Endogenous Protein Kinase Inhibitor γ Terminates Immediate-early Gene Expression Induced by cAMP-dependent Protein Kinase (PKA) Signaling

TERMINATION DEPENDS ON PKA INACTIVATION RATHER THAN PKA EXPORT FROM THE NUCLEUS*

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Expression of many genes induced by cAMP-dependent protein kinase (PKA) signaling is rapidly terminated. Although many mechanisms contribute to regulation of PKA signaling, members of the endogenous protein kinase inhibitor (PKI) family may be particularly important for terminating nuclear PKA activity and gene expression. Here we used both siRNA and antisense knockdown strategies to examine PKA signaling activated by parathyroid hormone or the β-adrenergic agonist, isoproterenol. We found that endogenous PKIγ is required for efficient termination of nuclear PKA activity, transcription factor phosphorylation, and immediate-early genes. Moreover, PKIγ is required for export of PKA catalytic subunits from the nucleus back to the cytoplasm following activation of PKA signaling because this is also inhibited by PKIγ knockdown. Leptomycin B blocks PKA nuclear export but has little or no effect on nuclear PKA activity or immediate-early gene expression. Thus, inactivation of PKA activity in the nucleus is sufficient to terminate signaling, and nuclear export is not required. These results were the first in any cell type showing that endogenous levels of PKI regulate PKA signaling.

Many genes are stimulated by cAMP-dependent protein kinase (PKA) signaling in an immediate-early fashion characterized by rapid but transient induction of gene transcription (1, 2). This transient gene transcription is associated with transient elevation of cAMP levels, transient PKA activation, and transient phosphorylation of both cAMP-responsive element-binding protein (CREB) and the related transcription factor, activating transcription factor-1 (ATF-1) (1, 3). A well studied example is the stimulation of immediate-early genes such as interleukin-6 (IL-6) and c-fos in osteoblasts by parathyroid hormone (PTH) (2–7). These immediate-early genes are induced in response to PTH by PKA-dependent phosphorylation of transcription factors, such as CREB and CCAAT enhancer binding protein (3, 4, 7, 8). Because these immediate-early genes mediate both the catabolic and anabolic effects of PTH (9), elucidation of the regulation of expression of these genes will substantially increase our understanding of the mechanism responsible for fine-tuning the balance between the catabolic and anabolic effects.

Transient regulation of gene transcription is most likely due to termination of one or more step(s) in the relevant signal transduction pathway. Consistent with this, multiple mechanisms contribute to precise regulation of both PKA signaling and the changes in gene expression induced by this signaling pathway (1, 3, 10). We have previously studied regulation of immediate-early gene expression following activation of PKA signaling by PTH or the β-adrenergic agonist, isoproterenol (ISO), in osteoblasts and fibroblasts (3). Those studies demonstrate that the primary mechanism responsible for termination of immediate-early gene expression acts downstream of receptor desensitization, adenyl cyclase activation, and cAMP degradation (3). Possible mechanisms therefore include inactivation of PKA itself (11), dephosphorylation or proteolysis of downstream transcription factors such as CREB (12–14), activation of inhibitory transcription factors such as inducible cAMP early repressor (15), histone deacetylation (13, 16), and co-activator methylation (17). We have focused on inactivation of PKA because it is the only one of these mechanisms that could account not only for termination of immediate-early gene transcription but also for the termination of both PKA activity and transcription factor phosphorylation that occur following activation of PKA signaling (3).

In the cytoplasm, activity of the catalytic subunit of PKA is terminated in response to reduced cAMP levels by its reassociation with regulatory subunits to form an inactive holoenzyme composed of two regulatory and two catalytic subunits (11). In contrast, the best candidates for terminating nuclear PKA catalytic subunit activity are protein kinase inhibitors (PKIs) (3, 11). The PKIs are a family of proteins that act as pseudosubstrates for PKA by binding to the PKA catalytic subunits and inhibiting their enzymatic activity (11, 18). Three PKI genes (PKIα, PKIβ, and PKIγ) have been identified (19). When PKI is overexpressed, it accumulates in the nucleus (20), binds and inactivates the catalytic subunits of PKA (11, 21–23), and transports them back to the cytoplasm (21, 23–27). This transport of PKA catalytic subunits depends on a leucine-rich nuclear export signal in PKI (26). Despite the large number of

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number(s) AY150308.

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† The abbreviations used are: PKA, cAMP-dependent protein kinase; PKI, protein kinase inhibitor; CREB, cAMP-responsive element-binding protein; ATF-1, activating transcription factor-1; IL-6, interleukin-6; PTH, parathyroid hormone; ISO, isoproterenol; BSA, bovine serum albumin; TNFα, tumor-necrosis factor α; ROS, rat osteosarcoma; PBS, phosphate-buffered saline.
Endogenous PKIγ Terminates PKA-induced Gene Expression

Endogenous PKIγ was required for efficient termination of immediate-early gene expression following activation of PKA signaling. Endogenous PKIγ was also required for export of PKA catalytic subunits from the nucleus back to the cytoplasm. However, inactivation of PKA in the nucleus by endogenous PKIγ was sufficient to terminate signaling, and nuclear export was not required. These results were the first in any cell type showing that endogenous levels of PKI regulate PKA signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—All cell culture media, supplements, and stimulators were screened for endotoxin contamination using the high sensitivity version of the colorimetric Limulus amoebocyte lystate assay (QCL-1000; Whittaker Bioproducts, Walkersville, MD) as described previously (34). Endotoxin levels were <0.004 endotoxin units/ml for the concentration of each stimulator used in the experiments. Rat ROS 17/2.8 osteoblastic osteosarcoma cells (35), murine MC3T3-E1 osteoblastic cells (36), and murine NIH3T3 fibroblastic cells (ATCC CRL 1658) were maintained as described previously (3). All cell lines were cultured in humidified atmosphere 5% CO2 and routinely passaged every 3 or 4 days.

siRNA Experiments—PKIγ cDNA from ROS 17/2.8 cells was amplified by PCR using primers 5'-GAAGAGATGCAGGAAG-3' and 5'-AGAAGATCGACAGGTC-3' based on the murine sequence (GenBankTM accession code U97100). PCR products were sequenced out RNA were used in all RT-PCR reactions. The identity of all PCR products was confirmed by sequencing core facility). Previously established stable ROS pTet-Off cells (3) were then transfected with pTRE-PKIγ-s, pTRE-PKIγ-AS, or control pTRE-Luc (Clontech) and selected with 20 μg/ml of hygromycin B (Sigma) to obtain double stable transfecant clones. In this system (40), withdrawal of tetracycline (Sigma) from the culture medium lowers the expression of sense or antisense PKIγ-mRNAs. Double stable transfecant clones were seeded into 60-mm dishes at a density of 2.5 × 10^4 cells/cm² and incubated for 3 days with tetracycline until they reached confluence. The cells were then cultured with or without tetracycline after being washed three times with PBS. After 48 h, the cells were incubated with 100 nM bPTH (1–34) in serum-free Ham’s F-12 medium containing 0.1% BSA for the indicated periods of time.

Leptomycin B Experiments—ROS 17/2.8 cells were seeded into 60-mm dishes at a density of 2.5 × 10^4 cells/cm², 24 h after reaching confluence, the cells were preincubated with or without 10 ng/ml leptomycin B (41) obtained from Minoru Yoshida (RIKEN, Wako, Japan) in serum-free Ham’s F-12 medium containing 0.1% BSA. After 1 h, 100 nM bPTH (1–34) or vehicle control (1 μg/ml acetic acid) were added and incubation continued for the indicated periods of time.

RT-PCR—mRNA levels were assessed by RT-PCR as described (2, 6). Briefly, total RNA was isolated using the TotalRALLY RNA kit (Ambion). 4 μg of total RNA were reverse transcribed to cDNA with RNase H free reverse transcriptase II (Invitrogen). The IL-6, c-fos, and actin primers were described previously (3). Additional primers were designed using Oligo 6 (Molecular Biology Insights, Inc., Cascade, CO). Murine/rat PKIγ/GAPDH primer was 5'-ATGCTAGTGTGGAAACT-3' and downstream was 5'-TTAGCTTTGAGGTCAACT-3'. RT-PCR products were amplified using 250 μM of each dNTP, 0.5 μM of each primer, 1.5 units of AmpliTaq Gold polymerase, and 1X AmpliTaq Gold buffer containing 2.0 mM MgCl₂ (Gibco BRL), 50 μM of each primer, and 1X AmpliTaq Gold buffer containing 2.5 mM Tris-HCl, pH 6.8, 50 mM KCl, 2.5 mM MgCl₂, 0.3% Triton X-100, and 0.5 mM dNTPs in a 50 μl reaction. Primers were used at a concentration of 0.1 μM. After an initial incubation at 95 °C for 2 min, the following cycles were used: 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. Following the reaction, 20 μl of the PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. bands were quantified with NIH Image software. Relative amounts of cDNA in each sample were calculated using the comparative CT method (2). Target mRNA levels were normalized to the corresponding GAPDH level.

Western Analysis—For total cell lysate preparation, cells were washed twice with ice-cold PBS and lysed with SDS lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 50 μM diethiothreitol) without bromphenol blue. Nuclear and cytosolic fractions were prepared using a nuclear and cytosolic fractionation kit (Pierce) as described previously (2). Cytosolic fractions were washed twice with PBS and then resuspended in lysis buffer (10 μg/ml of total protein) and scraped into lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.3% Nonidet P-40) containing protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitors (1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate). Cells were incubated on ice for 3 min followed by centrifugation at 5000 × g for 5 min at 4 °C. The supernatants contained the cytosolic fraction. Pellets containing cell nuclei were washed twice with lysis buffer without Nonidet P-40 and lysed with SDS lysis buffer. Nuclear and total cell lysates were sonicated on ice for 5 s three times to shear DNA and centrifuged at 12,000 × g for 10 min at 4 °C. The protein concentrations of all lysates were measured using the Bradford dye binding assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were electroblotted on SDC-PAGE gels (BioWhittaker Molecular Applications, Rockland, ME). Proteins were electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad) at 24 V for 1.5 h in 25 mM Tris-HCl, pH 8.5, 0.2 mM glycine, 20% methanol. The membranes were probed with affinity-purified rabbit polyclonal antibodies specific for AKT, CREB and phosphoCREB/phosphoATF-1 (Cell Signaling Technology, Beverly, MA), PKAα, PKAβ, and PKAγ (Santa Cruz Bio- technologies, Santa Cruz, CA), PKIγ (Clontech), PKIβ (obtained from Dr. Thomas R. Gingeras, University of Michigan (21)). The PKIγ antibody was affinity-purified using a PKIγ-maltose-binding protein fusion protein (22) (Santa Cruz Bio- technologies) and pho- phospho-anti-rabbit IgG (Cell Signaling Technology) and enhanced chemiluminescence (Amersham Biosciences).

PKA Activity Assays—Nuclear extracts were prepared for PKA as-
says as described previously (3). PKA activity was measured using Kemptide as a substrate. Briefly, 10 μl of each nuclear extract was incubated in duplicate for 5 min at 30 °C with [γ-32P]ATP (Amersham Biosciences), 50 μM Kemptide (Sigma), 100 μM ATP, 40 mM MgCl₂, 0.25 mg/ml BSA, and 50 mM Tris-HCl, pH 7.5, in the presence or absence of 1 μM PKI (6–22) amide (Sigma). Reactions were terminated by spotting 20 μl onto phosphocellulose discs, and peptide-incorporated 32P was determined by scintillation counting. PKA activity was calculated by subtracting activity measured in the presence of PKI from total activity of the nuclear extract. PKA activity was normalized to protein concentration as determined by Bradford dye binding assay.

**Statistical Analysis**—All presented RT-PCR and Western blot results are representative of at least three independent experiments. All quantitative data are presented as the mean ± S.E. of all available experiments (n = 3). Symbols without error bars represent S.E. smaller than the symbol. Statistical analyses were by analysis of variance with Fisher's protected least significant difference post hoc tests.

**RESULTS**

**PKIγ mRNA Is Strongly Expressed in ROS 17/2.8, MC3T3-E1, and NIH3T3 Cells, whereas Little or No mRNA Encoding the Other Family Members (PKIα and PKIβ) Is Expressed**—Each PKI family member has a unique pattern of tissue expression (19, 21, 29). We examined expression of mRNAs encoding each family member in rat ROS 17/2.8 osteoblastic cells (Fig. 1, A and B), murine MC3T3-E1 osteoblastic cells (Fig. 1C), and murine NIH3T3 fibroblastic cells (Fig. 1D). In all cases, PKIγ mRNA was strongly expressed, whereas little or no mRNA encoding PKIα or PKIβ was detectable (Fig. 1, A–D). The pattern of expression was not altered by activation of PKA signaling (Fig. 1, B–D). PKIγ is by far the least studied member of the PKI family, with only four citations in the literature (19, 21, 28, 43).

**PKIγ Knockdown by siRNA Substantially Delays Termination of Nuclear PKA Activity, CREB Phosphorylation, and Immediate-early Gene Expression following Stimulation with PTH**—Having shown that PKIγ is the primary member of the PKI family in osteoblastic cells, we performed knockdown experiments to determine whether PKIγ is required for termination of PKA signaling in these cells. For this purpose, rat PKIγ cDNA was sequenced (GenBank™ accession number AY150308). We found that the rat PKIγ coding region is 97.0 and 89.6% identical at the nucleotide level to murine and human PKIγ. At the amino acid level, rat PKIγ is 100 and 90.8% identical to murine and human PKIγ, respectively. Like PKIγ in other species (19, 21), the rat PKIγ sequence also contains the pseudosubstrate motif and nuclear export signal.

Five siRNA targets were selected in the coding region of rat PKIγ. Preliminary experiments showed that maximal knockdown was achieved with a mixture of the three siRNA duplexes depicted in Fig. 2A. The extent of knockdown of PKIγ by siRNA at both the mRNA and protein levels is shown in Fig. 2B, panels 1 and 2, respectively (see also Figs. 3A and 4A). Importantly, knockdown of PKIγ in these relatively short experiments did not result in compensatory up-regulation of PKIα or PKIβ mRNAs (Fig. 2B, panels 3 and 4) (see also Figs. 3A, 4A, and 5B). To assess whether endogenous PKIγ is required for termination of PKA signaling, we compared time course experiments in cultures stimulated with PTH (Fig. 2) or ISO (Fig. 3) following transfection either with PKIγ siRNA duplexes or with control siRNAs that target firefly luciferase. PKIγ knockdown by siRNA substantially delayed termination of nuclear PKA activity (Fig. 2C), phosphorylation of the transcription factors CREB and ATF-1 (Figs. 2D and 3B), and expression of the immediate-early genes IL-6 and c-fos (Figs. 2E and 3C).

The experiments just described compared cultures treated with PKIγ siRNA duplexes to cultures treated with an equal concentration of siRNA duplexes that targeted an irrelevant gene as a control for possible nonspecific effects of siRNA (39,
endogenous PKIγ knockdown by siRNA (A and B) substantially delays termination of nuclear PKA activity (C), CREB phosphorylation (D), and immediate-early gene expression (E) following stimulation with PTH. ROS 17/2.8 cells were transfected with a mixture of three siRNA duplexes (10 nM each) targeting the PKIγ coding region as depicted in panel A. Control siRNA cultures were transfected with duplexes (30 nM) targeting firefly luciferase. 24 h after transfection, cultures were incubated for the indicated times with 100 nM PTH or with vehicle control (C, 1 μM acetic acid). PKIγ knockdown was assessed by RT-PCR and by Western blotting (B), nuclear PKA activity was assessed biochemically (C), transcription factor phosphorylation was assessed by Western blotting (D), and immediate-early gene expression was assessed by RT-PCR (E). B, positive controls are total RNA from rat tissues that express PKIγ (skeletal muscle) and PKIβ (testes). C, asterisks indicate p < 0.001 as compared with the control siRNA groups at the same time points. D and E, equivalent loading is documented by examining levels of total CREB protein and actin mRNA, respectively.

PKIγ knockdown by siRNA affects expression of IL-6 and c-fos mRNAs induced specifically by PKA signaling. For this purpose, we compared the effect of PKIγ siRNA on gene expression induced by an agent that induces IL-6 and c-fos expression with a time course similar to that induced by PTH but through PKA-independent signaling pathways. TNFα was used for this purpose because it stimulates gene expression primarily through NFκB and mitogen-activated protein kinase pathways (45), and we have previously shown that a 5-min exposure to TNFα stimulates IL-6 gene expression with a time course that is indistinguishable from that induced by PTH but without PKA-dependent signaling pathways. PKIγ knockdown was assessed by Western blotting (B), nuclear PKA activity was assessed biochemically (C), transcription factor phosphorylation was assessed by Western blotting (D), and immediate-early gene expression was assessed by RT-PCR (E). B, positive controls are total RNA from rat tissues that express PKIγ (skeletal muscle) and PKIβ (testes). C, asterisks indicate p < 0.001 as compared with the control siRNA groups at the same time points. D and E, equivalent loading is documented by examining levels of total CREB protein and actin mRNA, respectively.

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An additional approach to confirm that the siRNA results are due to specific effects of PKIγ knockdown. First, we asked whether PKIγ knockdown by siRNA affects expression of IL-6 and c-fos mRNAs induced specifically by PKA signaling. For this purpose, we compared the effect of PKIγ siRNA on gene expression induced by an agent that induces IL-6 and c-fos expression with a time course similar to that induced by PTH but through PKA-independent signaling pathways. TNFα was used for this purpose because it stimulates gene expression primarily through NFκB and mitogen-activated protein kinase pathways (45), and we have previously shown that a 5-min exposure to TNFα stimulates IL-6 gene expression with a time course that is indistinguishable from that induced by PTH. TNFα did not detectably affect CREB or ATF-1 phosphorylation in either control or PKIγ knockdown cells (Fig. 4B), confirming that TNFα does not detectably activate PKA signaling. Importantly, PKIγ siRNA had no effect on expression of IL-6 or c-fos induced by TNFα (Fig. 4C). Documentation that PKIγ was efficiently knocked down in these experiments is shown at both the mRNA and protein levels (Fig. 4A). Moreover, both transcription factor phosphorylation and immediate-early gene expression were substantially increased in replicate cultures treated with PTH (Fig. 4, B and C). These results showed that PKIγ siRNA specifically affects PKA signaling and does not have detectable nonspecific effects either on phosphorylation of CREB or ATF-1 or on expression of IL-6 or c-fos.

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expression (Fig. 5D) in response to PTH. These sense and antisense transfection results, taken together with the siRNA results, strongly supported the hypothesis that endogenous PKIγ contributes to termination of PKA signaling.

PKA Catalytic Subunit Export from the Nucleus Depends on Endogenous PKIγ but Is Not Required for Termination of PKA Signaling—All three members of the PKI family contain functional CRM1-dependent, leucine-rich nuclear export signal motifs (21, 23–25). In contrast, the catalytic subunits of PKA do not contain nuclear export signal motifs themselves (24, 25) and overexpressed PKI can transport the catalytic subunits out of the nucleus (21, 23–25). We therefore asked whether endogenous levels of PKI contribute to termination of PKA signaling.

This study led to two major conclusions. First, endogenous PKIγ is necessary and sufficient for PKA catalytic subunit export from the nucleus. Second, PKIγ is necessary for nuclear export of PKA catalytic subunits, termination of PKA signaling does not depend on nuclear export. Instead, binding and inactivation of PKA by PKIγ in the nucleus is sufficient to inhibit signaling and expression of immediate-early genes.

DISCUSSION

These findings were the first in any cell type showing that PKIγ action in cells expressing endogenous levels of the protein. For this purpose, we inhibited PKIγ nuclear export using leptomycin B, which binds to CRM1 and specifically inhibits CRM1-dependent nuclear export (41, 47). As expected, leptomycin B completely blocked PKIγ nuclear export (Fig. 7A, panel 1). Leptomycin B also completely blocked nuclear export of the catalytic subunits of PKA (Fig. 7A, panels 2–4). In contrast, leptomycin B had little or no effect on termination of nuclear PKA activity (Fig. 7B), transcription factor phosphorylation (Fig. 7C), or gene expression (Fig. 7D). Thus, although PKIγ is necessary for nuclear export of PKA catalytic subunits, termination of PKA signaling does not depend on nuclear export. Instead, binding and inactivation of PKA by PKIγ in the nucleus is sufficient to inhibit signaling and expression of immediate-early genes.
endogenous PKI regulates PKA signaling.

The delayed termination of PKA signaling, transcription factor phosphorylation, and immediate-early gene expression because of PKIγ knockout demonstrates the important role of endogenous PKIγ in termination of these processes. PKIγ likely acts together with other more downstream mechanisms to maintain low levels of PKA-induced gene expression for longer time periods. For example, CREB dephosphorylation has been implicated in termination of PKA-induced gene expression in fibroblasts; however, it is controversial whether CREB dephosphorylation is primarily due to protein phosphatase-1 or to protein phosphatase-2A (reviewed in Ref. 3). An alternative mechanism for removal of phosphorylated CREB is ubiquitin-mediated proteasomal degradation (14). Such degradation may be especially relevant because activation of PKA signaling by PTH up-regulates proteasome activity (48). Our study confirmed that loss of phosphorylated CREB occurs rapidly in cells expressing wild type levels of PKIγ, although we did not investigate whether this is because of dephosphorylation or proteasomal degradation. Expression of inhibitory transcription factors, such as inducible cAMP early repressor, is another downstream mechanism that may act together with PKIγ to maintain low levels of PKA-induced gene expression (15). In this regard, PTH increases inducible cAMP early repressor (ICER) expression and ICER overexpression decreases stimulation of gene expression by PTH (49). Finally, histone deacetylation or co-activator methylation can also terminate PKA-induced gene expression (16, 17). It has recently been shown that histone deacetylase and protein phosphatase-1 form a complex that coordinately regulates CREB phosphorylation and histone acetylation (13). It is therefore interesting to speculate that PKIγ may also form complexes with molecules responsible for downstream mechanisms in order to coordinately regulate PKA-induced gene expression.

We also found that PKA catalytic subunit export from the nucleus depends on endogenous PKIγ. Moreover, inhibition of nuclear export signal activity blocks nuclear export of both PKIγ and PKA catalytic subunits despite the fact that the catalytic subunits do not contain nuclear export signal motifs (26). These results are consistent with the model that binding
to PKA catalytic subunits exposes the nuclear export signal on PKIγ, thereby inducing co-transport of the catalytic subunit-PKIγ complex out of the nucleus (25, 26). We also found that this nuclear export is not required for termination of PKA signaling and gene expression. Instead, it is likely that PKIγ-mediated nuclear export allows PKA catalytic subunits to re-associate with the regulatory subunits in the cytoplasm in preparation for subsequent rounds of cAMP signaling.

Regulation of PKA signaling and gene expression by PKIγ may have important physiological and clinical implications. For example, primary response genes induced by PKA signaling are responsible for both the catabolic and anabolic effects of PTH and β-adrenergic agonists on bone turnover (9, 50, 51). PTH dosing regimens are being developed to favor the anabolic effects of PTH over its catabolic effects as therapies for diseases that cause bone loss (52). Moreover, β-adrenergic antagonists increase bone mass in mice, and it has therefore been proposed that they may also be useful therapies for patients with bone loss (46). Thus, understanding and ultimately being able to manipulate the complex pathways that regulate the balance between the anabolic and catabolic responses induced by PTH and β-adrenergic signaling would have important clinical implications. PKIγ therefore represents a potentially important target for understanding and manipulating this balance between anabolic and catabolic responses to PTH and β-adrenergic signaling.

In summary, we found that endogenous levels of PKIγ are required for termination of both PKA signaling and immediate-early gene expression. We also found that nuclear export of PKA depends on PKIγ but is not required for termination of either PKA signaling or gene expression. Because this study focused on the role of PKI in osteoblasts, it is particularly relevant for understanding the complex balance between the catabolic and anabolic effects of PKA signaling on bone turn-

**Fig. 6.** PKA catalytic subunit export from the nucleus depends on endogenous PKIγ. PKIγ knockdown by siRNA (panels 1 and 2) blocks the export of PKA catalytic subunits Cα, Cβ, and Cγ back to the cytosol (panels 3–5) that occurs following nuclear translocation induced by PTH. Western blots were performed on nuclear and cytosolic fractions prepared from replicate cultures in the experiment described in Fig. 2. Identity and purity of the fractions were confirmed by assessing CREB as a nuclear marker (panels 6 and 7) and AKT as a cytosolic marker (panels 8 and 9) in aliquots representing equal cell numbers.

**Fig. 7.** PKA catalytic subunit export from the nucleus is not required for termination of PKA signaling. Inhibition of PKIγ export back to the cytosol by leptomycin B (LMB) (panel 1) blocks PKA catalytic subunit export back to the cytosol (panels 2–4) but does not alter termination of nuclear PKA activity (B), CREB phosphorylation (C), or immediate-early gene expression (D) following stimulation with PTH. ROS 17/2.8 cells were incubated for the indicated times with 100 nM PTH in the presence or absence of 10 ng/ml LMB. All cultures also received a mixture of vehicle controls, such that all contained 1 μM acetic acid and 0.2% ethanol. A, PKIγ and PKA levels in nuclear fractions were assessed by Western blotting. Identity and purity of the nuclear fractions were confirmed by assessing CREB as a nuclear marker (panel 5) and AKT as a cytosolic marker (panel 6) in aliquots representing equal cell numbers. B, nuclear PKA activity was assessed biochemically. There is no significant difference (p >0.28) between the two groups at any time point. C, transcription factor phosphorylation was assessed by Western blotting. D, immediate-early gene expression was assessed by RT-PCR. C and D, equivalent loading is documented by examining levels of total CREB protein and actin mRNA, respectively.
over described above. However, regulation of gene expression by PKA signaling controls diverse cellular processes in many cell types (10). It is therefore likely that endogenous levels of PKI have important effects on many of these processes.

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