Protein Kinase D Promotes *in Vitro* Osteoclast Differentiation and Fusion[*]

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Background: The role of PKD signaling in osteoclasts has not been reported previously.
Results: Inhibition of PKD expression or activity impaired formation of multinucleated osteoclasts from mononucleated precursors.
Conclusion: PKD activity is required for differentiation of osteoclast progenitors into mature osteoclasts.
Significance: PKD may be involved in pathological and physiological bone resorption.

Although PKD is broadly expressed and involved in numerous cellular processes, its function in osteoclasts has not been previously reported. In this study, we found that PKD2 is the main PKD isoform expressed in osteoclastic cells. PKD phosphorylation, indicative of the activated state, increased after 2–3 days of treatment of bone marrow macrophages with M-CSF and RANKL, corresponding to the onset of preosteoclast fusion. RNAi against PKD2 and treatment with the PKD inhibitor CID755673 showed that PKD activity is dispensable for induction of bone marrow macrophages into tartrate-resistant acid phosphatase-positive preosteoclasts in culture but is required for the transition from mononucleated preosteoclasts to multinucleated osteoclasts. Loss of PKD activity reduced expression of DC-STAMP in RANKL-stimulated cultures. Overexpression of DC-STAMP was sufficient to rescue treatment with CID755673 and restore fusion into multinucleated osteoclasts. From these data, we conclude that PKD activity promotes differentiation of osteoclast progenitors through increased expression of DC-STAMP.

Osteoclasts are large multinucleated cells responsible for breaking down bone (1). They do this as part of the repair mechanism in response to fracture or other mechanical trauma and in response to physiological cues such as systemic hypocalcemia. Osteoclasts are derived from cells of the monocyte/macrophage lineage. Stimulation of monocytes with M-CSF and RANKL (receptor activator of nuclear factor kappa-B ligand) directs them first toward a preosteoclast stage, characterized as mononucleated cells that express osteoclast marker genes such as *Nfatc1* and tartrate-resistant acid phosphatase (TRAP).[4] Mononucleated preosteoclasts subsequently fuse to give rise to multinucleated osteoclasts through a process that involves proteins such as DC-STAMP, MMP9, and V-ATPase V_0 subunit d2 (2). The osteoclast then binds tightly to bone and secretes acid and proteases that degrade the inorganic mineral and organic protein components of bone. Under physiological conditions, this process is closely coupled to bone formation to maintain proper bone structural integrity. Excessive bone resorption contributes to the bone-destructive pathologies of postmenopausal osteoporosis, arthritis, multiple myeloma, and other bone malignancies. Although much progress has been made, the intracellular signal transduction mechanisms governing osteoclast differentiation remain incompletely understood.

PKD consists of three related serine/threonine kinases (PKD1, PKD2, and PKD3) that are placed in the CAMK family of kinases (reviewed in Ref. 3). They have been implicated in numerous cell types with diverse cellular processes, including gene expression, cell adhesion and motility, Golgi-vesicle fission and trafficking, cellular proliferation, and apoptosis. PKD activity is stimulated by growth factors, hormones, and neurotransmitters, most commonly acting as second messengers downstream of diacylglycerol (DAG) and PKC (4). Monocytes, key lineage precursors of osteoclasts, are reported to express primarily PKD2, which is involved in their migration (5). However, the expression status and functional role of PKD during osteoclastogenesis have not been directly characterized.

The goal of this study was to determine the expression and functions of PKD in osteoclast differentiation and bone-resorptive activity. We found that PKD, primarily PKD2, is expressed in cells of the osteoclast lineage. We used overexpression, functional inhibition, and shRNA gene knockdown strategies to determine that PKD promotes differentiation of TRAP-positive preosteoclasts into multinucleated osteoclasts through regulation of DC-STAMP expression.

[4] The abbreviations used are: TRAP, tartrate-resistant acid phosphatase; DAG, diacylglycerol; BMM, bone marrow macrophage; DMSO, dimethyl sulfoxide; Ad5, adenovirus type 5; qRT-PCR, quantitative RT-PCR; HDAC, histone deacetylase.

EXPERIMENTAL PROCEDURES

Primary Osteoclast Cultures—Mouse primary osteoclasts were cultured as described previously (6). Briefly, bone marrow was flushed from mouse tibiae and femurs, treated with red blood cell lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA, pH 7.4), and cultured in α-minimal essential medium supplemented with 10% FBS and 10 ng/ml M-CSF on tissue culture-treated plastic. After overnight incubation, non-adherent bone marrow monocytes were collected and replated onto 24-well plates in α-minimal essential medium, 10% FBS, and 10 ng/ml M-CSF for a further 48 h to generate bone marrow macrophages (BMMs). Osteoclast differentiation was then stimulated by addition of 10 ng/ml M-CSF and 60 ng/ml RANKL. For inhibitor studies, cells were treated with 0.05% dimethyl sulfoxide (DMSO) or an equal volume of CID755673 or Gö 6976 (both from EMD Millipore) dissolved in DMSO to give the indicated final concentrations.

TRAP Staining—Cultures were fixed for 15 min in 4% paraformaldehyde in PBS and stained with 0.3 mg/ml Fast Red Violet, 0.1 mg/ml naphthol AS-MX, and 30 mM sodium tartrate in acetate buffer, pH 5.0. Cells were then counterstained with DAPI and photographed using phase-contrast and epifluorescence microscopy to facilitate counting nuclei.

Resorption Assay—Bone marrow monocytes were cultured on 24-well osteo assay plates (Corning). After differentiating for 5 days in standard α-minimal essential medium supplemented with M-CSF and RANKL, the cell medium was replaced with α-minimal essential medium, pH 7.0, M-CSF, and RANKL supplemented with 0.05% DMSO or 30 μM CID755673 for an additional 48 h. Cells were removed by incubation with 5% bleach for 5 min. Resorbed surface was photographed and analyzed using NIH ImageJ.

Apoptosis Assay—Bone marrow monocytes were cultured as described above. After differentiating for 3 or 4 days in M-CSF and RANKL, DMSO or 30 μM CID755673 was added, and the cultures were incubated overnight. The following day, caspase activity was measured using the Caspase-Glo® 3/7 assay (Promega) according to manufacturer's instructions.

RT-PCR—Total RNA was prepared from sets of three replicates using TRIzol (Invitrogen) according to the manufacturer's recommended protocol. cDNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) and amplified using iQ SYBR Green Supermix. Gene expression levels were normalized to GAPDH. For inhibitor studies, gene expression is presented relative to the mean expression in DMSO-treated cells.

Western Blotting—Cell lysates were prepared in modified radioimmune precipitation assay buffer supplemented with Halt™ protease and phosphatase inhibitor mixture (Pierce), resolved on 8% SDS-polyacrylamide gel, transferred to PVDF membranes, and subjected to immunoblotting. Membranes were blocked in TBS/Tween 20 and 5% BSA and incubated with primary antibodies against PKD1 (Cell Signaling), PKD2 (EMD Millipore), PKD3 (Bethyl Laboratories), phospho-PKD Ser-744/748 (Cell Signaling), phospho-PKD Ser-916 (Cell Signaling), HA (Sigma), and actin (Santa Cruz Biotechnology). Proteins were visu-

alized using HRP-conjugated secondary antibodies and ECL Plus chemiluminescent substrate (GE Healthcare).

Lentiviral Gene Suppression—Two lentiviral vectors encoding shRNAs against PKD2 and a control shRNA, together with a GFP marker, were purchased from Open Biosystems and used to produce replication-incompetent lentivirus according to the manufacturer's protocols. Viral stocks were titrated by infection in HeLa cells and assessed by GFP fluorescence. These stocks were used to infect murine primary osteoclasts. Following infection, primary osteoclast cultures were stimulated with M-CSF and RANKL, and after 5 days, cells were either harvested for RNA and protein or fixed and stained for TRAP.

Construction of Adenovirus Expressing DC-STAMP—The adenovirus type 5 (Ad5) vector expressing full-length *DC-STAMP* cDNA contains the CMV promoter-driven *DC-STAMP* transgene cassette inserted in place of the deleted E1 region of a common Ad5 vector. The full-length *DC-STAMP* cDNA was cloned into the pShuttle-CMV plasmid (7). The resultant plasmid, pShuttle-CMV-*DC-STAMP*, was linearized by PmeI digestion and subsequently cotransformed into *Escherichia coli* BJ5183 with the RGD fiber-modified Ad backbone plasmid (pAdEasy-RGD). After selection of recombinants, the recombinant DNA was linearized by PacI digestion and transfected into 911 cells to generate Ad-DC-STAMP. The virus was propagated in HEK293 cells, dialyzed in PBS with 10% glycerol, and stored at −80 °C. Titering was performed with an optical density-based measurement to determine the number of viral particles/ml. An identical replication-incompetent CMV promoter-driven luciferase expression vector (control virus) was used as a control vector.[5]

Adenovirus Infection—BMMs were isolated as described above. Prior to beginning stimulation with RANKL, the cells were incubated with adenovirus at a multiplicity of infection of 50 viral particles/cell of DC-STAMP-expressing or control adenovirus for 3 h at 37 °C in the presence of M-CSF. After 3 h, the adenovirus was removed from the osteoclasts, and the cells were treated with M-CSF and RANKL plus CID755673 or DMSO. Five days later, RNA was extracted for use in real-time quantitative RT-PCR (qRT-PCR), protein was extracted for Western blotting, or cells were stained for TRAP.

Statistical Analysis—Significance between groups was analyzed by Student's *t* test, with *p* values of 0.05 or less considered significant.

RESULTS

PKD Is Expressed and Active during Osteoclast Differentiation—To begin characterizing the role of PKD in osteoclasts, we determined the expression pattern of the three PKD isoforms during osteoclast differentiation. BMMs were generated by treatment of bone marrow monocytes with M-CSF for 48 h. Further stimulation with M-CSF and RANKL gave rise to TRAP-positive mononucleated preosteoclasts after 3 days and mature multinucleated osteoclasts after 5–6 days. The expected course of osteoclast differentiation was monitored by

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[5] Detailed information about the procedures used to construct the RGD fiber-modified Ad5 backbone plasmid (pAdEasy-RGD) and Ad control vector is available upon request.
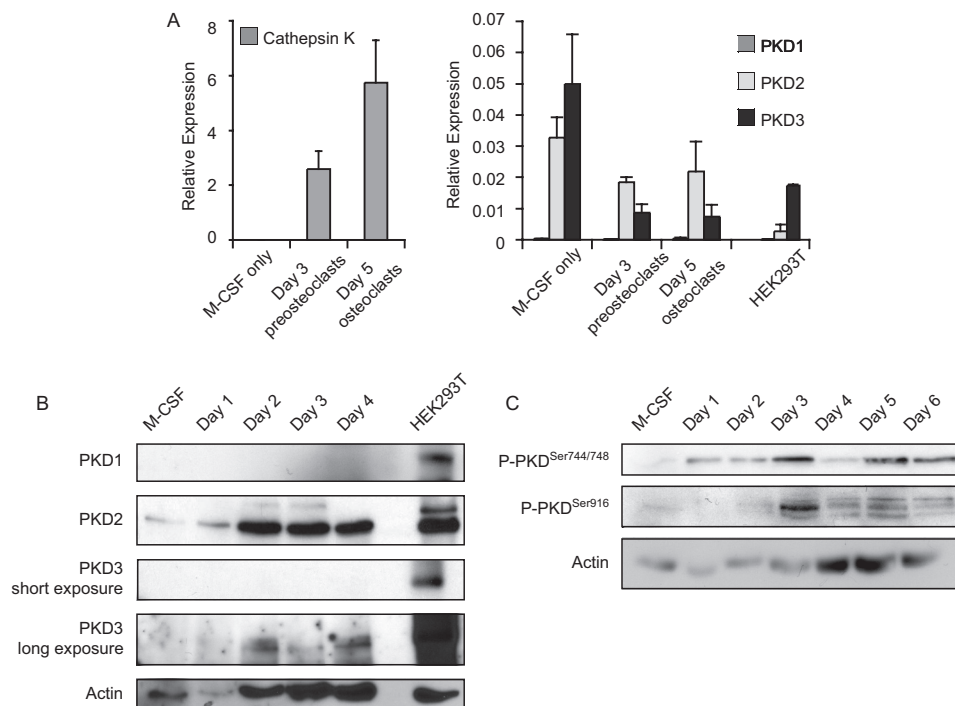
FIGURE 1. **PKD expression in osteoclastic cells.** *A*, qRT-PCR analysis of cathepsin K (*left*) and PKD1, PKD2, and PKD3 (*right*) mRNA expression in BMM cultures treated with M-CSF only or stimulated with M-CSF and RANKL for 3 days (preosteoclasts) and 5 days (osteoclasts) and in HEK293T cells. *B*, Western blot analysis of PKD1, PKD2, and PKD3 protein expression in osteoclast cultures grown in M-CSF only or in M-CSF and RANKL for the indicated times or in HEK293T cells. *C*, Western blotting with phospho-PKD Ser-744/748 (*P-PKD$^{Ser744/748}$*) and phospho-PKD Ser-916 (*P-PKD^{Ser916}*).

light microscopy (data not shown) and by qRT-PCR against the osteoclast marker gene *Ctsk* (c̲a̲t̲h̲e̲p̲s̲i̲n̲ K̲), which showed a strong increase during the culture period (Fig. 1*A*). qRT-PCR indicated that PKD2 was most highly expressed, with a lower level of PKD3 and very low levels of PKD1 expression in osteoclasts (Fig. 1*A*). A similar expression pattern was detected by Western blotting, with PKD2 and PKD3 (but not PKD1) expression detected in osteoclasts (Fig. 1*B*). In both assays, HEK293T cells were used as a positive control. PKD kinase activity is stimulated by phosphorylation of the kinase domain activation loop at Ser-744 and Ser-748 (using the amino acid numbering of PKD1). Active PKD then catalyzes phosphorylation at Ser-916 in PKD1 and PKD2, a residue not conserved in PKD3, thereby providing a further measure of PKD catalytic activity. Western blot analysis revealed an increase in phospho-PKD Ser-744/748 and Ser-916 at the preosteoclast stage at day 3 (Fig. 1*C*). Together, these data indicate that PKD proteins, particularly PKD2, are present and activated during osteoclast differentiation.

PKD Inhibition Does Not Alter Induction of TRAP-positive Mononucleated Preosteoclasts—We next asked whether treatment with the PKD inhibitor CID755673 inhibited differentiation of BMMs into preosteoclasts. BMM cultures were stimulated with M-CSF and RANKL for 3 days in the presence of DMSO vehicle or CID755673 at 10 and 30 μM. TRAP staining showed that each treatment group contained a similar number of TRAP-positive mononucleated cells (Fig. 2, *A* and *B*), whereas staining with DAPI revealed no significant change in the total number of nuclei (Fig. 2*C*). Similarly, we detected little change in expression of osteoclast markers *Nfatc1*, c-*fos*, or

Rank (Fig. 2*D*). Expression of *Rank* was reduced by 50% with 10 μM CID755673 ($p = 0.0004$), but was not significantly reduced with 30 μM CID755673. From these observations, we conclude that PKD inhibition has little effect on induction of TRAP-positive preosteoclasts by RANKL and M-CSF.

PKD Inhibition Decreases Maturation of Preosteoclasts into Multinucleated Osteoclasts—To further assess whether PKD regulates osteoclastogenesis, we continued culture of BMMs with M-CSF and RANKL in the presence of DMSO vehicle or CID755673 for 5–6 days, at which point the DMSO-treated cultures showed numerous large multinucleated osteoclasts (Fig. 3*A*). Cultures treated with 10 or 30 μM CID755673 showed a trend toward fewer TRAP-positive multinucleated cells with three or more nuclei (Fig. 3*B*). The CID755673-treated TRAP-positive osteoclasts were much smaller and had fewer nuclei compared with control cells. The median number of nuclei/osteoclast was reduced upon CID755673 treatment by ~30%, and the maximum number of nuclei/osteoclast was decreased by >50% (Fig. 3*B*), but there was no significant change in the total number of nuclei in CID755673 cultures (Fig. 3*B*), suggesting that this inhibition was not due to changes in proliferation or survival of osteoclast precursors. To further characterize the effect of CID755673 on osteoclastogenesis, we measured expression of a panel of genes involved in osteoclast differentiation (Fig. 3*C*). qRT-PCR analysis on day 5 showed relatively small or inconsistent effects on most osteoclast marker genes tested with the exception of *DC-STAMP* (a transmembrane protein involved in cell-cell fusion), which was consistently reduced, on average, by 36% in cultures treated with 10 μM CID755673 ($p = 0.0008$) and by 60% in those treated with 30 μM

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Day 3 Preosteoclasts
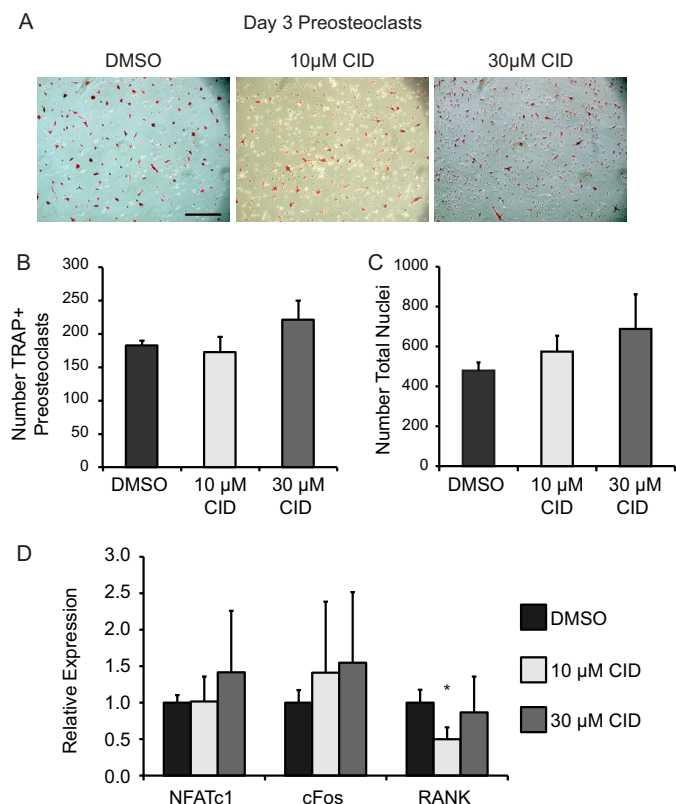
DMSO   10μM CID   30μM CID

FIGURE 2. **CID755673 does not inhibit preosteoclast induction.** BMM cultures stimulated with M-CSF and RANKL for 3 days in the presence of DMSO or 10 or 30 μM CID755673 (*CID*) were stained with TRAP (*A*) and counterstained with DAPI (not shown). *Scale bar = 500 μm*. CID755673 had no significant effect on either the total number of TRAP-positive cells (*B*) or the total number of nuclei/field (*C*). *D*, qRT-PCR analysis of *Nfatc1*, *c-fos*, and *Rank* expression in BMM cultures treated with CID755673 for 3 days. For each gene, expression in CID755673-treated cells is graphed as -fold change relative to DMSO-treated cells. *, $p < 0.001$ *versus* DMSO.

CID755673 ($p < 5 \times 10^{-7}$) (Fig. 3*C*). We observed a similar effect, *i.e.* impaired formation of multinucleated osteoclasts despite the presence of numerous TRAP-positive preosteoclasts, when BMM cultures were treated with the structurally distinct PKD inhibitor Gö 6976 (Fig. 3*D*).

To further test whether the impaired osteoclastogenesis from CID755673 and Gö 6976 was due to specific inhibition of PKD activity, we reduced PKD2 expression by infecting BMM cultures with lentiviruses encoding two distinct shRNAs against PKD2 or a negative control shRNA. qRT-PCR (Fig. 4*A*) and Western blotting (Fig. 4*B*) showed that both PKD2 shRNAs produced a strong reduction in endogenous PKD2 expression compared with cells infected with control shRNA lentivirus. Subsequent RANKL stimulation of the PKD2 shRNA-infected cultures showed that osteoclastogenesis was substantially impaired, with both of the PKD2 shRNAs giving a similar effect (Fig. 4*C*). Suppressing PKD2 had little effect on the total number of TRAP-positive cells induced by RANKL (Fig. 4*D*, *black bars*) but decreased the number of TRAP-positive multinucleated cells by 60% (Fig. 4*D*, *white bars*) and reduced their average size from 0.04 to ~0.015 mm^2 (Fig. 4*E*). Real-time qPCR analysis showed an ~80% reduction in *DC-STAMP* expression and a 59% inhibition of *Ctsk* (Fig. 4*E*). *Nfatc1* was reduced by ~20%, although this trend did not reach statisti-

cal significance. From the pharmacological inhibitor and RNAi studies, we conclude that PKD activity is required for osteoclast differentiation.

PKD Activity Is Required for Formation of Multinucleated Osteoclasts—The observation that BMMs treated with CID755673 and PKD2 shRNA generated many TRAP-positive mononucleated cells but few multinucleated osteoclasts suggested that PKD activity is not required until the TRAP-positive preosteoclast stage, which occurs on approximately day 3 under our culture conditions. To further define the CID755673-sensitive period, we treated BMM cultures with M-CSF and RANKL for 5 days and added or removed CID755673 during defined portions of the culture period such that all cells were exposed either to DMSO vehicle alone or to DMSO and CID755673 for the entire culture period. TRAP staining on day 5 showed that differentiation of multinucleated osteoclasts was inhibited when CID755673 was present on days 1–5 and 1–4 but was not inhibited by treatments on days 1–3 or 1–2 (Fig. 5). Similarly, inhibition was noted upon treatments on days 2–5 and 3–5 but not days 4–5. In all cases, numerous TRAP-positive mononucleated cells were detected, further demonstrating that CID755673 does not inhibit induction of preosteoclasts.

As these data suggest that PKD action is required after the TRAP-positive mononucleated preosteoclast stage, we asked whether CID755673 affected resorptive activity or survival of multinucleated osteoclasts. To measure the effects of CID755673 on resorptive activity, multinucleated osteoclasts were differentiated on osteo assay plates for 5 days and then treated with CID755673 for 48 h. We observed no change in either the number or average size of resorption pits or total resorbed area (Fig. 6, *A*–*C*). To measure apoptosis, BMMs were treated with M-CSF and RANKL for either 3 or 4 days and then treated overnight with 30 μM CID755673. We did not visually note any apparent cell death from CID755673 treatment (data not shown) or any change in caspase-3/7 activity following CID755673 treatment (Fig. 6*D*). These data do not provide evidence of PKD involvement in regulating osteoclast resorptive activity or survival.

Overexpression of DC-STAMP Rescues CID755673-inhibited Osteoclast Formation—DC-STAMP is a transmembrane protein required for osteoclast fusion (8). Because DC-STAMP expression was strongly reduced in CID755673-treated and PKD2-suppressed osteoclasts, we hypothesized that restoring DC-STAMP expression would restore fusion in CID755673-treated cultures. To test this, we infected BMMs with an adenoviral vector encoding HA-tagged DC-STAMP or a control adenovirus. Western blotting (Fig. 7*A*) and qRT-PCR (Fig. 7*B*) indicated successful expression of HA-DC-STAMP protein. Following viral transduction, cells were stimulated with M-CSF and RANKL and treated with either DMSO or CID755673 for 5 days (Fig. 7, *C*–*E*). As expected from a previous report (8), in DMSO-treated cultures, the DC-STAMP-overexpressing virus increased the number and size of osteoclasts. CID755673 impaired differentiation of control virus-infected cells. Importantly, infection of CID755673-treated cultures with the DC-STAMP-expressing virus significantly increased the size and number of multinucleated osteoclasts compared with
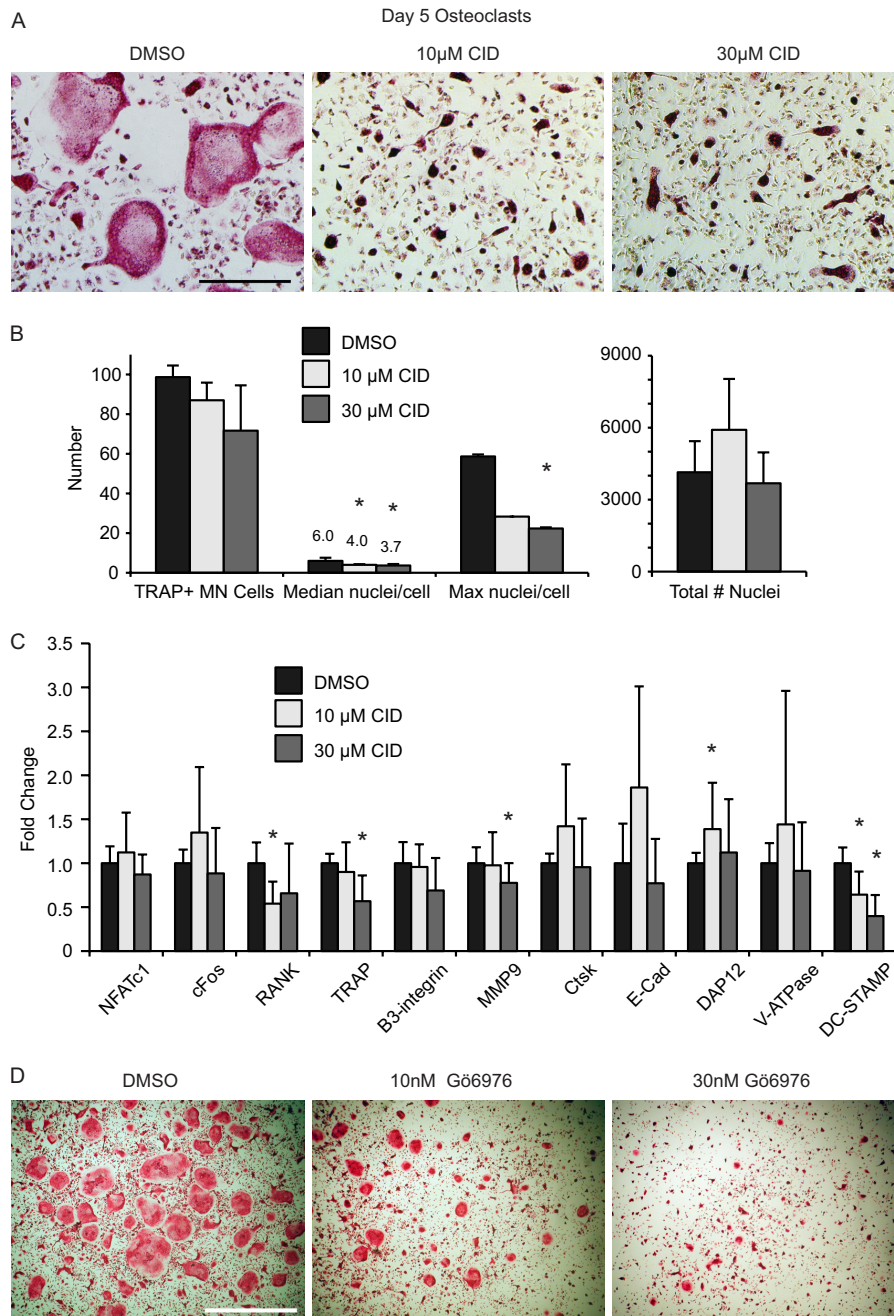
FIGURE 3. **PKD inhibitors CID755673 and Gö 6976 impair differentiation of multinucleated osteoclasts.** *A*, BMM cultures stimulated with M-CSF and RANKL for 5 days in the presence of DMSO or 10 or 30 μM CID755673 (*CID*) were stained with TRAP and counterstained with DAPI (not shown). *Scale bar* = 500 μm. *B*, CID755673 effects on the total number of TRAP-positive multinucleated (*TRAP+ MN*) cells, the median and maximum number of nuclei/multinucleated cell, and the total number of nuclei/field. *C*, qRT-PCR analysis of osteoclast gene expression in BMM cultures treated with CID755673 for 3 days. *, $p < 0.05$ *versus* DMSO. *E-Cad*, E-cadherin. *D*, TRAP staining of BMM cultures stimulated with M-CSF and RANKL for 5 days in the presence of DMSO or 10 or 30 nM Gö 6976. *Scale bar* = 1 mm.

CID755673-treated cells infected with control virus. Additionally, we examined expression of *Nfatc1* and *Ctsk* in these cells (Fig. 7*F*). In the case of *Nfatc1*, the DC-STAMP-expressing virus increased expression by 2.5-fold in DMSO-treated cultures, although this did not reach the level of statistical significance ($p = 0.18$), and in CID755673-treated cells, DC-STAMP overexpression did not alter *Nfatc1* levels. Expression of *Ctsk* was increased upon DC-STAMP overexpression by 5.6-fold in DMSO-treated cells ($p = 0.001$) and by 3.2-fold in CID755673-treated cells ($p = 0.03$). These data indicate that PKD contrib-

utes to osteoclast fusion at least in part by promoting DC-STAMP expression.

DISCUSSION

This study is the first to investigate the expression and functional role of PKD in osteoclasts. There is substantial sequence similarity between the PKD proteins and functional redundancy between family members. However, the phenotypes of knocking out individual PKD genes in mice are distinct from one another, indicating that non-redundant functions that are
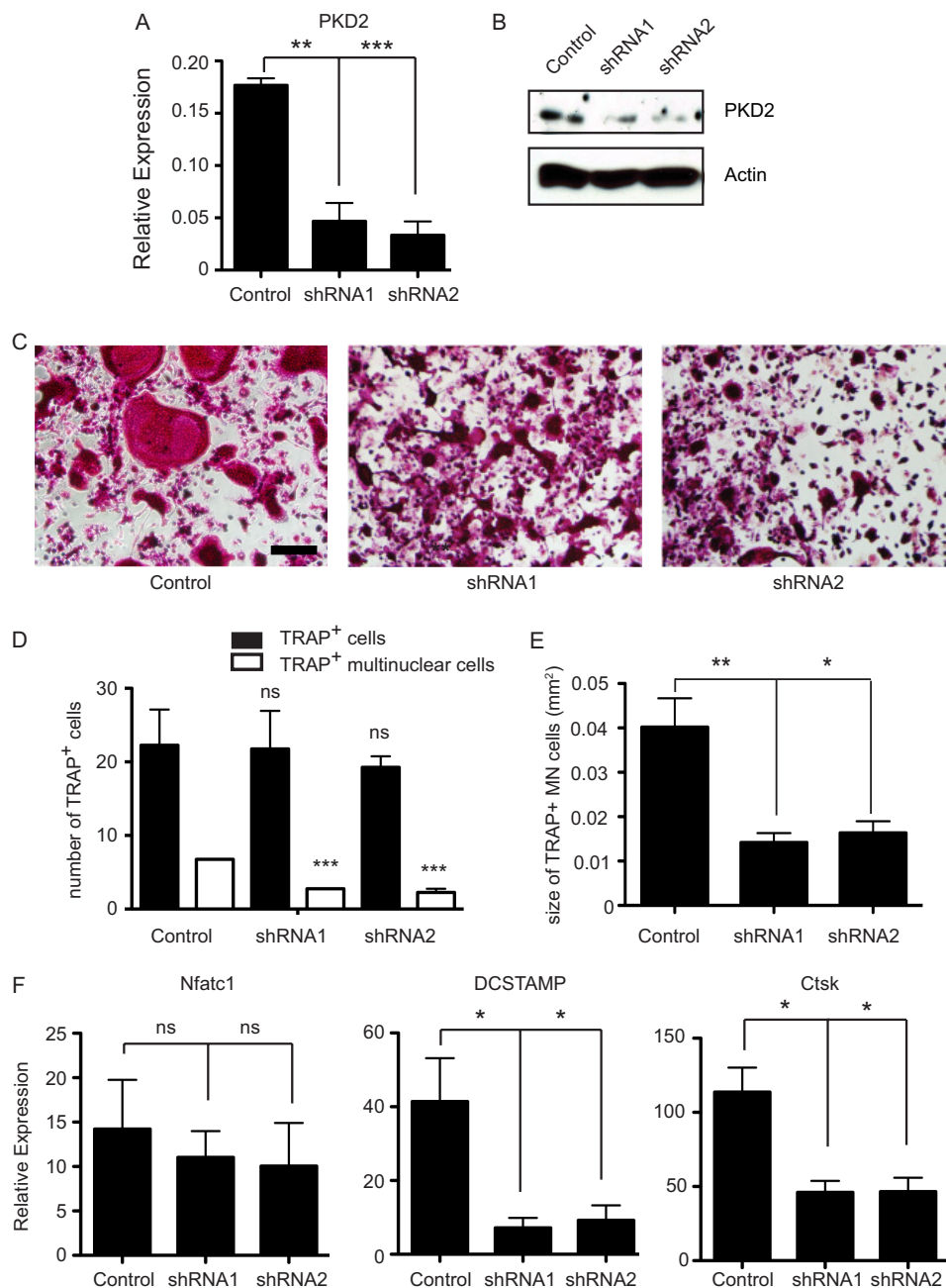
FIGURE 4. **PKD2 suppression reduces multinucleated osteoclast formation.** Lentiviruses expressing shRNAs against PKD2 reduced endogenous PKD2 expression as measured by qRT-PCR (*A*) and Western blotting with PKD2 and actin (*B*). *C*, TRAP staining of PKD2 suppressed BMMs stimulated with M-CSF and RANKL for 6 days. *Scale bar* = 500 μm. *D* and *E*, effect of PKD2 shRNA on the total number of TRAP-positive cells (*black bars*) and TRAP-positive multinucleated (*TRAP+ MN*) cells (*white bars*) and on the average size of the multinucleated cells, respectively. *F*, qRT-PCR analysis of *Nfatc1*, *DC-STAMP*, and *Ctsk* expression. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0005$ *versus* control virus; *ns*, not significant.

achieved by different expression patterns in specific cells or tissues, by differential recruitment to subcellular domains mediated by PKD interactions with scaffold proteins, or by differences in substrate specificity. Our data indicate that, of the three PKD isoforms, PKD2 is the most highly expressed in osteoclast precursors and during osteoclast differentiation, suggesting that PKD2 may play a unique role in osteoclastogenesis. This finding is in agreement with a previous report that indicates that PKD2 is the most abundant form of PKD in monocytes (5). Mice mutant for PKD2 are viable but display deficient T-cell function (9). It is unclear whether PKD2

knock-out mice display an *in vivo* defect in osteoclastogenesis or bone remodeling.

We found that inhibition of PKD kinase activity or RNAi suppression of its expression inhibited osteoclast formation, acting chiefly during the transition from TRAP-positive mononucleated preosteoclasts to multinucleated osteoclasts. Although blocking PKD impaired formation of mature multinucleated osteoclasts, numerous TRAP-expressing mononucleated cells were detected along with no change in expression of early osteoclast marker genes such as *Nfatc1* and c-*fos* following treatment of osteoclast cultures
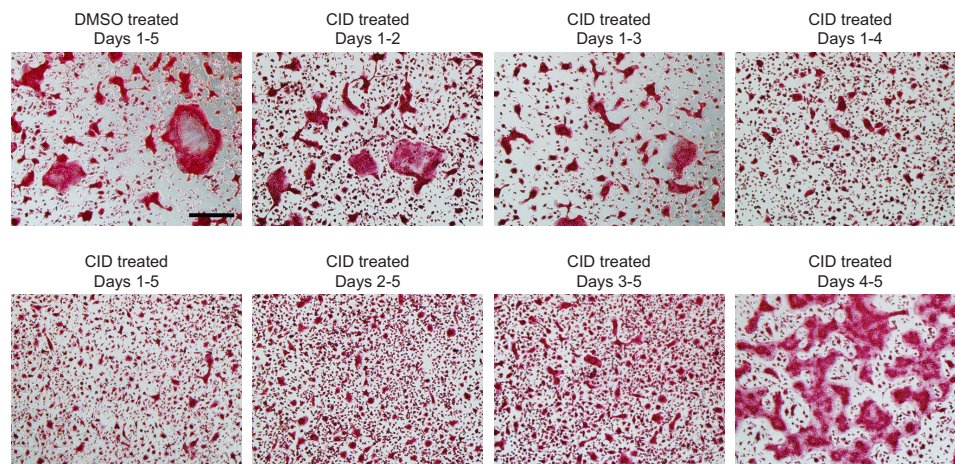
FIGURE 5. **Time sensitivity of osteoclast differentiation to PKD inhibition.** BMM cultures stimulated with M-CSF and RANKL were treated with DMSO for 5 days. At the indicated periods, the DMSO vehicle was replaced with 30 μM CID755673 (*CID*) in DMSO. Cells were fixed and stained for TRAP on day 5. *Scale bar* = 250 mm.
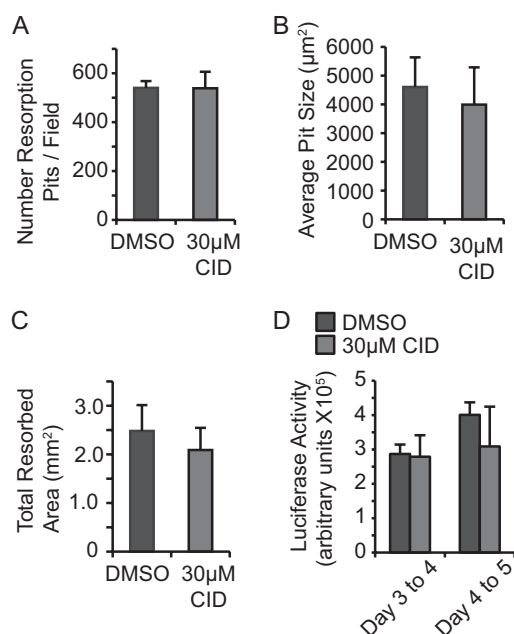

FIGURE 6. **Effect of CID755673 on osteoclastic bone resorption and osteoclast survival.** *A*, number of resorption pits. *B*, average pit size. *C*, total resorbed area for multinucleated osteoclasts plated on osteo assay substrate and treated with CID755673 (*CID*) for 48 h. *D*, caspase-3/7 activity in osteoclast cultures treated with DMSO or 30 μM CID755673 for 24 h beginning on day 3 or 4 differentiation with M-CSF and RANKL.

with the PKD inhibitor CID755673 or RNAi suppression of PKD2. PKD activity is regulated by phosphorylation of serine residues within the activation loop (10, 11), which enables phosphorylation of PKD substrates and autophosphorylation of Ser-873 at the extreme C terminus of PKD2 (corresponding to Ser-916 of PKD1) (12). Notably, we detected elevated PKD phosphorylation at these sites at roughly the same time as fusion began to take place, and this corresponded with sensitivity to inhibition of differentiation by CID755673. It is not yet known which pathways mediate activation of PKD in osteoclasts. PKD is activated by DAG and PKC family members, most commonly novel PKC isoforms that are themselves responsive to DAG but not to calcium. RANKL can activate phospholipase C to generate

DAG via DAP12 and Syk in preosteoclasts (13–15), suggesting that this pathway could represent a potential mechanism for PKD activation during osteoclastogenesis.

The change in cellular phenotype from preosteoclast to osteoclast is achieved by fusion of mononucleated precursors to form multinucleated syncytia. This process requires several steps: acquisition of fusion competence, chemotaxis, cell-cell recognition and adhesion, and finally fusion of the plasma membranes (2). DC-STAMP is a transmembrane protein that is crucial for fusion (8). Homozygous deletion of DC-STAMP has little effect on osteoclast marker gene expression but greatly inhibits fusion of preosteoclasts into polykaryons. We found that PKD inhibition decreased levels of *DC-STAMP* and that overexpressing DC-STAMP rescued fusion of CID755673-treated cells. We further detected increased cathepsin K levels following DC-STAMP overexpression and decreased cathepsin K levels upon PKD2 shRNA (but not CID755673) treatment. The significance of these observations is currently unclear. Taken together, our data led us to conclude that the impairment of osteoclast formation in PKD-inhibited cells is largely accounted for by the defect in DC-STAMP expression. PKD can act to enhance gene transcription by phosphorylating class IIa histone deacetylase (HDAC) corepressor proteins HDAC4, HDAC5, and HDAC7, causing them to be exported from the nucleus and freeing target genes from the repressive actions of HDACs (16–20). We demonstrated previously that suppression of HDAC7 acts in osteoclast lineage cells to inhibit their differentiation (21). Perhaps PKD attenuates the repressive effects of HDAC7 by such a mechanism. However, it is not yet known whether this is the mechanism through which PKD regulates *DC-STAMP* expression.

This study establishes an important role for PKD signaling in osteoclast differentiation. Our findings also raise a number of questions. What are the upstream signals that activate PKD activity? What proteins are phosphorylation substrates of PKD in osteoclasts? How does PKD regulate *DC-STAMP* expression? We hope that understanding these questions will give a greater understanding of the molecular mechanisms that govern osteoclast formation and activity *in vivo* and may enable
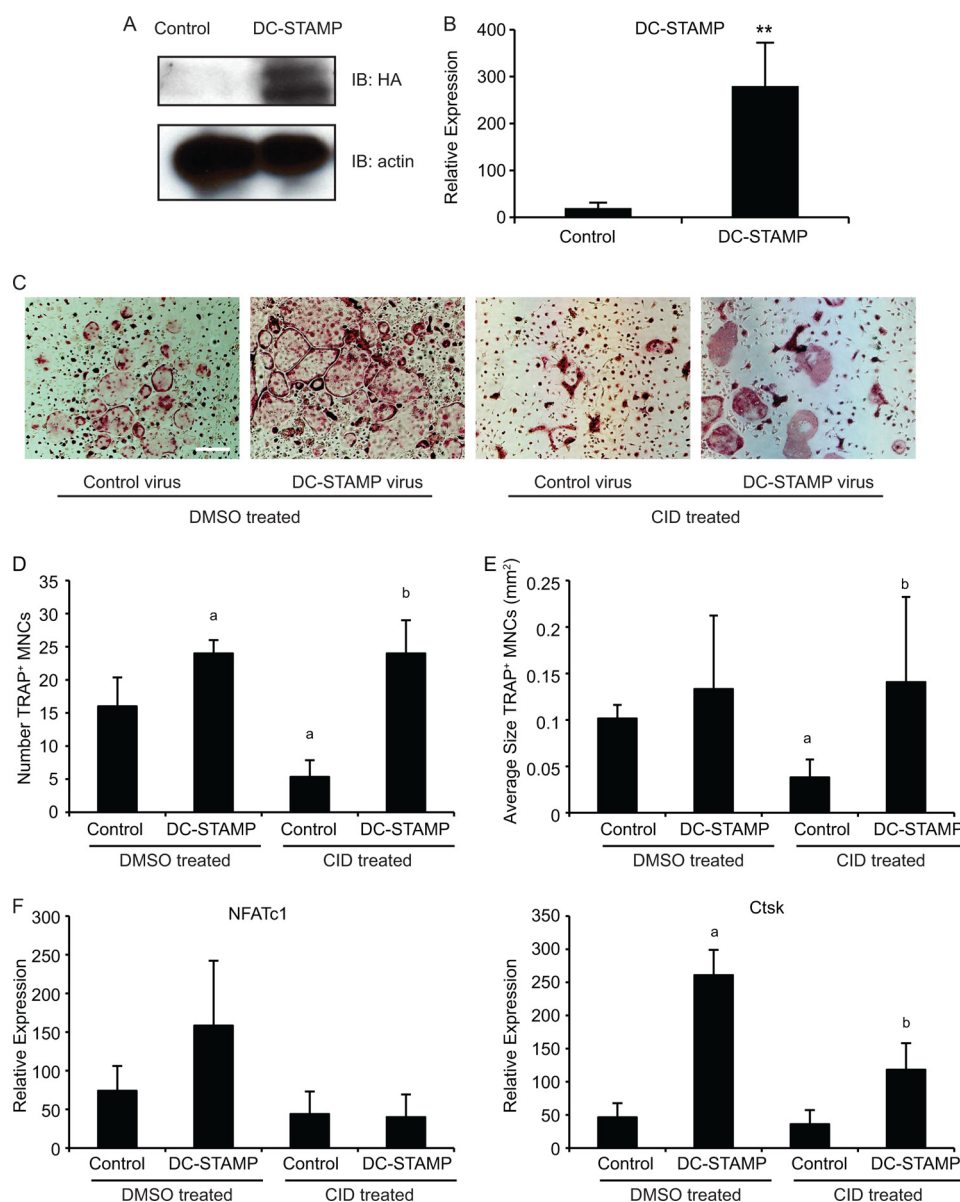
FIGURE 7. **DC-STAMP rescues osteoclast differentiation in CID755673-treated cells.** *A*, Western blotting of cells infected with control or HA-DC-STAMP-expressing adenoviruses blotted for HA or actin. *IB*, immunoblot. *B*, qRT-PCR measurement of *DC-STAMP* expression in control and DC-STAMP-encoding adenoviruses. *C*, TRAP staining of cultures infected with either control adenovirus or DC-STAMP-expressing virus and then treated with DMSO vehicle or 30 μM CID755673 for 5 days. *Scale bar* = 500 μM. *D* and *E*, quantitative analysis of cells shown in *B* measuring the average number and size of TRAP-positive multinucleated cells (*TRAP$^+$ MNCs*), respectively. *F*, qRT-PCR analysis showing the effect of DC-STAMP overexpression on cells treated with CID755673. *a, p* < 0.05 *versus* control virus + DMSO; *b, p* < 0.005 *versus* control virus + CID755673.

novel opportunities for better diagnostics or therapeutics targeting osteoclasts and bone resorption.

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