

Evolutionarily Conserved Role of Calcineurin in Phosphodegron-Dependent Degradation of Phosphodiesterase 4D[∇]

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Calcineurin is a widely expressed and highly conserved Ser/Thr phosphatase. Calcineurin is inhibited by the immunosuppressant drug cyclosporine A (CsA) or tacrolimus (FK506). The critical role of CsA/FK506 as an immunosuppressant following transplantation surgery provides a strong incentive to understand the phosphatase calcineurin. Here we uncover a novel regulatory pathway for cyclic AMP (cAMP) signaling by the phosphatase calcineurin which is also evolutionarily conserved in *Caenorhabditis elegans*. We found that calcineurin binds directly to and inhibits the proteasomal degradation of cAMP-hydrolyzing phosphodiesterase 4D (PDE4D). We show that ubiquitin conjugation and proteasomal degradation of PDE4D are controlled by a cullin 1-containing E₃ ubiquitin ligase complex upon dual phosphorylation by casein kinase 1 (CK1) and glycogen synthase kinase 3 β (GSK3 β) in a phosphodegron motif. Our findings identify a novel signaling process governing G-protein-coupled cAMP signal transduction—opposing actions of the phosphatase calcineurin and the CK1/GSK3 β protein kinases on the phosphodegron-dependent degradation of PDE4D. This novel signaling system also provides unique functional insights into the complications elicited by CsA in transplant patients.

Calcineurin (also known as protein phosphatase 2B) was first identified as a calcium/calmodulin-binding protein in brain extracts (40, 57). Further analysis demonstrated that calcineurin is a widely expressed and highly conserved phosphatase (5, 19, 21, 25, 53). Calcineurin is inhibited by the immunosuppressant drug cyclosporine A (CsA) or tacrolimus (FK506) (17, 24, 43). CsA (or FK506) forms complexes with cyclophilins (or FK506-binding proteins) and inhibits calcineurin. The critical role of CsA and FK506 as immunosuppressants following transplantation surgery (16, 45, 46, 48) provides a strong incentive to understand the phosphatase calcineurin.

At the molecular level, calcineurin contributes to immune regulation by inhibiting the transcription factor NFAT (nu-

clear factor of activated T cells). The transcription factor NFAT family was first identified as an important regulator of interleukin-2 (IL-2) gene expression (23). NFAT is located in the cytosol of resting cells (20, 30). Increased intracellular calcium activates the phosphatase calcineurin, which binds to a calcineurin docking motif (PXIXIT) (4, 6, 15) and dephosphorylates NFAT. Administration of CsA to transplant patients inhibits calcineurin and blocks the nuclear translocation of NFAT. Hence, NFAT remains in the cytosol and the expression of cytokine genes such as that for IL-2 is reduced. Since IL-2 is a critical mitogen of T cells, a reduced level of IL-2 abrogates T-cell differentiation and minimizes the incidence of host-versus-graft reactions in transplant patients.

Despite the tremendous advantages of CsA and FK506 inhibition of calcineurin after transplantation surgery, prolonged treatment with CsA or FK506 produces many side effects, including diabetes mellitus, hyperlipidemia, nephrotoxicity, neuronal damage, and cardiovascular disease (3, 26, 38, 48). The current model indicates that calcineurin-NFAT signaling regulates the growth and differentiation of beta cells in the pancreatic islets (27, 28). In the presence of CsA, changes in

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insulin signaling could account for the complications found in transplant patients.

Here we uncover a novel signaling system which centers on calcineurin controlling ubiquitin conjugation and proteosomal degradation of cyclic AMP (cAMP)-hydrolyzing phosphodiesterase 4D (PDE4D). Degradation of PDE4D is channeled through a cullin 1 (Cul1)-containing E₃ ubiquitin ligase complex and is dependent on dual phosphorylation carried out by casein kinase 1 (CK1) and glycogen synthase kinase 3 β (GSK3 β). Integration of calcineurin, CK1, GSK3 β , and Cul1 is also evolutionarily conserved in *Caenorhabditis elegans*. This new signaling system expands the repertoire of calcineurin substrates and indicates that calcineurin impinges on phosphodependent protein degradation. We propose that this novel calcineurin signaling system provides new insights into alternative therapeutic targets for a range of adverse pathological complications elicited by CsA in transplant patients.

MATERIALS AND METHODS

Mice. Animal experiments were performed in accordance with the guidelines of the Albert Einstein College of Medicine Institute of Animal Studies. *CnA β ^{-/-}* mice were generated as described previously, by the insertion of a neomycin resistance gene to disrupt exon 2 (12). *CnA β ^{-/-}* mice were backcrossed with C57BL/6 mice at least 10 times before use. Backcrossed *CnA β ^{-/-}* mice were also used for the isolation of mouse embryonic fibroblasts (MEFs).

Worm strains. *C. elegans* strains were maintained under standard culture conditions on NGM agar plates with *Escherichia coli* OP50 as a food source (10). Wild-type animals were of Bristol strain N2 (a kind gift from Ji Ying Sze). Loss-of-function calcineurin *tax-6(p675lf)* (41) and PDE-4 *pde-4(ok1290)* mutants were a kind gift from Ji Ying Sze or obtained from the *Caenorhabditis* Genetics Center.

Reagents. Mouse *CnA β* was amplified by PCR from mouse mammary gland cDNA and hemagglutinin (HA) tagged using primers 5'-AGGAATTCGCCAC CATGTACCCATACGATGTTCCAGATTA CGCTGCCGCCCGGAGCCG GCC-3' and 5'-CTCGACTCGGCGACTATGGTT-3'. The PCR product was subcloned into the vector pCDNA3 using the EcoRI and XhoI sites. *C. elegans* PDE4 was amplified by PCR from plasmid KM#203 (a kind gift from Ken Miller) (13) and FLAG tagged using primers 5'-GATCGGATCCCGGCA GCCAACATGCCAGGAAGACGCGCTCGTCGTCGTCGTCG-3' and 5'-GATCGAATTCTCATTGTGTCATCATCGTCCTTATAGTCTTTGTGCTC GTCATCTTCTGTTACAGT-3'. The PCR product was subcloned into the vector pCDNA3 using the BamHI and EcoRI sites. Expression vectors for VSV-G-tagged PDE4D3, VSV-G-tagged PDE4D5, FLAG-tagged GSK3 β , HA-tagged CK1, FLAG-tagged dominant negative Cul1, HA-tagged ubiquitin, and *C. elegans* PDE4 (KM#203) have been described previously (13, 37, 44, 59, 60, 63). Point mutations were generated by the QuikChange protocol and sequenced. Recombinant PDE4D was expressed using the vector pGEX-5X1 by cloning into the BamHI and XhoI sites. Antibodies for PDE4D (sc25814 and sc25097), the VSV-G tag (sc66180), the HA tag (sc7392), the FLAG tag (F3185), calcineurin A (sc6124 and sc17808), and calcineurin B (sc6119) were obtained from Santa Cruz or Sigma. Polyclonal antibodies against phospho-Thr were obtained from Invitrogen (71-8200). Tubulin antibody (clone E7) was obtained from the monoclonal antibody facility at the University of Iowa. Polyclonal antibodies against PDE4D and *C. elegans* PDE4 were described previously (9, 13). Antibodies to phospho-Ser⁶¹⁶ were generated by using the synthetic phosphopeptide Cys-Glu-Ser-Asp-Thr-Glu-Lys-Asp-Ser(P)-Gly-Ser-Gln-Val-Glu-Glu-Asp and conjugated to keyhole limpet hemocyanin. The phosphospecific antibody was affinity purified from rabbit serum as described previously (62). The PDE inhibitors IBMX and rolipram, the calcium ionophore ionomycin (Ion), the calcineurin inhibitor CsA, the GSK3 β inhibitor lithium chloride (LiCl), the CK1 inhibitor D4476, the CK2 inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT), and the proteasome inhibitor MG132 were obtained from Calbiochem, Sigma, and/or Fisher Thermo Scientific.

Cell culture. MEFs were prepared from E13.5/14.5 embryos after trypsin digestion as described previously (61). MEFs and COS7 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml). COS7 cells were transfected using Lipofectamine (Invitrogen) according to the manu-

facturer's protocol. Cells were harvested in Triton lysis buffer (20 mM Tris [pH 7.4], 134 mM NaCl, 2 mM EDTA, 25 mM β -glycerophosphate, 2 mM NaPP_i, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 10 μ g/ml leupeptin).

Semiquantitative RT-PCR. Total RNA was isolated from MEFs using TRIzol reagent (Invitrogen). Five micrograms of RNA was reverse transcribed into cDNA using superscript II reverse transcriptase (Invitrogen). Semiquantitative reverse transcription (RT)-PCR was performed for each sample in triplicate with two different concentrations of cDNA to establish linear amplification. Twenty PCR cycles were amplified. The primers used for RT-PCR were as follows: for PDE4A, 5'-CCTGCCTGACAAGTCCAAT-3' and 5'-AGCAGAGATGACG GCAGAAT-3'; for PDE4B, 5'-ATCACCTTGCTGTGGGATTC-3' and 5'-AA CCAACCTGGGATTTTCC-3'; for PDE4D, 5'-GTCCCATGTGTGACAAG CAC-3' and 5'-CAAGTTTCAGGCTGGCTTTC-3'; for PDE7A, 5'-TGCTGC AGACGTTACTCAGG-3' and 5'-AGCATTTCAGGCTTTC-3'.

Half-life determination. The plasmid for VSV-G-tagged PDE4D3 expression was transfected into COS7 cells. Cells were treated with cycloheximide (CHX; 0.5 mM) in the presence or absence of CsA (5 μ M) for 0, 1, 2, or 4 h before harvest. The effect of the transcriptional inhibitor actinomycin D (ActD; 0.5 μ g/ml) was also examined.

Protein kinase assays. Glutathione S-transferase (GST) recombinant protein (2 μ g) encompassing either the NH₂ or COOH terminus of the catalytic core of PDE4D was incubated with purified GSK3 β or CK1 (5 U) in the presence of [γ -³²P]ATP for 30 min. Phosphorylation of PDE4D was examined by sodium dodecyl sulfate-gel electrophoresis and autoradiography.

Binding assays. The plasmid for VSV-G-tagged PDE4D3 expression was cotransfected with HA-tagged CnA β into COS7 cells. Cell extracts prepared using Triton lysis buffer were incubated with protein G-agarose and HA antibody to immunoprecipitate CnA β in coimmunoprecipitation assays. Binding assays were also performed by using GST or GST-PDE4D recombinant protein and purified calcineurin. After three washes with Triton lysis buffer, the bound proteins were detected by immunoblot analysis.

Statistical analysis. The data shown are means \pm standard errors of the means. The Student *t* test was performed, and *P* values of <0.05 were considered to be significant.

RESULTS

Posttranscriptional regulation of PDE4D expression by calcineurin. We initially set out to determine whether calcineurin contributes to the regulation of cAMP-hydrolyzing PDE4D, given that targeted disruption of calcineurin led to metabolic complications with dysregulation in the β -adrenergic G-protein-coupled receptor (GPCR)/cAMP signaling pathway (data available on request). Among the 11 PDE families, PDE4 was chosen because of its widespread expression in different cells and tissues (18, 32). Indeed, PDE4 accounts for the bulk of the total cAMP-hydrolyzing PDE activity in primary MEFs (11). Furthermore, PDE4 can be regulated upon phosphorylation by protein kinase A (PKA) and/or ERK (29, 55), which are downstream effectors of the β -adrenergic receptor signaling pathway.

Unexpectedly, we found that the levels of PDE4D expression were potentiated upon coexpression with CnA β (Fig. 1). Increased protein expression was found in at least two different members of the PDE4D family, including PDE4D3 and PDE4D5 (Fig. 1A). CnA β , however, affected neither the level of expression of exogenous β -arrestin 1 (β Arr-1) nor the levels of expression of endogenous tubulin and insulin growth factor receptor. These data indicate that CnA β regulates PDE4D3 and PDE4D5 expression.

We further ascertained calcineurin regulation of PDE4D expression by using the calcium ionophore ionomycin to activate calcineurin and the calcineurin inhibitor CsA (Fig. 1B and C). In the presence of ionomycin, the expression levels of endogenous PDE4D in MEFs were potentiated (Fig. 1B). The

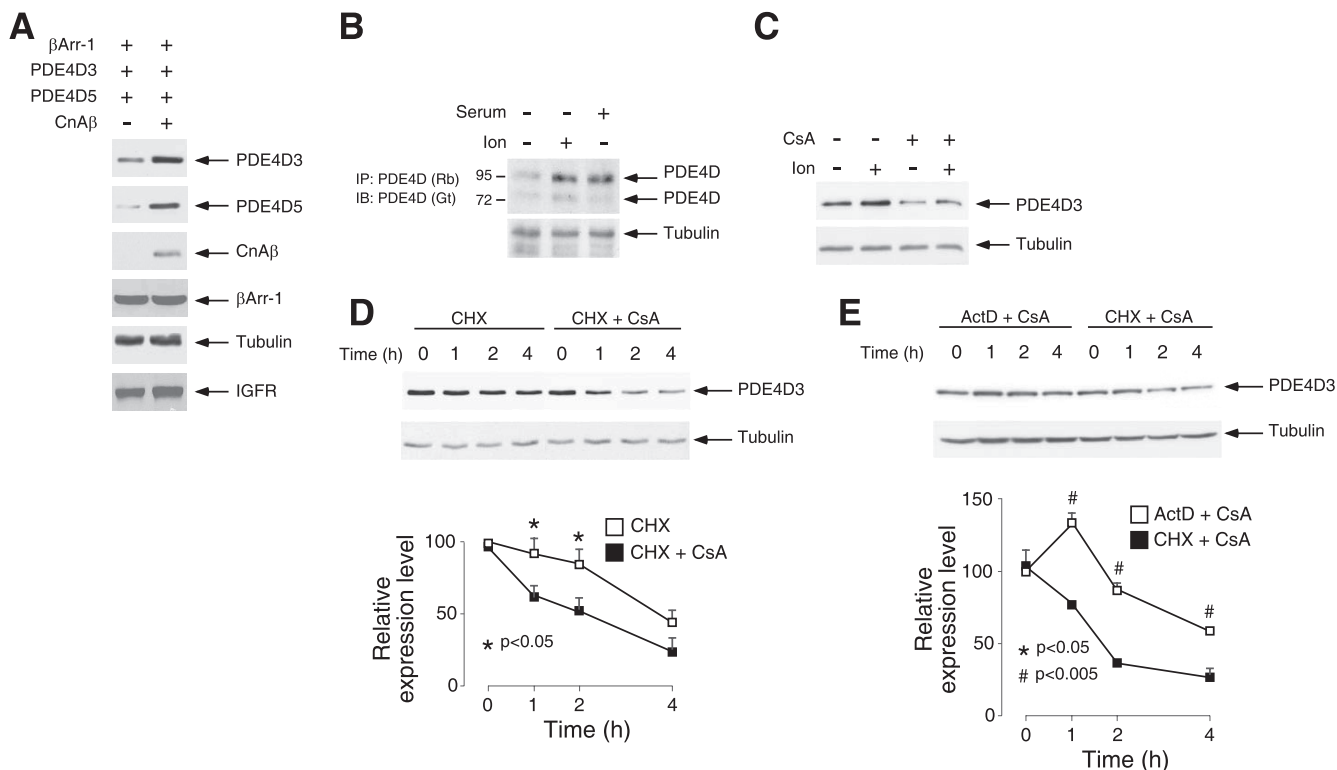


FIG. 1. Posttranscriptional regulation of PDE4D expression by calcineurin. (A) Extracts prepared from COS7 cells transiently transfected with PDE4D3, PDE4D5, or βArr-1 were examined by immunoblot analysis. The effect of coexpression of CnAβ is also shown. Expression of endogenous IGF-1 receptor (IGF-1) and tubulin is shown as a control. (B) Expression of endogenous PDE4D in MEFs was detected by immunoprecipitation (IP) using rabbit (Rb) polyclonal PDE4D antibody (sc25814) and subsequent immunoblot analysis (IB) using goat (Gt) polyclonal PDE4D antibody (9). The effect of the calcium ionophore ionomycin and mitogenic stimuli (20% fetal calf serum) is also shown. (C) Extracts prepared from COS7 cells transiently transfected with PDE4D3 were treated with the calcium ionophore Ion (2 μM) and/or the calcineurin inhibitor CsA (5 μM). Expression of PDE4D3 was determined by immunoblot analysis. (D) COS7 cells were transiently transfected with PDE4D3. The half-life of PDE4D3 was determined in the presence CHX (0.5 mM). The effect of CsA (5 μM) is also shown. Expression of tubulin was used as a control. (E) COS7 cells were transiently transfected with PDE4D3. The half-life of PDE4D3 was determined in the presence of CsA (5 μM). The effect of the transcription inhibitor ActD (0.5 μg/ml) or the translational inhibitor CHX (0.5 mM) was examined. *, *P* < 0.05; #, *P* < 0.005.

expression levels of endogenous PDE4D were also potentiated by serum stimulation, which induced a Ca²⁺ influx (Fig. 1B). Administration of CsA, however, reduced PDE4D3 expression (Fig. 1C). Notably, CsA abrogated the potentiation of PDE4D3 expression elicited by ionomycin (Fig. 1C). These data confirm that calcineurin regulates PDE4D expression.

To investigate the role of calcineurin in PDE4D protein expression, we determined the half-lives of PDE4D3 in the presence and absence of CsA (Fig. 1D). In the presence of the translational inhibitor CHX, we noted that the half-life of PDE4D3 was ~4 h (Fig. 1D). Administration of CsA reduced the half-life of PDE4D3 to ~1 h (Fig. 1D). These data indicate that calcineurin regulates the degradation of the PDE4D3 protein.

Given the prominent role of calcineurin in transcription regulation (20, 30), we asked whether the role of calcineurin in PDE4D3 potentiation was also transcription dependent (Fig. 1E). Unlike the effect of a translation inhibitor, administration of CsA in the presence of the transcription inhibitor ActD did not further reduce the half-life of PDE4D3 (Fig. 1E). These data indicate that calcineurin regulates the posttranscriptional degradation of the PDE4D3 protein.

Ubiquitin-mediated degradation of PDE4D via a phosphodegron motif. Ubiquitin conjugation and subsequent proteasome-mediated degradation play a critical role in protein turnover (49, 50). We have identified a potential phosphodegron (DSGSQVEED⁶²³) located in the COOH-terminal conserved catalytic core of PDE4D (Fig. 2A). In general, a phosphodegron (e.g., DpSGX₂₋₄pS) promotes the polyubiquitin-mediated degradation of phosphoproteins via the Skp1-Cul-F box receptor (SCF) E₃ ubiquitin ligase complex (34, 36). Phosphodependent degradation of PDE4D has also been implicated in a recent genome-wide identification of Cul1 substrates (65, 66). The location of the phosphodegron in the COOH-terminal conserved catalytic core further suggests that all PDE4D isoforms are regulated similarly.

Here we show that PDE4D degradation can be channeled through the polyubiquitin-dependent proteasome pathway (Fig. 2B). Indeed, in the presence of the proteasome inhibitor MG132, degradation was reduced while ubiquitin conjugation of PDE4D was increased (Fig. 2B). Notably, expression of dominant negative Cul1 (ΔCul1), which blocks recruitment and subsequent protein degradation mediated by the SCF E₃ ubiquitin ligase complex, also reduced PDE4D degradation

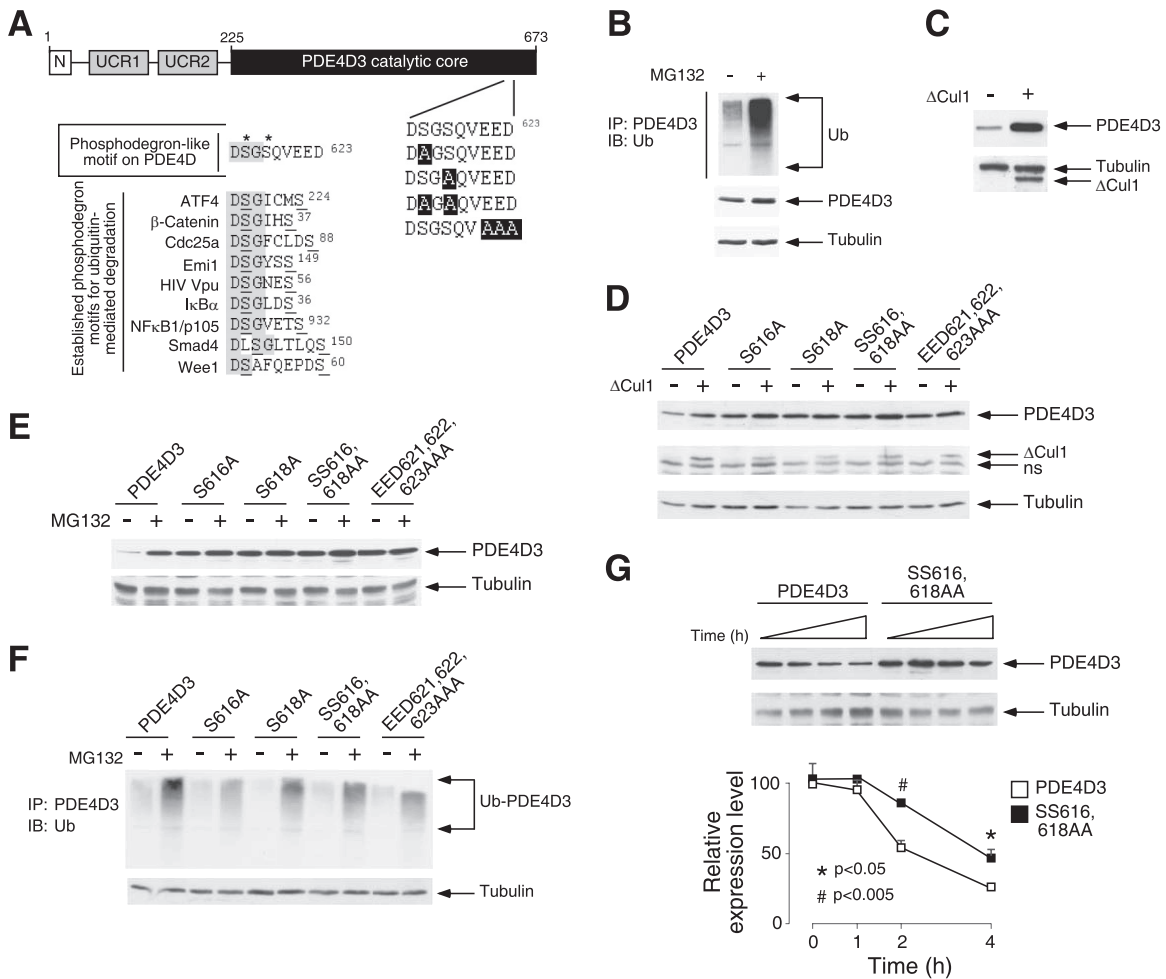


FIG. 2. Ubiquitin-mediated degradation of PDE4D via a phosphodegron motif. (A) Schematic illustration of the location of the DSGSQVEED phosphodegron in PDE4D3. Sequences of known phosphodegrons are also shown. Conserved amino acid residues and phosphorylated Ser are highlighted and underlined, respectively. A phosphodegron-like motif (DSGSQVEED) and potential phosphorylation sites (Ser⁶¹⁶ and Ser⁶¹⁸) in PDE4D3 are also shown. (B) COS7 cells were transiently transfected with PDE4D3. Ubiquitin conjugation of PDE4D3 was determined by coimmunoprecipitation assays. The presence of ubiquitin in PDE4D3 immunoprecipitates (IP) was analyzed by immunoblot (IB) analysis. The effect of the proteasome inhibitor MG132 (10 μ M) is also shown. (C) COS7 cells were transiently transfected with PDE4D3. The expression levels of PDE4D3 in the presence and absence of dominant negative Cul1 (Δ Cul1) were determined by immunoblot analysis. (D) COS7 cells were transiently transfected with wild-type or phosphodegron-mutated (Ala⁶¹⁶, Ala⁶¹⁸, Ala^{616,618}, or Ala^{621,622,623}) PDE4D3. The expression levels of wild-type and phosphodegron-mutated PDE4D3 were determined in the presence and absence of Δ Cul1. (E) COS7 cells were transiently transfected with wild-type or phosphodegron-mutated (Ala⁶¹⁶, Ala⁶¹⁸, Ala^{616,618}, or Ala^{621,622,623}) PDE4D3. The expression levels of wild-type and phosphodegron-mutated PDE4D3 were determined in the presence and absence of the proteasome inhibitor MG132 (10 μ M). (F) COS7 cells were transiently transfected with wild-type or phosphodegron-mutated (Ala⁶¹⁶, Ala⁶¹⁸, Ala^{616,618}, or Ala^{621,622,623}) PDE4D3. Ubiquitin conjugation of wild-type and phosphorylation-defective PDE4D3 was determined by coimmunoprecipitation assays. The presence of ubiquitin in PDE4D3 immunoprecipitates was analyzed by immunoblot analysis. The effect of the proteasome inhibitor MG132 (10 μ M) is also shown. (G) COS7 cells were transiently transfected with wild-type or phosphodegron-mutated (Ala^{616,618}) PDE4D3. The half-lives of wild-type and phosphorylation-defective PDE4D3 were determined in the presence of CHX (0.5 mM) and CsA (5 μ M). *, $P < 0.05$; #, $P < 0.005$.

(Fig. 2C). These data indicate that the degradation of PDE4D is mediated by a Cul1-containing SCF E₃ ubiquitin ligase complex.

Next, we determined the role of the phosphodegron in PDE4D degradation (Fig. 2D to F). Phosphodegron-defective PDE4D (Ala⁶¹⁶, Ala⁶¹⁸, Ala^{616,618}, or Ala⁶²¹⁻⁶²³) exhibited increased expression compared to the wild type (Fig. 2D to F). In the presence of CHX, the half-life of phosphodegron-defective PDE4D was potentiated (Fig. 2G). Expression of Δ Cul1 reduced PDE4D degradation (Fig. 2D). The effect of Δ Cul1 on PDE4D degradation, however, was abrogated in phosphode-

gron-defective PDE4D (Fig. 2D). These data indicate that degradation of PDE4D by the SCF E₃ ubiquitin ligase complex is phosphodegron dependent.

Reduced degradation of phosphodegron-defective PDE4D in the presence of the proteasome inhibitor MG132 was also confirmed. In the presence of MG132, the expression and ubiquitin conjugation of PDE4D were potentiated (Fig. 2E and F). Phosphodegron-defective PDE4D, however, exhibited reduced ubiquitin conjugation despite potentiated expression (Fig. 2E and F). Together, these data indicate that the phosphodegron is required for ubiquitin-mediated degradation of

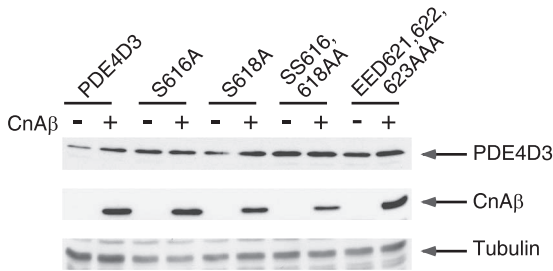


FIG. 3. Calcineurin regulation of PDE4D degradation is phosphodegrogen dependent. Wild-type or phosphodegrogen-mutated (Ala⁶¹⁶, Ala⁶¹⁸, Ala^{616,618}, or Ala^{621,622,623}) PDE4D3 was coexpressed with CnAβ in COS7 cells. The expression levels of PDE4D3 and CnAβ were determined by immunoblot analysis. The expression level of tubulin was used as a control.

PDE4D via a Cul1-containing SCF E₃ ubiquitin ligase complex.

Calcineurin regulation of PDE4D degradation is phosphodegrogen dependent. Given that calcineurin potentiates PDE4D expression (Fig. 1), we therefore asked whether calcineurin regulation is also phosphodegrogen dependent (Fig. 3). Expression of calcineurin reduced PDE4D degradation (Fig. 3). The effect of calcineurin on PDE4D degradation, however, was abrogated in phosphodegrogen-defective PDE4D (Fig. 3). Re-

duced degradation of phosphodegrogen-defective PDE4D in the presence of pharmacological agents that modulate endogenous calcineurin activity was also confirmed (data available on request). Administration of the calcium ionophore ionomycin reduced, while the calcineurin inhibitor CsA potentiated, PDE4D degradation (data available on request). The effect of ionomycin and CsA on PDE4D degradation, however, was abrogated in phosphodegrogen-defective PDE4D (data available on request). These data illuminate the role of calcineurin, via the phosphodegrogen, on PDE4D protein degradation.

Mapping of functional PxIXIT-like calcineurin docking motifs in PDE4D. Previous studies established that calcineurin interacts with its substrates via a PXIXIT docking motifs (4, 6, 15). For example, conserved PXIXIT docking motifs are found in all four calcineurin-regulated NFAT family members (Fig. 4A), which play an important role in cytokine gene transcription and contribute significantly to the success of transplant surgery (30). Replacement of the PXIXIT motif with Ala attenuates the dephosphorylation of NFAT by calcineurin (4, 6, 15). In addition, the PXIXIT-containing peptide domain acts in a dominant negative fashion and interferes with calcineurin interaction and subsequent dephosphorylation (4, 6, 15), thereby inhibiting NFAT-mediated cytokine gene transcription. Similar PXIXIT motifs are found in calcineurin substrates in yeast (Fig. 4A) (21), indicating an evolutionarily conserved

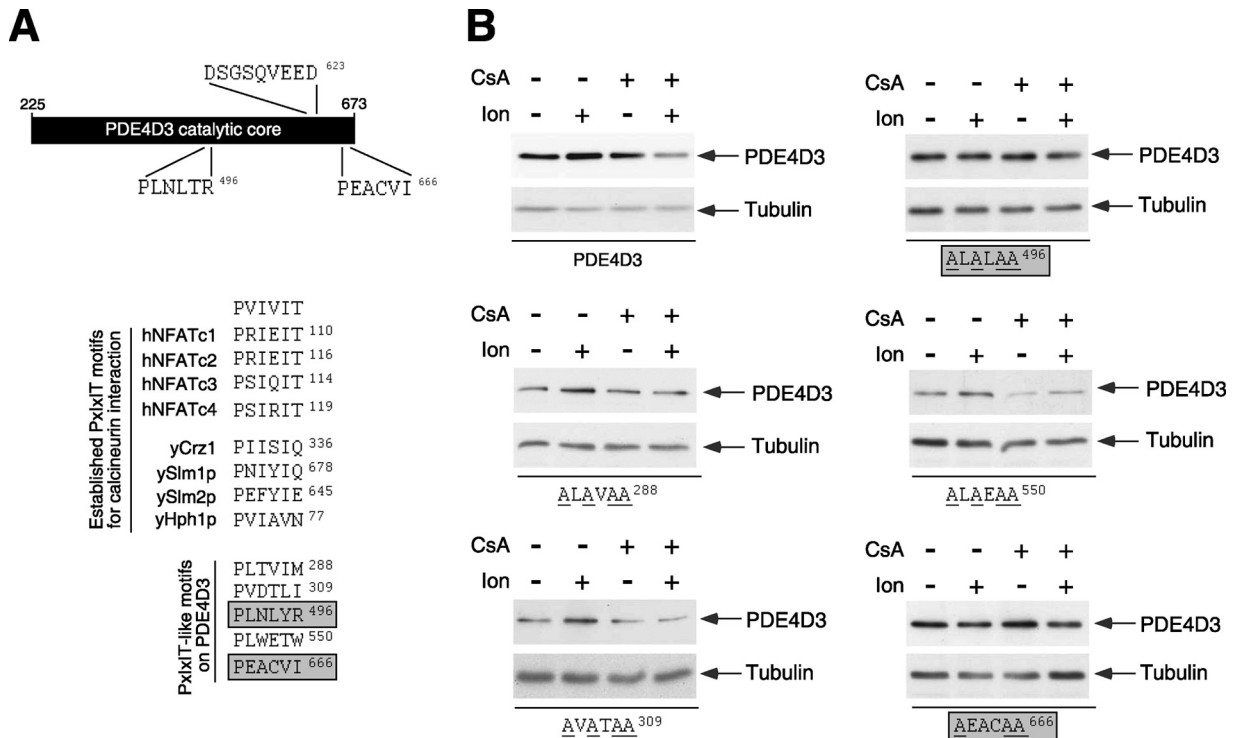


FIG. 4. Mapping of functional PXIXIT-like calcineurin docking motifs in PDE4D. (A) Schematic illustration of the locations of the two functional PXIXIT-like motifs (PLNLYR⁴⁹⁶ and PEACVI⁶⁶⁶) in the catalytic core of PDE4D3. The location of the DSGSQVEED phosphodegrogen in PDE4D3 is also indicated. A sequence comparison of known calcineurin docking PXIXIT motifs found in human NFAT and yeast proteins is shown. Potential PXIXIT-like motifs in PDE4D3 and an optimized PXIXIT motif (PVIVIT) are also shown. Functional PXIXIT-like motifs in PDE4D3 are highlighted. (B) Wild-type and PXIXIT-mutated PDE4D3 were transiently transfected into COS7 cells. The expression levels of wild-type and PXIXIT-mutated PDE4D3 were determined in the presence and absence of Ion (2 μM) and/or CsA (5 μM). The expression of tubulin was used as a control. Replacements of critical amino acid residues with Ala in potential PXIXIT-like motifs of PDE4D3 are underlined. Functional PXIXIT-like motifs in PDE4D3 are highlighted.

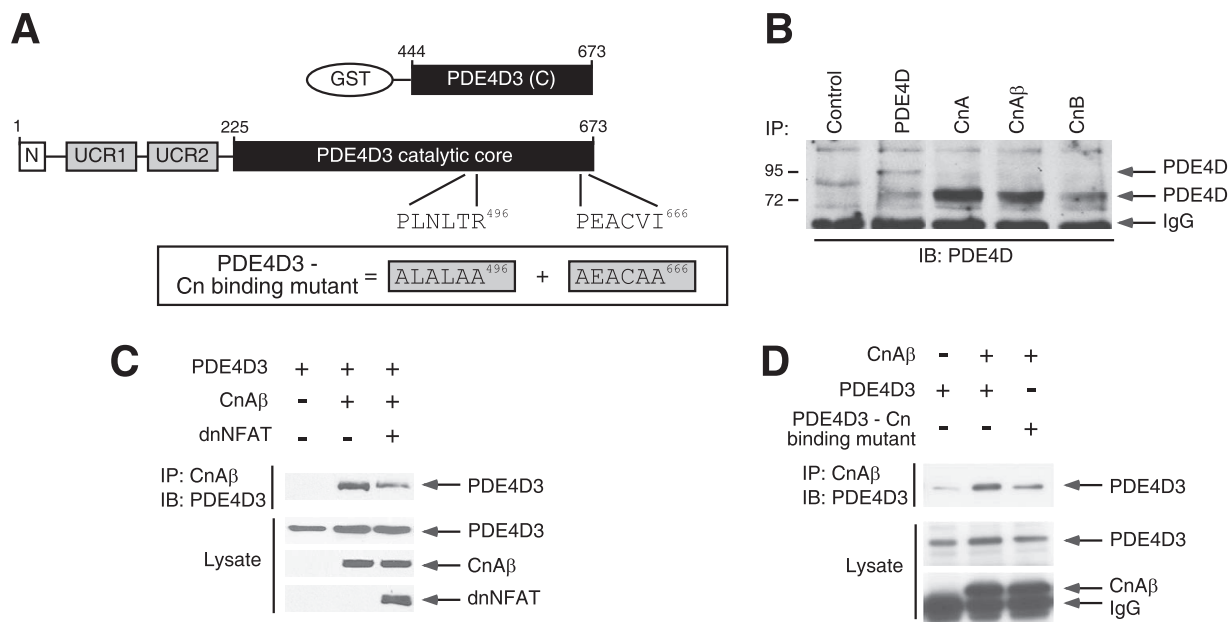


FIG. 5. Direct interaction between calcineurin and PDE4D. (A) Schematic illustration of PDE4D3 and PDE4D3 GST fusion protein. Functional calcineurin docking motifs (PLNLTR⁴⁹⁶ and PEACVI⁶⁶⁶) are indicated. Calcineurin binding-defective PDE4D3 (ALALAA⁴⁹⁶ and AEACAA⁶⁶⁶) is also shown. (B) Binding of endogenous PDE4D and calcineurin was determined by coimmunoprecipitation assays. Endogenous PDE4D in COS7 cells was immunoprecipitated (IP) using goat polyclonal PDE4D antibody (sc25097), mouse monoclonal calcineurin A antibody (sc17808), goat polyclonal calcineurin Aβ antibody (sc6124), or goat polyclonal calcineurin B antibody (sc6119). The presence of endogenous PDE4D in calcineurin immunoprecipitates was analyzed by immunoblot (IB) analysis using rabbit polyclonal PDE4D antibody (sc25814). Mouse M2 monoclonal antibody was used as a control. (C) PDE4D3 was coexpressed with CnAβ in COS7 cells. Binding of PDE4D3 and CnAβ was determined by coimmunoprecipitation assays. The presence of PDE4D3 in CnAβ immunoprecipitates and in cell extracts was analyzed by immunoblot analysis. The effect of the PXIXIT-containing calcineurin docking inhibitor dnNFAT is also shown. (D) Wild-type and calcineurin binding-defective PDE4D3 were coexpressed in the presence and absence of CnAβ in COS7 cells. The presence of PDE4D3 in CnAβ immunoprecipitates and in cell extracts was analyzed by immunoblotting.

enzyme-substrate mechanism mediated by calcineurin docking via the PXIXIT motif.

We have identified several putative PXIXIT-like calcineurin docking motifs in the COOH-terminal conserved catalytic core of PDE4D (Fig. 4A). We surmised that some of these motifs are functional and are critical in mediating the effect of calcineurin on PDE4D degradation. Thus, we performed site-directed mutagenesis to replace consensus amino acids of the PXIXIT-like motif with Ala (AXAXAA) and tested whether the expression of the Ala-substituted PDE4Ds was regulated by ionomycin or CsA (Fig. 4B). Immunoblot analysis indicated that PLNLTR⁴⁹⁶ and PEACVI⁶⁶⁶ are functional PXIXIT-like calcineurin docking motifs in PDE4D (Fig. 4B). Replacement of PLNLTR⁴⁹⁶ or PEACVI⁶⁶⁶ with Ala abrogated the stabilization of PDE4D expression by ionomycin (Fig. 4B). Similarly, increased degradation of PDE4D in the presence of CsA was attenuated in Ala-substituted PLNLTR⁴⁹⁶ or PEACVI⁶⁶⁶ PDE4D (Fig. 4B). These data indicate that there are two functional PXIXIT-like calcineurin docking domains in PDE4D. The location of these functional calcineurin docking domains in the COOH-terminal conserved catalytic core of PDE4D implies that all PDE4D isoforms are subject to similar regulation.

Direct interaction between calcineurin and PDE4D. Next, we examined whether calcineurin interacts with PDE4D and whether such interaction requires the two identified PXIXIT-like motifs (PLNLTR⁴⁹⁶ and PEACVI⁶⁶⁶) (Fig.

5A). Coimmunoprecipitation assays showed that endogenous calcineurin interacts with endogenous PDE4D (Fig. 5B). Notably, the calcineurin-PDE4D interaction was attenuated by coexpression of a PXIXIT-containing calcineurin docking inhibitor, dnNFAT (6, 15) (Fig. 5C). Furthermore, the calcineurin-PDE4D interaction was impaired upon the replacement of critical residues in the PXIXIT-like motifs with Ala (ALALAA⁴⁹⁶ and AEACAA⁶⁶⁶) in PDE4D (Fig. 5D). Finally, binding assays using purified proteins supported the concept that calcineurin interacts directly with PDE4D (data available on request).

CK1 or GSK3β phosphorylates the PDE4D phosphodegron.

Interaction with and stabilization of PDE4D by calcineurin suggest that the phosphodegron motif (DSGSQVEED⁶²³) might be phosphorylated. We therefore sought protein kinases that might counterbalance the role of calcineurin in PDE4D stabilization (Fig. 6). Common Ser/Thr protein kinases that preferentially phosphorylate target sites encompassing acidic amino acid residues include CK1, CK2, and GSK3β. Phosphorylation mediated by GSK3β and/or CK1 can promote protein degradation (34, 60). Since integration of calcineurin, GSK3β, and CK1 regulates NFAT activation (7, 67), we asked whether GSK3β and/or CK1 phosphorylate PDE4D.

In vitro protein kinase assays indicated that GSK3β and CK1 phosphorylated the COOH-terminal portion of the PDE4D catalytic core (Fig. 6A and B), which encompasses the DSGSQVEED phosphodegron. Replacement of Ser⁶¹⁶ and/or

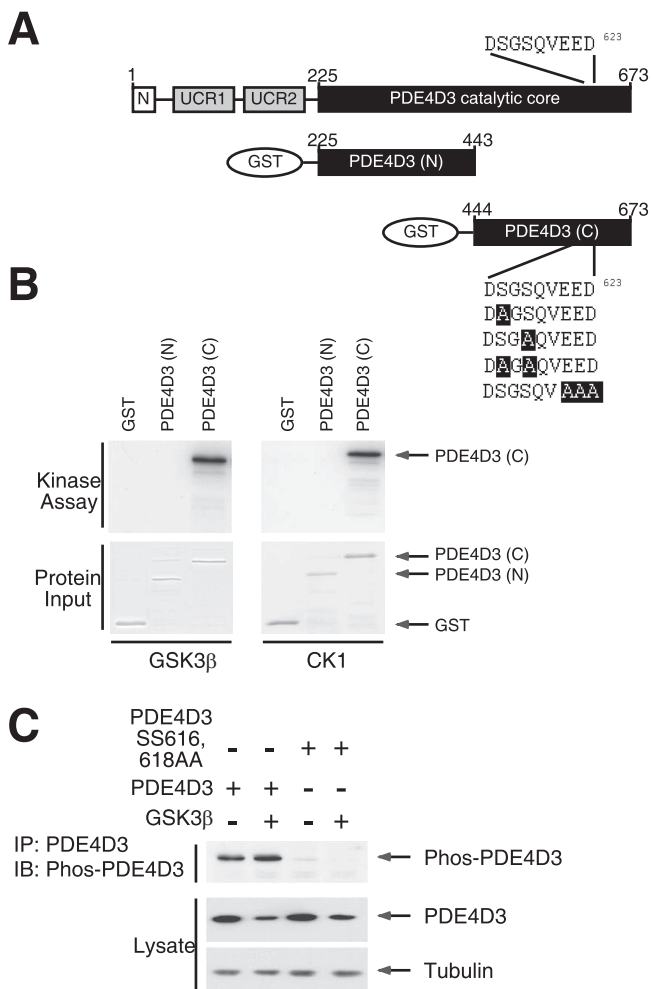


FIG. 6. CK1 and GSK3β phosphorylate PDE4D phosphodegron. (A) Schematic illustration of PDE4D3 and PDE4D3 GST fusion protein. The location of the DSGSQVEED phosphodegron in PDE4D3 are also shown. Replacements of critical amino acid residues in the DSGSQVEED phosphodegron of PDE4D3 with Ala are highlighted. (B) GST recombinant proteins encompassing either the NH₂-terminal or the COOH-terminal portion of the catalytic core of PDE4D3 were subjected to *in vitro* protein kinase assays in the presence of recombinant GSK3β or CK1 and [γ-³²P]ATP. Autoradiography and Coomassie blue staining of recombinant PDE4D3 are shown. (C) COS7 cells transiently transfected with wild-type or Ala^{616,618} mutant PDE4D3. The effect of GSK3β was also examined. Phosphorylation of Ser⁶¹⁶ of PDE4D3 was assessed by phospho-PDE4D antibody (Phos-PDE4D) in immunoblot analysis. Expression of PDE4D3 and tubulin is also shown.

Ser⁶¹⁸ in the phosphodegron with Ala reduced PDE4D phosphorylation mediated by GSK3β or CK1 (data available on request). Replacement of Glu⁶²¹, Glu⁶²², and Asp⁶²³ with Ala also reduced GSK3β- or CK1-mediated PDE4D phosphorylation (data available on request), indicating that these acidic amino acid residues are critical for optimal phosphorylation. These data indicate that CK1 and GSK3β phosphorylate Ser⁶¹⁶ and/or Ser⁶¹⁸ at the phosphodegron in PDE4D.

To elucidate the phosphorylation of the phosphodegron of PDE4D *in vivo*, we developed phosphospecific antibodies to detect phospho-Ser⁶¹⁶. Immunoblotting analyses demon-

strated phosphorylation of PDE4D3, which increased in intensity upon the coexpression of GSK3β (Fig. 6C). Notably, GSK3β reduced the expression of PDE4D3, despite increased phosphorylation of PDE4D3. The replacement of Ser^{616,618} of PDE4D3 with Ala, however, abrogated the detection of PDE4D3 by the phosphospecific antibodies (Fig. 6C). In the presence of MG132 to inhibit proteosomal degradation, phosphorylated PDE4D3 accumulated (data available on request). Together, these data confirm phosphorylation at the phosphodegron of PDE4D3 *in vivo*. These data also support the concept that phosphorylation of PDE4D at the phosphodegron is a prerequisite for subsequent degradation via the proteosome.

CK1 or GSK3β promotes PDE4D degradation via the phosphodegron motif. Given that calcineurin reduces PDE4D degradation, we asked whether GSK3β or CK1 mediates the opposing effect and potentiates PDE4D degradation (Fig. 7). Coexpression of either GSK3β or CK1 potentiated the degradation of PDE4D (Fig. 7A). Expression of exogenous βArr-1 and endogenous tubulin, however, was not affected by GSK3β or CK1 coexpression (Fig. 7A). We further ascertained that endogenous GSK3β or CK1 regulates PDE4D degradation using the protein kinase inhibitors LiCl and D4476, respectively (Fig. 7B). In the presence of LiCl or D4476, degradation of PDE4D was reduced (Fig. 7B). Inhibition of CK2 with DMAT did not affect the degradation of PDE4D (Fig. 7B). These data demonstrate that the endogenous protein kinase GSK3β or CK1 phosphorylates PDE4D and promotes its degradation.

Next, we examined whether GSK3β or CK1 regulation of PDE4D is phosphodegron dependent (Fig. 7C and D). Expression of GSK3β or CK1 potentiated PDE4D degradation (Fig. 7C and D). The effect of GSK3β or CK1 on PDE4D degradation, however, was abrogated in phosphodegron-defective PDE4D (Fig. 7C and D). Conversely, administration of the inhibitor LiCl or D4476 to block endogenous protein kinase activity reduced PDE4D degradation (data available on request). The effect of LiCl or D4476 on PDE4D degradation, however, was abrogated in phosphodegron-defective PDE4D (data available on request). These data demonstrate that protein kinase GSK3β or CK1 phosphorylates PDE4D and promotes its degradation via the phosphodegron.

Evolutionarily conserved regulation of PDE4D degradation via calcineurin, CK1, and GSK3β. Both PDE4 and calcineurin are highly conserved in evolution from *C. elegans* to mammals (18, 21, 41, 53) (Fig. 8A). Indeed, we have identified similar phosphodegron and calcineurin docking motifs in the *C. elegans* PDE4 counterpart (cePDE4) (Fig. 8A). In addition, we have recently found that ablation of CnAβ led to sustained β-adrenergic receptor/PKA activation (data available on request). We found that sustained elevation of cAMP in the absence of CnAβ was due in part to impaired cAMP hydrolysis (data available on request).

Next, we asked whether, in analogy to calcineurin inhibition by CsA, the expression of PDE4D is altered upon ablation of CnAβ. Immunoblot analysis indicated reduced expression of PDE4D upon the ablation of CnAβ (Fig. 8B). Expression of PDE4D, as well as PDE4A and PDE4B, mRNA in *CnAβ*^{+/+} and *CnAβ*^{-/-} cells, however, was indistinguishable (data available on request). These data indicate that, similar to calcineurin

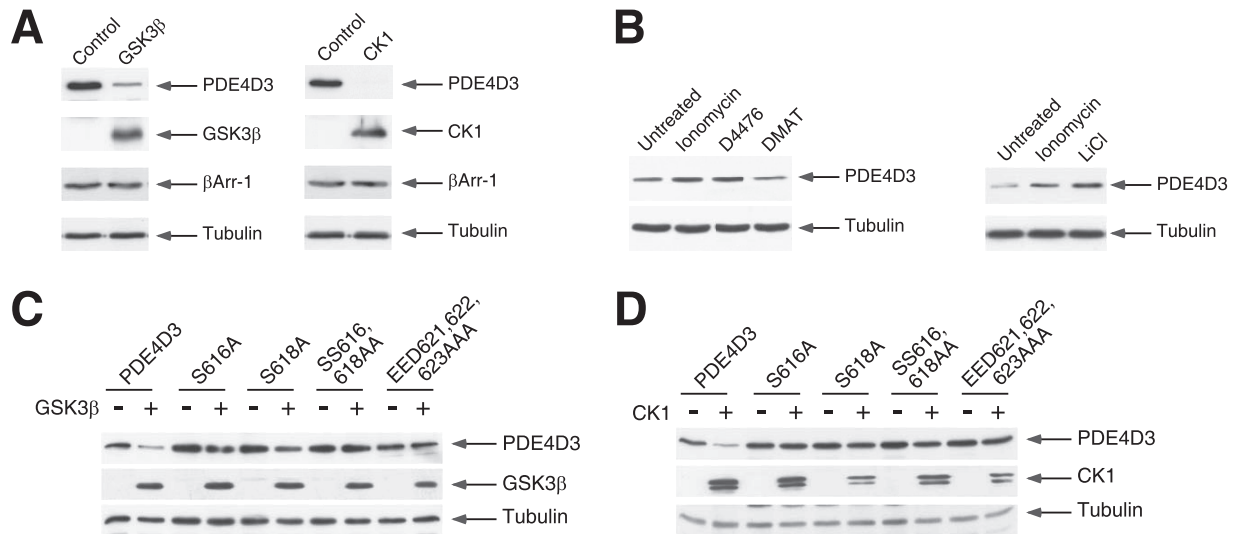


FIG. 7. CK1 and GSK3 β promote PDE4D degradation. (A) PDE4D3 or β Arr-1 was coexpressed with GSK3 β or CK1 in COS7 cells. The expression levels of PDE4D3, β Arr-1, GSK3 β , and CK1 were determined by immunoblot analysis. The expression level of tubulin was used as a control. (B) PDE4D3 was transiently expressed in COS7 cells. The expression levels of PDE4D3 in the presence and absence of protein kinase inhibitors for 4 h were determined by immunoblot analysis. The effect of Ion (2 μ M) is also shown. The protein kinase inhibitors administered included the GSK3 inhibitor LiCl (20 mM), the CK1 inhibitor D4476 (1 μ M), and the CK2 inhibitor DMAT (400 nM). (C and D) Wild-type or phosphodegrom-mutated (Ala⁶¹⁶, Ala⁶¹⁸, Ala^{616,618}, or Ala^{621,622,623}) PDE4D3 was coexpressed with GSK3 β (C) or CK1 (D) in COS7 cells. The expression levels of PDE4D3, GSK3 β , and CK1 were determined by immunoblot analysis. The expression level of tubulin was used as a control.

inhibition, ablation of CnA β reduces PDE4D expression at the posttranscriptional level.

To further demonstrate the relevance of the calcineurin-PDE4D signaling cross talk, we used *C. elegans* as a model system. Immunoblot analysis demonstrated that the expression of endogenous cePDE4 was reduced in worms bearing a loss-of-function mutation in calcineurin [*tax-6(p675lf)*] (Fig. 8C) (41). Evolutionarily conserved regulation of PDE4 by calcineurin indicates the importance of this novel signaling cross talk.

At the molecular level, similar to that of mammalian PDE4D, degradation of cePDE4 was channeled through the Cul1-containing SCF E₃ ubiquitin ligase complex (data available on request). Degradation of cePDE4 was reduced by either expression or activation of calcineurin (data available on request). Administration of CsA to inhibit calcineurin, however, potentiated cePDE4 protein degradation (data available on request). Expression of either CK1 or GSK3 β protein kinase also potentiated cePDE4 degradation (Fig. 8D). Notably, expression of CK1 or GSK3 β increased the phosphorylation of cePDE4, despite the reduced cePDE4 expression in the presence of these protein kinases (Fig. 8D). Together, these data demonstrate that regulation of PDE4 protein degradation by Cul, calcineurin, CK1, and GSK3 β is evolutionarily conserved.

DISCUSSION

Calcineurin in cell signaling regulation. In this report, we uncover a novel calcineurin signaling system (Fig. 9). We find that calcineurin regulates phosphodependent degradation of PDE4D, a widely expressed cAMP-degrading PDE that is well known to underpin key functions of cAMP signaling (18, 33). We demonstrate that calcineurin binds directly to PDE4D in

order to oppose the novel action of the CK1 and GSK3 β protein kinases in phosphorylating a phosphodegrom motif in PDE4D. Such phosphorylation by CK1 and GSK3 β allows the SCF E₃ ubiquitin ligase to promote ubiquitin conjugation, which then targets PDE4D for proteosomal degradation. Intriguingly, our study of *C. elegans* indicates that calcineurin regulation of PDE4D degradation by a phosphodegrom is evolutionarily conserved, supporting the importance of this novel calcineurin-PDE4D signaling system in GPCR/cAMP signal transduction. Thus, in addition to subcellular compartmentalization and phosphorylation-dependent activation (18, 33), controlling the stability of PDE4D by targeted ubiquitination involving CK1 and GSK3 β phosphorylation and subsequent recruitment of the SCF E₃ ubiquitin ligase complex provides a novel, evolutionarily conserved control point at which to regulate cAMP signaling in cells.

Our studies also expand the repertoire of calcineurin function in regulating protein stability via phosphodegroms. Identification of substrates targeted for phosphodegrom-mediated degradation has been proven difficult because of complex phosphorylation events for substrate recognition. These substrates are often phosphorylated at multiple sites and low in abundance, as they are rapidly degraded once phosphorylated. Therefore, identification of these substrates becomes a "catch-22" event, as phosphorylation induces binding to the SCF adaptors, which facilitate ubiquitin conjugation and promote degradation. Conversely, interaction with and subsequent dephosphorylation by calcineurin will increase the protein stability of some phosphodegrom-mediated substrates. Future systematic investigations to identify such substrates will provide new insights for the pleiotropic effect of calcineurin.

The opposing actions of the phosphatase calcineurin and the CK1/GSK3 β protein kinases on phosphodegrom-dependent

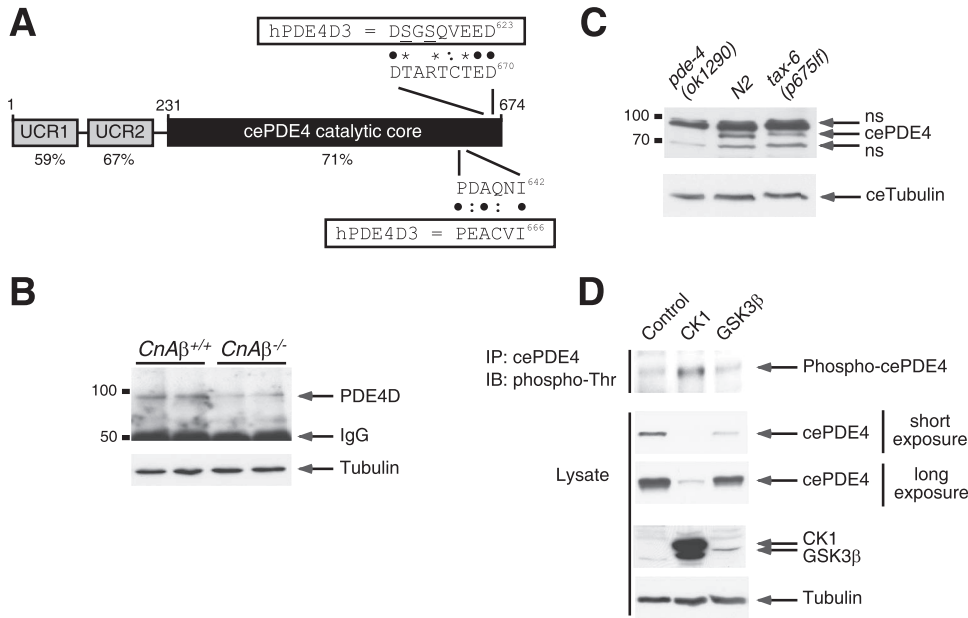


FIG. 8. Evolutionarily conserved regulation of PDE4D degradation via calcineurin, CK1, and GSK3 β . (A) Schematic illustration of *C. elegans* PDE4 (cePDE4). The locations of the potential phosphodegron and calcineurin docking motif are also shown. Sequence alignment and percent identity with human PDE4D (hPDE4D) are indicated. Bullets indicated conserved amino acid residues. Colons indicated similar amino acid residues. Asterisks indicated potential phosphorylation sites. (B) Extracts prepared from *CnA β ^{-/-}* and *CnA β ^{+/+}* mouse skeletal muscle were subjected to immunoprecipitation and immunoblot analysis to determine the endogenous expression of PDE4D. The expression level of tubulin was used as a control. (C) Extracts prepared from N2 wild-type control *C. elegans* and loss-of-function calcineurin mutant worms [*tax-6(p675lf)*] were subjected to immunoblot analysis to determine the endogenous expression of cePDE4. Extracts prepared from PDE4 null worms [*pde-4(ok1290)*] were used to show the specificity of the cePDE4 antibody. The expression level of *C. elegans* tubulin was used as a control. (D) cePDE4 was transiently cotransfected with either CK1 or GSK3 β into COS7 cells. Phosphorylation of cePDE4 was determined by immunoprecipitation (IP) using M2 monoclonal antibody against FLAG-tagged cePDE4 and subsequent immunoblotting (IB) analysis using phospho-Thr polyclonal antibodies. The expression levels of cePDE4, CK1, GSK3 β , and tubulin are also shown.

degradation of PDE4D are analogous to their roles in regulating the nucleocytoplasmic shuttling of NFAT transcription factors. Coordinated regulation by the phosphatase calcineurin (and CK1/GSK3 β protein kinases) on NFAT-mediated transcription and on PDE4D protein stability would elicit intricate biological responses. For example, cAMP-dependent protein kinase (PKA) opposes calcineurin-mediated nuclear NFAT accumulation (7, 14). Stabilization of PDE4D, and hence dampening of PKA activation via increasing cAMP hydrolysis, would facilitate nuclear accumulation of NFAT upon activation of calcineurin. Induction of NFAT target genes (e.g., T-cell mitogens) and downregulation of cAMP concentration/localization in specific cellular compartments by calcineurin may also contribute to overall T-cell function and proliferation (1, 2, 56). A similar role for calcineurin in NFAT and PDE4D regulation may also be found in endocrine secreting cells, such as adipocytes and pancreatic β cells, where NFAT mediates the expression of secretory factors (e.g., adiponectin, resistin, and insulin) (28, 61), while PDE4D regulates exocytic release via compartmentalized cAMP signaling (58).

Calcineurin in posttransplantation complications. Our findings on calcineurin regulation of the stability of PDE4D have clinical significance in providing a new perspective on the molecular pathology of posttransplantation complications elicited by the widely used calcineurin inhibitor and immunosuppressant CsA. Suppression of immune function by CsA during and after transplant surgery minimizes the incidence of host-ver-

sus-graft reactions in transplant patients and increases the success of organ transplantation. Many of these transplant patients, however, develop posttransplantation complications even without a history of such pathologies. Posttransplantation complications include a broad range of pathologies, including insulin resistance, hyperlipidemia, hypertension, and nephro- and neurotoxicity (3, 26, 38, 48). Their etiology, however, remains elusive.

Current models suggest that CsA disrupts the mitochondrial permeability transition pore function and causes cellular dysfunction in β cells (22). CsA also inhibits calcineurin (54), which dephosphorylates the transcription factor NFAT (17, 35). Calcineurin-NFAT signaling is critical for pancreatic β -cell growth and adipokine gene transcription (28, 61). Upon calcineurin inhibition by CsA, dysregulated pancreatic function and loss of insulin sensitivity can account for the metabolic complications found in transplant patients.

The effect of sustained elevation of cAMP upon the loss of calcineurin function in stabilizing PDE4D expression can also contribute to the adverse pathological complications found in CsA-treated transplant patients. The diverse functions of GPCR signal transduction and an array of effectors regulated by the second messenger cAMP, e.g., cAMP-dependent protein kinase (PKA) (39, 47), cAMP-gated ion channels (8), and cAMP-regulated guanine nucleotide exchange factors (Epac) (31), provide a wide base to account for the pathologies found in CsA-treated transplant patients. Notably, GPCR antago-

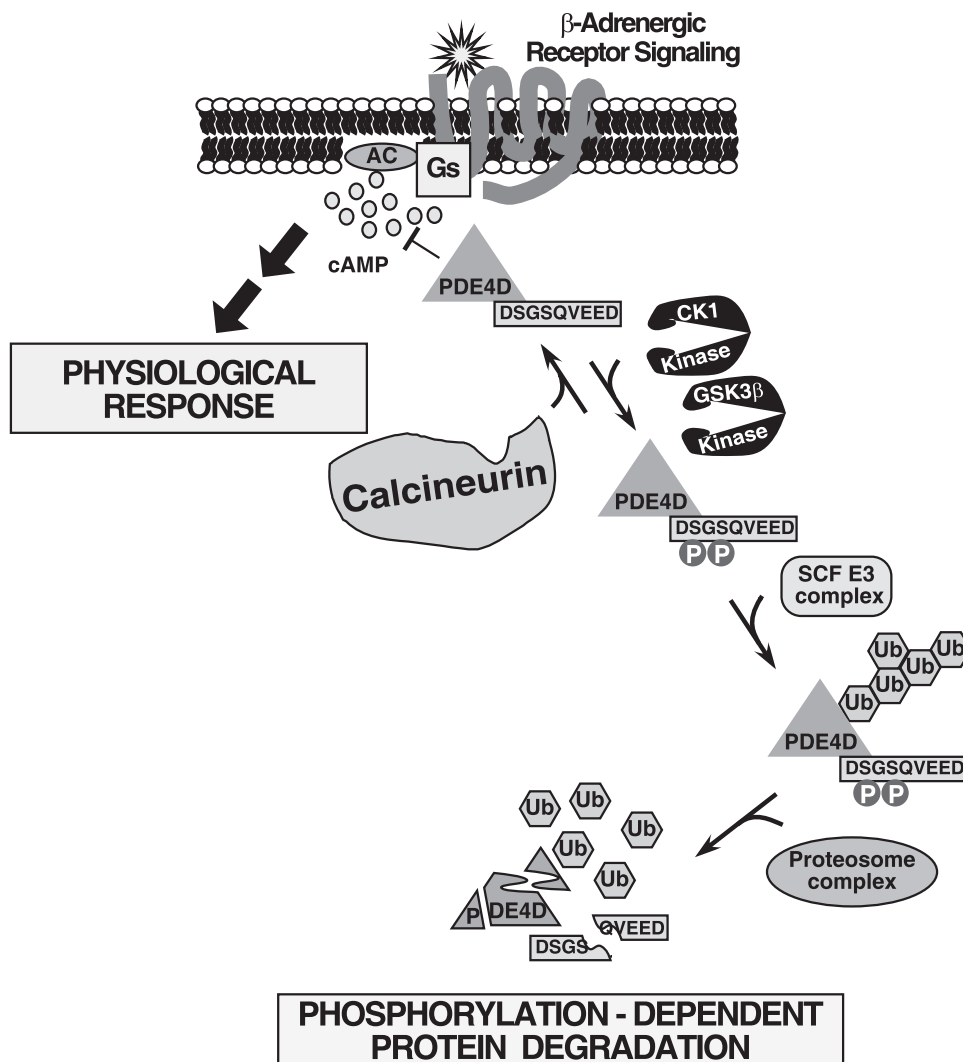


FIG. 9. Model of the phosphatase calcineurin in the β -adrenergic signaling pathway via regulation of PDE4D degradation. PDE4D encompasses a DSGSQVEED phosphodegron. Phosphorylation at the phosphodegron by the protein kinases GSK3 β and CK1 promotes PDE4D degradation, which is mediated by the SCF E3 ubiquitin (Ub) ligase complex. Ubiquitinated PDE4D was degraded by the proteasome complex. Conversely, dephosphorylation mediated by the phosphatase calcineurin opposes PDE4D degradation. Thus, phosphorylation-dependent degradation controls the expression level of PDE4D, which in turn regulates the intracellular concentration of the second messenger cAMP upon activation of the β -adrenergic receptor signaling pathway—a key component of the control of diverse physiological responses. The calcineurin/ubiquitin/PDE4D signaling axis is also evolutionarily conserved in *C. elegans*, indicating its important role in critical cellular processes to regulate cAMP duration. We propose that potentiation of the G_s -coupled signaling pathway due to sustained elevation of cAMP, which is a consequence of reduced PDE4D expression in the absence of calcineurin function, accounts for the metabolic complications (e.g., hyperlipidemia and hemodynamic dysregulations) found in transplant patients treated with CsA. AC, adenylyl cyclase.

nists and calcium channel blockers, seemingly unrelated to calcineurin function, have shown certain success in attenuating pathological complications elicited by CsA (42, 51, 52, 64). We propose that metabolic complications found in transplant patients treated with CsA may be a consequence of not only pancreatic dysregulation via the insulin pathway but also of potentiation of the GPCR signaling pathway due to sustained elevation of cAMP.

Conclusions. In conclusion, we have uncovered a novel, evolutionarily conserved calcineurin signaling system that regulates SCF-mediated ubiquitin conjugation and proteosomal degradation of PDE4D upon dual phosphorylation by CK1 and GSK3 β . The calcineurin/ubiquitin/PDE4D signaling system

impinges on diverse cellular processes by regulating cAMP responses. This new calcineurin signaling system provides new insights and points to novel therapeutic targets for a range of adverse pathological complications elicited by CsA in transplant patients.

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