The retinoblastoma protein (pRB) is a tumor suppressor and key regulator of the cell cycle. We have previously shown that pRB interacts with phosphatidylinositol-4-phosphate 5-kinases, lipid kinases that can regulate phosphatidylinositol 4,5-bisphosphate levels in the nucleus. Here, we investigated pRB binding to another lipid in the phosphoinositide cycle, diacylglycerol kinase (DGK) that phosphorylates the second messenger diacylglycerol to yield phosphatidic acid. We found that DGKζ, but not DGKκ or DGKθ, interacts with pRB in vitro and in vivo. Binding of DGKζ to pRB is dependent on the phosphorylation status of pRB, since only hypophosphorylated pRB interacts with DGKζ. DGKζ also binds to the pRB-related pocket proteins p107 and p130 in vitro and in cells. Although DGKζ did not affect the ability of pRB to regulate E2F-mediated transcription, we found that pRB, p107, and p130 potently stimulate DGKζ activity in vitro. Finally, overexpression of DGKζ in pRB-null fibroblasts reconstitutes a cell cycle arrest induced by γ-irradiation. These results suggest that DGKζ may act in vivo as a downstream effector of pRB to regulate nuclear levels of diacylglycerol and phosphatidic acid.

Diacylglycerol (DAG) regulates many cellular processes, including proliferation, differentiation, and cell migration, by modulating the activity of several proteins, such as protein kinase C (PKC), Ras guanyl nucleotide-releasing proteins, chimaerins, and Munc 13 (1). DAG can be produced by the action of several different signal transduction pathways, including phospholipase C-mediated hydrolysis of phosphoinositides or phosphatidylinositol-choline and phosphatidylcholine-mediated hydrolysis of phosphatidylcholine followed by dephosphorylation of phosphatidic acid (PA), and during de novo synthesis of phospholipids (2).

DAG is not only produced at the plasma membrane but at other intracellular sites as well, including the nucleus. Nuclear DAG levels are increased in liver as a consequence of two-thirds partial hepatectomy (3) and in cell cultures treated with insulin-like growth factor 1, which stimulates proliferation (4, 5). This suggests that nuclear DAG levels are intimately linked with cell cycle progression, but a causal relationship has not been firmly established. An attractive hypothesis is that nuclear DAG stimulates cell cycle progression via a DAG-binding protein such as PKC (1, 6). Indeed, DAG in the nucleus recruits and activates PKC in response to insulin-like growth factor 1 stimulation of Swiss 3T3 cells, which is required for G1 to S phase transition (4, 7). However, the role of PKC in regulating the cell cycle is complex, with different PKC isoforms inducing a cell cycle arrest or stimulation of cell cycle progression. Furthermore, the same PKC isoform is able to induce both an arrest and progression through the cell cycle when expressed in different cell types (8).

In the nucleus, DAG kinase (DGK) controls the levels of DAG generated from PI-phospholipase C-mediated hydrolysis of PI(4,5)P2 (9), and nuclear DGK activity can be stimulated in response to both growth factor (10) and peptide-hormone treatment (11). DGKζ (Fig. 1A) is one of 10 different DGK isoforms identified to date (12, 13). DGKζ contains a nuclear localization signal (14, 15), and DGKζ has indeed been shown to be nuclear in some cell types (16, 17). The nuclear localization signal sequence in DGKζ overlaps with a motif similar to the PKC phosphorylation site domain (PSD) within the myristoylated alanine-rich protein kinase C substrate (MARCKS) protein (DGKζ-MARCKS-PSD). The DGKζ-MARCKS-PSD can be phosphorylated by PKC, which prevents nuclear accumulation of DGKζ (15). Furthermore, PKC-mediated phosphorylation of the DGKζ-MARCKS-PSD also inhibits DGKζ activity (18). Importantly, overexpression of DGKζ within the nucleus inhibits cell cycle progression (15). Thus, the levels and activity of DGKζ in the nucleus are subject to regulation by PKC, whereas, conversely, DGKζ may regulate nuclear DAG levels and consequently PKC activity.

We previously demonstrated that, in vivo, the level of nuclear PI(4,5)P2 can be modulated by the interaction of Type I PI kinases (enzymes that synthesize PI(4,5)P2) with pRB (19). Together with its family members p107 and p130, pRB regulates cell cycle progression by interacting with and attenuating the activity of the E2F transcription factor family (20, 21). Since PI(4,5)P2 is hydrolyzed by phospholipase C, which generates DAG, and since this nuclear DAG was shown to be subsequently phosphorylated by a DGK (9, 10), we questioned whether pRB may act as a nuclear scaffold to regulate PI signaling and DAG phosphorylation.

In this study, we show that GST-pRB fusion proteins can bind and extract DGK, PI-kinase, and PI-kinase activities from cell lysates. We identify DGKζ as the DGK isoform that specifically interacts both in vitro and in vivo with pRB and its family members p107 and p130 and show that this interaction potently enhances DGK activity. Finally, we demonstrate that DGKζ probably lies downstream of pRB signaling in a DNA damage signaling pathway. Our data would imply that disruption of pRB function, which frequently occurs in human cancers, may lead to enhanced nuclear DAG levels and, in turn, to uncontrolled nuclear PKC activity.

**EXPERIMENTAL PROCEDURES**

*Expression Plasmids—Wild-type DGKζ, catalytic inactive DGKζ, the DGKζ-MARCKS-PSD deletion mutant, and COOH-terminal FLAG-

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3 The abbreviations used are: DAG, diacylglycerol; DGK, diacylglycerol kinase; MEF, mouse embryonic fibroblast; MEL, murine erythroleukemia; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; pRB, retinoblastoma protein; PSD, phosphorylation site domain; MARCKS, myristoylated alanine-rich protein kinase C substrate; HA, hemagglutinin; GFP, green fluorescent protein; wtDGKζ, wild type DGKζ; kdDGKζ, kinase-dead DGKζ.
tagged DGKζ were published previously (15, 22). NH2-terminal HA-tagged DGKζ and GFP-DGKζ were cloned via three-point ligations into pMT2SM-HA and pEGFP-C2, respectively, using internal NdeI and Xmal sites, respectively. The 5′ fragment was generated by PCR, and the 3′ fragment was digested from wild-type DGKζ, GST-DGKζ and VSV-DGKζ (both COOH terminus) were cloned by inserting DGKζ in pMT2SM-GST and pMT2SM-tag, respectively, via a three-point ligation using the internal SphI site. The 3′ fragment was generated by PCR, and the 5′ fragment was derived from wild-type DGKζ. The GST-pRBC terminus (amino acids 767–928) was generated by PCR and inserted into pGEX-4T-2. Wild-type and kinase-inactive DGKζ were cloned into pBabe by PCR.

Cell Culture and Transfection—COS-7, HEK293T, Phoenix, MCF7, MEF, MEL, SAOS-2, and C33A cells were grown in Dulbecco’s modified Eagle’s medium containing 8% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics. COS-7 cells were transfected using the DEAE-dextran method; HEK293T, Phoenix and C33A cells were transfected using the calcium phosphate precipitation method; and SAOS-2 cells were transfected using FuGene (Roche Applied Science) according to the manufacturer’s instructions.

Cellular Lysates and Immunoprecipitations—Rat brain lysates were prepared as described (19). Cells were lyzed 48 h after transfection in 1% Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 50 mM KCl, 10 mM EDTA, 1% Nonidet P-40, complete protease inhibitor mixture (Roche Applied Science)). Immunoprecipitations were performed overnight using an anti-DGKζ polyclonal antibody (23), anti-pRBC polyclonal antibody C-15 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-FLAG monoclonal antibody M2 (Sigma), or anti-HA monoclonal antibody 12CA5 (Roche Applied Science). Antibodies were captured using Protein A- or G-Sepharose beads (Amershams Biosciences) and washed with 1% Nonidet P-40 lysis buffer. Endogenous immunoprecipitates were then washed once with PIPkinase buffer (25 mM Tris pH 7.4, 10 mM MgCl2, 80 mM KCl, 1 mM EDTA), and 15 or 20% was resuspended in 20 μl of 10 mM Tris pH 7.4 for the DGK activity assay, whereas 85 or 80% was analyzed by Western blotting. Immunoprecipitates or total lysates were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with anti-DGKζ polyclonal antibody, anti-pRBC monoclonal antibody G3-245 (Pharmingen), anti-p107 polyclonal antibody C-18 (Santa Cruz Biotechnology), anti-p130 monoclonal antibody C-20 (Santa Cruz Biotechnology), or anti-cyclin E monoclonal antibody sc-248 (Santa Cruz Biotechnology). Blots were stained with secondary antibodies (DAKO) and visualized using ECL (Amersham Biosciences) or Super Signal (Pierce).

Affinity Purifications—GST-DGKζ (expressed in COS-7 cells) and GST fusion proteins of pRBC, p107, and p130 (expressed in bacteria and induced with 200 μM isopropyl 1-thio-β-D-galactopyranosidase) were purified using glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s instructions. Approximately 200 μg of cell lysate was incubated with 4 μg of immobilized GST fusion proteins for 2 h at 4 °C, and beads were then washed with 1% Nonidet P-40 lysis buffer. For DGK activity assays, equal amounts of GST–protein complexes were washed once in PIPkinase buffer, resuspended in 20 μl of 10 mM Tris (pH 7.4), and assayed for DGK activity. For Western blotting, affinity-purified proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an anti-DGKζ polyclonal antibody or anti-VSV monoclonal antibody P5D4 (Roche Applied Science).

MCF7 lysate (450 μg) or 250 ng of eluted GST–pRBC or GST–Cdc42 was incubated overnight with 100 μg of biotinylated TAT-DGKζ-MARK5S peptide (YARAAAARQARAGASKKKRKSSSSK) or TAT control peptide (YARAAAARQARAG), which were immobilized on streptavidin-agarose (Sigma) and washed with wash buffer (50 mM Tris, pH 7.4, 140 mM NaCl, 10 mM MgCl2, 0.1% Tween 20). Affinity-purified pRBC or GST fusion protein was visualized by immunoblotting.

DGK Activity Assay—Immunoprecipitates, GST–protein complexes, or purified proteins were assayed for DGK activity as described by Divecha et al. (4). Lipid vesicles were prepared by sonication 1 nmol of dioleoylglycerol (from Sigma), 1 nmol of PIP (Biomol), 1 nmol of PI (Sigma), and 3 nmol of PA (Sigma) in 10 mM Tris (pH 7.4). Reactions were performed at 30 °C for 10 min (GST pull-downs and immunoprecipitates) or 5 min (purified proteins) in PIPkinase buffer containing 10 μM cold ATP and 5 μCi of [γ-32P]ATP in a final volume of 100 μl. Lipids were extracted with 0.5 ml of chloroform/methanol (1:1, v/v), followed by the addition of 125 μl of 2.4 M HCl and phase separation. Lipid extracts were dried and separated by thin layer chromatography (silica gel 60 TLC plates (Sigma) soaked in 1 mM EDTA and 1 mM potassium oxaloacetate and heat-activated) using chloroform/methanol/water/ammonia (45:35:7.5:2.5, v/v/v/v). Lipids were visualized by autoradiography and quantified using phosphoimaging.

E2F Luciferase Activity Assay—C33A cells were seeded in 6-well plates and transiently transfected with the indicated plasmids using the calcium phosphate precipitation method. 48 h after transfection, cells were lysed in 250 μl of 1X passive lysis buffer (Promega), and 50 μl was used to measure first firefly luciferase and then Renilla luciferase activity using the dual luciferase reporter assay system (Promega). Luciferase activity was detected using a Wallac 1420 multilabel counter (PerkinElmer Life Sciences) according to the manufacturer’s instructions. To control for transfection efficiency, firefly luciferase activity was corrected for Renilla luciferase activity.

Preparation of Purified Proteins for DGK Activity Assay—in order to determine DGK activation as a result of direct protein–protein interaction (DGKζ and GST fusion proteins of protein 4), the following purification steps were performed. HA-DGKζ was immunoprecipitated from 600 μg of COS-7 lysate and eluted from the beads using elution buffer (50 mM Tris, pH 8.0, 300 mM NaCl) containing 1 mg/ml HA peptide (YPYDVPDYA). Purified GST fusion proteins were eluted using GST elution buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM glutathione) and quantified using a bovine serum albumin standard on a Coomassie-stained gel. Purified HA-DGKζ was incubated with GST fusion proteins at 4 °C for 1 h. All samples contained equal amounts of GST elution buffer and 5 μg of bovine serum albumin.

Generation of pRBC—MEFs Stably Expressing DGKζ and Cell Cycle Analysis after γ-Irradiation—To immortalize mouse embryonic fibroblasts (MEFs) and to generate cells stably expressing DGKζ, LZRSTBX2-iresGFP (kindly provided by M. van Lohuizen (The Netherlands Cancer Institute)) or DGKζ-pBabe constructs were transfected in Phoenix packaging cells and used to transduce MEFs. Ecotropic retroviral supernatants were collected 48 h after transfection, filtered through a 0.45-μm filter, and incubated with 4 μg/ml Polybrene (Sigma) before adding to the cells. Viral supernatants were diluted 6 h after transduction. After immortalizing cells with TBX2, cells were transduced with DGKζ and, after 48 h, were selected with 200 μg/ml puromycin (Sigma). For irradiation experiments, 50,000 cells were plated per well (6-well plates). Two days after seeding, cells were γ-irradiated using a 137Cs radiation source. 30 min after irradiation, cells were treated with nocodazole (1 μM/mL Sigma) for 30 h. For cell cycle analysis, cells were trypsinized, fixed in ice-cold 70% ethanol, and resuspended in 200 μl of phosphate-buffered saline containing 50 μg/ml propidium iodide and 50 μg/ml RNase. Cell cycle distribution was determined by FACScan analysis and quantified using FCS Express 2.
pRB-type Proteins Bind to and Activate DGKζ

RESULTS

DGKζ Interacts with pRB, p107, and p130 in Vitro—To determine which lipid kinases interact with pRB, we used three different GST-pRB fusion proteins (Fig. 1B): the large pocket region that includes the small pocket domain and the C terminus (GST-pRB), the small pocket domain (GST-pRB(A+B)), and the pRB C terminus (GST-pRB(C)). These GST fusion proteins were used to affinity-purify lipid kinases from lysates of rat brain, murine erythroleukemia (MEL) cells, or human MCF7 breast cancer cells. GST-Cdc42 served as a negative control for nonspecific binding. Fig. 2A shows that, in each cell lysate, each of the three pRB constructs bound three different lipid kinase activities (i.e. PI-kinase (yielding PIP), PIP-kinase (yielding PIP2), and DGK (yielding PA)), as assessed by 32P incorporation into the respective lipid products. The ability of pRB to interact with PIP-kinases is in agreement with our previous data (19). Here, we focus on the novel finding that pRB binds to DGK.

Fig. 2A shows that rat brains contain much more pRB-binding lipid kinase activity than MEL and MCF7 cells. In each of the cell lysates, GST-pRB(A+B) (the small pocket of pRB; lanes 2, 6, and 10) shows slightly less binding of each of lipid kinase activities than the other two pRB constructs (large pocket and C terminus) (for rat brain, this is better seen at the lower exposure; lanes 1*-4*). Thus, in three different cell lysates, three different lipid kinases of the canonical PI cycle (i.e. a DGK, PI-kinase, and PIP-kinase) all associate with GST-pRB fusion proteins. These data suggest that pRB may act as a scaffold protein in order to regulate nuclear PI signaling.

To define which DGK isoform interacts with pRB, we used GST-pRB to extract DGKs from lysates of COS-7 cells overexpressing VSV-tagged DGKζ, DGKζ, or DGKθ. Interaction between DGKζ and GST-pRB was assessed using in vitro DGK activity assays and Western blotting. GST-pRB extracted 30-fold more DGK activity from lysates expressing DGKζ compared with those expressing DGKζ or DGKζ (Fig. 2B, top, compare lane 3 with lanes 1 and 5), although less DGKζ activity was present in the lysates compared with DGKζ or DGKζ activity (compare lane 10 with lanes 9 and 11). Similarly, more DGKζ bound to GST-pRB than DGKζ or DGKθ, as revealed by Western blotting, despite the lower expression of DGKζ in cell lysates (Fig. 2B, bottom). These data indicate that DGKζ is the predominant isoform that binds to pRB.

To assess if DGKζ activity was required for interaction with pRB, we mutated a conserved glycine residue within the ATP binding site. This mutant showed less than 1% of the activity in vitro but was still able to interact with GST-pRB as well as the wild-type enzyme (data not shown).

To more carefully examine the region of pRB where DGKζ binds, we performed a titration experiment in which increasing amounts of GST
fusión protéicos se utilizaron para inducir DGKxz de COS-7 líquidos. DGKxz tiene afinidades similares para GST-pRB y GST-pRB(C) (Fig. 2C, compare lanes 3 y 8), mientras que GST-pRB(A+B) sólo se une débilmente a DGKxz. La actividad DGKz substancialmente baja de GST-pRB(A+B) en células endógenas de sistemas (Fig. 2A) se compara con las mínimas cantidades de DGKxz que se unen a pRB(A+B) halladas en el transflectograma pueden ser debido a diferencias en los ensayos utilizados. Basado en el análisis de Western blot, concluimos que el término pRB es el sitio de unión mayor para DGKxz.

Desde pRB se incluye en el conjunto de la familia de proteínas pRB que también incluyen p107 y p130, evaluamos si p107 y p130 también interactúan con DGKxz. Dado que GST-p130 y GST-p130 tienen las mismas moléculas de peso, utilizamos líquidos expresando proteínas con DGKxz para discriminar entre ellos en SDS-PAGE. Líquidos del GST-p130 y del GST-p130 también se unen con GST-p130 (Fig. 3A). Sin embargo, GST-p130 extrae menos DGKxz comparado con GST-pRB y GST-p130, que reflejan la menor cantidad de GST proteínas utilizado o que p130 tiene una menor afinidad por DGKxz. Estas data que DGKxz puede interactuar con todos los miembros de la familia pRB pocket proteínas.

DGKxz Binds pRB, p107, and p130 in Cells—To demonstrate que DGKxz se une a pRB también en células, expresamos pRB y FLAG-tagged DGKxz (FLAG-DGKxz) y/o combinaciones en HEK293T células y inmunoprecipitamos la linaza de las células usando un método anti-FLAG. pRB y DGKxz se expresaron en líquidos total de células transfletadas HEK293T células (Fig. 3B, bottom), y FLAG-DGKxz podría ser inmunoprecipitado. Sin embargo, pRB solo se detectó en las inmunoprecipitaciones de líquidos expresando ambas pRB y DGKxz (Fig. 3B, top). También probamos las pRB familia miembros p107 y p130 para DGKxz bindings in cells. Total líquidos reveló que DGKxz, p107, y p130 se expresaron en líquidos total de células transfletadas HEK293T células (bottom of Fig. 3, C and D). Similar a pRB, p107 y p130 se usaron en la anti-FLAG inmunoprecipitaciones cuando se expresaron con FLAG-DGKxz (top of Fig. 3, C and D). El interacción de p130 con DGKxz se vio en ambos en vitro GST pull-downs and in the co-immunoprecipitations, suggesting that p130 binds DGKxz with a lower affinity than pRB and p107.

To demonstrate that endogenous DGKxz and pRB interact with each other, inmunoprecipitado DGKxz in MEL líquidos y sujetamos el precipitado inmunoblotting with a pRB-specific antibody. pRB was present in the DGKxz inmunoprecipitado but no in a control precipitado (Fig. 4A, top). Fig. 4A (bottom) confirma la presencia de DGKxz protein in the DGKxz inmunopre-
pRB-type Proteins Bind to and Activate DGKζ

FIGURE 5. DGKζ associates with pRB via its MARCKS-PSD. A, HEK293T cells were transfected with wtDGKζ, a MARCKS-PSD deletion mutant (DGKζ-ΔMARCKS), a MARCKS-PSD mutant in which all basic amino acids are substituted for alanines (DGKζ-K/R→A), or empty vector as indicated. Lysates were incubated with the indicated GST fusion proteins, and associated proteins were analyzed by immunoblotting using a DGKζ-specific antibody. GST fusion protein precipitates are shown in lanes 1–16, and total lysates are shown in lanes 17–20. B, DGKζ-MARCKS-PSD peptide specifically binds to pRB. Biotinylated DGKζ-MARCKS-PSD peptide or biotinylated control peptide were incubated overnight with MCF7 cell lysates and immobilized on streptavidin-agarose beads. Associated pRB was visualized by immunoblotting using an anti-pRB antibody. Total lysates contain one-twentieth of input lysate. C, full-length DGKζ binding to pRB is blocked by a DGKζ-MARCKS-PSD peptide. Lysates of COS-7 cells overexpressing DGKζ were incubated for 2 h with 1 or 10 μg of indicated peptides together with GST-pRB immobilized on glutathione-Sepharose 4B beads. Affinity-purified DGKζ was visualized by immunoblotting using an anti-DGKζ antibody (lanes 1–6). 10% of input lysates are shown in lanes 7 and 8. D, purified eluted GST-pRB directly binds to DGKζ-MARCKS-PSD peptide. 250 ng of purified eluted GST-pRB or GST-Cdc42 was incubated overnight with 100 μg of biotinylated DGKζ-MARCKS-PSD or control peptide. Peptides were immobilized on streptavidin-agarose beads and associated GST proteins were analyzed by Western blotting using a GST-specific antibody. The lanes marked input contain one-tenth of the GST fusion proteins used in the assay.

cipitate but not in the control precipitate. To successfully immunoprecipitate pRB and determine DGKζ co-immunoprecipitation, we used MEL lysates of differentiated cells that contain pRB predominantly in the hypophosphorylated status, the status of pRB that binds DGKζ (see Fig. 6). A small fraction of pRB was immunoprecipitated (Fig. 4B, top), and DGKζ protein and DGK activity were specifically co-immunoprecipitated (Fig. 4B, bottom). These results indicate that endogenous pRB and DGKζ interact with each other.

The MARCKS-PSD of DGKζ Is a pRB-binding Site—The MARCKS-PSD of DGKζ has previously been shown to be a major determinant for the localization of DGKζ in the nucleus (15). We therefore postulated that the MARCKS-PSD may be important in the interaction of DGKζ with pRB. To test this hypothesis, we tested the interaction between pRB and a DGKζ mutant in which the MARCKS domain was deleted (DGKζ-ΔMARCKS). Whereas both wild type (wtDGKζ) and DGKζ-ΔMARCKS were equally expressed (Fig. 5A, compare lane 17 with lane 18), DGKζ-ΔMARCKS was hardly detectable compared with wtDGKζ in the GST-pRB fusion protein precipitates (lanes 5–8). Since the MARCKS-PSD contains a large number of basic amino acids that might be important for electrostatic interaction between pRB and DGKζ, we tested a DGKζ-MARCKS-PSD mutant in which all basic amino acids of the nuclear localization signal were substituted for alanines (DGKζ-K/R→A) for pRB binding. Similar to the MARCKS-PSD deletion mutant, the DGKζ-K/R→A mutant was unable to interact with GST-pRB (lanes 9–12), although the protein was expressed at a higher level compared with wtDGKζ (compare lane 19 with lane 17).

To test whether the DGKζ-MARCKS-PSD was sufficient to mediate interaction with pRB, we used a biotinylated DGKζ-MARCKS-PSD peptide to affinity-purify pRB from MCF7 cell lysates. The DGKζ-MARCKS-PSD peptide bound to pRB, whereas a biotinylated control peptide was unable to bind pRB (Fig. 5B). Furthermore, the interaction between full-length DGKζ and GST-pRB was inhibited by the DGKζ-MARCKS-PSD peptide (Fig. 5C, lane 3) but not by the control peptide (Fig. 5C, lane 4). Together, these results indicate that the DGKζ-MARCKS-PSD is important in mediating the interaction between pRB and DGKζ.

The MARCKS-PSD of DGKζ Binds to pRB Directly—To determine whether pRB directly binds to DGKζ, we tested whether purified and eluted GST-pRB could be extracted by the biotinylated DGKζ-MARCKS-PSD peptide. Purified GST-pRB specifically bound to the DGKζ-MARCKS-PSD peptide coupled to streptavidin-agrose (Fig. 5D), whereas GST-Cdc42 did not bind. GST-pRB was not extracted by a control peptide. These results suggest that pRB binds to the MARCKS-PSD of DGKζ directly.

DGKζ Binds Hypophosphorylated pRB—pRB regulates cell cycle progression through its interaction with the transcription factor E2F. During G1, pRB exists in a hypophosphorylated state and can bind to and inactivate E2F. When cells progress to S-phase, pRB becomes highly phosphorylated by cyclin-cyclin-dependent kinase complexes, which leads to the release of E2F, enabling E2F-mediated transcription of genes required for S-phase progression (24). In total cell lysates, pRB is
pRB-type Proteins Bind to and Activate DGKζ

Figure 6. DGKζ binds to hypophosphorylated pRB. GST-DGKζ was isolated from COS-7 cells and incubated with lysates of SAOS-2 cells that were transfected with pRB and/or cyclin E as the indicated constructs. Affinity-purified proteins (lanes 1–6) and total lysates representing one-twentieth of input lysates (lanes 7–9) were subjected to Western blotting using a pRB-specific (top), GST-specific (bottom left), or cyclin E-specific antibody (cyclin E, top right).

Figure 7. DGKζ does not affect regulation of E2F by pRB. C33A cells were co-transfected with indicated constructs. Cells were lysed 48 h after transfection and assayed for firefly lucerase and Renilla lucerase activity using a luminometer. The firefly lucerase data were corrected for Renilla lucerase activity and plotted in the histogram shown as means ± the range of the duplicates (n = 2).

Present in both the low and highly phosphorylated state (see the doublet in Fig. 3B, bottom), whereas only one band is detectable in the DGKζ immunoprecipitate (Fig. 3B, top), suggesting that the interaction between DGKζ and pRB may be dependent on the phosphorylation status of pRB. To further test this, we used the osteosarcoma cell line SAOS-2 that lacks functional pRB. When pRB is overexpressed in SAOS-2 cells, it is not phosphorylated (Fig. 6, lane 7) and causes a cell cycle arrest. Co-expression of pRB with cyclin E, however, leads to hyperphosphorylation of pRB (lane 8), which attenuates the pRB-mediated cell cycle arrest (25). We purified GST-DGKζ from COS-7 cells and used it to affinity-purify pRB from lysates of SAOS-2 cells expressing pRB alone, or co-expressing pRB and cyclin E. Hypophosphorylated pRB (lane 1) specifically bound to GST-DGKζ, but in the presence of cyclin E when pRB is hyperphosphorylated (lane 2), binding was almost undetectable (the minor amount of pRB in lane 2 is hypophosphorylated). These results indicate that, similar to E2F, DGKζ preferentially binds to the hypophosphorylated form of pRB.

DGKζ Does Not Affect Sequencing and Inactivation of E2F by pRB—Since DGKζ interacts specifically with hypophosphorylated pRB, we questioned if DGKζ might influence pRB-mediated regulation of E2F transcriptional activity. Therefore, we used a reporter construct with a promoter containing six E2F binding sites upstream from the firefly lucerase reporter coding region. Binding of E2F to the promoter drives transcription of the lucerase reporter. C33A cells were co-transfected with the E2F lucerase reporter construct, a control Renilla lucerase reporter construct, E2F1, and DP1, in the absence or presence of pRB and/or DGKζ. E2F and DP1 caused a 4.5-fold stimulation of E2F promoter activity compared with background E2F activity. This stimulation was 50 and 75% reduced by co-transfection of 100 and 250 ng, respectively, of pRB expression plasmid (Fig. 7). The addition of DGKζ plasmid did not affect basal E2F activity or pRB-mediated inhibition of E2F activity, indicating that DGKζ does not affect the regulation of E2F activity by pRB.

pRB, p107, and p130 Stimulate DGKζ Activity—To explore the function of the interaction between DGKζ and pRB, we tested whether pRB could regulate DGKζ activity. Therefore, we compared the activity of HA-DGKζ immunoprecipitated with an anti-HA antibody with HA-DGKζ affinity-purified by GST-pRB. Immunoprecipitation with the anti-HA antibody yielded DGKζ that was not bound to pRB, whereas affinity purification with GST-pRB ensured that all of the DGKζ present on the beads interacted with pRB. The amount of DGKζ protein on the beads was determined by immunoblotting, whereas DGKζ activity was assessed by an in vitro assay. As shown in Fig. 8A, the amount of immunoprecipitated HA-DGKζ in lane 2 was comparable with the amount of HA-DGKζ affinity-purified by GST-pRB in lane 6. However, 7.5-fold more DGKζ activity was associated with GST-pRB-bound HA-DGKζ compared with HA-immunoprecipitated DGKζ. Since the HA tag antibody did not interfere with HA-DGKζ activity (data not shown), these results suggest that DGKζ is more active when in a complex with pRB.

To further verify that all of the pocket protein family members could stimulate DGKζ activity, we purified GST-pRB, -p107, and -p130 and assessed their effects on DGKζ activity in vitro. HA-DGKζ was immunoprecipitated from COS-7 cell lysates and eluted from the beads using an HA-peptide. Purified GST-pRB, GST-p107, and GST-p130 were eluted from the beads using glutathione. Purified HA-DGKζ and GST fusion proteins were combined on ice, and complexes were allowed to form prior to the DGKζ activity assay. GST-pRB, GST-p107, and GST-p130 enhanced DGKζ activity 5-, 3.5-, and 4.5-fold, respectively, in a concentration-dependent manner, whereas a GST-Cdc42 control did not affect DGKζ activity (Fig. 8B). Together, these results indicate that pRB family members activate DGKζ in vitro.

Overexpression of DGKζ Can Partially Rescue the Loss of a G1 Arrest after γ-Irradiation in pRB-null MEFs—In order to establish a physiological role for DGKζ in pRB-dependent signaling pathways, we studied the G1 arrest induced by γ-irradiation. Cell cycle arrest in response to ionizing radiation is a well established tumor-suppressive pathway that is dependent on the growth-suppressive activity of pRB. This pathway is completely blocked in pRB-null mouse embryonic fibroblasts (MEFs) (26). We postulated that, if DGKζ kinase activity is enhanced by pRB, then overexpression of DGKζ may partially substitute for the loss of pRB in γ-irradiation-induced cell cycle arrest. MEFs isolated from pRB-null mice were transduced with viral constructs encoding vector (pRB+/+ vector), kinase-inactive DGKζ (pRB~/~/−/− /koDGKζ), or wild-type DGKζ (pRB−/−/− wtDGKζ). As a control, MEFs were isolated from wild-type mice. In all cases, MEFs were immortalized by prior transduction with TBX2, which blocks passage-induced senescence. MEFs were γ-irradiated and, after 30 min, treated with nocodazole to arrest them in G2/M. Cells arrested in G1 were assessed by fluorescence-activated cell sorting analysis. As shown in Fig. 9, γ-irradiation of wild-type MEFs (pRB+/+) led to a dose-dependent increase in the number of cells arrested in G1. As expected, irradiation of pRB-null MEFs (pRB−/−/− vector) did not lead to any increase in the number of cells in G1. Consistent with a role for DGKζ in pRB signaling, overexpression of DGKζ in pRB-null MEFs led to a partial rescue of the arrest defect at all doses tested. The rescue was dependent on the activity of DGKζ, since it was not observed in cells transduced with the kinase-inactive DGKζ.
pRB-type Proteins Bind to and Activate DGKζ

In this study, we show that pRB specifically interacts with DGKζ in vitro and in vivo. The C terminus of pRB is required for the interaction with DGKζ, whereas the MARCKS-PSD of DGKζ is sufficient to mediate the interaction. Furthermore, the interaction between pRB and DGKζ is dependent on the phosphorylation status of pRB, since DGKζ only binds active hypophosphorylated pRB. The interaction between DGKζ and pRB does not appear to modulate the repression of E2F transcriptional activity by pRB. However, we show that pRB and other pocket protein family members are potent activators of DGKζ activity in vitro.

We previously demonstrated that Type I phosphatidylinositol 4-phosphate 5-kinase, an enzyme that converts phosphatidylinositol 4-phosphate into PI(4,5)P2, interacts with and is activated by pRB (19). In this paper, we now demonstrate that a PI 4-kinase that will generate phosphatidylinositol 4-phosphate and a DGK that phosphorylates DAG to PA can also interact with pRB. Furthermore, the p55 subunit of PI 3-kinase, which can generate PI(3,4,5)P3 via the 3’-phosphorylation of PI(4,5)P2, was also shown to interact with pRB (27). We suggest that pRB may act as a scaffold protein to integrate nuclear PI signaling and may provide a link between cell cycle regulation and changes in nuclear signaling lipids. For example, as cells progress from G1 to S phase, nuclear levels of DAG, PA, PIP, and PIP2 have been shown to change.

In addition to pRB binding, DGKζ also interacts with the pRB-related pocket proteins p107 and p130. pRB, p107, and p130 are highly similar within the pocket region, but also regions beyond the pocket domain are conserved. To date, almost all p107- and p130-binding proteins also bind to pRB, whereas most pRB-binding proteins have not been tested for binding to p107 and p130 (21). All pocket proteins show substantial functional overlaps as well as some unique functions (21, 29). When overexpressed, they all can arrest the appropriate cells in G1-phase of the cell cycle and inhibit E2F-mediated gene transcription and are all phosphorylated by cyclin-cyclin-dependent kinase complexes. However, p107 and p130 bind different E2F family members than pRB and therefore regulate transcription of different sets of genes. Also, their expression patterns differ during the cell cycle; the levels of pRB are

DISCUSSION

FIGURE 8. pRB stimulates DGKζ activity. A, COS-7 cells were transfected with either HA-DGKζ or vector as indicated and lysed after 48 h. HA-DGKζ was immunoprecipitated from 20 µg (lane 1) and 40 µg (lane 2) of cell lysate, using a fixed amount of anti-HA antibody and affinity-purified by 1, 2, or 3 µg of GST-pRB (lanes 4–6) from 80 µg of cell lysate. Negative controls are included in lanes 3, 7, and 8. Immunoprecipitates and affinity-purified proteins were split, and 20% was assayed for DGK activity, with GST-pRB by immunoblotting using a DGKζ-specific polyclonal antibody (bottom). Note that lane 2 and lane 6 contain the same amount of DGKζ protein, whereas the activity of HA-DGKζ bound to GST-pRB was 7.5-fold higher than the immunoprecipitated HA-DGKζ. B, HA-DGKζ overexpressed in COS-7 cells was immunoprecipitated using an anti-HA antibody and eluted from the beads using HA peptide. Eluted HA-DGKζ was incubated with the indicated amounts of GST fusion proteins that were isolated from bacteria, eluted from the beads using glutathione, and quantified relative to bovine serum albumin standards on a Coomassie-stained gel. In vector controls, 100 ng of the indicated GST fusion proteins was added. All samples contained 5 µg of carrier bovine serum albumin. Mixtures were assayed for DGK activity as in A. Results are the means ± the range of the duplicates (n = 2) and representative of three experiments.

FIGURE 9. DGKζ reconstitutes a cell cycle arrest induced by γ-irradiation in pRB-null MEFs. pRB+/+ and pRB−/− mouse embryonic fibroblasts (MEFs) were immortalized by TβX, and pRB−/− MEFs were transduced with empty vector, wtDGKζ, or kdDGKζ. Stable cell lines were irradiated with the indicated doses of γ-irradiation, and 30 min after irradiation, cells were treated with nocodazole (1 µg/ml) for 3 h. Cells arrested in G2 phase were quantified using flow cytometry. A significant radiation-induced increase in the percentage of cells in G1 phase above basal levels (subtracted in the figure) is apparent only in pRB+/+ cells and in wtDGKζ-transduced pRB−/− cells (black bars). Data are means ± S.E. (n = 3) and representative of three separate experiments. Significance was as follows: *, p < 0.05; **, p < 0.01 (Student’s t test). Inset, Western blot showing DGKζ expression in stable cell lines. Gy, gray.

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constant during the cell cycle, whereas p107 expression peaks during S-phase of the cell cycle and p130 is highly expressed in quiescent cells. Furthermore, deletion of pRB attenuates adipocyte differentiation, whereas deletion of p107 acts to sensitize MEFs to adipocyte differentiation (30).

In this study, we show that overexpression of DGKζ is able to partially rescue a cell cycle arrest defect in response to γ-irradiation in pRB-null MEFs. Irradiation of MEFs is known to induce pRB activity, which leads to a cell cycle arrest required to prevent cells from entering S-phase with damaged DNA (26, 31). The arrest is thought to allow DNA repair and thus ensure the survival of the cell. How irradiation induces a cell cycle arrest is not clear. In response to irradiation, p53 is induced and up-regulates the levels of the cyclin kinase inhibitor p21WAF1/CIP1. This inhibits phosphorylation of pRB, leading to its activation. The cell cycle arrest is thought to be induced by pRB-mediated attenuation of E2F transcriptional activity. However, in pRB-negative C3A cells, expression of a mutant of pRB in the LXCXE binding site, while maintaining the ability of pRB to interact with and repress E2F activity, is unable to reconstitute a DNA damage arrest, whereas wild-type pRB expression can (32). This suggests that yet another factor besides E2F repression determines induction of the cell cycle arrest in response to DNA damage. This factor could be DGKζ, since overexpression of DGKζ can partially rescue the loss of a cell cycle arrest in pRB-null cells. This would suggest that DGKζ acts either on a parallel pathway or lies directly downstream of pRB. Since our studies also demonstrate that the active (hypophosphorylated) form of pRB interacts with and stimulates DGKζ activity, we favor the latter suggestion. Furthermore, since a kinase-inactive DGKζ is unable to reinitiate a cell cycle arrest, it appears that either the removal of DAG or the generation of PA is important for the cell cycle arrest.

An interesting possibility by which overexpression of DGKζ allows regulation of a cell cycle arrest in response to irradiation may be through the other pocket protein family members, p107 and p130. We suggest that pocket proteins act as scaffolds to regulate nuclear inositol metabolism and to regulate the levels of the second messengers DAG and PA. Our data are consistent with a physiological role for either DAG or PA in modulating a pRB-mediated cell cycle arrest in response to DNA damage.

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