cGMP-dependent protein kinase I interacts with TRIM39R, a novel Rpp21 domain-containing TRIM protein

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Roberts JD Jr, Chiche J-D, Kolpa EM, Bloch DB, Bloch KD. cGMP-dependent protein kinase I interacts with TRIM39R, a novel Rpp21 domain-containing TRIM protein. Am J Physiol Lung Cell Mol Physiol 293: L903–L912, 2007. First published June 29, 2007; doi:10.1152/ajplung.00157.2007.—Nitric oxide modulates vascular smooth muscle cell (SMC) cytoskeletal kinetics and phenotype, in part, by stimulating cGMP-dependent protein kinase I (PKGI). To identify molecular targets of PKGI, an interaction trap screen in yeast was performed using a cDNA encoding the catalytic region of PKGI and a human lung cDNA library. We identified a cDNA that encodes a putative PKGI-interactor that is a novel variant of TRIM39, a member of the really interesting new gene (RING) finger family of proteins. Although this TRIM39 variant encodes the NH2-terminal RING finger (RF), B-box, and coiled-coil (RBBC) domains of TRIM39, instead of a complete COOH-terminal B30.2 domain, this TRIM39 isoform contains the COOH-terminal portion of Rpp21, a component of RNase P. RT-PCR demonstrated that the TRIM39 variant, which we refer to as TRIM39R, is transcribed in the human fetal lung and in rat pulmonary artery SMC. Indirect immunofluorescence using an antibody generated against the conserved domains of TRIM39 and TRIM39R revealed the proteins in speckled intranuclear structures in human acute monocytic leukemia (THP-1) and human epidermal carcinoma line (HEP-2) cells. PKGI phosphorylated a typical PKGI/PKA phosphorylation domain in a conserved region of TRIM39 and TRIM39R. Additional studies demonstrated that PKGI interacts with both isoforms of TRIM39 in yeast cells and phosphorylates both isoforms of TRIM39 in human cell lines. Although PKGI has been observed to interact with proteins that regulate cytoskeletal function and gene expression, this investigation shows for the first time that PKGI interacts with tripartite motif (TRIM) proteins, which, through diverse molecular pathways, are often observed to regulate important aspects of cellular homeostasis.

Pulmonary; RING finger protein; RNase P

NITRIC OXIDE (NO) has an important role in regulating pulmonary function. NO that is synthesized in pulmonary endothelial cells diffuses into subjacent smooth muscle cell (SMC) where it stimulates soluble guanylate cyclase to produce cGMP from GTP and thereby affects the activity of cGMP-binding proteins (29). Although NO and cGMP cause pulmonary vasodilatation, several studies indicate that they also modulate lung structure by regulating the proliferation, differentiation, and apoptosis of pulmonary endothelial cells and vascular SMC. It has been observed that decreased pulmonary vascular NO and cGMP signaling is associated with diminished pulmonary microvascular and alveolar development in the injured premature lung (3, 47, 65) and with hyperplasia of vascular SMC and the development of pulmonary hypertension in the injured newborn and infant lung (54–56). Moreover, in many of these conditions, inhalation of low levels of NO gas increases NO and cGMP signaling in the lung and diminishes abnormal pulmonary cell proliferation and development (2, 4, 60, 61). Although NO and cGMP might regulate lung maturation by causing vasodilatation, data indicate that their protective mechanisms might also involve direct regulation of pulmonary cell proliferation and phenotype. For example, NO and cGMP have been observed to inhibit vascular SMC proliferation in culture (19), and inhaled NO decreases abnormal cell proliferation in the injured developing lung through mechanisms that do not require vasodilatation (60).

Several recent studies suggest that cGMP-dependent protein kinase I (PKGI), in part, mediates the regulation of pulmonary cell proliferation and phenotype caused by NO and cGMP (reviewed in Ref. 46). In cells that lack PKGI such as highly passaged vascular SMC (14) and baby hamster kidney cells (24), NO and cGMP do not regulate cell proliferation and phenotype. In contrast, in cells with PKGI, such as in freshly dispersed SMC (14) or passaged SMC exposed to PKGI-encoding expression plasmids (5) or adenoviruses (12), NO and cGMP readily inhibit proliferation and modulate cell phenotype. Moreover, specific inhibitors of PKGI have been observed to diminish the effects of cGMP on vascular SMC phenotype (15).

PKGI regulates cell function by interacting with and phosphorylating protein targets. Through alternate splicing of the 5′ end of a pre-mRNA encoded by a single gene (79), PKGI exists in SMC in two isoforms, PKGIα and PKGIβ, that have different NH2-terminal leucine zipper (LZ) domains. The variability of these domains facilitate the homodimerization of the PKGI isoforms (59, 67) and mediate isoform-specific interactions with heterologous protein. At the COOH-terminal end of PKGI, there is a catalytic region that transfers the γ-phosphate of ATP to the hydroxyl side group of the amino acids threonine and serine in target proteins. Many of the known PKGI phosphorylation targets in SMC attenuate cellular contraction by decreasing intracellular free Ca2+ levels, diminishing the calcium sensitivity of contractile proteins, and by affecting SMC thin filament binding proteins (reviewed in Ref. 46).

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The protein targets through which PKGI regulates SMC proliferation and phenotype are less well-known than those targets phosphorylated by PKGI that affect cytoskeletal function. Recently, PKGI has been observed to phosphorylate several growth factor signal transduction proteins and transcriptional regulators (30, 31, 46, 52). For example, upon stimulation with cGMP, PKGI has been reported to phosphorylate TFIIF-I, a transcription factor, and thereby enhance the transactivation of gene expression by TFIIF-I (10, 11). Hypothesizing that important phosphorylation targets might directly interact with the PKGI substrate recognition domain, the PKGI catalytic region was used in an interaction trap assay to identify novel PKGI targets that are expressed within the human lung. Through this investigation, PKGI was observed for the first time to interact and phosphorylate a tripartite motif (TRIM) protein. TRIM proteins constitute a class of regulators that contain a really interesting new gene (RING) finger domain, zinc-binding B-box motifs, and associated coiled-coil regions (71). Because TRIM proteins are involved in regulating a broad range of biological processes, including transcription, ubiquitination, and viral protein processing (49), the observation that PKGI interacts with a member of this family suggests that PKGI has an important role in regulating these processes within cells.

MATERIALS AND METHODS

DNA constructs. pGBT9·PKGICR, a yeast expression vector encoding the human PKGICR, the PKGI substrate recognition and kinase domains, was constructed from pRc/RSV·PKGIB (12). pMal·TRIM39R and pGEX·TRIM39R were constructed using pMal (New England Biolabs) and pGEX-KG (Amersham Pharmacia Biotech), respectively, and the BamHI restriction fragment of pGAD10·TRIM39R, the plasmid encoding the putative PKGI-interacting protein isolated in the interaction screen. Plasmids encoding mutant TRIM39R were generated as described in DETAILED MATERIALS AND METHODS section available in the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site, using mutagenic primers and PCR (17, 43, 48, 77). pFLAG·TRIM39R was produced by ligating the EcoRI restriction fragment of TRIM39B and a HindIII·EcoRI linker into HindIII and EcoRI cut p3xFLAG·myc- CMV-24 (Sigma). PCR was used to generate TRIM39R mutants: pMal·TRIM39R, which contains nucleotides encoding the B-box and coiled-coil domains of TRIM39B and TRIM39R. *S. cerevisiae* and RT-PCR. The 5′ end of cDNA encoding TRIM39R was specified using RNA ligase-mediated 5′ rapid amplification of cDNA ends (RACE) as described by Troutt and coworkers (72). cDNA specifying the Rpp21 domain were transcribed using a primer complementary to Rpp21, a component of RNase P. The cDNA were linked, using T4 RNA ligase, 83% (wt/vol) PEG, and 1 mM hexamine cobalt chloride, to an anchoring oligonucleotide (72). The ligation product was used directly in a PCR using REDTaq DNA polymerase (Sigma) and primers described in the DETAILED MATERIALS AND METHODS section available in the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site. The amplions were detected, after gel electrophoresis and transfer to a charged nylon membrane, using a radiolabeled cDNA fragment encoding the TRIM39B RF domain, and autoradiography.

Genomic organization of TRIM39. The genomic structure of TRIM39 and TRIM39B was determined using their dyeodeoxy chain termination method DNA sequencing information, database searching (1), and genomic alignment algorithms (BLAT; Ref. 38).

Reverse-transcription PCR. cDNA were transcribed using human fetal lung and rat pulmonary artery SMC RNA from a previous study (12). Moloney murine leukemia virus RT (MMLV), and random primers. PCR was performed using REDTaq DNA polymerase (Sigma) and sense primers, described in the DETAILED MATERIALS AND METHODS section available in the data supplement online at the AJP-Lung Cellular and Molecular Physiology, common to both TRIM39 isoforms and antisense primers specific to the Rpp21-like domain in TRIM39R. Sequence analysis of the amplions confirmed that the PCR products were of a fragment of TRIM39R. Amplions resolved using agarose gel electrophoresis were stained with ethidium bromide and detected using epifluorescence. In each case, a prominent single band of expected molecular weight was observed; the sequence of the rat pulmonary artery SMC homolog was confirmed by DNA sequencing. In silico RT-PCR was performed using the GeneNote search engine (66) and the National Center for Biotechnology Information (NCBI) Unigene dataset.

RNA blot hybridization. TRIM39 expression was detected using membranes containing human organ and cell RNA (Human MTN Blot I and II; Clontech), a radiolabeled EcoRI-SalI fragment of pGAD10·TRIM39R, which is common to both TRIM39 isoforms, and autoradiography.

Antibody generation. Chicken polyclonal antibodies were generated using a purified maltose binding protein (MBP)-TRIM39 fusion protein, which contains the TRIM39 B-box and coiled-coil domains that are common to both TRIM39 isoforms. IgY were purified using chloroform extraction and PEG precipitation as described by Polson (53). The specificity of the anti-TRIM39 antibody was tested using HEP-2 cell protein, IgY purified from a preimmune egg yoke, and immunoblotting.
**Immunoblotting.** To assess TRIM39 solubility, HEp-2 cell protein was solubilized in lysis buffers containing 50 mM Tris, pH 8.0, 1.0% Igepal CA-630, low (0 mM), medium (150 mM), and high (500 mM) levels of NaCl, and RIPA buffer, which contained PBS, 0.5% sodium deoxycholate (DOC), and 0.1% SDS, and protease inhibitors (Sigma). Protein (15 μg) was resolved using SDS-PAGE, transferred to a nitrocellulose membrane, blocked in 5% milk TBS containing 0.1% Tween-20 (TBST), and incubated overnight with the anti-TRIM39 antibody or preimmune IgG. After exposing the membrane to rabbit anti-chicken IgY-horseradish peroxidase conjugates (Promega), the antigen-antibody complexes were detected using chemiluminescence.

**Immunocytofluorescence.** Cells were fixed in 4% formaldehyde in PBS, permeabilized in methanol, and blocked with 1% goat serum in PBS. After incubation with chicken anti-TRIM39 or preimmune IgY, the cells were exposed to biotinylated goat anti-chicken IgY (Vector Laboratories), Alexa 488-labeled streptavidin (Molecular Probes, Eugene, OR), and 4,6-diamidino-2-phenylindole (DAPI), a fluorescent DNA-binding dye. Using laser scanning confocal microscopy (LSM 5 Pascal; Zeiss), images were captured of 1-μm-thick sections of the nucleus and surrounding cytoplasm of the cells. In experiments conducted to examine the colocalization of TRIM39 and Ro52/SS-A, the cells were incubated with the chicken anti-TRIM39 antibodies and human serum containing anti-Ro52/SS-A before exposure to biotinylated goat anti-chicken IgY (Vector Laboratories). FITC-labeled avidin, and Texas Red-labeled anti-human antibodies (Vector Laboratories). The TRIM39 and Ro52/SS-A immunoreactivities were assessed using epifluorescence microscopy.

**Protein-RNA interaction assay.** A biotylated, complementary H1 RNA probe (biotin-ch1) was generated, after digesting pcDNA3-H1 with BamHI, using SP6 RNA polymerase in the presence of biotin-14-CTP. After extracting with phenol/chloroform/amyl alcohol, the probe was washed with CHCl3, dried, and resuspended in diethyl pyrocarbonate-treated water. One day after transfecting HEp-2 cells with p3xFLAG-myc-CMV24, pFLAG-Rpp21, and pFLAG-TRIM39R using Effectene (Qiagen), the cells were lysed in RIPA buffer containing 0.1 mM EDTA and protease inhibitors (Sigma). After washing the beads with TBS, they were resuspended in TBS containing 0.05% SDS and 300 mM Na acetate, pH 5.7, and extracted using phenol/chloroform/amyl alcohol, and the RNA was precipitated in the presence of 1 μg of tRNA with ethanol and dried. Subsequently, the RNA was dissolved in 1× RIPA buffer containing deionized formamide and biotin-ch1 heated at 85°C for 10 min and incubated at 42°C overnight. After digesting single-stranded RNA using RNase A and RNase T1, the RNA was extracted and precipitated and dried, as described above, and resolved using a 5% Tris-borate-EDTA gel containing urea. The biotin-ch1 RNA probe was detected using the Phototope-Star detection kit (New England Biolabs) according to the manufacturer’s instructions.

**In vitro and in vivo phosphorylation assays.** In vitro phosphorylation of native and mutant TRIM39 was examined using the method described by Francis and coworkers (18). Purified MBP-TRIM39 transgenes were phosphorylated in the presence of 20 μM Tris, pH 7.4, 200 μM ATP, 30,000 counts·min⁻¹ μl⁻¹ [γ-32P]ATP, 20 mM MgCl2, 10 μM cGMP, and 500 units of bovine PKGI (Calbiochem). After terminating the reaction with SDS protein sample-loading buffer and resolving the mixture using polyacrylamide gel electrophoresis, the phosphorylated products were identified using autoradiography.

The in vivo phosphorylation of TRIM39 was detected using a nonradioactive assay. HEp-2 cells were reacted with an adenovirus encoding FLAG-PKGIβ and plasmids encoding FLAG-TRIM39R, FLAG-TRIM39B, and FLAG-VASP, a protein known to be phosphorylated by PKGI (13). Two days later, the cells expressing PKGIβ were treated for 2 h with 1 mM 8-Br-cGMP, a membrane-permeable cGMP compound. The FLAG-tagged proteins were collected from cell lysates using biotinylated anti-FLAG antibody (BioM2; Sigma) and streptavidin beads (Sigma) and resolved using SDS-PAGE. Phosphoproteins were detected in the gel using a fluorescent small molecule phosphate sensor (Pro-Q Diamond; Molecular Probes) and detected using epifluorescence according to the manufacturer’s instructions.

**RESULTS**

**PKGI<sub>CR</sub> interacts with TRIM39R, a novel RF protein.** A yeast two-hybrid screen was conducted to identify proteins encoded in a human lung cDNA library that interact with the constitutively active catalytic region of human PKGI (PKGI<sub>CR</sub>). Of ~10<sup>6</sup> yeast clones screened, 3 clones were identified that exhibited histidine prototrophy in the presence of 3-AT. One of the yeast clones contained a plasmid that encoded a TRIM protein, which is a member of a class of proteins that were not previously known to interact with PKGI. DNA sequence analysis revealed that the cDNA encoded the B-box and coiled-coil domains and the LDPD and WEVE, but not the LDYE, amino acid sequence motifs of the B30.2 domain of TRIM39 (GenBank accession no. NM_172016.1; Ref. 50). The B30.2 domain has no known function but first was observed to be encoded within a single exon (named B30.2-2) in the major histocompatibility complex of human chromosome 6p21.3 (73). The 3’ end of the cDNA that was identified in the library screen encoded 135 amino acids of the COOH-terminal end of Rpp21, a constituent of RNase P (32). The interaction screen also identified yeast clones that contained a plasmid that encoded the regulatory domain of cAMP-dependent protein kinase (R1α), which is a known phosphorylation target of PKGI (20), and a cDNA that encoded a portion of axotrophin.

The novel TRIM39 isoform characterized in this study was designated TRIM39R because it encoded a portion of Rpp21. Likewise, the previously reported TRIM39 now is referred to as TRIM39B because it encoded an isoform of TRIM39 that had a full-length B30.2 domain.

PKGI<sub>CR</sub> and TRIM39R interacted in yeast cells (Fig. 1). When AH109 yeast cells, which have HIS3 under the control of a GAL4-responsive promoter, were transformed with plasmids that encoded GAL4 binding domain-PKGI<sub>CR</sub> and TRIM39R-GAL4 activation domain fusion proteins, they exhibited histidine prototrophy, suggesting that PKGI<sub>CR</sub> and TRIM39R interacted within these cells. In addition, when both plasmids were transformed in Y187 yeast cells, which contain the LacZ reporter under control of a GAL4-responsive promoter, the interaction between PKGI<sub>CR</sub> and TRIM39R was confirmed because β-gal activity was detected. The levels of cell growth of AH109 cells in selective media and of β-gal activity in Y187 cells cotransformed with the PKGI<sub>CR</sub> and TRIM39R encoding plasmids were similar to that observed in yeast cells that incorporated plasmids encoding SV40 T large antigen and murine p53, two proteins previously known to interact in vivo (44). Using a similar approach, an interaction between PKGI<sub>CR</sub> and TRIM39B was also observed in yeast cells (data not shown). These results suggest that PKGI interacts with both TRIM39 isoforms in the shared region of the molecules.

**Genomic organization of TRIM39.** TRIM39R and TRIM39B appear to be pre-mRNA splicing variants of the same gene. Genome-wide interrogation using BLAT (38) revealed that TRIM39R, TRIM39B, and Rpp21 reside on human...
chromosome 6p21.3. The genomic structure of TRIM39B, TRIM39R, and Rpp21 was analyzed using nucleotide sequence information obtained in this study and reported by Jarrous, Orimo, and their coworkers (32, 50) and is shown in Fig. 2A. The sequence of the 5′ portion of the TRIM39R cDNA isolated from the lung library was nearly identical to that of TRIM39B cDNA, which was isolated from the human testis library and reported by Orimo and coworkers (50). However, TRIM39R did not contain nucleotides encoding a RF domain or the nucleotides observed in exon 7 of TRIM39B. A screen of a human spleen cDNA library using a radioactively labeled cDNA that contained exon 7 and was similar to a TRIM39B cDNA identified by Orimo et al. (50) in a murine testis library. The exon containing the 5′ end of TRIM39B was observed to be located 2,455 bases from the 5′ end of Rpp21 (Homo sapiens genomic assembly; hg17). Sequence analysis of the TRIM39R cDNA isolated from the human lung library revealed that it does not include a complete B30.2 domain (Fig. 2A). The 3′ end of TRIM39R appeared to result from the splicing of mRNA encoded within exon 10 of TRIM39B, just after the nucleotides that encode an incomplete B30.2 domain and a large portion of Rpp21. The previously published cDNA sequence indicates that TRIM39B contains an additional exon (gray box) that was not observed in the TRIM39R cDNA isolated from the human lung or the TRIM39B cDNA isolated from the human spleen library. The exon containing the 5′ end of Rpp21 is ~2,455 nucleotide bases downstream from the exon containing the 3′ end of TRIM39B. Moreover, expressed sequence tag (EST) database searching reveals a cDNA (AI805914) that is encoded by exons shared by TRIM39B, TRIM39R, and Rpp21, an isoform of Rpp21 in which the intervening nucleotides between exon 1 and 2 are expressed. The nucleotides encoding TRIM39R and Rpp21 were aligned and compared with those specifying typical splice donor and acceptor junctions in primates. The nucleotides corresponding to the exons are in boxes, the splicing sites are shown by arrows, and the homologous putative donor and acceptor splice junction nucleotides are in uppercase text. The nucleotides guiding pre-mRNA splicing for TRIM39R and Rpp21 are similar to what is generally observed in primates.
TRIM39R contains a RF. Because the plasmid encoding TRIM39R that was isolated from the human lung library did not contain a ribosomal binding consensus sequence (42) or RF domain, RNA ligase-mediated 5' RACE was used to define the 5' end of TRIM39R mRNA. The primers used to reverse-transcribe Rpp21-specific cDNA from RNA prepared from HL60 cells, a human promyelocytic leukemia cell line, and to detail the 5' end of TRIM39R are shown in Fig. 3 and described in MATERIALS AND METHODS. TRIM39R was observed to encode a 5' RF domain. A radiolabeled deoxynucleotide fragment encoding the RF domain of TRIM39R identified sequence encoding this domain in the cDNA of TRIM39R (Fig. 3, arrow). Moreover, this study suggested that the mRNA for TRIM39R begins ~200 nucleotide bases 5' of the putative translational start point and RF-encoding DNA.

TRIM393 is widely expressed. RNA blot hybridization using a radiolabeled probe that identifies both TRIM39 isoforms revealed that TRIM39R/TRIM39B are expressed in many human organs (Fig. 4A). The highest expression levels appeared in tissues with high cell proliferation such as the testis and spleen. In addition, RT-PCR using oligonucleotide probes specifying sequence in exon 10a of TRIM39 and exon 2 of Rpp21 revealed that TRIM39R was expressed in the human fetal lung (Fig. 4B). Because PKGI is abundant in lung SMC, the expression of TRIM39R was evaluated in pulmonary artery SMC. RT-PCR using TRIM39R-specific primers and cDNA generated from rat pulmonary SMC RNA yielded a single amplicon (Fig. 4B) that had a DNA sequence consistent with a rat homolog of TRIM39R. The DNA sequence of this rat homolog of TRIM39R was 87% identical to the sequence that we observed in human TRIM39R in the range of nucleotides amplified during this experiment.

The RBBC domains of TRIM proteins affect their compartmentalization within cells (57). As demonstrated in Fig. 5, A and C, indirect immunofluorescence revealed that in HEp-2 and THP-1 cells, the TRIM39 isoforms are organized in fine and coarse intranuclear speckles. Furthermore, in cells undergoing mitosis, as demonstrated in THP-1 cells (Fig. 5C, arrow), the nuclear TRIM39 isoform immunoreactivity was abolished suggesting that their overall expression level in the dividing cell was diminished. The RBBC domains of TRIM proteins mediate their participation in macromolecular complexes and decrease their solubility in lysis buffers. Immunoblotting of proteins derived from HEp-2 cells revealed that the TRIM39 isoforms, like other RBBC proteins (36), required buffers containing high salt levels or detergent to be solubilized (Fig. 5B).
Several other TRIM proteins have been observed to reside in intranuclear structures. For example, the TRIM protein Ro52/SS-A, which might have E3 ubiquitin ligase activity (75), has been identified in nuclear speckles (27). The promyelocytic leukemia protein (PML), a tumor suppressor protein, is observed in 5–20 discrete nuclear dots (78). In THP-1 cells, TRIM39 was detected in coarse nuclear speckles, similar to the staining pattern produced by anti-Ro52/SS-A antibodies (Fig. 5C). Using serum from patients with autoimmune disease, PML was observed in THP-1 cells to be localized in ≤20 discrete dots that were larger than, and did not appear to colocalize with, speckles that were identified with TRIM39 and Ro52/SS-A immunoreactivity (data not shown).

**TRIM39R does not interact with H1 RNA in cells.** Previous studies showed that Rpp21 is an integral protein of RNase P (26, 32–34). Interaction trap studies in yeast and UV-cross-linking studies in vitro suggest that Rpp21 associates with H1 RNA, the catalytic RNA component of RNase P (34). Although the domains of Rpp21 required for interaction with H1 RNA are unknown, analysis of the crystal structure of Ph1601p, an archaeal homolog of Rpp21 from *Pyrococcus horikoshii* OT3, suggests that the COOH-terminal end of Rpp21 folds into a zinc ribbon domain that might interact with RNA (35). Because this portion of Rpp21 is present in TRIM39R, we tested whether TRIM39R interacted with H1 RNA in vivo. As shown in Fig. 6, transient transfection of HEP-2 cells caused the expression of NH2-terminal FLAG-tagged Rpp21 and TRIM39R that could be collected with immobilized anti-FLAG antibodies and detected using immunoblotting methods. However, although H1 RNA coprecipitated with FLAG-Rpp21, as detected using an RNase protection assay, H1 RNA was not coprecipitated with the FLAG-TRIM39R. These studies suggest that TRIM39R does not associate with H1 RNA.

**PKGI interacts with TRIM39R in a kinase phosphorylation domain.** A survey of amino acid consensus sequences that are phosphorylated by PKGI reveals that, in most cases, PKGI phosphorylates a threonine or serine within the domain (R/K)2X(T/S) (Ref. 37). Inspection of the nucleotides of the TRIM39 isoforms revealed a sequence on exon 10 that would be expressed as RRFT. In vitro studies were performed to investigate whether PKGI phosphorylates the Thr347 encoded in this putative phosphorylation domain. As shown in Fig. 7A, PKGI interacts with TRIM39R in a kinase phosphorylation domain.
TRIM39R mutants and PKGICR was assessed in yeast using the interaction trap method. As shown in Fig. 7B, PKGICR interacts with native TRIM39R and the mutant form in which Thr347 was mutated to alanine. The interaction persisted when the TRIM39R kinase phosphorylation domain was removed. However, when the phosphorylation domain and 10–11 adjacent amino acids were deleted, PKGICR was observed to no longer interact with TRIM39R. These results suggest that the kinase and substrate recognition domains are separable in PKGI.

Additional studies were performed to assess whether PKGI phosphorylates the TRIM39 isoforms in cells. In this assay, HEp-2 cells were transiently transfected with plasmids encoding NH2-terminal FLAG-tagged TRIM39R, TRIM39B, and vasodilator-stimulated phosphoprotein (VASP). VASP is known to be phosphorylated by PKGI (13). In some experiments, the cells were also infected with an adenovirus encoding an NH2-terminal FLAG-tagged human PKGIB. After the PKGIB-transduced cells were stimulated with 8-Br-cGMP, a membrane-permeable cGMP analog, the proteins were collected using an immobilized anti-FLAG antibody and resolved using polyacrylamide gel electrophoresis. The phosphorylation state of the proteins was then detected using epifluorescence following exposure to a fluorescent phosphate-binding molecule. As shown in Fig. 7C, cGMP-stimulated PKGIB phosphorylated TRIM39R, TRIM39B, VASP, and itself in vivo.

**DISCUSSION**

In the present study, PKGI was observed to interact with and to phosphorylate TRIM39R, a novel TRIM protein encoded by human lung gene transcripts. Although TRIM39R shares the RBCC domains of TRIM9B (50), instead of having a complete B30.2 domain, TRIM39R contains ~75% of the COOH-terminal end of Rpp21, a component of the ribonucleoprotein enzyme RNase P (32–34) that processes 5' leader sequences of precursor tRNAs. TRIM proteins constitute a class of molecules that, through their membership in large multimeric protein complexes, regulate a plurality of important cellular functions (6, 57, 62). The interaction between PKGI and TRIM39R observed in this investigation support accumulating evidence indicating that PKGI interacts with a diverse set of proteins that function to regulate cytoskeletal dynamics, growth factor signaling transduction, gene transcription, and cell proliferation and phenotype.

Because of the importance of PKGI in regulating SMC function, several investigators have used interaction screens to identify PKGI targets (11, 63, 68, 76, 80). Surks and coworkers (68) used a cDNA encoding bovine PKGI and a human activated T cell library to identify an association between PKGI and the myosin-binding subunit of myosin phosphatase, a modulator of cell contraction. Of particular interest, Casteel et al. (11) employed a cDNA encoding human PKGIB and a mouse embryo cDNA library to detect an interaction between PKGI and TFII-I, a transcriptional regulator. These latter data provide evidence that PKGI might modulate cell proliferation and phenotype by directly phosphorylating proteins that regulate gene expression (51). In each of these screens, full-length PKGI was used as bait, and the NH2-terminal LZ domain of PKGI was observed to facilitate the interaction of PKGI with the identified target proteins. Wang and associates (76) exam-
ined the role of the PKGI LZ domain in mediating protein-protein interactions. In a screen using a cDNA encoding the LZ domain of human PKG\(\alpha\), they identified several proteins encoded in a human aortic cDNA library that interact with PKGI (76). In our studies, PKGI\(_{CR}\) was used to identify potential PKGI interactors instead of full-length PKGI for three reasons. First, the COOH-terminal substrate recognition and kinase domains of the catalytic region of PKGI have been observed to retain substrate specificity and phosphotransferase activity in vivo. Therefore, it was expected that a screen using this portion of PKGI would likely identify PKGI-interacting proteins that might not require association with the LZ domain of PKG\(\alpha\) and PKGI\(\beta\). Second, because cytosolic PKGI LZ domain-interacting proteins are likely to anchor PKGI in the cytosol and PKGI that lacks the LZ domain has been observed to diffuse into the nucleus (9, 25), we thought that this approach might permit the identification of novel PKGI-interacting proteins residing in the nucleus. Third, because PKGI\(_{CR}\) is conserved in the PKGI isoforms, we reasoned that a bait protein using this portion of PKGI would permit the identification of functional targets that might interact with and be phosphorylated by either isoform of PKGI. Indeed, using this strategy, PKGI was observed to interact with two novel PKGI targets. Results from this investigation suggest that the substrate recognition domain of PKGI is separable from its catalytic domain. Comparison of amino acid sequences in protein targets near typical PKGI phosphorylation sites suggests that PKGI phosphorylates threonines and serines that reside within a \((R/K)_2X(T/S)\) domain, where \(X\) is generally a hydrophobic amino acid (22, 23, 45). We observed that PKGI phosphorylated a threonine within the sequence RRFT in TRIM39R in vitro, which is a typical PKGI phosphorylation domain. In an attempt to map the PKGI-TRIM39R interaction site in vivo, it was noted that when the \((R/K)_2X(T/S)\) domain in TRIM39R was deleted, PKGI\(_{CR}\) still interacted with the mutant TRIM39R in cells. In addition, when this domain and adjacent amino acids were deleted, the interaction between PKGI\(_{CR}\) and mutant TRIM39R was abolished. Because PKGI interacted with amino acids in TRIM39R that are not constituents of the TRIM39R putative PKGI phosphorylation domain, these data suggest that PKGI substrate recognition and kinase sites are separate. This is supported by in vitro studies using peptide libraries that indicate that amino acids outside of the PKGI \((R/K)_2X(T/S)\) phosphorylation domain influence PKGI-substrate interaction (16, 70). Although the structures of the substrate recognition and kinase domains of PKGI have not been elucidated, studies of the structure of the PKA, a kinase with strong active site amino acid sequence homology (69) and substrate specificity similarities (39) with PKGI, suggest that the substrate recognition and kinase domains reside on adjacent parts of the catalytic region (40, 41). The catalytic region of PKA is bilobal in shape; substrate recognition by PKA occurs through interaction of amino acids in the target with amino acids on the surface of one of the lobes, whereas interaction between amino acids in the cleft between the lobes in PKGI and that of the protein target permits phosphotransferase activity. The resolution of the PKGI structure will more precisely delineate the molecular mechanisms involved in PKGI phosphorylation of protein targets. However, studies performed in this investigation suggest that the interaction trap methodology might be useful to define amino acids required for the interaction between kinases and substrates. In addition, this approach might be useful to identify novel substrate decoys that can be used to inhibit PKGI activity in vivo.

Although the interaction between PKGI and TRIM39R is carefully characterized in this investigation, a limitation of the study is that the functions of TRIM39R and their modulation by PKGI phosphorylation are unknown. Although the genomic structure of TRIM39B has been characterized and its mRNA identified in many organs, like many other TRIM proteins, its function has not been identified. Moreover, although Rpp21 has been observed to be a critical component of RNase P, and its interaction with the protein and RNA constituents of RNase P has been carefully determined, its function within that ribozyme is unknown. However, the possibility that TRIM39R has a similar cellular distribution was examined. It was noted that although Rpp21 was reported to be abundant in the dense fibrillar component of cell nucleoli, the distribution of TRIM39R and TRIM39B was in fine intranuclear speckles. Moreover, although Rpp21 was observed to interact with H1 RNA, the catalytic component of RNase P, TRIM39R did not appear to interact with the catalytic RNA component of RNase P (32).

In summary, during this investigation, PKGI was observed to interact with and phosphorylate a novel splice variant of a TRIM protein that is expressed in the human lung and resides in the nucleus of several cell lines. Future studies defining the function of this new PKGI target are likely to further delineate the important role of PKGI in regulating cell function in the lung.

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The nucleotide sequence for TRIM39R has been submitted to the GenBank Data Bank and has been granted accession no. EU012025.

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