho-H3 Thr11 sera (Active Motif) in 5% nonfat milk in TBS for 1 h, followed by staining with 4-
or GST, despite near equivalent expression of all proteins (Fig. 2A). The ability of full-length PRK1 (residues 1–942, herein referred to as PRK1), expressed as a FLAG epitope-tagged form in mammalian HEK 293 cells, to bind GST.TPα312–343 and GST.TPβ312–407 was also investigated. PRK1 showed a specific interaction with GST.TPα312–343 and GST.TPβ312–407 but not with GST.HIP299–386, a GST protein expressing the C-tail domain of the human prostacyclin receptor (Fig. 2B).

The ability of PRK1 to associate with HA-tagged forms of TPα or TPβ stably expressed in the previously characterized HEK.TPα and HEK.TPβ cell lines (27) was examined through co-immunoprecipitations. PRK1 was detected in the anti-HA immunoprecipitates from HEK.TPα and HEK.TPβ cells but not in corresponding immunoprecipitates from the HEK.βGal cells, encoding HA-tagged β-Gal, acting as an additional/alternative control (Fig. 2C). Thereafter, the effect of short term agonist exposure (10 min) on the interaction between TPα/TPβ and PRK1 was investigated in response to stimulation of cells with the selective TXA₂ mimetic U46619. As described previously, in the absence of agonist, PRK1 was specifically detected in the anti-HA immunoprecipitates from HEK.TPα and HEK.TPβ cells. Stimulation of cells with U46619 led to an alteration in the amount of PRK1 associated with GST.TPβ (Fig. 2D). In all cases, failure to detect PRK1 in GST.TPβ immunoprecipitates from HEK.TPβ cells (Fig. 2, C and D, mid-panels) or failure of the PRK1 antibody to detect PRK1 in immunoprecipitates from HEK.TPβ cells (Fig. 2, B and D, lower panels) was not because of lack of PRK1 expression in those cells (27) was examined through co-immunoprecipitations, where possible interactions with GST.TPβ and, to an even lesser extent, in the TPβ312–343 fragment that lacks the common region involved in contributing to their interaction with PRK1 was demonstrated (36). Hence, here the interaction between TPα and TPβ with PRK1 or with three of its subfragments comprising either the kinase domain (PRK1361–942), the ACC/HR1 domains alone (PRK11–357), or ACC/HR1 domains in the AA-binding site, as in the PRK1–357 subfragments lacking both of these regions and the TPβ-H8 residues impaired the interaction (supplemental Fig. 1). The role of individual residues within the common region of TPα/TPβ in contributing to this interaction with PRK1 was also investigated (supplemental Fig. 1). This region is of particular interest as much of it (residues 316–323) is predicted to be in the α-H8, a structural feature of many GPCRs (32, 33). Ala-scanning SDM and Y2H-based screening established that mutation of Leu316, Arg318, and Leu323 abolished the interaction of TPα312–343 with PRK1, although mutation of all other residues only impaired it (supplemental Fig. 1). In contrast, whereas mutation of each of the α-H8 residues impaired the interaction of TPβ312–407 with PRK1460–942, none of those mutations per se completely disrupted the interaction (supplemental Fig. 1).

Confirmation of the Association between TPα/TPβ and PRK1 Using Glutathione S-Transferase Pulldown Assays and Co-immunoprecipitations in Mammalian Cells—To further examine the interaction between PRK1 and TPα/TPβ, in vitro GST pull-down assays were performed. Consistent with the Y2H studies, PRK1361–942 was found to bind GST.TPβ312–407, GST.TPβ329–407, and GST.TPα312–343 but not to GST.TPα329–343 or GST, despite near equivalent expression of all proteins (Fig. 2A).

FIGURE 1. Interaction of PRK1 with the intracellular domains of TPα and TPβ. A and B, S. cerevisiae Y187/pACT2.TPα162–392 prey strain and S. cerevisiae AH109 bait strain, were transformed with the pGBT7 and pGAL4 vectors encoding the listed TPs or TPβ subfragments and pGBT7:: activating domain plasmids. Diploids were selected on QDO media due to the GAL4-dependent transcriptional activation of the HIS3 reporter genes because of positive interaction between PRK1 and TPβ. C, S. cerevisiae TPα15445 and HEK.TPβ2AR cells were transfected with the PRK1-Flag HA epitope-tagged form referred to as PRK1, expressed as a FLAG epitope-tagged form in mammalian HEK 293 cells, to bind GST.TPα312–343 and GST.TPβ312–407 but not with GST.HIP299–386, a GST protein expressing the C-tail domain of the human prostacyclin receptor (Fig. 2B).

The ability of PRK1 to associate with HA-tagged forms of TPα or TPβ stably expressed in the previously characterized HEK.TPα and HEK.TPβ cell lines (27) was examined through co-immunoprecipitations. PRK1 was detected in the anti-HA immunoprecipitates from HEK.TPα and HEK.TPβ cells but not in corresponding immunoprecipitates from the HEK.βGal cells, encoding HA-tagged β-Gal, acting as an additional/alternative control (Fig. 2C). Thereafter, the effect of short term agonist exposure (10 min) on the interaction between TPα/TPβ and PRK1 was investigated in response to stimulation of cells with the selective TXA₂ mimetic U46619. As described previously, in the absence of agonist, PRK1 was specifically detected in the anti-HA immunoprecipitates from HEK.TPα and HEK.TPβ cells. Stimulation of cells with U46619 led to an alteration in the amount of PRK1 associated with GST.TPβ (Fig. 2D). In all cases, failure to detect PRK1 in GST.TPβ immunoprecipitates from HEK.TPβ cells (Fig. 2, C and D, mid-panels) or failure of the PRK1 antibody to detect PRK1 in immunoprecipitates from HEK.TPβ cells (Fig. 2, B and D, lower panels) was not because of lack of PRK1 expression in those cells (27) was examined through co-immunoprecipitations, where possible interactions with GST.TPβ and, to an even lesser extent, in the TPβ312–343 fragment that lacks the common region involved in contributing to their interaction with PRK1 was demonstrated (36). Hence, here the interaction between TPα and TPβ with PRK1 or with three of its subfragments comprising either the kinase domain (PRK1361–942), the ACC/HR1 domains alone (PRK11–357), or ACC/HR1 domains in the AA-binding site, as in the PRK1–357 subfragments lacking both of these regions and the TPβ-H8 residues impaired the interaction (supplemental Fig. 1). The role of individual residues within the common region of TPα/TPβ in contributing to this interaction with PRK1 was also investigated (supplemental Fig. 1). This region is of particular interest as much of it (residues 316–323) is predicted to be in the α-H8, a structural feature of many GPCRs (32, 33). Ala-scanning SDM and Y2H-based screening established that mutation of Leu316, Arg318, and Leu323 abolished the interaction of TPα312–343 with PRK1, although mutation of all other residues only impaired it (supplemental Fig. 1). In contrast, whereas mutation of each of the α-H8 residues impaired the interaction of TPβ312–407 with PRK1460–942, none of those mutations per se completely disrupted the interaction (supplemental Fig. 1).

Confirmation of the Association between TPα/TPβ and PRK1 Using Glutathione S-Transferase Pulldown Assays and Co-immunoprecipitations in Mammalian Cells—To further examine the interaction between PRK1 and TPα/TPβ, in vitro GST pull-down assays were performed. Consistent with the Y2H studies, PRK1361–942 was found to bind GST.TPβ312–407, GST.TPβ329–407, and GST.TPα312–343 but not to GST.TPα329–343 or GST, despite near equivalent expression of all proteins (Fig. 2A). The ability of full-length PRK1 (residues 1–942, herein referred to as PRK1), expressed as a FLAG epitope-tagged form in mammalian HEK 293 cells, to bind GST.TPα312–343 and GST.TPβ312–407 was also investigated. PRK1 showed a specific interaction with GST.TPα312–343 and GST.TPβ312–407 but not with GST.HIP299–386, a GST protein expressing the C-tail domain of the human prostacyclin receptor (Fig. 2B).

The ability of PRK1 to associate with HA-tagged forms of TPα or TPβ stably expressed in the previously characterized HEK.TPα and HEK.TPβ cell lines (27) was examined through co-immunoprecipitations. PRK1 was detected in the anti-HA immunoprecipitates from HEK.TPα and HEK.TPβ cells but not in corresponding immunoprecipitates from the HEK.βGal cells, encoding HA-tagged β-Gal, acting as an additional/alternative control (Fig. 2C). Thereafter, the effect of short term agonist exposure (10 min) on the interaction between TPα/TPβ and PRK1 was investigated in response to stimulation of cells with the selective TXA₂ mimetic U46619. As described previously, in the absence of agonist, PRK1 was specifically detected in the anti-HA immunoprecipitates from HEK.TPα and HEK.TPβ cells. Stimulation of cells with U46619 led to an alteration in the amount of PRK1 associated with GST.TPβ (Fig. 2D). In all cases, failure to detect PRK1 in GST.TPβ immunoprecipitates from HEK.TPβ cells (Fig. 2, C and D, mid-panels) or failure of the PRK1 antibody to detect PRK1 in immunoprecipitates from HEK.TPβ cells (Fig. 2, B and D, lower panels) was not because of lack of PRK1 expression in those cells (27) was examined through co-immunoprecipitations, where possible interactions with the unrelated HA-tagged β₂AR served as an additional independent control. As described previously, PRK1 associated strongly and specifically with TPα and TPβ as evidenced by its detection in immunoprecipitates from HEK.TPα and HEK.TPβ but not from control HEK.β₂AR cells (Fig. 3B, upper panels). The kinase only PRK1361–942 subfragment was also abundantly expressed in TPα and TPβ, but not β₂AR, immunoprecipitates and at levels comparable with that of PRK1. Conversely, lower levels of PRK11–357 were detected in the TPβ and, to an even lesser extent, in the TPα immunoprecipitates. Furthermore, removal of the AA-binding site, as in the PRK11–357 subfragment, completely abolished binding to TPα and substantially reduced binding to TPβ. The observed differences in immunoprecipitation of PRK1 or its subfragments were not due to variations in their expression levels in the cell lines used or in the efficiency of the immunoprecipitations per se (Fig. 3B,
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**FIGURE 2. Interaction of PRK1 with TPα and TPβ in vitro and in mammalian cells.** A GST product (3 μl) was incubated at room temperature for 2 h with glutathione-Sepharose beads preloaded with purified GST alone (control) or the indicated GST fusion proteins (5 μg). IB, immunoblot.

**A** and **B**, cellular lysates (500 μg) obtained from HEK 293 cells, transiently transfected with pCMVTag2b:PRK1 (PRK1) or pCMVTag2b, were subject to immunoprecipitation with anti-HA (3F10), anti-FLAG, or anti-GST. To verify expression of the FLAG PRK1, an aliquot of whole cell lysates (500 μl) was immunoblotted with anti-FLAG antibody (101R) or 12CA5, anti-HA (3F10), or anti-GST. To verify expression of endogenous RhoA in all cell lines (supplemental Fig. 1), cell lysates (500 μl) were also immunoblotted with anti-GST antibody (B, lower panel). The relative positions of molecular size standards are indicated.

**C** and **D**, HEK.TPα, HEK.TPβ, or as controls HEK.β-Gal cells, were subject to immunoprecipitation with anti-HA (101R) antibody (C, C-terminal region of PRK1, comprising its AA-binding domain of PRK1 in interacting with the TPs was established and, to a lesser extent, with TPα (Fig. 3C). Collectively, these data confirm a constitutive physical interaction between TPα/TPβ and PRK1 in mammalian cells and point to a critical role for the C-terminal region of PRK1, comprising its AA-binding C2-like domain and its catalytic kinase domain, in that interaction.

**RhoA Association with the TP-PRK1 Complex**—PRK1 is a downstream effector of the GTPase RhoA (37). Moreover, both TPα and TPβ modulate RhoA signaling (23). Hence, it was sought to further examine the interaction of TPα/TPβ with PRK1 and to establish whether endogenous RhoA may associate with the complex. Initially, HEK.TPα, HEK.TPβ, and the control HEK.β-Gal cell lines, co-transfected with pCMVTag2b:PRK1, were stimulated with U46619 for 0–120 min, and the presence of PRK1 and endogenous RhoA in the anti-HA immunoprecipitates was examined. In the absence of agonist, PRK1 was detected in the anti-HA TPα and TPβ, but not β-Gal, immunoprecipitates, although neither short term nor more prolonged U46619 stimulation led to measurable changes in the amount of PRK1 associated with the TPs (supplemental Fig. 2, A–D). Moreover, RhoA was detected in the anti-HA TPα and TPβ immunoprecipitates and at levels that were unaffected by U46619 stimulation (supplemental Fig. 2, A–D). Conversely, RhoA was not detected in HA-β-Gal immunoprecipitates despite its efficient immunoprecipitation and equivalent expression of endogenous RhoA in all cell lines (supplemental Fig. 2, A and B).

To exclude the possibility that the observed associations of PRK1 or RhoA with TPα or TPβ may be an artifact of overexpression of PRK1, the ability of endogenous PRK1 and endogenous RhoA to associate with the TP isoforms was examined. As with the overexpressed PRK1, similar levels of association between TPα/TPβ and PRK1 were observed in both nonstimulated and U46619-stimulated cells (Fig. 4, A and B; supplemental Fig. 2, E and F). Moreover, consistent with previous findings, RhoA was associated with the TPα/TPβ-PRK1 immunoprecipitates in ternary complexes that were not affected by U46619 stimulation (Fig. 4, A and B; supplemental Fig. 2, E and F). Collectively, these data confirm a physical interaction between TPα/TPβ and PRK1 in a constitutive ternary complex with the PRK1 effector RhoA in mammalian cells that is independent of TP agonist activation.
TPα and TPβ Are Not Phosphorylation Targets of PRK1—Several functional targets of PRK1 have been identified, ranging from its ligand-dependent interaction and activation of the nuclear androgen receptor (8) to its interaction and phosphorylation of vimentin and glial fibrillary acidic protein, to inhibit filament formation (38). Moreover, both TPα and TPβ are recognized targets of PKC phosphorylation (24–26, 28). Hence, in view of its many regulatory functions, it was sought to investigate whether PRK1 might target TPα and/or TPβ by direct phosphorylation. To this end, the ability of purified preparations of PRK1 to phosphorylate GST fusion proteins encoding the intracellular loop domains (IC1–IC3) and the C-tail domains of TPα or TPβ were examined in vitro. The known PRK1 substrate histone H1 served as a control in the in vitro kinase assays (39). Although histone H1 was readily phosphorylated, none of the purified recombinant GST proteins encoding the respective intracellular subdomains of TPα/TPβ were phosphorylated in vitro by PRK1 (supplemental Fig. 3). Moreover, despite repeated attempts, whole cell phosphorylations in HEK.TPα or HEK.TPβ cells established that PRK1 did not lead to phosphorylation of TPα or TPβ either in the absence or presence of U46619 stimulation or following overexpression of PRK1 (data not shown).

PRK1 Activity Is Increased in Response to U46619 Stimulation—As stated, it has previously been established that agonist activation of TPα results in robust activation of RhoA (23). Moreover, it has been established here that PRK1 constitutes a component of a complex that also contains RhoA. Hence, we sought to investigate here whether the immune complexes is recruited to and may respond to agonist stimulation of TP, as suggested by the prior observation that PRK1 associates with SHIP (21). In the presence of U46619, with maximal responses occurring at 10–30 min for both TPα and TPβ (Fig. 5, A and B). Conversely, relative levels of histone H1 phosphorylation were significantly lower when PRK1 was precipitated from the control HEK.TPβ-Gal cells despite near equivalent immunoprecipitation of PRK1 in all cell types (Fig. 5, A and B). Moreover, time course assays established that, in the absence of agonist, PRK1 resulted in histone H1 phosphorylation, which was transiently increased in the presence of U46619, with maximal responses occurring at 10–30 min for both TPα and TPβ (Fig. 5, C and D). The precise physiologic impact of the observed increase in PRK1 activity in response to TP agonist activation, as determined by analysis of histone H1 phosphorylation, is unclear.

PRK1 immunoprecipitated from EA.hy926, 1° h.CoASM cells, or 1° HUVECs also led to increases in histone H1 phosphorylation in vitro in the response to agonist stimulation (supplemental Fig. 4, A and B; and data not shown) and were lower than those observed in HEK.TPα and HEK.TPβ cells. Such differences are most likely reflective of the levels of endogenous TPα and TPβ expressed in the former cell types (10–20 fmol/mg cell protein (40)) relative to those levels in the clonal.
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(A) HEK.TPα cells
(B) HEK.TPβ cells

FIGURE 4. Agonist-dependent interaction of PRK1 and RhoA with TPα and TPβ. HEK.TPα (A) and HEK.TPβ (B) or, as controls, HEK.β-Gal (A and B) cells were incubated with vehicle (−) or 1 μM U46619 (+) for 10–120 min prior to immunoprecipitation with anti-HA (101R) sera. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB) versus anti-FLAG, anti-HA (3F10), anti-Rho, or anti-PRK1 sera. Aliquots of whole cell lysates (50 μg/lane) were also immunoblotted with anti-FLAG, anti-RhoA, or anti-PRK1 sera. The relative positions of the molecular size standards (kDa) are indicated. Data; n = 3.

As a means of verifying that the increased PRK1 phosphorylation of histone H1 observed in the presence of U46619 was due to activation of TPα and TPβ in the presence of androgens (8, 9), increased TXA2 production induced by agonist stimulation was tested in the in vitro assays (Fig. 5, A and F, upper right panels). Endogenous PRK1 present in the anti-TPα and anti-TPβ precipitates resulted in increased phosphorylation of histone H1, although, as stated previously, phosphorylation was detected using the anti-HA H11011 blot from HEK.β-Gal cells (Fig. 5, E and F, upper right panels). Moreover, whereas PRK1 present in the anti-TPα and anti-TPβ precipitates phosphorylated histone H1 in vitro in the absence of agonist, phosphorylation was increased following U46619 stimulation in both cases (Fig. 5, E and F, upper right panels). Hence, collectively, these data confirm that PRK1 associated in immune complexes with TPα and TPβ is functionally active and that it undergoes enhanced activation in response to receptor stimulation.

H3 Thr11 Phosphorylation in Prostate PC-3 and LNCaP Cells—PRK1 has been established to play a key role in the regulation of transcription by the nuclear androgen receptor (AR) through its specific phosphorylation of histone H3 at a critical Thr11 residue (H3 Thr11), thus initiating chromatin remodeling and potentiating androgen-induced gene expression, such as within the prostate (8, 9). Furthermore, this occurs through androgen-induced association of PRK1 with the AR (8, 9). Hence, because of the findings herein demonstrating a direct interaction between TPα/TPβ with PRK1, it was sought to explore whether U46619-mediated activation of PRK1 might actually induce phosphorylation at Thr11 (H3 Thr11) in 1° HUVECs and 1° hCoASMCs, somewhat similar to that now established for the androgens (8, 9). To this end, H3 Thr11 phosphorylation was assessed by SDS-PAGE and immunoblotting (IB) versus anti-phospho-H3 Thr11 blots with specific anti-phospho-H3 Thr11 antibodies. In metaphase with colcemid-arrested cells served as a positive control for the assay in both cell lines (Fig. 6, A and B). Hence, in view of the direct interaction of PRK1 with TPα and TPβ and of the by now established role of androgen-activated PRK1 in inducing H3 Thr11 phosphorylation (8, 9), it was sought to investigate whether U46619-mediated activation of TP/PRK1 signaling might induce H3 Thr11 phosphorylation in the human prostate carcinoma PC-3 and LNCaP cell lines. Moreover, it was also sought to investigate whether TP-mediated PRK1 activation might affect androgen-induced responses by comparing the effect of U46619 on dihydrotestosterone (DHT)-induced H3 Thr11 phosphorylation in the androgen-insensitive PC-3 relative to the androgen-sensitive human adenocarcinoma LNCaP cell lines, where colcemid-arrested cells served as a positive control for the assay in both cell lines (Fig. 6, A and B). In contrast to 1° HUVECs and 1° hCoASMCs, stimulation of both PC-3 and LNCaP cell types with U46619 led to significant increases in H3 Thr11 phosphorylation with maximal responses occurring at 30–60 min in both cell lines (Fig. 6, A and B). In all cases, rescreening of the anti-phospho-H3 Thr11 blots with...
anti-histone H3 itself confirmed that any differences in H3 Thr\(^{11}\) phosphorylation in either PC-3 or LNCaP cells, e.g. in response to U46619, were not due to discrepancies in histone H3 levels (Fig. 6, A and B). Although treatment of PC-3 cells with DHT did not induce a significant increase in H3 Thr\(^{11}\) phosphorylation \textit{per se} (Fig. 6A), co-stimulation with U46619 led to similar and time-dependent increases in H3 Thr\(^{11}\) phosphorylation but to levels that were not significantly different from those in the absence of DHT (Fig. 6A, \(F\)-test analysis, \(p = 0.9039\)). Conversely, pretreatment of LNCaP cells with DHT led to a significant increase in H3 Thr\(^{11}\) phosphorylation (Fig. 6B). Moreover, co-stimulation of LNCaP cells with U46619 in the presence of DHT led to robust, time-dependent increases in H3 Thr\(^{11}\) phosphorylation relative to those levels in the presence of U46619 alone (Fig. 6B, \(F\)-test analysis, \(p < 0.0001\)), with maximal responses occurring at 30–60 min post-U46619-treatment. In point of fact, at 30 min of post-agonist stimulation, maximal levels of H3 Thr\(^{11}\) phosphorylation in LNCaP

\[\text{FIGURE 5. Agonist-induced activation of PRK1 by TP}\alpha\text{ and TP}\beta\text{. HEK.TP}\alpha\text{, HEK.TP}\beta\text{, and/or HEK.β-Gal cells were incubated with 1 \(\mu\text{M}\) U46619 (+) for 10 min (A and B) or with 1 \(\mu\text{M}\) U46619 for 0–60 min (C and D) prior to immunoprecipitation (IP) with anti-PRK1 antibody. E and F, HEK.TP}\alpha\text{, HEK.TP}\beta\text{, and/or HEK.β-Gal were incubated with 1 \(\mu\text{M}\) U46619 for 10 min (left panels) or with 1 \(\mu\text{M}\) U46619 for 0 or 10 min (right panels) prior to immunoprecipitation with anti-HA (101R) antibody. Resulting anti-PRK1 (A–D) or anti-HA (E and F) immunoprecipitates were used as a source of PRK1 to examine U46619-induced \textit{in vitro} phosphorylation of histone H1 (10 \(\mu\text{g}\); 30 °C for 30 min). Phosphorylated histone H1 was visualized by autoradiography, although all immunoprecipitates were immunoblotted (IB) with anti-PRK1 antibody (A–F, upper and lower panels, respectively). The relative positions of the 30- and 104-kDa molecular size markers are to the left of the panels. The \textit{bar charts} represent mean percentage changes in phosphorylation relative to PRK1 expression in the anti-PRK1 immunoprecipitates, where basal levels in the absence of U46619 are assigned a value of 100%. The \textasterisks indicate that levels of phosphorylation were significantly increased in response to U46619 stimulation, relative to vehicle-treated cells, where ** signifies \(p = 0.01\) for post hoc Dunnett’s multiple comparison \(t\) test analysis. Data; \(n = 3\).} \]
cells were up to 3-fold greater in the presence of DHT plus U46619 relative to basal levels in the absence of either agent (Fig. 6B, two-way ANOVA, \( p < 0.0001 \)). In addition, to investigate whether the increased H3 Thr11 phosphorylation in the latter cell types is specific to the TPs or a more general phenomenon, both PC-3 and LNCaP cells were stimulated with 17-phenyl trinor PGE2 or cicaprost, selective agonists of the \( G_q \)-coupled EP1 subtype of the PGE2 receptor or the \( G_s \)-coupled prostacyclin receptor, respectively, and their effect on H3 Thr11 phosphorylation was compared with that of the TP agonist U46619 (supplemental Fig. 5, A and B). The precise physiologic impact of the observed TP-mediated increase in H3 Thr11 phosphorylation, as determined herein by analysis of H3 Thr11 phosphorylation, is not fully evident at this time. However, because of the serious nature of enhanced H3 Thr11 phosphorylation as a key marker of androgen-dependent gene expression, any change in H3 Thr11 phosphorylation in response to TP activation, possibly leading to enhanced AR-dependent gene expression, is likely to be of substantial significance, such as in the context of the enhanced TP expression associated with prostate cancer (12–14).

To investigate whether this receptor actually interacts with the TP or other signalling partners, transfection of PC-3 and LNCaP cells was performed with an affinity-purified antibody to immunoprecipitate the preimmune serum (IgG). Immunoprecipitates (IP) and/or aliquots of whole cell lysates (50 \( \mu g/lane \)) were resolved by SDS-PAGE and immunoblotted (IB) with anti-PRK1. The relative positions of the molecular size markers (kDa) are to the left of the panels. Data: \( n \geq 3 \). C, confocal image analysis of GFP:PRK1 nuclear translocation in LNCaP cells stimulated with U46619 (1 \( \mu M \); 0–240 min). Images were captured at \( \times 63 \) magnification using Zeiss LSM imaging software, with horizontal bars representing 10 \( \mu m \). Data are representative of at least three independent experiments.

FIGURE 7. Interaction of PRK1 with TPα and TPβ in PC-3 and LNCaP cells. PC-3 (A) and LNCaP (B) cells were preincubated with vehicle, U46619 (1 \( \mu M \); 30 min), DHT (1 \( nM \); 24 h), or U46619 + DHT (1 \( nM \) DHT for 24 h followed by 1 \( \mu M \) U46619 for 30 min), as indicated. Thereafter, lysates (~500 \( \mu g/assay \)) were subject to immunoprecipitation with affinity purified anti-TPα/TPβ (No. 168; 20 \( \mu g \)) or with an equivalent concentration of the preimmune serum (IgG). Immunoprecipitates (IP) and/or aliquots of whole cell lysates (50 \( \mu g/lane \)) were resolved by SDS-PAGE and immunoblotted (IB) with anti-PRK1. The relative positions of the molecular size markers (kDa) are to the left of the panels. Data: \( n \geq 3 \). C, confocal image analysis of GFP:PRK1 nuclear translocation in LNCaP cells stimulated with U46619 (1 \( \mu M \); 0–240 min). Images were captured at \( \times 63 \) magnification using Zeiss LSM imaging software, with horizontal bars representing 10 \( \mu m \). Data are representative of at least three independent experiments.

FIGURE 6. Effect TP activation on H3 Thr11 phosphorylation in PC-3 and LNCaP cells. A–D, immunoblot analysis of H3 Thr11 phosphorylation in PC-3 (A and C) and LNCaP (B and D) cells were performed with vehicle, U46619 (1 \( \mu M \); 0–60 min), as indicated, following preincubation with either vehicle (0.01% EtOH; 24 h), DHT (1 \( nM \); 24 h), and/or the PRK1 inhibitor RO-31-8221 (10 \( \mu M \); 1 h), as indicated. As a control for H3 Thr11 phosphorylation, cells were growth-arrested by treatment with colcemid (50 ng/ml) for 24 h. E and F, immunoblot analysis of H3 Thr11 phosphorylation in PC-3 (E) and LNCaP (F) cells transiently co-transfected with either pCMVTag2b (Ø), or pCMVTag2b:PRK1K644E (PRK1K644E) and stimulated 48 h post-transfection with U46619 (1 \( \mu M \); 30 min). In all cases, isolated histones were resolved by SDS-PAGE and immunoblotted (IB) with anti-phospho-H3 Thr11 (upper panel) or anti-histone H3 (lower panel) antibody followed by chemiluminescence detection. The bar charts represent mean percentage changes in H3 Thr11 phosphorylation relative to histone H3 levels and are expressed in arbitrary units (± S.E., \( n = 3 \)), where basal levels in vehicle-treated cells and in the absence of U46619 are assigned a value of 100%. The asterisks indicate that levels of H3 Thr11 phosphorylation were significantly increased in response to U46619 stimulation, relative to vehicle-treated cells. # signifies that levels of H3 Thr11 phosphorylation were significantly increased in the presence of DHT, and $$$ indicates \( p < 0.001 \) for unpaired Student’s t test analysis. $ signifies that levels of H3 Thr11 phosphorylation were significantly increased in the presence of DHT, and $$$ indicates \( p < 0.001 \) for unpaired Student’s t test analysis. The insets in E and F confirm overexpression of FLAG-tagged dominant negative PRK1K644E variant, and Ø signifies the empty vector pCMVTag2b.
Translocation from the cytosolic to the nuclear fraction following stimulation of LNCaP (Fig. 7C) and PC-3 (supplemental Fig. 6) cells with U46619, where maximal nuclear localization was observed at 30 min post-agonist stimulation.

Further confirmation that the agonist-induced increases in H3 Thr\(^{11}\) phosphorylation observed in both PC-3 and LNCaP cells are due to PK1-induced mechanisms was established whereby the broad spectrum PK1 inhibitor RO-31-8220 partially inhibited colcemid- and U46619 phosphorylation and the DHT responses in LNCaP cells (Fig. 6, C and D). As the PRK1 inhibitor RO-31-8220 can also inhibit other kinases, including GSKβ, S6K, RSK, MSK, and PKCα (42), additional approaches were used to examine PK1 specificity. Although overexpression of the wild type PK1 led to modest increases in basal H3 Thr\(^{11}\) phosphorylation, which was further increased on U46619 stimulation (supplemental Fig. 5, C and D), overexpression of a kinase-defective dominant negative PK1KD44E (9) impaired U46619-induced H3 Thr\(^{11}\) phosphorylation in both PC-3 and LNCaP cells (Fig. 6, E and F, respectively). Furthermore, siRNA directed to PK1 (siRNA\(_{PK1}\)), but not to the scrambled control sequence (siRNA\(_{Control}\)), substantially reduced PK1 expression (Fig. 8, A and B). U46619-induced H3 Thr\(^{11}\) phosphorylation was significantly increased in the presence of DHT, in PC-3 and LNCaP cells (Fig. 8, E and F). Moreover, the DHT-induced increase was observed in the absence and presence of siRNA\(_{Control}\) partially, but not completely, impaired by the siRNA\(_{PK1}\). In all cases, the agonist-induced increase in the presence of DHT was significantly greater than in vehicle-treated cells and unequal loading of the protein samples was excluded by immunoblotting of membranes for the ubiquitously expressed molecular chaperone HDJ-2/ DNA-J protein, which served as an internal loading control (Fig. 7C and D).

U46619-induced activation of TP/TPβ endogenously expressed in PC-3 cells has been previously shown to increase cell motility and migration, possibly accounting for the increased correlation between TP expression and signaling in prostate cancers (14). Hence, here it was sought to explore TP-mediated cell migration in the androgen-responsive LNCaP and nonresponsive PC-3 cell lines and to investigate whether or siRNA\(_{Control}\). Some 48 h post-transfection, cells, preincubated with either vehicle (veh) (0.01% EtOH; 24 h) or DHT (1 nM; 24 h), were treated with U46619 (1 μM; 30 min). The bar charts represent mean percentage changes in H3 Thr\(^{11}\) phosphorylation relative to histone H3 levels and are expressed in arbitrary units (± S.E., n = 3), where basal levels in siRNA\(_{Control}\)-transfected vehicle-treated cells and in the absence of U46619 are assigned a value of 100%. E and F, PC-3 (E) and LNCaP (F) cells were either transfected for 24 h with siRNA\(_{PK1}\) or siRNA\(_{Control}\), or, as a control, nontransfected, followed by pretreatment for a further 24 h with either vehicle (0.01% EtOH) or 1 nM DHT prior to assessment of migration for 4 h in either the vehicle (0.01% EtOH), 1 μM U46619, 1 nM DHT, or 1 μM U46619 plus 1 nM DHT. In all cases, mean cell migration in vehicle-treated cells was assigned a value of 100% and agonist-stimulated migration expressed as a relative percentage. * and signify that levels of H3 Thr\(^{11}\) phosphorylation are significantly increased in response to U46619, U46619 + DHT, or 1 μM U46619 plus 1 nM DHT. In all cases, single and double symbols signify p ≤ 0.05 and p ≤ 0.01, respectively, for post hoc Dunnett’s multiple comparison t test analysis. The insets in E and F represent immunoblot analysis of endogenous PK1 expression in PC-3 and LNCaP cells, where blots were screened with anti-HDJ2 to confirm uniform protein loading.
PRK1 expression may influence that migration (Fig. 8, E and F). Stimulation with U46619 led to substantial increases in migration of both LNCaP and PC-3 cells (Fig. 8, E and F; Nontransfected). Furthermore, DHT also increased migration of LNCaP but not of PC-3 cells, and this effect was augmented by co-stimulation of the former cells with U46619 (Fig. 8, E and F; Nontransfected; two-way ANOVA, $p = 0.0004$). Disruption of PRK1 expression with the siRNA_{PRK1}, but not the siRNA_{Control}, specifically impaired U46619-induced cell migration in both PC-3 and LNCaP cells (Fig. 8, E and F). In addition, siRNA_{PRK1} also partially impaired DHT-induced cell migration in LNCaP cells both in the absence and presence of U46619 (Fig. 8F).

Taken together, these data establish that agonist-induced activation of the TPs endogenously expressed in the prostate adenocarcinoma PC-3 and LNCaP cell lines leads to H3 Thr$^{11}$ phosphorylation, a previously recognized marker of chromatin remodeling exclusively associated with androgen-induced responses. Moreover, the TXA$_2$ mimetic U46619 can significantly augment the androgen-induced H3 Thr$^{11}$ phosphorylation in LNCaP but not in PC-3 cells. It was established that PRK1 directly interacts with TP$\alpha$/TP$\beta$ endogenously expressed in both PC-3 and LNCaP cells and disruption of PRK1, such as through targeted siRNA, substantially impairs TP$\alpha$/TP$\beta$-mediated H3 Thr$^{11}$ phosphorylation and cell migration in response to U46619. Collectively, these data provide a potential molecular basis for the role of TXA$_2$ and its receptor in prostate cancer, and in other conditions in which aberrant TXA$_2$/RhoA signaling is implicated.

**DISCUSSION**

In this study, we report the discovery of a novel interaction between the TP$\alpha$ and TP$\beta$ subfamilies of monomeric GTPases. TP$\alpha$- and TP$\beta$-mediated signaling in the prostate is well characterized (15, 23). However, less is known about their signaling to extracellular stimuli that do not involve direct coupling to heterotrimeric G proteins. TP$\alpha$/TP$\beta$ can form homo/heterodimers or oligomers, raising the possibility of multiple protein associations at a single TP complex. Moreover, the roles of various GPCR-interacting proteins are increasingly recognized in regulating novel intracellular cascades through direct protein-protein interaction, most often involving the intracellular loops and/or C-tail domain(s) of the GPCR, and which do not necessarily involve classic G protein signaling (44). A number of novel associations with either TP$\alpha$ or TP$\beta$ have been previously identified. The proteasome activator PA28$\gamma$ specifically interacts with TP$\beta$, enhancing its degradation by a proteasome-dependent mechanism (45). Interaction of TP$\beta$ with the nucleoside diphosphate kinase Nm23-H2 leads to its Rac1-dependent endocytosis (46), although its interaction with Rab11 participates in its agonist-induced trafficking through the slow endosome pathway (47). More recently, interactions between TP$\alpha$/TP$\beta$ with angio-associated migratory cell protein (48) and with KIAA1005 were reported (49). Interestingly, both PRK1 and KIAA1005 contain C2 domains. However, the significance of these conserved functional domains in the two TP interactants was not examined in this study and will be investigated in future studies.

The direct interaction between TP$\alpha$/TP$\beta$ and PRK1 identified in this study was found to be constitutive in mammalian cells. Although there was no agonist-dependent alteration in the association of TP$\alpha$/TP$\beta$ with either PRK1 or RhoA, TP agonist stimulation enhanced PRK1 activation leading to phosphorylation of its general substrate histone H1. Although the precise nature of the interaction with PRK1 remains to be determined, Y2H studies identified two regions of importance within the intracellular C-tail domains of TP$\alpha$/TP$\beta$, namely the common region (residues 312–328), proximal to transmembrane (TM) 7, and a more distal region of TP$\beta$ (residues 366–392). In the absence of one or both of these regions, binding to PRK1$^{640–942}$ in yeast is severely reduced or completely abolished. Using GST-based in vitro approaches, the specific requirement of the common 312–328 region within the C-tail domains of TP$\alpha$ and TP$\beta$ as a critical binding determinant with both the kinase domain and PRK1 was confirmed. Examination of the subdomains of PRK1 reaffirmed an essential role for the C-terminal domain, incorporating the AA-binding site and the kinase domain, in the interaction with TP$\alpha$/TP$\beta$, although the contribution of the PRK1 domains, involved in Rho/Rac binding, were not required. Moreover, ectopic expression of the C-terminal domain of PRK1$^{1–594}$, specifically through interaction with TP$\beta$ and, to a lesser extent to TP$\alpha$, in addition with binding of the full-length isoform.

Dissection mapping of the regions within TP$\alpha$/TP$\beta$ and its subdomain that contribute to the interaction is beyond the scope of this study and will be the subject of further investigations. Notably, the three IC loops in the interaction with PRK1 and its subdomains. However, because of their limited sizes for Y2H-type interaction studies, results generated were inconclusive, and therefore, the possibility that any one of all of the IC domains may contribute to the interaction with PRK1 in mammalian cells cannot be excluded.

The role of the common 312–328 region, encoding the $\alpha$-H8 domain (32, 33) of TP$\alpha$/TP$\beta$, in contributing to their interaction with PRK1 was also investigated herein. Located proximal to TM7, perpendicular to the heptahelical TM bundles, the $\alpha$-H8 domain can play an essential role in mediating interactions between certain GPCRs and their GPCR-interacting proteins in addition to influencing receptor expression and/or trafficking (50–52). Moreover, it may act as a conformational switch between the active and inactive states of certain GPCRs. Hence, our discovery of a role for the putative $\alpha$-H8 in the interaction of TP$\alpha$/TP$\beta$ with PRK1 is indeed consistent with its functional importance and in mediating protein:protein interactions. Although mutation of certain residues (Leu$^{316}$, Arg$^{318}$, and Leu$^{323}$) within the $\alpha$-H8 domain completely disrupted the interaction between TP$\alpha$ and PRK1, mutation of other residues only impaired it. In contrast, mutation of residues within $\alpha$-H8 impaired the interaction of TP$\beta$ with PRK1 in each case, although no single mutant completely disrupted
the interaction per se. This is entirely consistent with the role of other residues, namely 366–392, within the distal C-tail domain of TPβ in contributing to its interaction with PRK1. Greater insights into the molecular components contributing to the specificity of interaction of TPα and/or TPβ with PRK1 will require further investigations involving alternative approaches outside of the Y2H system, including detailed biochemical approaches. However, in terms of specificity, of particular note was the finding that although residues within the common (312–328) and more distal (366–392) domains have also recently been implicated in the interaction of TPβ with angio-associated migratory cell protein (48), Ala-scanning SDM of the α-H8 domain within that region does not influence the interaction of TPβ with angio-associated migratory cell protein in yeast. Moreover, the finding that the α-H8 domain contributes to the interaction of TPα/TPβ with PRK1 is indeed consistent with the growing body of evidence of its general importance in mediating, at least in part, protein-protein interactions between certain GPCRs and specific GPCR interacting protein(s) (50–52).

The cellular responses to TXA2 are subject to regulation by both agonist-dependent homologous desensitization (24, 25) and cross-talk with other signaling systems (23, 27, 29), which mainly occur through direct phosphorylation of the TPα and/or TPβ isoforms themselves. For example, both TPα and TPβ undergo PKC phosphorylation within their IC (e.g. Ser145, IC2) and unique C-tail (e.g. TPα at Thr337, TPβ at Thr399) domains (24–26). As PRK1 is closely related to other members of the PKC family, particularly in its kinase domain known to phosphorylate many, but not all, of its interactants, it was sought to establish whether PRK1 is capable of phosphorylating any of the intracellular domains of TPα/TPβ or all of the intracellular domains of both TPα and TPβ, including Ser145 (Thr337) and Thr399, individually or in combination. To this end, neither TPα nor TPβ are phosphorylated by PRK1 in whole cell phosphorylation assays. The finding that the agonist-induced phosphorylation of PRK1 occurs through a combined PKC and PKG feedback mechanism, although that of TPβ occurs through the GRK2/3 mechanism (24, 25), is entirely consistent with previous reports by us that established agonist-dependent homologous desensitization (24, 25) and/or TPα-mediated PRK1 activation might possibly lead to H3 Thr11 phosphorylation and resulted in only marginal increases in histone H1 phosphorylation by PRK1 immunoprecipitated from those cell lines. In contrast, as stated, evidence of U46619-induced PRK1 activation and H3 Thr11 phosphorylation and the presence of PRK1 in anti-TPα/TPβ immune complexes were readily detected in the prostate PC-3 and LNCaP cell lines. Furthermore, DHT induced H3 Thr11 phosphorylation in the androgen-responsive LNCaP cells but not in the androgen-insensitive PC-3 cell line, an effect that was substantially augmented by the TXA2 mimetic U46619. Moreover, a direct constitutive interaction between PRK1 and TPα/TPβ endogenously expressed in both PC-3 and LNCaP cells was confirmed, and disruption of PRK1 activity (RO-31-8220 or overexpression of PRK1(K646E)) or PRK1 expression (siRNA

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impaired TP-mediated H3 Thr11 phosphorylation. Collectively, these data establish that TP-mediated PRK1 activation can independently lead to H3 Thr11 phosphorylation in prostate carcinoma cell lines but that it can also cooperate to augment androgen-induced H3 Thr11 phosphorylation. These findings are entirely significant in that they are the first to establish that agents other than androgens can induce H3 Thr11 phosphorylation and that it occurs through a similar PRK1-dependent mechanism. Furthermore, in preliminary experiments, it was established that similar to that observed in prostate carcinoma cell lines, agonist (U46619)-dependent activation of TPα and/or TPβ leads to activation of PRK1 and also increased H3 Thr11 phosphorylation, enhancing transcriptional activation of AR-responsive genes such as within the prostate. Additionally, in the androgen-responsive LNCaP cells, co-stimulation with the TP agonist and DHT augments PRK1-dependent H3 Thr11 phosphorylation.

FIGURE 9. Model of AR- and TP-dependent H3 Thr11 phosphorylation in response to PRK1 activation. The cell-permeable androgen testosterone (T) is converted to its active metabolite DHT by the cellular 5-β-reductase. DHT, in turn, binds to the hormone binding domain of the AR, leading to its release from an inactive complex with HSPs, promoting AR dimerization and activation. The ligand-bound AR can activate gene expression by (i) translocating to the nucleus leading to transcriptional activation of target genes, such as within the prostate. Herein, it was established that PRK1 is recruited into a complex with AR, leading to activation of PRK1 and also increased H3 Thr11 phosphorylation, enhancing transcriptional activation of AR-responsive genes such as within the prostate. Additionaly, in the androgen-responsive LNCaP cells, co-stimulation with the TP agonist and DHT augments PRK1-dependent H3 Thr11 phosphorylation.
signaling, transducing TNFα signaling to its many functional targets, including activation of NF-κB and c-Jun kinase (JNK)-mediated inflammatory responses and/or apoptotic cascades. Disruption of PRK1 expression impairs TRAF2-induced NF-κB activation linking PRK1 to the regulation of TNFα-mediated inflammatory responses (54). Interestingly, in a follow-up study, PRK1 was found to specifically phosphorylate the related TRAF1, which lacks the JNK/IKK signaling effector domain but not its interacist TRAF2 or other TRAF members, leading to the recruitment of TRAF1 to the TNF receptor, silencing the receptor complex by PRK1 (55). Moreover, within the vasculature, PRK1 has been implicated in the mediation of VSM-specific gene expression promoting VSM differentiation, such as in response to transforming growth factor-β1 (56, 57). Keeping in mind the central role of TXA2 within the vasculature, including the mediation of inflammatory disease, coupled with the finding herein of a direct interaction with and activation of PRK1, it will be of significant interest to investigate the possible interplay between those critical pathways, be it at the cellular or (patho)physiologic levels.

In conclusion, as outlined in the model presented in Fig. 9, this study provides evidence of a novel constitutive interaction between TPα/TPβ and PRK1, in complex with RhoA. Furthermore, results demonstrate an agonist-dependent increase in PRK1 activity and a significant TP-dependent increase in H3 Thr11 phosphorylation and associated cell migration in prostate carcinoma cell lines, a modification that was until this study almost exclusively viewed as an androgen-induced marker of chromatin remodeling and transcription. Although the involvement of RhoA in H3 Thr11 phosphorylation by PRK1 linking additional experimental evidence of such an interaction between TPα/TPβ and PRK1, such a discovery hints at a range of (patho)physiologic processes behind certain neoplasms and in vascular inflammatory disease. Further investigation will reveal an increased understanding of the physiologic, and possibly (patho)physiologic or clinical, significance of this interaction and whether antagonism might offer a therapeutic advantage in such conditions.

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Identification of an Interaction between the TPα and TPβ Isoforms of the Human Thromboxane A2 Receptor with Protein Kinase C-related Kinase (PRK) 1: IMPLICATIONS FOR PROSTATE CANCER

Elizbeth C. Turner, David J. Kavanagh, Eamon P. Mulvaney, Cailtriona McLean, Katarina Wikström, Helen M. Reid and B. Therese Kinsella

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