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Identification of Cyclin A_2 as the Downstream Effector of the Nuclear Phosphatidylinositol 4,5-Bisphosphate Signaling Network*[S]

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In addition to the well characterized phosphoinositide second messengers derived from the plasma membrane, increasing evidence supports the existence of a nuclear phosphoinositide signaling network. The aim of this investigation was to dissect the role played by nuclear phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) in cell cycle progression and to determine the cell cycle regulatory component(s) that are involved. A number of cytosolic/nuclear PtdIns(4,5)P_2-deficient Swiss 3T3 cell lines were established, and their G_0/G_1/S cell cycle phase transitions induced by defined mitogens were examined. Our results demonstrate that nuclear PtdIns(4,5)P_2 down-regulation caused a delay in phorbol ester-induced S phase entry and that this was at least in part channeled through cyclin A_2 at the transcriptional level. In summary, these data identify cyclin A_2 as a downstream effector of the nuclear PtdIns(4,5)P_2 signaling network and highlight the importance of nuclear PtdIns(4,5)P_2 in the regulation of mammalian mitogenesis.

Activation of diverse signal transduction pathways can cause quiescent cells to re-enter the cell cycle. Among these pathways, phosphoinositides (PIs)[2] represent a major class of mitogenic mediators. One of the recent developments in the field of PI signaling was the discovery of a discrete PI signaling network in the nucleus (1–4). Among the various PI species, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is particularly important because it is the precursor molecule of diacylglycerol (DAG), inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). The known signaling functions of nuclear PtdIns(4,5)P_2 include cell proliferation and differentiation, mRNA export/processing, chromatin remodeling, and transcriptional control (5–7). In addition, many of the PtdIns(4,5)P_2-metabolizing enzymes such as phospholipase C (PLC), type II PI kinase, protein kinase C (PKC), DAG kinases, and PDK-1 have also been shown to exist in nuclei (5, 7).

In Swiss 3T3 cells, stimulation with insulin-like growth factor I (IGF-I) causes a decrease in nuclear PtdIns(4)P and PtdIns(4,5)P_2 accompanied by an increase in nuclear DAG (6). These data contrasted with those obtained when cells are stimulated with the neuropeptide bombesin, which results in similar PI changes in the plasma membrane while the nuclear PI pool was unaffected (6). These results clearly demonstrate the distinct regulation of cytosolic and nuclear PtdIns(4,5)P_2. More recently, the synchrony between cell cycle progression and nuclear PI turnover has been demonstrated (8, 10). The fact that nuclei isolated from Swiss 3T3 cells stimulated with IGF-I exhibit a decrease in PtdIns(4,5)P_2 accompanied by an increase in DAG strongly suggests the involvement of PLC activity (6). Indeed, it was later shown that nuclear PLCβ1 activity is up-regulated 2-fold in Swiss 3T3 cells stimulated with IGF-I (11), and down-regulation of PLCβ-1 markedly reduces the sensitivity of Swiss 3T3 cells to IGF-I (12). IGF-I induces the phosphorylation of PLCβ1, and this phosphorylation can be blocked by MAPK cascade inhibitors (PD98059 and U0126, which target MEK (MAPK/extracellular signal-regulated kinase kinase)) (13). In support of this observation, Xu *et al.* (9) showed that PLCβ1 can be phosphorylated/activated by ERK both *in vitro* and *in vivo* in its regulatory domain. PLCδ, PLCγ, and PLCζ have also been found in the nucleus of various cell types. Although the different PLC family members share the same enzymatic function, given that these PLCs have distinct regulatory mechanisms and that they translocate to the nucleus at different cell cycle phases (14, 15), it is reasonable to speculate that cells utilize specific PLC isoforms according to specific signaling cues (1).

Collectively, the evidence accumulated over the past decade has firmly established the link between nuclear PtdIns(4,5)P_2 signaling and cell cycle control, although the underlying mechanisms remain to be defined. The aim of this study was to determine cell cycle regulatory component(s) that are under the influence of nuclear PtdIns(4,5)P_2. As a model system, a number of recombinant Swiss 3T3 cell lines with down-regulated cytosolic/nuclear PtdIns(4,5)P_2 were engineered via cell com-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Figs. S1–S6.

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[2] The abbreviations used are: PI, phosphoinositide; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C; PKC, protein kinase C; IGF-I, insulin-like growth factor I; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SKIP, skeletal muscle and kidney enriched 5-phosphatase; BSA, bovine serum albumin; GST, glutathione *S*-transferase; NCS, newborn calf serum; PDB, phorbol 12,13-dibutyrate; BrdUrd, 5-bromo-2′-deoxyuridine; PH, pleckstrin homology; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; NLS, nuclear localizing sequence.

partment-specific (nuclear *versus* cytosolic) expression of SKIP, a PtdIns(4,5)P_2/PtdIns(3,4,5)P_3-specific phosphatase (16, 17). Because PtdIns(4,5)P_2 is the major precursor molecule for the synthesis of PtdIns(3,4,5)P_3 (18), SKIP-mediated down-regulation of PtdIns(4,5)P_2 will concurrently limit the generation of PtdIns(3,4,5)P_3. Unlike PtdIns(4,5)P_2, which is a housekeeping PI species and present in both resting and stimulated cells, PtdIns(3,4,5)P_3 generation is tightly coupled with the activation of PI 3-kinase and is rapidly hydrolyzed by PI phosphatases such as PTEN and SHIP1/2 (19–22). Hence, in this study, any phenotypical changes observed in the recombinant cell lines are interpreted as being channeled through PtdIns(4,5)P_2 down-regulation. In addition, to dissociate the cytosolic and nuclear PtdIns(4,5)P_2-mediated signaling pathways more systematically, defined mitogens such as bombesin and phorbol esters were utilized to induce G_0/G_1/S cell cycle phase transition.

Here, we report that both cytosolic and nuclear PtdIns(4,5)P_2 down-regulation resulted in a delay in S phase entry; however, this inhibitory effect was achieved via different routes. Our results show that in cytosolic PtdIns(4,5)P_2-deficient cells, the delay in bombesin-induced S phase entry was channeled through cyclin D_1, whereas in nuclear PtdIns(4,5)P_2-deficient cells, the delay in phorbol ester-induced S phase entry was channeled through cyclin A_2.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cells—The antibodies used for immunoblotting were as follows: anti-cyclin D_1 (72-13G), anti-p27 (C-19G), anti-Cdk2 (C-163), anti-Cdk4 (C-22), anti-Cdk6 (C-21), anti-$p70^{S6K}$ (C-18), and anti-Erk-1 (C-16) (Santa Cruz Biotechnology, Inc.); anti-α-tubulin (TAT-1), anti-cyclin A (E23.1), and anti-cyclin D_3 (DCS28) (Cancer Research UK); anti-phospho-Akt (Ser-473; 587F11) (Cell Signaling Biotechnology); and anti-pRb (G3-245) (Pharmingen). Swiss 3T3 cells were employed for all experiments. SKIP-expressing cells were constructed by infection of Swiss 3T3 cells with appropriate recombinant retroviruses and isolated by puromycin selection (for details of constructs, see supplemental "Experimental Procedures").

Determination of SKIP Phosphatase Activity by Phosphate Release Assay—PtdIns(4,5)P_2 was dissolved in 1% octyl glucoside at 400 μM. To form PtdIns(4,5)P_2 micelles that the phosphatase could utilize, the lipid suspension was bath-sonicated (Ultrawave Ltd.) for 10 min. The reaction was carried out with 100 μM PtdIns(4,5)P_2, 50 μg of bovine serum albumin (BSA), 4 mM magnesium chloride, and 30 ng of glutathione *S*-transferase (GST)-SKIP in a 30-min incubation at 37 °C; terminated by addition of 100 μl of malachite dye (6 mM malachite green, 3.6 N HCl, and 17 mM ammonium molybdate); and allowed to develop for 30 min prior measuring the absorbance at 625 nm.

Swiss 3T3 Fibroblast Stimulation—For experimental purposes, 5×10^5 cells were plated on 9-cm plates in Dulbecco's modified Eagle's medium and 10% newborn calf serum (NCS). Cells were allowed to quiesce over the following 7–9 days. Dishes of confluent and quiescent cells were incubated in Dulbecco's modified Eagle's medium containing various supplements at the following concentrations: NCS (10%), insulin (1 μg/ml), phorbol 12,13-dibutyrate (PDB; 100 nM), and bombesin (100 nM).

Measurement of S Phase Entry by 5-Bromo-2′-deoxyuridine (BrdUrd) Incorporation into Cellular DNA—Swiss 3T3 fibroblasts were seeded on 35-mm dishes containing 10-mm coverslips. When cells were confluent and quiescent, they were stimulated and supplemented with 10 μM BrdUrd (Sigma). 32 h later, cells on coverslips were fixed and stained for BrdUrd as described previously (25).

Measurement of Cyclin A_2 mRNA Transcript Level by Real-time Quantitative PCR—Total RNA was isolated using RNeasy columns (Qiagen). 1 μg of RNA was treated with DNase (Invitrogen), and cDNA was prepared by using the SuperScript first-strand synthesis system (Invitrogen) for reverse transcription-PCR. Detection of ribosomal L19 and target gene expression was performed with SYBR Green Master Mix (Applied Biosystems) and an ABI PRISM 7700 sequence detection system (Applied Biosystems) using the relative standard curve method. L19 was used to normalize for variances in input cDNA. All measurements were performed in duplicate. The sequences of the primers used for cyclin A_2 were 5′-GCAGTTTTGAATCACCACATGC-3′ and 5′-TGGCTGCCTCTTCATGTAACC-3′, and those used for L19 were 5′-GGAAAAAGAAGGTCTGGTTGGA-3′ and 5′-TGATCTGCTGACGGGAGTTG-3′.

PtdIns(4,5)P_2 Detection Using Recombinant GST-tagged PLCδ1 Pleckstrin Homology (PH) Domain—Adherent cells growing on glass coverslips were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 10 min, followed by quenching with 50 mM NH_4Cl/PBS for 15 min. Next, a permeabilization/blocking step was performed with either 0.25% Triton-X100 (nuclear PtdIns(4,5)P_2 staining) or 120 μg/ml digitonin (cytoplasmic PtdIns(4,5)P_2 staining) in 3% fatty acid-free BSA/PBS for 30 min. 100 ng/ml GST-PLCδ1 PH domain in 3% fatty acid-free BSA/PBS was then applied to the cells and incubated for 30 min. (For the competition experiment, the GST-PLCδ1 PH domain/BSA/PBS solution was preincubated with 100 ng of Ins(1,4,5)P_3 (Sigma) for 30 min.) After the incubation, coverslips were washed (3 × 10 min) with PBS with gentle rocking and then incubated for 60 min with anti-GST antibody (Novagen) in 3% fatty acid-free BSA/PBS (1:10,000 dilution), followed by PBS washes as before. Cells were incubated with TRITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) in 3% fatty acid-free BSA/PBS (1:100 dilution) for 60 min. Residual antibody was washed away with PBS, followed by washing once with sterile distilled water before mounting with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using a Nikon TE2000 fluorescence microscope equipped with a Hamamatsu charge-coupled device camera. Images were analyzed using IPLab software. DAPI was visualized at 460 nm after excitation at 350 nm, TRITC at 572 nm after excitation at 547 nm, and enhanced green fluorescent protein (EGFP) at 508 nm after excitation at 490 nm. All immunofluorescent images shown were acquired with the same sensitivity/exposure settings.

Cyclin A_2 Immunofluorescence—Cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100, and treated with blocking buffer (5% bovine serum albu-

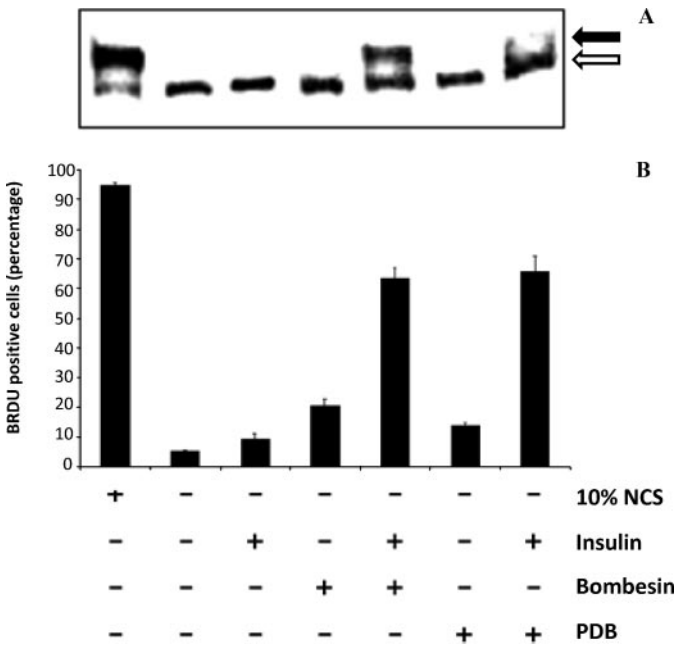

FIGURE 1. **Efficiencies of bombesin or PDB (±insulin) in inducing S phase entry in Swiss 3T3 cells.** Quiescent Swiss 3T3 cells were treated with the indicated agents plus BrdUrd (*BRDU*) for 24 h. Cells were either lysed and immunoblotted for pRb (*A*) or analyzed for BrdUrd incorporation (*B*). Hyperphosphorylated pRb is indicated by the *black arrow*, and hypophosphorylated pRb is indicated by the *white arrow*. For BrdUrd incorporation, the results are presented as the means ± S.E. of three independent experiments.

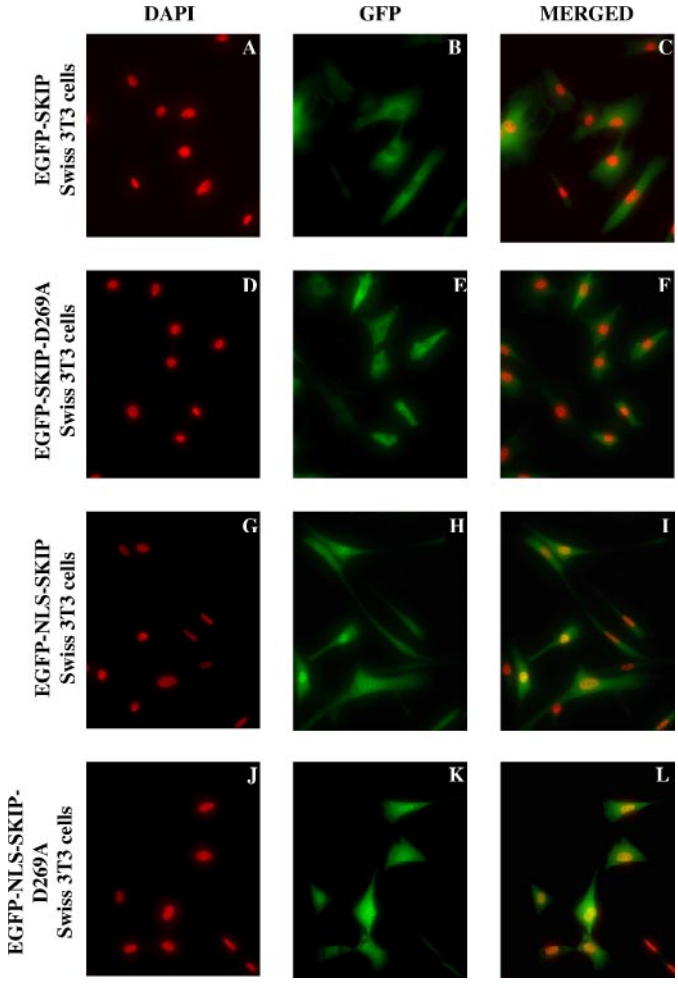

FIGURE 2. **Localization of EGFP-SKIP and EGFP-NLS-SKIP in Swiss 3T3 cells.** Swiss 3T3 cells infected with pBabe-puro-EGFP-SKIP, pBabe-puro-EGFP-NLS-SKIP, and the corresponding EGFP-SKIP-D269A mutants were grown on poly-L-lysine-coated coverslips and counterstained with the DNA stain DAPI. The pseudo colors assigned for DAPI and EGFP are *red* and *green*, respectively. All images shown are typical results obtained from at least 10 different fields.

min) for 45 min at room temperature to block nonspecific interactions prior to addition of the primary antibody. Cells were incubated with the primary antibody, anti-cyclin A_2 (1:200; Abcam), for 2 h at room temperature; washed; and incubated with the secondary antibody, Alexa Fluor 555-labeled goat anti-mouse IgG (1:3000; Molecular Probes), for 45 min at room temperature. Following all fixation, permeabilization, and antibody incubations, cells were washed (3 × 5 min) with PBS. Coverslips were mounted with Mowiol containing DAPI. Images were captured using an AxioCam camera on a Zeiss Axiovert 200M inverted microscope. For quantitation of the immunofluorescence, 150–200 cells in random fields of view across the entire coverslip of each cell line were scored for the presence of cyclin A_2 in the nucleus. For the SKIP-expressing cell lines, only GFP-positive cells were scored for the presence of cyclin A_2 in the nucleus. All immunofluorescent images shown were acquired with the same exposure settings.

RESULTS

G_0/G_1/S Phase Transition of the Cell Cycle in Response to Bombesin/PDB—We first examined the mitogenic activities of bombesin and PDB in Swiss 3T3 cells. Consistent with previous reports (26–28), the supplementation of insulin markedly increased the potency of bombesin/PDB-induced S phase entry (Fig. 1). Quiescent fibroblasts were stimulated with the indicated mitogens for 24 h, and the hyperphosphorylated form of pRb was clearly detectable in cells stimulated with 10% NCS, with bombesin and insulin, and with PDB and insulin (Fig. 1*A*). Results obtained from BrdUrd incorporation assays showed good agreement with the pRb mobility shift assay, in which the treatment of cells with 10% NCS, bombesin + insulin, and PDB + insulin resulted in the highest levels of BrdUrd incorporation (Fig. 1*B*). In terms of phospholipid signaling, the major difference between the mitogens bombesin and PDB is that they have a differential dependence on cytosolic PtdIns(4,5)P_2. For instance, bombesin and PDB activate PKC in a PtdIns(4,5)P_2-dependent and PtdIns(4,5)P_2-independent manner, respectively (29, 30).

Targeting SKIP to the Nuclei of Swiss 3T3 Fibroblasts—Accumulating evidence has firmly established that cytosolic and nuclear PtdIns(4,5)P_2 can have distinct influences on cell cycle progression. To investigate this signaling with respect to cell cycle control, SKIP was employed as a molecular tool with which to manipulate PtdIns(4,5)P_2 levels in Swiss 3T3 fibroblasts in different cellular compartments. Native SKIP is predominantly cytosolic with no apparent nuclear localization (16, 23, 24). To target SKIP to the nucleus, a nuclear localizing sequence (NLS) derived from SV40 T-antigen was incorporated into the sequence of SKIP along with a GFP tag to allow identification of SKIP subcellular localization. We constructed an enzymatically inactive version of SKIP as a control through

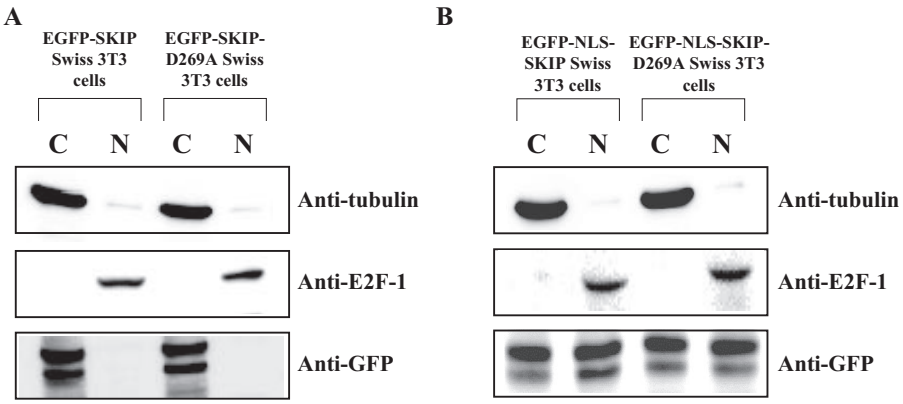

FIGURE 3. **Subcellular distribution of EGFP-SKIP and EGFP-NLS-SKIP in Swiss 3T3 cells.** Cell lysates derived from EGFP-SKIP- and EGFP-SKIP-D269A-expressing Swiss 3T3 cells (*A*) and EGFP-NLS-SKIP- and EGFP-NLS-SKIP-D269A-expressing Swiss 3T3 cells (*B*) were fractionated into cytosolic (*C*) and nuclear (*N*) fractions. The fractionated samples were normalized according to protein concentrations and were separated on 10% SDS-polyacrylamide gels. Immunoblotting was performed using antibodies against α-tubulin, E2F-1, and GFP. The results shown are typical results from three independent experiments.

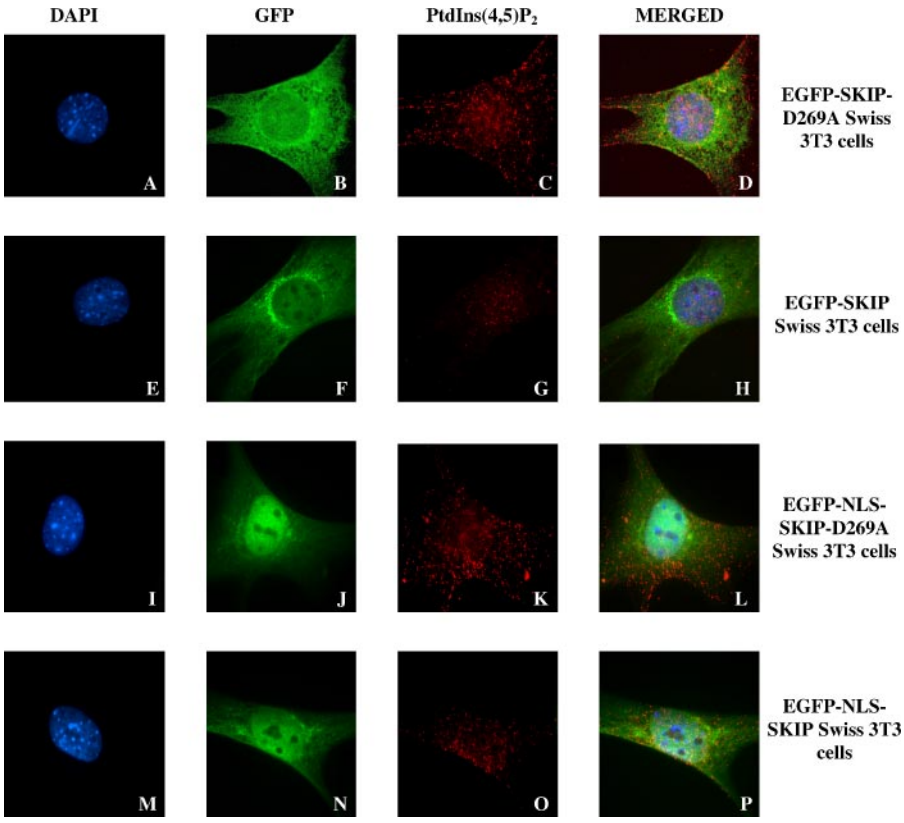

FIGURE 4. **Effect of overexpressing EGFP-SKIP/EGFP-NLS-SKIP on cytosolic PtdIns(4,5)P_2 levels in Swiss 3T3 cells.** EGFP-SKIP-, EGFP-NLS-SKIP-, or the corresponding EGFP-SKIP-D269A-overexpressing Swiss 3T3 cells were grown on poly-L-lysine-coated coverslips, and PtdIns(4,5)P_2 staining was performed as described under "Experimental Procedures." The pseudo colors assigned for DAPI, EGFP, and PtdIns(4,5)P_2 are *blue*, *green*, and *red*, respectively. All images shown are typical results obtained from at least 10 different fields.

mutation of a key catalytic Asp residue in motif 2 (Asp^{269} in SKIP) previously identified in other lipid phosphatases (supplemental Fig. S1). By infecting wild-type Swiss 3T3 cells with the retroviral EGFP-SKIP cDNA constructs, recombinant Swiss 3T3 cell lines overexpressing each EGFP-SKIP fusion protein were established. Infected cell pools were used for all experiments presented here. The cellular distribution of EGFP-SKIP, EGFP-NLS-SKIP, and the corresponding SKIP-D269A variants was first analyzed using fluorescence microscopy (Fig. 2). EGFP-SKIP and EGFP-SKIP-D269A were distributed evenly throughout the cytoplasm with no specific subcellular localization (Fig. 2, *C* and *F*). In contrast, enrichment of SKIP in nuclei was observed when the NLS was incorporated into SKIP (Fig. 2, *I* and *L*). These results were validated using subcellular fractionation techniques (Fig. 3). Taken together, these data indicate that NLS versions of SKIP are substantially elevated in nuclei, whereas other variants are exclusively cytosolic.

Overexpression of SKIP Down-regulates PtdIns(4,5)P_2 Levels in Swiss 3T3 Fibroblasts—To monitor the effect of SKIP overexpression on cytosolic or nuclear PtdIns(4,5)P_2 levels in the recombinant Swiss 3T3 cell lines, we employed the PLCδ1-PH domain as a PtdIns(4,5)P_2 probe for staining fixed cells. The integrity of this PtdIns(4,5)P_2 detection method was examined, and the results are shown in supplemental Figs. S2 and S3. A decrease in cytosolic PtdIns(4,5)P_2 labeling was observed in the cells that overexpressed EGFP-SKIP (Fig. 4*G*) or EGFP-NLS-SKIP (Fig. 4*O*). Notably, among all the cell lines examined, a decrease in nuclear PtdIns(4,5)P_2 levels was observed exclusively in the cells that overexpressed EGFP-NLS-SKIP (Fig. 5*O*). Based on the results obtained from the subcellular localization studies (Fig. 3), the observed cytosolic PtdIns(4,5)P_2 down-regulation in the nuclear SKIP cell line was most likely the consequence of the incomplete nuclear targeting of NLS-SKIP. Overexpression of the catalytically inactive forms of SKIP had no discernible effect on cytosolic or nuclear PtdIns(4,5)P_2 staining, indicating that the decrease in PtdIns(4,5)P_2 staining was the direct effect of the overexpressed SKIP activity.

Negative Effects of SKIP Overexpression on PtdIns(4,5)P_2-dependent Signaling Events—Prior to dissecting the signaling role(s) of cytosolic/nuclear PtdIns(4,5)P_2 in cell cycle regulation, a number of well characterized PtdIns(4,5)P_2-dependent signaling pathways were analyzed with the recombinant cell lines. Down-regulation of cytosolic PtdIns(4,5)P_2 levels had a

pronounced inhibitory effect on insulin-induced Akt activation and bombesin-induced Erk-1 activation (Fig. 6*B* and supplemental Fig. S4). These results are not unexpected, as the mitogens chosen to activate these signaling components are known to be dependent on the signals derived from PtdIns(4,5)P_2 (29). In contrast, PDB elicits its signaling function by activating PKC directly (*i.e.* PtdIns(4,5)P_2-independent) (30) and was therefore employed as a mitogen likely to bypass the inhibitory effect of cytosolic SKIP overexpression. Indeed, the results obtained from the PDB-induced Erk-1 mobility shift experiment (Fig. 6*B* and supplemental Fig. S4) clearly show that PDB treatment circumvented the inhibitory effect of cytosolic SKIP overexpression. Although PDB-induced Erk-1 phosphorylation is expected to be channeled predominantly through the activity of PKC, the slight delay observed in the SKIP-overexpressing cells (especially at 10 min) indicates that the down-regulation of PtdIns(4,5)P_2 in cells might have some unintentional side effects (see "Discussion"). As a complementary readout for the phospho-Erk-1 assay, bombesin/PDB-induced $p70^{S6K}$ phosphorylation was also analyzed with the recombinant cell lines. Treatment of resting cells with bombesin and PDB resulted in the phosphorylation of $p70^{S6K}$ as demonstrated by the appearance of the slower migrating bands (Fig. 6*C*). A decrease relative to the control cell lines in bombesin-induced $p70^{S6K}$ phosphorylation was observed with the EGFP-SKIP- and EGFP-NLS-SKIP-overexpressing cells, but the relative inhibitory effect was again less pronounced in the PDB treatment. Among all the cytosolic signaling events examined, results comparable with those found for wild-type cells were obtained with all other SKIP-D269A cell lines under all stimulatory conditions, thus suggesting that the observed

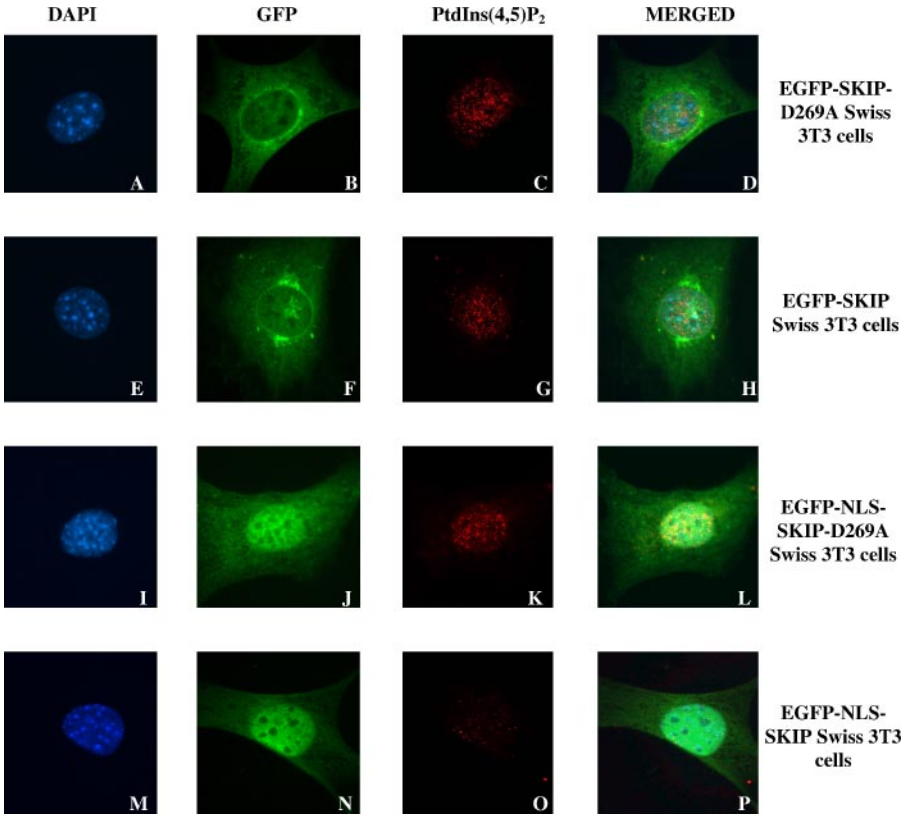

FIGURE 5. **Effect of overexpressing EGFP-SKIP/EGFP-NLS-SKIP on nuclear PtdIns(4,5)P_2 levels in Swiss 3T3 cells.** Nuclear PtdIns(4,5)P_2 staining was performed as described under "Experimental Procedures." The pseudo colors assigned for DAPI, EGFP, and PtdIns(4,5)P_2 are *blue*, *green*, and *red*, respectively. All images shown are typical results obtained from at least 10 different fields.

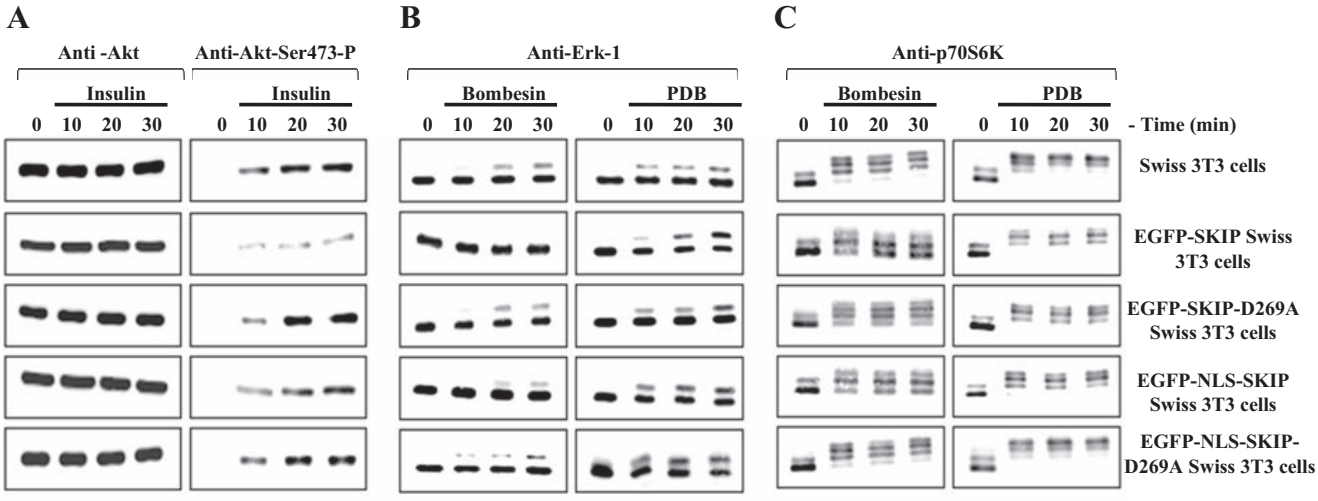

FIGURE 6. **Overexpression of EGFP-SKIP or EGFP-NLS-SKIP down-regulates PtdIns(4,5)P_2-dependent signaling pathways.** Wild-type Swiss 3T3 cells and the indicated SKIP-overexpressing Swiss 3T3 cells were rendered quiescent by serum deprivation, followed by treatment with the indicated stimulants. Cells were lysed at the time points indicated, and immunoblotting was performed. All immunoblots shown are representative of typical results from three independent experiments.

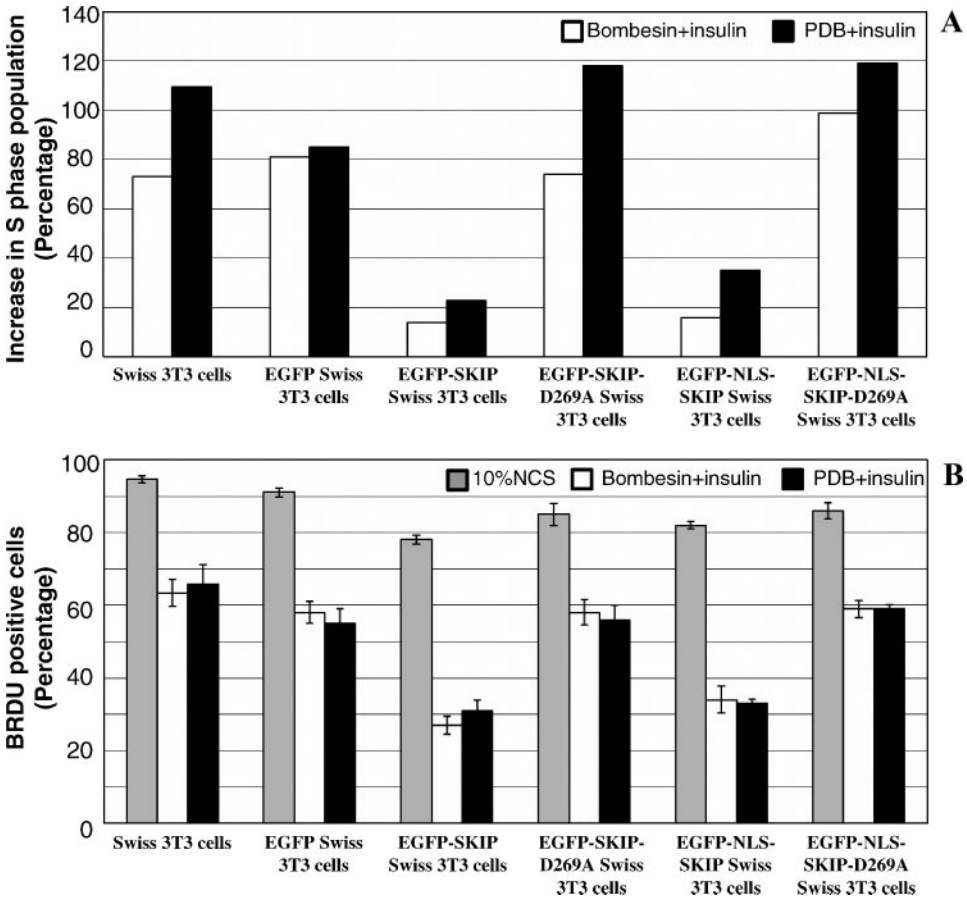

FIGURE 7. **Overexpression of EGFP-SKIP or EGFP-NLS-SKIP delays S phase entry induced by bombesin/PDB + insulin in Swiss 3T3 cells.** *A*, wild-type (*Wt*) Swiss 3T3 cells and SKIP-overexpressing lines were rendered quiescent by serum deprivation, followed by stimulation using bombesin + insulin or PDB + insulin for 24 h. Ethanol-fixed cells were then analyzed by flow cytometry with 1×10^4 cells recorded per treatment. Data are presented as the percentage of increase in S phase population relative to the non-stimulated cells. *B*, quiescent cells were stimulated using 10% NCS, bombesin + insulin, or PDB + insulin in the presence of BrdUrd (*BRDU*) for 32 h. BrdUrd immunohistochemistry was performed as described under "Experimental Procedures." The results are presented as the means ± S.E. of three independent experiments.

inhibitory effects on these signaling pathways were the direct effect of PtdIns(4,5)P_2 down-regulation.

Cytosolic/Nuclear PtdIns(4,5)P_2 Down-regulation Results in the Delay of S Phase Entry—The effect of cytosolic/nuclear PtdIns(4,5)P_2 depletion on S phase entry induced by bombesin/PDB + insulin was investigated using two quantitative approaches: flow cytometry and BrdUrd incorporation. In the flow cytometry experiments, quiescent cells were stimulated for 24 h, and a significant decrease in S phase entry was observed in the cells that overexpressed EGFP-SKIP or EGFP-NLS-SKIP after bombesin/PDB + insulin treatment (Fig. 7*A*). Results similar to those with wild-type Swiss 3T3 fibroblasts were obtained with cells expressing catalytically inactive SKIP. Next, to investigate whether the observed S phase entry delay imposed by overexpressed SKIP can be circumvented by a stronger mitogen or prolonged stimulation, 10% NCS was included, and the stimulation time was extended to 32 h in the BrdUrd incorporation assays. Treatments of cells with 10% NCS induced high levels of BrdUrd incorporation in all cell lines tested, indicating that the overexpressed SKIP activity could not counteract this potent mitogen. However, even with the prolonged stimulation time, bombesin/PDB + insulin failed to induce a similar level of BrdUrd incorporation in the EGFP-SKIP- and EGFP-NLS-SKIP-expressing Swiss 3T3 cells in contrast to their SKIP-D269A-expressing counterpart cell lines (Fig. 7*B*).

Overexpression of Cytosolic/Nuclear SKIP Delays the Expression of Cyclins D_1 and A_2 Induced by Bombesin and Insulin—The data obtained from the S phase entry analyses clearly demonstrate that cytosolic/nuclear PtdIns(4,5)P_2 down-regulation resulted in delayed S phase entry induced by bombesin/PDB + insulin. To investigate the underlying molecular mechanism, time course experiments were carried out, and immunoblot analyses were performed to detect key cell cycle regulatory proteins. Following stimulation, cells were harvested at 8-h intervals from 8 to 32 h. Treatment of quiescent wild-type Swiss 3T3 fibroblasts with bombesin + insulin (Fig. 8) resulted in the up-regulation of all cyclins tested. The induction of cyclin A_2 began 16 h post-stimulation, reaching a plateau after 24 h. Cyclins D_1 and D_3 were detected at all time points after stimulation. A time-dependent phosphorylation pattern was observed for Cdk2 (the phosphorylated form is the faster migrating species), where its phosphorylation was detectable 16 h post-stimulation. Cdk4 and Cdk6 levels remained constant over the time frame tested. The highest level of p27 was detected in the resting cells, and down-regulation was observed 8–16 h after stimulation. Analysis of pRb phosphorylation status by mobility shift indicated that the hyperphosphorylated species was detectable from 16 h post-stimulation and increased in abundance thereafter. The onset of pRb hyperphosphorylation coincided with Cdk2 phosphorylation and the up-regulation of cyclin A_2. Comparable results were obtained when EGFP-SKIP- and EGFP-NLS-SKIP-expressing Swiss 3T3 cells were treated with bombesin + insulin (Fig. 8). In comparison with wild-type Swiss 3T3 cells, less up-regulation of cyclins D_1 and A_2 was observed in both cell lines expressing active SKIP. The induction of cyclin A_2 was delayed, being detectable only 24 h post-stimulation. Quantitation of these blots (supplemental Fig. S5) indicated that cyclin D_1 expression was retarded in both lines expressing active SKIP. No notable difference was observed for cyclin D_3, Cdk4, Cdk6, and p27. Consistent with the expression levels of cyclin A_2 and the phosphorylation of Cdk2, pRb hyperphosphorylation was also markedly delayed,

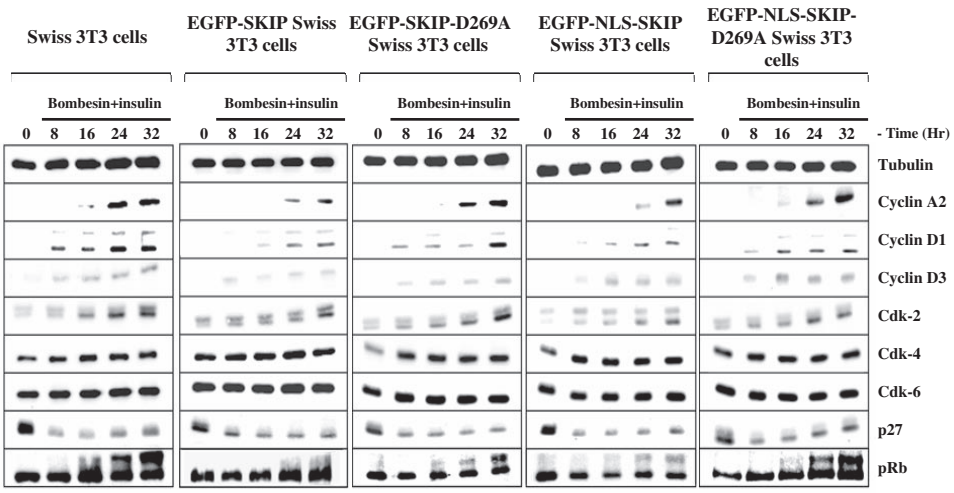

FIGURE 8. **Overexpression of cytosolic/nuclear SKIP delays the expression of cyclins D_1 and A_2 induced by bombesin + insulin.** Quiescent Swiss 3T3 and SKIP-overexpressing cells were stimulated with bombesin + insulin for the indicated time periods. Cells were then lysed and immunoblotted using antibodies against the indicated proteins. The immunoblots shown are representative of typical results from three independent experiments.

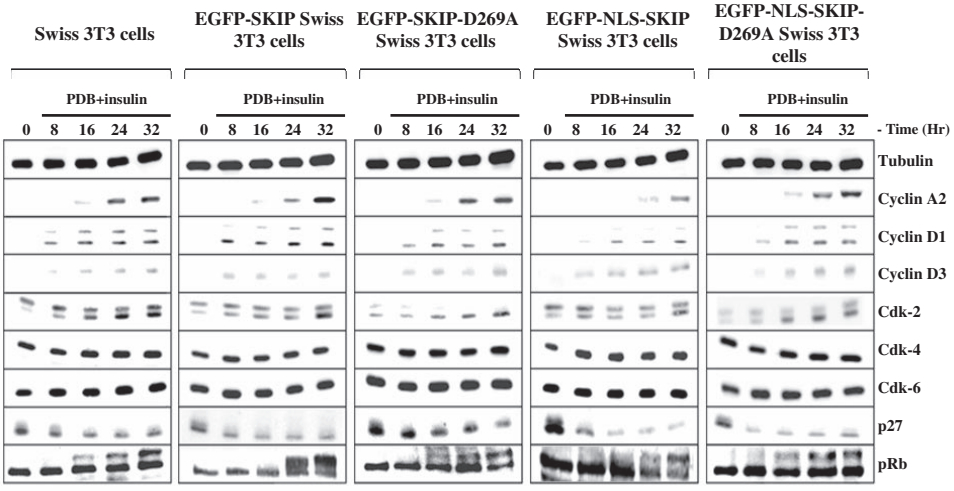

FIGURE 9. **Overexpression of nuclear SKIP delays the expression of cyclin A_2 induced by PDB + insulin.** Cells were processed as described in the legend to Fig. 8, that except PDB + insulin was used as a stimulant. The immunoblots shown are representative of typical results from three independent experiments.

being weakly detectable only 24 h post-stimulation. Results obtained with EGFP-SKIP-D269A- and EGFP-NLS-SKIP-D269A-expressing Swiss 3T3 cells (Fig. 8) showed close resemblance to those obtained with wild-type Swiss 3T3 cells, thus illustrating that overexpression of the inactive SKIP mutant has little effect on cell cycle progression in these cells.

Identification of Cyclin A_2 as the Specific Downstream Effector of Nuclear PtdIns(4,5)P_2—We repeated the above analysis using PDB + insulin treatment. PDB + insulin stimulation of Swiss 3T3 cells resulted in changes to cell cycle regulators (Fig. 9) similar to those seen with bombesin + insulin (Fig. 8). Consistently, the results obtained with EGFP-SKIP-D269A- and EGFP-NLS-SKIP-D269A-expressing Swiss 3T3 cells were most similar to those obtained with the parental cells (Fig. 9), thus reinforcing the lack of interference of overexpression of the inactive SKIP mutant in cell cycle progression. As predicted and in agreement with the results obtained from the Erk-1 and $p70^{S6K}$ mobility shift experiments (Fig. 6, *B* and *C*), the retardation of mitogenesis imposed by cytosolic SKIP was rescued by the direct activation of PKC using PDB as indicated by the restoration both of the normal expression patterns of cyclins D_1 and A_2 and of pRb phosphorylation. Although EGFP-SKIP- and EGFP-NLS-SKIP-expressing Swiss 3T3 cells exhibited similar inhibitory effects (relative to the parental cells) on the cell cycle regulatory components in the bombesin + insulin experiments, distinct cyclin A_2 expression patterns were observed when PDB + insulin were used (Fig. 9). In all other cell lines, cyclin A_2 expression induced by PDB + insulin was detectable 16 h post-stimulation, but was weakly discernible only at 24 h in EGFP-NLS-SKIP-expressing Swiss 3T3 cells (see also supplemental Fig. S5, *C* and *D*). Next, to determine whether the observed cyclin A_2 down-regulation occurs at the mRNA level, real-time quantitative PCR was performed. Quiescent cells were stimulated with PDB + insulin for 16, 24, and 32 h, and their total RNA was extracted and analyzed (Fig. 10*A*). The ribosomal gene L19 was used as the internal control. Relative to the wild-type and mutant SKIP cell lines, lower levels of cyclin A_2 transcripts were detected in the cytosolic/nuclear SKIP-overexpressing cells at all time points. Consistent with the immunoblot results (Fig. 9), the lowest levels of cyclin A_2 transcripts were detected in the nuclear SKIP-overexpressing cells. In addition, immunohistochemistry of these cells indicated that nuclear SKIP overexpression preferentially reduced the accumulation of cyclin A_2 in the nuclei of the stimulated cells (Fig. 10*B* and supplemental Fig. S6). Thus, taken together, the data shown in this work demonstrate a specific effect of PtdIns(4,5)P_2 down-regulation on cyclin A_2 at both the mRNA and protein levels.

DISCUSSION

To determine the cell cycle regulatory component(s) that are under the influence of nuclear PtdIns(4,5)P_2 in a mammalian cell system, recombinant Swiss 3T3 cell lines with their cytosolic/nuclear PtdIns(4,5)P_2 down-regulated were engineered as the study model. Using this cell system, the PtdIns(4,5)P_2-dependent signaling pathways activating Akt via PI 3-kinase and MAPK/$p70^{S6K}$ via PKC were examined. As expected, the

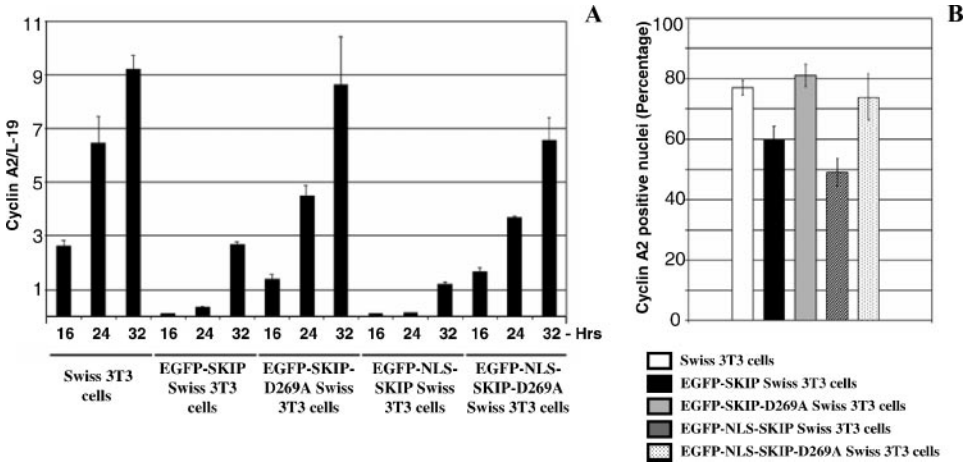

FIGURE 10. **Overexpression of nuclear SKIP specifically down-regulates cyclin A_2 mRNA and nuclear accumulation.** Quiescent wild-type Swiss 3T3 cells and the various recombinant Swiss 3T3 cells were stimulated with PDB + insulin for the indicated times. *A*, real-time quantitative PCR for cyclin A_2 transcripts was performed, and measurements are presented as the means ± range of two independent experiments normalized against the L19 transcript. *B*, cells were fixed and stained for cyclin A_2. The results show the percentage of cells with nuclear cyclin A_2 (means ± S.D.) from three independent experiments with at least 200 cells counted per experiment.

results obtained from these signaling studies reveal that the down-regulation of cytosolic PtdIns(4,5)P_2 has a pronounced inhibitory effect on Akt/MAPK/p70^{S6K} activation. In all three signaling pathways studied, the level of inhibition was more pronounced in the cytosolic SKIP cell line compared with the nuclear SKIP cell line, and this is most likely a reflection of the lower cytosolic PtdIns(4,5)P_2 level found in the cytosolic SKIP-overexpressing cells. When the cytosolic SKIP-overexpressing cells were challenged with PDB, MAPK/p70^{S6K} activations were partially restored to levels similar to those in wild-type cells. The incomplete rescue observed (especially at the 10-min time point) could be related to the intracellular calcium level in the SKIP-overexpressing cells. One might expect the basal Ins(1,4,5)P_3 level in cells overexpressing cytosolic SKIP to be lower than that in normal cells. Because the classic PKCs (α, β, and γ) require calcium as their cofactor (33), the decrease in intracellular calcium in the cytosolic SKIP-overexpressing cells may lead to reduced PKC activation. In line with this assumption, preincubation with calcium chelators attenuates PDB signaling in osteoblast and smooth muscle cells (34, 35).

In terms of cell cycle progression, overexpression of cytosolic or nuclear SKIP delayed S phase entry induced by bombesin/PDB + insulin to a similar level. Because cytosolic and nuclear SKIP-overexpressing cells responded to bombesin and PDB differently (as shown by the MAPK/p70^{S6K} experiments), the comparable retardation of S phase entry strongly implies that nuclear SKIP interferes with the $G_0/G_1/S$ transitions at a different level compared with cytosolic SKIP. More detailed analyses of the cell cycle regulatory components revealed that the delay in bombesin-induced S phase entry in the cytosolic PtdIns(4,5)P_2-deficient cells was channeled through cyclin D_1, whereas the delay in PDB-induced S phase entry in the nuclear PtdIns(4,5)P_2-deficient cells was channeled predominantly through cyclin A_2. Although the link between cytosolic PtdIns(4,5)P_2 and cyclin D_1 has been highlighted by studies on Erk-1-mediated cyclin D_1 gene activation (36–40), Akt-mediated cyclin D_1 gene activation (41–43), and Akt-mediated cyclin D_1 protein stabilization (44), the relationship between nuclear PtdIns(4,5)P_2 and cyclin A_2 is currently unknown. From the classical signaling perspective, one of the main functions of PtdIns(4,5)P_2 is the generation of the second messengers DAG and Ins(1,4,5)P_3 through its hydrolysis by PLC. The best characterized signaling function of DAG is its activation of PKC, and nuclear DAG seems likely to conform to this role. Apart from the nuclear translocation of PKCα demonstrated in IGF-I-stimulated Swiss 3T3 cells (6), other known nuclear PKC isoforms include PKCβ, PKCδ, and PKCϵ and PKCζ (45, 46). However, to date, few of the nuclear substrates of the PKCs have been rigorously defined. Because at present the link between nuclear PKCs and cyclins is not evident, it remains to be seen whether signals downstream of nuclear PKCs have a direct role in regulating the expression levels of cell cycle regulatory components such as cyclin A_2.

Another important second messenger generated by PLC is Ins(1,4,5)P_3, a well known Ca^{2+} liberator. The binding of nuclear Ins(1,4,5)P_3 to the Ins(1,4,5)P_3 receptors increases intranuclear Ca^{2+} levels and consequently leads to the activation of Ca^{2+}-dependent proteins. Apart from its role in controlling nuclear calcium levels, another emerging function of nuclear Ins(1,4,5)P_3 is that of transcriptional regulation as exemplified by the control of genes involved in arginine metabolism in *Saccharomyces cerevisiae* (47). Interestingly, genetic evidence has implicated nuclear inositol polyphosphate in the export of mRNA through the nuclear pore complex (48). Therefore, in the context of this investigation, it is reasonable to speculate that the down-regulation of PtdIns(4,5)P_2 (and hence, the inositol polyphosphate species) might impair in part the export of cyclin A_2 transcripts, resulting in the down-regulation of the biosynthesis of the gene product.

The assignment of any physiological outcome as a direct consequence of nuclear PI metabolism remains difficult because most of the extracellular stimuli that can lead to nuclear PI turnover inevitably affect cytosolic PI homeostasis. Nevertheless, from the literature on nuclear PI signaling and the data presented here, it is clear that the signals generated from the cytosolic/nuclear PtdIns(4,5)P_2 systems can be at least partially dissociated with cyclin A_2 being preferentially targeted by nuclear PtdIns(4,5)P_2 modulation at the mRNA and protein levels. The data from this study give new mechanistic insights on how nuclear PtdIns(4,5)P_2 interacts with the cell cycle machinery and should therefore aid the subsequent investigation in this field.

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