Proprotein convertases play an important role in regulating PKGI endoproteolytic cleavage and nuclear transport

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Kato S, Zhang R, Roberts, Jr. JD. Proprotein convertases play an important role in regulating PKGI endoproteolytic cleavage and nuclear transport. Am J Physiol Lung Cell Mol Physiol 305: L130–L140, 2013. First published May 17, 2013; doi:10.1152/ajplung.00391.2012.—Nitric oxide (NO) and cGMP regulate vascular SMC phenotype primarily by stimulating cGMP-dependent protein kinase I (PKGI) (10, 15, 47, 95) through mechanisms that are incompletely understood. PKGI is expressed in vascular SMC as two isoforms, PKGIa and PKGIb, which are translated from alternatively spliced PKGI mRNA (85). These PKGI isoforms differ in their first ~100 amino acids, which harbor a leucine zipper-like (LZ) domain (69) that directs PKGI isoform homodimerization (65) and interactions with cytosolic anchoring proteins (13, 50, 89). This amino acid region also contains an autoinhibitory (AI) domain that interacts with the substrate recognition domain in the PKGI catalytic region and thereby inhibits PKGI kinase activity in the absence of cGMP stimulation (30). The PKGI COOH-terminal region, which is identical in the PKGI isoforms, has cGMP- and ATP-binding domains and a catalytic region. The phosphorylation of cytosolic targets regulates SMC cytoskeletal activity by regulating intracellular Ca2+ levels and sensitization and possibly the thin filament (reviewed in Ref. 46).

Several studies suggest that PKGI nuclear localization is required for the regulation of gene expression and the modulation of cell phenotype by NO and cGMP. For example, PKGI has been detected in the nucleus of several cell types, including vascular SMC (17, 28, 62). PKGI also has been shown to phosphorylate or regulate the activity of several proteins that reside primarily in the cell nucleus that modulate gene expression (60). For example, PKGI has been reported to phosphorylate and activate cAMP response-binding protein (CREB) (28, 60, 81), activating transcription factor-1 (70), TFII-I (14), and CRP4 (94) and to increase the expression of transcription regulators, such as the activator protein 1 constituents c-FOS and JunB (26, 27), growth arrest-specific homeobox gene (91), and peroxisome proliferator-activated receptor-γ (90). Moreover, in several cell types, nuclear PKGI translocation has been observed to be required for the regulation of gene expression by cGMP (13, 26, 28, 81). Although it is possible that cytoplasmic targets of PKGI could regulate gene expression, one study suggested that PKGI regulates nuclear signaling through mechanisms that are independent of Ca2+, cAMP-dependent protein kinase, and MAPK signaling pathways (26). However, the lack of nuclear PKGI localization and the variability in PKGI-regulated gene expression observed in some experiments (12, 16, 22, 37) and the observation of SMC dedifferentiation and proliferation in some vascular tissues with increased PKGI expression suggest that additional mechanisms might control nuclear PKGI function. Understanding what regulates nuclear PKGI translocation and activity will likely provide important insights into how NO and cGMP signaling influence health and disease.

Nitric oxide (NO) and cGMP play an important role in regulating vascular function and structure. In several disease models, decreased NO and cGMP signaling are associated with dedifferentiation and excessive proliferation of vascular smooth muscle cells (SMC) (9, 60), which can contribute to vasoconstriction and hypertension. In models of newborn lung disease, for example, pulmonary injury often causes decreased NO and cGMP signaling, pulmonary SMC hyperplasia, pulmonary hypertension, right heart failure, and reduced somatic growth. In such cases, selective pulmonary NO treatment has been observed to decrease abnormal pulmonary artery SMC proliferation (66, 67), improve alveolar development (4, 8, 45, 52), and prevent pulmonary hypertension (66, 67).

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We recently observed that PKGI proteolysis regulates PKGI nuclear localization and cGMP’s regulation of gene transcription (81). PKGI epitope mapping studies in intracellular compartments and in purified subcellular protein fractions suggested that proteolysis removes the NH2-terminal LZ and AI domains in the PKGI isoforms and generates a constitutively active COOH-terminal kinase fragment, which we termed PKGIy, that localizes in the nucleus in a variety of cell types. Moreover, scanning mutagenesis studies of the putative PKGI cleavage site and intracellular compartmentation studies revealed that PKGI proteolysis was critical for cGMP’s stimulation of nuclear PKGIy localization and the activation of CREB phosphorylation and transactivation of gene expression. However, the enzymes that regulate PKGI cleavage had not been identified. Here we report that proprotein convertases (PCs) have an important role in mediating PKGI proteolysis.

MATERIALS AND METHODS

Antibodies and reagents. Biotinylated monoclonal mouse anti-FLAG was obtained from Sigma (M2; F9291), monoclonal mouse anti-actin was obtained from EMD Millipore (clone AT6/172, 05-384), rabbit anti-COOH-terminal PKGI (anti-PKGIκ) was obtained from Enzo (ADI-KAP-0005), anti-furin was obtained from Pierce (PA1062), anti-V5 epitope was obtained from Sigma (V8137), and anti-insulin-like growth factor I receptor (IGF-IR) was from Cell Signaling (3018). Alexa Fluor 546 anti-rabbit antibody was obtained from Invitrogen (A-11035). Peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch (715-035-150 and 711-035-152, respectively). To stimulate PKGI, the membrane-permeant cGMP analog 8-(p-chlorophenylthio)-cGMP (8-pCPT-cGMP; C5438; Sigma) was used. To inhibit PCs in the aortic SMC, the membrane-permeable PC inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-CMK; Bachem) was used.

Plasmid and adenovirus construction and characterization. pcDNA3·PKGI·FLAG, which contains cDNA inserted into the BamHI-Apal sites of pcDNA3.1 and encodes murine PKGI with a C-terminal FLAG epitope, was generated and characterized as described previously (81). pcDNA3·PKGI·FLAG was constructed by exchanging the HindIII-EcoRl fragment of pcDNA3·PKGI·FLAG with cDNA that encodes the 5’-terminal sequence of PKGI that was generated using mouse lung mRNA, and RT-PCR. pcDNA3·PKGI·FLAG·5KEVVT, which encodes PKGI·FLAG with alanines substituted for the KVEVTK in pcDNA3·PKGI·FLAG and PCR-mediated site-directed mutagenesis as described previously (81). pSVL-furin, which encodes mature ~87 kDa furin, was generated and characterized by Van de Ven and colleagues using a mouse cDNA library (87) and was obtained from American Type Culture collection (ATCC; 63248). pcDNA3-furin·V5 was constructed by using DNA primers (sense: c acc atg gag ctc gag tca ttc ggt tct gca gaa gtc and antisense: aag ggc gct ctc gtt gtt gaa ggc) and PCR to amplify the coding region of furin from pSVL-furin while adding a Kozak consensus sequence and removing the stop codon. The furin-V5 encoding cDNA was then cloned into pENTR using In-Fusion (Clontech) and subsequently inserted into pcDNA3.2-V5-DEST (Invitrogen) using recombination. pCMV SPORT6-PC7, which contains a mammalian gene collection (MGC) clone that encodes murine PCI (70), was obtained from ATCC (MGC-11587). pcDNA3·PC5A was constructed using a commercially available human PC5A MGC clone in pTO7B (BO12006; Open Biosystems MHS1011-75908) by recombining the PC5A-encoding cDNA into pcDNA3.2-V5-DEST (Invitrogen) through pDONR221 (Invitrogen). DNA sequencing revealed that this clone encodes the PC5A isoform of PC5 (49). pRCMV-α1-PDX was kindly provided by Dr. Gary Thomas (Vollum Institute, Oregon Health & Science University); the in vitro PC inhibitory activity of the encoded α1-PDX is characterized elsewhere (11, 36).

An adenovirus that encodes murine PKGIβ with a COOH-terminal FLAG epitope (Ad·PKGIβ·FLAG) was constructed in the following manner. First, PCR was used to introduce a furin mutation that deleted the Pael site in PKGIβ in pcDNA3·PKGI·FLAG and then the Asp718- Pmde1 digested fragment of the plasmid, which encodes PKGIβ·FLAG, was ligated into the Asp718-EcoRV sites of PENTR2B in the presence of 6% polyethylene glycol 8000. Next, pAd·PKGIβ·FLAG was generated using this new plasmid, pAd·CMV·V5-DEST (Invitrogen), and recombination. Finally, Ad·PKGIβ·FLAG was generated after transfecting 293A packaging cells (Invitrogen) with Pael-digested pAd·PKGIβ·FLAG using Lipofectamine 2000 (Invitrogen). The Ad·PKGIβ·FLAG was purified using end-point dilution (57) and characterized by detecting PKGIβ·FLAG transgene expression using M2- and anti-PKGIκ antibodies and immunoblotting. The adenovirus titer was determined using the cytopathic effect assay described by O’Carroll and coworkers (58). Endonuclease mapping and automated DNA sequencing were used, when appropriate, to verify the authenticity of all the plasmid constructs.

Cell culture and transfection. The investigations were approved by the Subcommittee for Research Animal Studies at the Massachusetts General Hospital. Rat aortic SMC were isolated using the following method that was described previously (81). Aortas from eight rats were minced, combined, and treated with type 2 collagenase (Worthington Biochem) in 10% fetal bovine serum (vol/vol; FBS HyClone SH30088.03) in DMEM supplemented with antibiotics and glucose. Subsequently, the cells were resuspended in 10% FBS DMEM supplemented with antibiotics and glucose, allowed to seed onto cell culture plates, and then expanded. The SMC identity was confirmed based on their typical morphology and expression of α-smooth muscle actin as determined by reactivity with an antibody (1A4, Sigma) and epifluorescence microscopy, as described previously (5). The rat aortic SMC were used before reaching the eighth passage. All other cells were obtained from commercial sources: human embryonic kidney (HEK) 293 cells (ATCC CRL-1573), and LoVo metastatic colon adenocarcinoma cells (ATCC CCL-229).

The rat aortic SMC, HEK293, and LoVo cells were maintained in DMEM; media was supplemented with 10% FBS (GIBCO), penicillin, and streptomycin. Glutamine was added to the DMEM media. The cells were passaged before becoming confluent using EDTA-trypsin. Cells were transfected using Lipofectamine 2000 and methods detailed by the manufacturer.

Immunodetection of proteins and quantification of proteolysis. The protein expression levels of PKGI and PKGIγ were detected using immunoblotting. After being washed gently with ice-cold (PBS) buffered saline, the cells were collected in lysis buffer containing 50 mM Tris-HCl, pH 7, 4% (wt/vol) sodium dodecyl sulfate, and 10% (vol/vol) glycerol and sonicated. Following centrifugation to remove insoluble material, the protein concentration in the supernatant was determined using a bicinchoninic acid-based (BCA) protein assay method (Pierce). Finally, 0.01 vol of β-mercaptoethanol and 1% (wt/vol) bromphenol blue were added, and the sample was heated to 95°C and then cooled on ice. We found that PKGIγ protein levels decreased in frozen cell lysates so PKGIγ immunoreactivity was examined soon after the samples were collected. To detect PKGIγ proteolysis and furin abundance, protein fractions were resolved using SDS-PAGE and then transferred to a polyvinylidene fluoride membrane. After the protein blot was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20, it was incubated with a primary antibody and exposed to a horseradish peroxidase-conjugated secondary antibody before the antigen-antibody complexes were detected using chemiluminescence. In the cases in which the biotinylated anti-FLAG antibody was used to detect the FLAG epitope, the blots were exposed to complexed avidin-biotin-peroxidase (PK-6200; Vector Laboratories) instead of the secondary antibody before detection using chemiluminescence.
The percent PKGI proteolysis was quantified using immunoblot chemiluminescence. Unlabeled chemiluminescent signals were acquired using a cooled charge-coupled camera (ChemiDoc XRS; Bio-Rad) and analyzed using ImageJ (63). The percent PKGI cleavage was determined by dividing the density of the PKGIγ-FLAG fragment by the sum of those associated with the PKGIβ-FLAG and PKGIγ-FLAG fragments, which represent the total amount of PKGI transgene expressed in the cell.

**PC enzyme activity.** PC activity was determined in the cell lysates of HEK293 cells that were transfected with either pcDNA3, pSVL-furin, or pCMV-SPORT6-PC7 using Pyr-Arg-Thr-Lys-Arg-AMC (I-1650; Bachem), an internally quenched fluorogenic substrate, fluorescence spectroscopy, and methods described by others (6, 35). HEK293 cells seeded at $2 \times 10^5$ cells/cm² in 12-well plates were transfected with 1.5 µg of plasmid using 3 µl of Lipofectamine 2000 in Opti-MEM (Invitrogen). The next day, the cells were scraped into 50 mM Tris·HCl, pH 7.4, 1% (wt/vol) Triton X-100, 10% (vol/vol) glycerol, and 1% (vol/vol) protease inhibitors (P8340; Sigma). After sonication and centrifugation, the protein concentration was determined in soluble cell fractions using the BCA protein assay method, and PKGI activities were determined in the cell lysates of HEK293 cells seeded at 0.25 × 10⁵ cells/cm² in glass bottom chamber slides in 10% FBS DMEM containing antibiotics and supplemental glutamine. The following day, the cells were briefly washed with 0.1% FBS DMEM containing antibiotics and supplemental glutamine, and then the media was exchanged with media containing 0 or 50 µM dec-RVKR-CMK. After overnight incubation, the cells were exposed to a fresh aliquot of the solutions with and without dec-RVKR-CMK and 200 µM 8-pCPT-cGMP for an additional 2 h. This level of dec-RVKR-CMK has been reported to inhibit PC activity in several cell types for several hours without causing appreciable toxicity (38, 78). Nuclear PKGIγ was detected using the following method. The cells were gently washed with PBS and fixed in 4% formaldehyde in buffered PBS for 15 min, and then the nuclei were permeabilized by treating the cells with methanol for 10 min. After blocking with 1% goat serum in PBS for 1 h, the cells were exposed to rabbit anti-PKGIγ serum diluted in PBS containing 1% bovine serum albumin and incubated at 4°C overnight. After being washed with PBS, the cells were exposed to Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) for 1 h and then PBS containing 4’,6-diamidino-2-phenylindole before being mounted under a glass cover slip. Nuclear COOH-terminal PKGI immunoreactivity was then detected using laser scanning confocal microscopy (LSMS Pascal; Zeiss) with the pinhole set to sample a 2-µm-thick slice through the center of the nucleus.

**Data analysis.** The hydrophobicity plot was generated using the predicted amino acid sequence flanking putative scissile amino acid bond site in murine PKGI using the method detailed by Grantham (25) and implemented by ProtScale (24).

Experiments were repeated at least three times, and typical data are shown. Numerical data are represented as means ± SD. Enzyme activity data were compared using a one-way model of ANOVA; a Tukey range test was used post hoc. Significance was determined at $P < 0.05$. Statistical analysis was performed using R (34).

**RESULTS**

PKGI isoforms encode a minimum PC consensus recognition site in the putative cleavage area that is adjacent to the NH2-terminal portion of PKGIγ. To gain insight into candidate protease recognition sites in PKGI, we previously performed amino acid microsequencing of PKGIγ that was immunopurified from nuclei of SMC (81). Although it was not possible to obtain unambiguous amino acid sequence information, the data obtained from one immunopurified protein fragment suggested that PKGI is cleaved at a scissile amino acid bond that is COOH-terminal to the amino acid sequence KVEVTK. The relationship between this sequence and the functional domains of the PKGI isoforms and PKGIγ is depicted in Fig. 1A. This site resides within the cGMP-binding domain, which is identical in the PKGI isoforms, and is COOH-terminal to a PKGIα and PKGIβ hinge region that is depicted in the figure as the narrow box between the LZ and AI domains (LZ-AI domains) of PKGIα and PKGIβ. This hinge region has been reported to be sensitive to in vitro proteolysis (55, 84). Recent PKGI crystal-structure studies indicated that this cleavage site resides in a portion of amino acids in PKGI that form a beta-sheet that projects into the extramolecular milieu (40). It is likely that this molecular configuration makes the PKGI KVEVTK domain accessible to proteases. Moreover, the potential importance of this putative protease recognition sequence is supported by analysis of PKGI amino acid sequence data obtained from the NCBI database that reveals that this site is conserved in diverse species (Fig. 1A).

Interrogation of a proteolytic enzyme database (64) suggested that the putative PKGI cleavage site that we identified is...
recognized by PCs. This is because the PKGI amino acid sequence predicted to reside NH2-terminal to the detected cleavage site is of the form (K/R)-Xn-(K/R), where n = 0, 2, 4, or 6 and X is any amino acid but not generally C, which usually directs PC-mediated substrate cleavage (72). PCs are a family of Ca2+-dependent proteases that are expressed in several cell types. PC members include furin, PC1/3, PC2, PC4, PC5, PACE4, and PC7, all of which cleave proteins at a COOH-terminal basic amino acids, and subtilisin kexin isoenzyme-1 and PCSK9, which are involved in fatty acid metabolism (3, 74, 86). PCs proteolyze and activate a variety of proteins that regulate angiogenesis and the response of SMC to vascular injury. Furin (31), and likely the other homologous PCs (32), contains a negatively charged substrate binding/catalytic pocket that interacts with basic residue-rich amino acid sequences in target proteins. As depicted in Fig. 1B, PKGI has several basic residues in the putative cleavage site that increase protein polarity and might facilitate the interaction between PKGI and the PC catalytic domain.

PC overexpression is associated with increased PKGI proteolysis. Because furin is a prototypical PC that is widely expressed in cells and whose proteolytic activity is well defined, we first tested whether or not furin overexpression increases PKGI proteolysis. We used HEK293 cells as a model system for these experiments because in pilot studies we observed PKGI proteolysis in these cells, which suggests that they have functional systems that facilitate PKGI transport to the protease-containing compartment, and because these cells are readily transfected. We examined the cleavage of COOH-terminal FLAG epitope-tagged PKGI isoforms because the sensitivity and specificity of the FLAG-detection system allowed us to detect steady-state levels of PKGIγ, and thereby PKGI proteolysis, without needing to expose the cells to radiochemicals. For these studies, we studied primarily the proteolysis of the PKGIγ isoform because PKGIβ was better expressed than PKGIγ in this experimental model, and, as noted before (81), PKGIβ proteolysis is often more readily detected in cells.

Overexpression of furin increased PKGI proteolysis. HEK293 cells transfected with pcDNA3-PKGIβ-FLAG exhibited a low level of PKGIβ-FLAG proteolysis in soluble cell lysates examined with immunoblotting and an anti-FLAG antibody (Fig. 2A). This is shown by the detection of an ~60-kDa protein band with anti-FLAG antibody immunoreactivity that migrates faster in the polyacrylamide gel than the ~78-kDa protein species detected with the antibody that has a molecular mass consistent with uncleaved PKGIβ-FLAG. No bands with anti-FLAG immunoreactivity were detected in the lysates of cells transfected with control plasmids (data not shown). The ~60-kDa molecular mass protein was similar in size to the only PKGI fragment that we identified previously in the nuclei of vascular SMC and PKGI-expressing cell lines (81). This nuclear PKGI fragment was determined to be a COOH-terminal PKGI fragment using differentiation antibody epitope mapping and amino acid microsequencing, and was defined as PKGIγ (81). Importantly, transfection of the HEK293 cells with a plasmid that encoded a furin transgene with a COOH-terminal V5 tag (pcDNA3-furin-V5) caused a nearly 50% increase in the levels of this PKGI cleavage product (Fig. 2B).

Although PCs often exhibit functional redundancy, in some cases they do not share the same protein targets and thereby may proteolyze target proteins differently. PC-substrate specificity is influenced by differences in substrate affinity and by the colocalization of PCs and protein targets. PC5, PC7, and furin are all expressed in vascular SMC (77, 79). PC5 is expressed as two isoforms, PC5A and PC5B (also referred to as PC6A and PC6B), that result from the alternate splicing of PC5 mRNA (56). PC5A (but not PC5B) expression is modulated by injury in vascular SMCs (79). Because of the expression pattern of these PCs, we tested whether PC5A and PC7 overexpression is associated with increased PKGI proteolysis. As shown in Fig. 3A, cotransfection of HEK293 cells with plasmids that encode PKGIβ-FLAG and increasing amounts of PC5A were associated with a dose-dependent increase in PKGIγ-FLAG immunoreactivity. In contrast, transfection of cells with a PC7-encoding plasmid (pCMV-SPORT6-PC7) did not increase PKGI cleavage. To examine whether proteolytic activity was increased in this PC overexpression model,
HEK293 cells were transfected with pCMV-SPORT6-PC7, pSVL-furin, or a control plasmid, and PC activity was examined in cell lysates using an internally quenched PC substrate probe. We observed that transfection with the PC7-encoding plasmid led to nearly a 1.5-fold increase in PC activity, suggesting that active PC7 was expressed from this plasmid (PC activity, arbitrary units: pcDNA3 transfected 7,581 ± 554 vs. pCMV-SPORT6-PC7 transfected 10,675 ± 903, n = 6 each group, P < 0.05; Fig. 3B). Moreover, we confirmed that active furin was expressed from the pSVL-furin plasmid. The relatively low level of cell lysate PC activity observed in the furin-overexpressing cells might be because of secretion of some of the furin into the tissue culture media (88) or possibly reduced furin pro-segment-mediated activation of the encoded mature furin (1). As shown in Fig. 3C, and in agreement with data from others (61), transfection of cells with furin-encoding plasmids results in the secretion of a proteolyzed furin fragment (“shed furin”) in the cell culture media.

**Mutation of a minimum PC recognition site decreases PKGI proteolysis.** Using alanine-stretch scanning mutagenesis, we previously observed that amino acids in the putative PKGI cleavage area regulated nuclear PKGI tyrosine phosphorylation and gene transactivation in SMC (81). To define the role of the putative PC consensus sequence in directing PC-regulated PKGI proteolysis, we examined whether furin cleaves PKGIβ-FLAG–KVEVTK, which is generated by plasmid that harbors mutations that encode alanines instead of the KVEVTK sequence. In pilot studies using immunoblotting, we observed PKGIβ-FLAG–KVEVTK had approximately the same molecular weight and in vivo kinase activity (measured by ability to phosphorylate VASP) as PKGIβ-FLAG (data not shown). As shown in Fig. 4, overexpression of PKGI and mutant PKGI was associated with a minor level of proteolysis. However, we observed that increased cleavage of wild-type PKGIβ-FLAG with furin overexpression was not observed in cells expressing PKGIβ-FLAG–ΔKVEVTK and the PC. These studies support the role of the KVEVTK consensus sequence in directing PKGI proteolysis by PCs. It is also interesting to note that overexpression of PKGIβ-FLAG was associated with the generation of an ~50-kDa fragment with FLAG immunoreactivity. We do not know the identity of this protein fragment; however, its apparent molecular weight suggests that it might be a further cleavage product of PKGI.

**Furin overexpression increases PKGI proteolysis in furin-deficient cells.** LoVo cells are a human colon adenocarcinoma cell line (20) that expresses furin with mutations in the homo
B domain and therefore has deficient furin activity (82, 83). Previous investigators have used LoVo cells to test whether furin overexpression rescues cleavage of putative PC protein targets (e.g., see Ref. 43). Accordingly, to further examine the role of PCs in PKGI proteolysis, we tested whether furin overexpression increases PKGI cleavage in LoVo cells. Because LoVo cells were difficult to cotransfect, they were first infected with an adenovirus that encoded PKGI·FLAG and later transfected with furin-encoding or control plasmids. As shown in Fig. 5, despite overexpressing PKGI·FLAG at high levels, little PKGI proteolysis was detected in the lysates of cells transfected with furin-encoding or control plasmids. A small level of PKGI proteolysis was observed in the LoVo cells, probably because they express other functional PCs (41, 73). Importantly, PKGI·FLAG proteolysis was increased in LoVo cells transfected with pSVL-furin. These data support the role of furin in directing PKGI proteolysis in cells.

PC inhibition decreases PKGI proteolysis. α1-PDX is a serpin-like α1-antitrypsin Pittsburgh mutant that was engineered to contain a furin consensus recognition sequence (2). In a variety of cells, α1-PDX binds tightly to the furin catalytic site and inhibits its endoproteolytic activity [IC50 = 0.6 nM (2)]. The furin·α1-PDX complex is stable during SDS-polyacrylamide gel electrophoresis, and the level of this complex detected using immunoblotting corresponds with the extent of α1-PDX-mediated furin inhibition (36). α1-PDX also inhibits other PCs (19); the level of α1-PDX-inhibited PC activity depends on the cells studied (7). To test whether α1-PDX decreases PC proteolytic activity in our model system, we examined whether transfection of HEK293 cells with an α1-PDX-encoding plasmid (pRcCMV·α1-PDX) inhibits autoproteolysis of endogenous profurin and forms an inhibitory complex with overexpressed mature furin. As shown in Fig. 6A, α1-PDX partially inhibits endogenous furin activity, and it also forms an SDS-stable complex with overexpressed furin in HEK293 cells. With α1-PDX expression, the steady-state level of endogenous profurin (Fig. 6A) was increased, whereas that of the autoproteolyzed mature furin product was decreased.

Fig. 6. PC inhibition decreases PKGI proteolysis in cells. Cells were transfected with plasmids that encode α1-PDX, an inhibitor of PC activity, PKGI isoforms with a COOH-terminal FLAG tag, or control plasmids. Later, soluble proteins in cell lysates were denatured and resolved using gel electrophoresis and transferred to a charged membrane for immunoblotting. In some experiments, furin and a furin-α1-PDX complex were detected with an anti-furin antibody. In others, PKGI proteolysis was detected using an anti-FLAG antibody. A: HEK293 cells express profurin (♦) and mature furin (open arrow), which results from profurin autoproteolysis, and associated N-glycosylated species. Transfection with pSVL-furin increased the level of the encoded mature ~85-kDa furin protein. Coexpression of α1-PDX and furin caused the formation of a higher-molecular-weight anti-furin antibody-reactive band (closed arrow) that is indicative of the formation of an α1-PDX-furin complex and PC inhibition. Similar α-tubulin immunoreactivity showed equal protein transfer. B: in HEK293 cells overexpressing PKGI·FLAG, transfection with the α1-PDX-encoding plasmid caused inhibition of PKGI transgene proteolysis and PKGIγ generation.
brane-permeable PC inhibitor dec-RVKR-CMK (38, 39, 53, 78). First, we tested whether dec-RVKR-CMK inhibits PC activity in our model system by determining whether it decreases serum-stimulated pro-IGF-IR proteolysis. As shown in Fig. 7A, in the lysates of serum-starved SMC, the endoplasmic reticulum (ER) form of pro-IGF-IR and the cleaved β-subunit of IGF-IR (IGF-IRβ) were observed using an antibody against the COOH-terminal portion of IGF-IR and immunoblotting, whereas the slower-migrating Golgi apparatus (GA) form of pro-IGF-IR, which is cleaved by PCs residing in the GA, was not detected. Moreover, with serum stimulation, the amount of IGF-IRβ was increased, whereas there was no change in the abundance of the ER form of pro-IGF-IR, indicating that serum stimulated the production of pro-IGF-IR, which was fully cleaved by GA PCs. However, in the serum-stimulated SMC, dec-RVKR-CMK treatment inhibited PCs and caused the accumulation of the GA form of pro-IGF-IR and a decreased abundance of IGF-IRβ. Others (78) have described a similar degree of dec-RVKR-CMK-mediated PC inhibition of pro-IGF-IR cleavage in vascular SMC.

Importantly, dec-RVKR-CMK was observed to decrease nuclear PKGIγ localization in cGMP-stimulated rat aortic SMC (Fig. 7B). In these studies, the SMC were treated with a membrane-permeable cGMP (8-pCPT-cGMP) because this has been observed to increase PKGI cleavage (81) and the fraction of cells with nuclear PKGI localization (13, 28, 81). Moreover, to localize cleaved PKGIγ in the nuclear compartment, anti-COOH-terminal PKGI immunoreactivity was detected in cell sections that transect the nucleus using laser scanning confocal microscopy. In these studies, whereas treatment of rat aortic SMC with dec-RVKR-CMK alone did not modulate nuclear PKGI immunoreactivity, exposure to the PC inhibitor decreased cGMP-stimulated nuclear PKGIγ localization in many of the cells. Because previously PKGI proteolysis was observed to be critical for nuclear PKGIγ compartmentation (81), the results detailed here suggest that PC activity plays an important role in processes that regulate nuclear PKGI localization.

**DISCUSSION**

The objective of this investigation was to identify mechanisms that regulate PKGI proteolysis and nuclear localization. Inspection of a likely cleavage site in PKGI and protease database interrogation (64) revealed an amino acid sequence in PKGI isoforms that is reminiscent of a PC minimum consensus sequence (72) and is conserved in several species. Therefore, we tested the hypothesis that PCs modulate PKGI proteolysis in cells. We observed that overexpression of furin or PC5, but not of PC7, increases PKGI cleavage. Using furin as a prototypical PC and PKGI with mutations in the putative PC consensus sequence, we demonstrated that amino acids just NH2-terminal to the putative PKGI proteolysis site are required for its cleavage. In addition, we observed that PKGI proteolysis

![Fig. 7](http://ajplung.physiology.org/)

**Fig. 7.** PC inhibition decreases PC activity and proteolysis-dependent nuclear PKGIγ localization in vascular SMC. A: rat aortic SMC were serum starved and then treated in media containing 10% FBS with and without 50 μM decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-CMK), a membrane-permeable PC inhibitor. Subsequently, COOH-terminal insulin-like growth factor I receptor (IGF-IR) immunoreactivity was detected in cell lysates using a specific antibody and immunoblotting. Whereas the endoplasmic reticulum-like (ER) form of pro-IGF-IR and cleaved IGF-IRβ was detected in all cell lysates, the latter being increased with serum stimulation, the Golgi apparatus (GA) form of uncleaved pro-IGF-IR was detected in cells treated with dec-RVKR-CMK. B: rat aortic smooth muscle cells were pretreated with and without 50 μM dec-RVKR-CMK and then with and without the inhibitor and 200 μM 8-(p-chlorophenylthio)-cGMP (8-pCPT-cMP), a membrane-permeable cGMP analog. Endogenous PKGI immunoreactivity was detected using an antibody that binds to the COOH-terminal PKGI domain (PKGCR), a fluorescently tagged secondary antibody, and laser-scanning confocal microscopy with optical sections through the cell nucleus. Proteolysis-dependent nuclear PKGIγ translocation stimulated by 8-pCPT-cGMP was inhibited by treatment with dec-RVKR-CMK.
was increased by overexpression of furin in an adenocarcinoma cell line that has defective furin and decreased when PC activity was inhibited by expression of \( \alpha_1 \)-PDX. Last, in testing the role of PCs in regulating nuclear PKGI function in vascular SMC, we observed that exposure to a membrane-permeable peptide-based PC inhibitor inhibited proteolysis-dependent nuclear localization of PKGI\(_{\gamma} \) in early passage vascular SMC. Overall, these data indicate that PCs play an important role in regulating PKGI proteolysis and nuclear translocation in cultured cells.

The investigations detailed here provide new insights into key mechanisms that determine how cGMP regulates SMC phenotype. Following the studies by Garg and Hassid, which indicated that NO and cGMP decrease vascular SMC proliferation (23), much work has been devoted to elucidating the intracellular pathways through which these molecules modulate SMC phenotype. Several lines of evidence suggest that PKGI mediates the effects of NO and cGMP on cell phenotype (47). Studies indicate that cells that are deficient in PKGI, such as baby hamster kidney fibroblast (BHK) cells, and highly passaged vascular SMC acquire an undifferentiated and proliferative phenotype, whereas those in which PKGI activity is stimulated or reconstituted, such as some low-passage vascular SMC stimulated with cGMP, 3T3 fibroblasts, BHK cells, and highly passaged vascular SMC in which PKGI is expressed from plasmids or adenoviruses, appear more differentiated and less proliferative. The relationship between PKGI expression and vascular SMC phenotype has been further demonstrated in studies in which the decrease in differentiation protein markers, such as smooth muscle myosin heavy chain, vimentin, and calponin in vascular SMC with diminished PKGI expression or activity, was reversed by overexpression of PKGI (93–95). These studies have stimulated intensive efforts to identify mechanisms through which PKGI regulates cell phenotype.

The observation that PCs differentially regulate PKGI proteolysis provides insight into potential mechanisms through which they might regulate nuclear cGMP signaling. PCs exhibit substrate specificity that is based on their subcellular colocalization with and relative affinity for target proteins. Furin, PC5, and PC7 harbor a COOH-terminal transmembrane domain and a cytosolic tail that regulate their residence in the ER, GA, cytosol, and cell surface. Although active furin (11, 54) and PC5 (18) have been observed in the GA, the majority of active PC7 does not reside in that compartment. PC7 has been observed to transit from the ER directly to the plasma membrane through non-COPI-coated vesicles (68). We noted that PKGI is cleaved by furin and PC5A but not PC7. It is possible that PC7 does not cleave PKGI because plasma membrane-bound PC7 does not encounter PKGI. Also, it is possible that the catalytic domain of PC7 does not have a high affinity for PKGI. Contrasting substrate affinity between furin and PC5 and PC7 has been observed by others. For example, one study found that integrin pro-\( \alpha \)-subunits are cleaved by \( \alpha_1 \)-PDX and PC5A but not PC5B and PC7 (48). The mechanisms regulating differential PKGI proteolysis by PCs warrant further investigation.

The studies detailed here support accumulating evidence that PCs play an important role in vascular biology during health and disease (3). GA fragmentation has been observed in pulmonary arterial lesion cells isolated from animals with monocrotaline-induced lung injury (71) and from patients with idiopathic pulmonary hypertension (42). However, whether the GA disruption observed in these models inhibits proteolytic processing of proteins by GA-resident PCs has not been determined. Recent data suggest that PCs play an important role in vascular homeostasis. PCs activate several growth factors, including transforming growth factor beta-1 (21), endothelin-1, platelet-derived growth factor-B (76), and vascular endothelial growth factor-C (75), as well as extracellular matrix proteins (39) and metalloproteinases (51, 92) that have critical roles in regulating blood vessel assembly and function. Moreover, abnormalities in PC function might contribute to vascular disease. Genetic variations in furin and PC5 have been linked to hypertension and dyslipidemias (33, 44). Furin, PC5, and PC7 are detected in vascular SMC (79), and it is possible that vascular injury disrupts not only intracellular localization of PC but also their endoproteolytic function and regulation of signaling systems such as those involving PKGI.

The mechanisms through which PCs modulate PKGI proteolysis require further investigation. For example, although the GA-resident PCs furin and PC5 appear to have a role in PKGI proteolysis, it is not known whether PKGI translocation to the GA is required for PKGI proteolysis. In addition, the mechanisms regulating how PKGI traffics to the GA and how PKGI\(_{\gamma} \) leaves this organelle and enters the nucleus are not defined at this time. The identification of PCs as critical regulators of PKGI proteolysis and nuclear translocation provides a starting point for in-depth investigation of the mechanisms that regulate PKGI and PKGI\(_{\gamma} \) transport and topology.

In summary, we identified PCs as critical mediators of PKGI proteolysis and nuclear translocation in cultured cells. These findings support an important role for PCs in regulating nuclear cGMP signaling and SMC phenotype specification.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.K., R.Z., and J.D.R. conceived and designed the experiments; S.K., R.Z., and J.D.R. performed the experiments; S.K., R.Z., and J.D.R. analyzed the data; S.K., R.Z., and J.D.R. interpreted results of experiments; S.K., R.Z., and J.D.R. drafted manuscript.

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