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Research Article

Combined Inhibition of MEK and Mammalian Target of Rapamycin Abolishes Phosphorylation of Cyclin-Dependent Kinase 4 in Glioblastoma Cell Lines and Prevents Their Proliferation

Sabine Paternot and Pierre P. Roger

Institute of Interdisciplinary Research, Université Libre de Bruxelles, Campus Erasme, Brussels, Belgium

Abstract

The Ras/Raf/MEK/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) signaling pathways are aberrantly activated in many tumors, including highly proliferative glioblastomas, but how they are wired with the cell cycle remains imperfectly understood. Inhibitors of MEK/ERK and mTOR pathways are tested as anticancer agents. They are generally considered to induce a G_1 cell cycle arrest through down-regulation of D-type cyclins and up-regulation of $p27^{kip1}$. Here, we examined the effect of targeting mTOR by rapamycin and/or MEK by PD184352 in human glioblastoma cell lines. In combination, these drugs cooperatively and potently inhibited the G_1-S transition and retinoblastoma protein phosphorylation. Their cooperation could not be explained by their partial and differential inhibitory effects on cyclin D1 or D3 but instead by their synergistic inhibition of the activating T172 phosphorylation of cyclin-dependent kinase (CDK) 4. This appeared independent of p27 and unrelated to weak modulations of the CDK-activating kinase activity. The T172 phosphorylation of CDK4 thus appears as a crucial node integrating the activity of both MEK/ERK and mTOR pathways. Combined inhibition of both pathways should be considered as a promising strategy for treatment of tumors harboring a deregulated CDK4 activity. [Cancer Res 2009; 69(11):4577–81]

Introduction

The Ras/Raf/MEK/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) signaling pathways are aberrantly activated in many tumors including glioblastomas, leading to deregulation of various metabolic processes, including proliferation and cell cycle progression. Inhibitors of these cascades are thus of great interest for cancer therapy and are, or have been, used in clinical trials (1–5). Rapamycin very specifically inhibits mTOR-raptor complex (mTORC1), resulting in dephosphorylation of p70 S6 kinase and 4EBP1 and inhibited protein translation (4). Moreover, MEK inhibitors and rapamycin induce G_1 cell cycle arrests in various cell systems, which is associated with inhibition of retinoblastoma protein (pRb) phosphorylation through inactivation of cyclin-dependent kinases (CDK) 4/6 and 2 (6). This inhibition of CDK4 activity is believed to result from down-regulation of D-type cyclins (7–10) and/or accumulation of the CDK inhibitor $p27^{kip1}$ (11).

CDK4 couples mitogenic/oncogenic signaling pathways with the core cell cycle regulation. Its activation is essential for many oncogenic processes (9, 12). Critical features of CDK4 activation remain poorly known or debated, including its regulated association with D-type cyclins, subcellular location, activating T172 phosphorylation, and the roles of Cip/Kip CDK "inhibitors" in these processes (13). We have recently shown CDK4 T172 phosphorylation as a crucial target for both mitogenic and antimitogenic signals (14–16). Here, we identify it as the target of strong inhibitions of S-phase entry in glioblastoma cell lines by a combined inhibition of mTORC1 and MEK1/2 by rapamycin and PD184352.

Materials and Methods

Cell culture. T98G, U-87 MG, and U-138 MG (American Type Culture Collection) are tumorigenic human glioblastoma cell lines. T98G cells retain serum dependence of proliferation but lack both p16 and functional p53, whereas U-87 MG cells express wild-type p53. They were cultured in DMEM + 10% fetal bovine serum (FBS). After starvation in 0.2% FBS for 3 days, cells were growth stimulated by 15% FBS and DNA replicating cells were identified by 30 min incubation with BrdUrd. Rapamycin was from Calbiochem and PD184352 was kindly provided by Dr. Philip Cohen (University of Dundee). They were dissolved in ethanol and DMSO, which were thus added in all the treatments at concentrations of 0.2% and 0.1%, respectively.

Immunoblot analyses. Equal amounts of whole-cell extract proteins were separated according to molecular mass and immunodetected using the following antibodies: monoclonal antibodies against cyclin D1, cyclin D3, and p27 (all from Neomarkers); anti-total pRb monoclonal antibody (BD Pharmingen); polyclonal anti-phospho-pRb (T826) antibody (Biosource/Invitrogen); polyclonal antibodies against phospho-p70 S6 kinase (T389), phospho-Akt (T308), and total Akt, rabbit monoclonal antibody against phospho-S6 ribosomal protein (S235/236), and monoclonal antibody against phospho-p42/44 mitogen-activated protein kinases (T202/Y204; all from Cell Signaling Technology); and polyclonal antibodies against CDK4, cyclin H, ERK2, and monoclonal anti-CDK7 antibody (all from Santa Cruz Biotechnology). The monoclonal antibody from BD Pharmingen was used for simultaneous detection of both poly(ADP-ribose) polymerase and its cleavage product and the anti-α-tubulin antibody was from Neomarkers. Secondary antibodies were coupled either to horseradish peroxidase (Amersham Biosciences) for detection by enhanced chemiluminescence (Western Lightning; Perkin-Elmer) or to DyLight 680 and 800 (Pierce Biotechnology) for infrared fluorescence detection using the Odyssey scanner (LI-COR).

Immunoprecipitation. Coimmunoprecipitations were done as described (14, 15) using monoclonal antibodies against cyclin D1 and D3 (Neomarkers), a mixture of the K25020 anti-p27 monoclonal antibody (BD Pharmingen) and the C-15 p27 polyclonal antibody (Santa Cruz Biotechnology), or polyclonal antibody against cyclin H (Santa Cruz Biotechnology).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Pierre P. Roger, Institute of Interdisciplinary Research, Université Libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Brussels, Belgium. Phone: 32-2-555-41-53; Fax: 32-2-555-46-55; E-mail: proger@ulb.ac.be.

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pRb kinase assay. As described (14, 15), immunoprecipitated protein complexes were incubated with ATP and a recombinant pRb fragment (QED) before SDS-PAGE separation of the incubation mixture and Western blotting detection of the S780 phosphorylation of the pRb fragment, cyclin D1, cyclin D3, CDK4, and p27.

Separation of phospho-CDK4 by two-dimensional gel electrophoresis. As described (14), immunoprecipitated protein complexes were denatured in a buffer containing 7 mol/L urea and 2 mol/L thiourea. Proteins were separated by isoelectric focusing on immobilized linear pH gradient (pH 3-10) strips. After SDS-PAGE separation and blotting, CDK4 was immunodetected and enhanced chemiluminescence detections were quantified using a GS-800 densitometer and the Quantity One software (Bio-Rad Laboratories).

CDK-activating kinase activity assay. As described (16), inactive cyclin D3 complexes containing nonphosphorylated CDK4 or CDK6 were immunoprecipitated from serum-starved T98G cells (14) and used as a substrate for activation by recombinant CDK-activating kinase (CAK; positive control) or CAK complexes immunoprecipitated from the assayed T98G cells. Their *in vitro* activation was then shown by their pRb kinase activity assessed as above.

Transfection. During starvation in 0.2% FBS, T98G cells were transfected for 12 h using Lipofectamine with 2 μg/mL pcDNA3 vectors encoding HA-tagged wild-type CDK4 or T172ACDK4 (14). Cells were then stimulated by 15% FBS for 16 h with bromodeoxyuridine (BrdUrd) during the last 30 min. For double immunofluorescent detection of incorporated BrdUrd and the HA epitope of CDK4 using two mouse monoclonal antibodies, cells were fixed with 2% paraformaldehyde for 90 s at 4°C and then with methanol for 10 min at -20°C, permeabilized with 0.1% Triton X-100 in PBS (pH 7.5) at room temperature, and blocked for 30 min with 5% normal sheep serum. BrdUrd was unmasked by 30 min incubation with 2 mol/L HCl. After washings, cells were then incubated overnight at 4°C with anti-HA antibody (Santa Cruz Biotechnology) and then for 2 h with Cy3-conjugated anti-mouse immunoglobulin (Jackson Immunoresearch). Washed cells were then successively incubated for 30 min with 1% normal mouse serum for 2 h with unconjugated anti-IgG F(ab) fragment (50 μg/mL; Jackson Immunoresearch) and then overnight at 4°C with mouse anti-BrdUrd monoclonal antibody (Becton Dickinson) followed by biotinylated anti-mouse immunoglobulin (Amersham) and fluorescein-conjugated streptavidin (Amersham).

Results

Complete inhibition of DNA synthesis and pRb phosphorylation requires the combined inhibition of MEK1/2 and mTOR-raptor pathways. Rapamycin and PD184352 have been tested as potential anticancer drugs in various phase I and II clinical trials (1, 2, 4). In serum-stimulated T98G and other glioblastoma cell lines, inhibition of MEK1/2 by PD184352 resulted in a selective dose-dependent decrease of ERK1/2 phosphorylation, whereas rapamycin completely and selectively inhibited the phosphorylation of $p70^{S6\ kinase}$ and S6 protein, reflecting mTORC1 activity (Fig. 1*A*; Supplementary Fig. S1). Used alone, rapamycin and PD184352 at concentrations that completely blocked ERK phosphorylation only partly inhibited the serum-induced DNA synthesis (Fig. 1*B*; Supplementary Fig. S1). Nevertheless, the combination of both drugs cooperatively blocked serum-stimulated S-phase entry (Fig. 2*A*) and proliferation (Fig. 2*B*) in T98G cells. In U-87 MG and U-138 MG cells that maintained a somewhat higher rate of DNA synthesis on serum deprivation, combined rapamycin and PD184352 in the presence of serum similarly cooperated to inhibit DNA replication, although the inhibition by PD184352 alone was more pronounced in U-87 MG cells (Supplementary Fig. S1*A* and *D*). On the other hand, rapamycin and PD184352, alone or in combination, did not increase the proportion of apoptotic cells in the three cell lines as detected by immunofluorescent detection of active caspase-3 and cleaved poly(ADP-ribose) polymerase (data not shown) and Western blotting detection of poly(ADP-ribose) polymerase and its cleavage product (Fig. 2*C*; Supplementary Fig. S1*B* and *E*).

Rapamycin and PD184352 similarly cooperated to inhibit pRb phosphorylation, including at T826, which is specifically targeted by CDK4 (Fig. 2*C*; Supplementary Fig. S1*B* and *E*). We thus analyzed the expression of CDK4 and its regulatory proteins (Fig. 2*C*; Supplementary Fig. S1*B* and *E*). CDK4 levels were not affected. Serum induction of cyclin D1 in T98G cells was partially inhibited by PD184352 and slightly more by combined drugs (Fig. 2*C*). In the other two cell lines, cyclin D1 accumulation was constitutive and only slightly reduced by PD184352 in U-87 MG cells (Supplementary Fig. S1*B* and *E*). By contrast, in the three cell lines, rapamycin but not PD184352 reduced cyclin D3 expression, which was essentially independent of serum (Fig. 2*C*; Supplementary Fig. S1*B* and *E*). In T98G cells, PD184352 and, more weakly, rapamycin prevented the serum-induced down-regulation of p27 (Fig. 2*C*). However, in the other two cell lines, serum and the inhibitors had a much weaker effect on p27 levels (Supplementary Fig. S1*B* and *E*), which thus unlikely mediated the strong modulations of DNA synthesis and pRb phosphorylation.

Rapamycin and PD184352 cooperatively inhibit CDK4 activity. We next analyzed the formation and pRb kinase activity of D-type cyclin-CDK4 complexes in T98G cells. Serum induced the pRb kinase activity associated with cyclin D1 and D3 (Fig. 2*D*). In cyclin D1 complexes, this was in part explained by increased cyclin

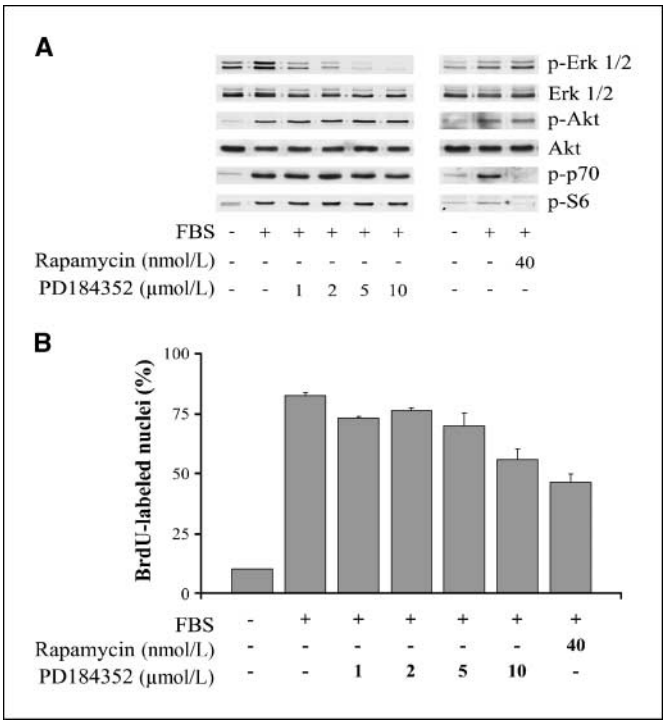

Figure 1. Suppression of MEK or mTOR activities only partially prevents DNA synthesis in T98G cells. Cells were stimulated (+) or not (-) with 15% FBS for 30 min (*A*) or 18 h (*B*) in the presence (+) or absence (-) of rapamycin or PD184352. *A*, T202/Y204 phosphorylation of ERK1/2 (*p-Erk 1/2*), T308 phosphorylation of Akt (*p-Akt*), T389 phosphorylation of p70 S6 kinase (*p-p70*), and S235/236 phosphorylation of S6 ribosomal protein (*p-S6*) as well as total Erk 1/2 and Akt were immunodetected. *B*, DNA synthesis was evaluated from duplicate dishes by counting the percentage of nuclei having incorporated BrdUrd (*BrdU*) during the last 30 min of stimulation.

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D1 expression, but inactive cyclin D3-CDK4 complexes were already present in serum-starved cells as described previously (14). Rapamycin alone reduced both the presence and the activity of cyclin D3-CDK4 complexes in serum-stimulated cells. However, the reduced presence of cyclin D3 appeared to reorient CDK4 toward cyclin D1 complexes, which remained active (Fig. 2*D*). Conversely, PD184352 weakly decreased the amount of cyclin D1 and cyclin D1-CDK4 complexes, but CDK4 association to cyclin D3 increased, and the pRb kinase activity of both cyclin D1-CDK4 and cyclin D3-CDK4 was only partially inhibited (Fig. 2*D*). An almost complete inhibition of pRb kinase activity was achieved in both cyclin D1 and D3 complexes only when rapamycin and PD184352 were combined (Fig. 2*D*).

In both cyclin D1 and D3 immunoprecipitations and all the different cell treatments, the presence of p27 and CDK4 closely correlated (Fig. 2*D*). Even at its reduced levels in serum-stimulated T98G cells (as seen in Fig. 2*C*), p27 is not limited for its association with cyclin D1/3-CDK4 complexes (14). Therefore, the observed modulations of p27 accumulation in response to rapamycin and/or PD184352 (Fig. 2*C*) had no effect on p27 association to CDK4 complexes (Fig. 2*D*) and thus unlikely explained the inhibition of the activity of cyclin D1/3-CDK4 complexes by these drugs.

Rapamycin and PD184352 cooperatively inhibit CDK4 activating phosphorylation. In several systems including T98G cells, we have identified the activating T172 phosphorylation of CDK4 as a crucial target for regulation of the activity of cyclin D1/3-CDK4 complexes (13–16). The relative presence of phosphorylated and nonphosphorylated CDK4 forms in coimmunoprecipitated complexes was assessed as previously (14) using two-dimensional gel electrophoresis (Fig. 3*A*). We have previously identified the most negatively charged form as the T172 phospho-CDK4 using [^{32}P]phosphate incorporation, a new phosphospecific antibody, *in vitro* phosphorylation by recombinant CAK, and analysis of T172A-mutated CDK4 (14).

Serum stimulation of T98G cells induced the T172 phosphorylation of CDK4 associated not only with cyclins D1 and D3 but also with p27 (Fig. 3*A*). In the other two cell lines, T172 phospho-CDK4 was also abundantly associated with p27 (Supplementary Fig. S1*C* and F), as we have observed it in a variety of cell types and situations (14, 15). At variance with initial claims (17), p27 thus unlikely prevented the activating phosphorylation of CDK4 (14). In T98G cells, combined rapamycin and PD184352 totally inhibited the phosphorylation of CDK4 coimmunoprecipitated with cyclin D1, cyclin D3, or p27 (Fig. 3*A*), thus explaining the total inhibition of pRb kinase activity (Fig. 2*D*). Both drugs used alone induced partial inhibition of CDK4 phosphorylation (Fig. 3*A*). In U-87 MG and U-138 MG cells, the phosphorylation of CDK4 associated to cyclin D1 or D3 was also strongly inhibited only when rapamycin and PD184352 were combined (Supplementary Fig. S1*C* and *F*). Nonphosphorylated T172A CDK4 but not wild-type CDK4 prevented serum-induced DNA synthesis in T98G cells (Fig. 3*B*), showing that inhibition of CDK4 T172 phosphorylation can suffice to explain the cell cycle arrest by combined rapamycin and PD184352.

Combined rapamycin and PD184352 do not block CAK activity. The activating T172 phosphorylation of CDK4, like the analogous T-loop phosphorylation of other CDKs, is considered to be done only by CAK, the cyclin H-CDK7-Mat1 complex (18, 19). However, the activity of CAK is generally constitutive and nonregulated (13, 14, 18). We thus assessed the activity of cyclin H-CDK7 complexes from cells treated or not with rapamycin and/or PD184352. As shown in Fig. 3*C*, cyclin H-CDK7 complexes from serum-stimulated T98G cells had only a weak intrinsic pRb kinase activity (*lane 9*) but strongly increased the pRb kinase activity of inactive cyclin D3-CDK4/6 complexes from quiescent T98G cells used as a substrate (*lanes 2* and *8*). Unstimulated cells (*lane 1*) also contained (somewhat less abundant) active cyclin H-CDK7 complexes (*lane 2*). Rapamycin (*lane 3*) but not PD184352 (*lane 4*)

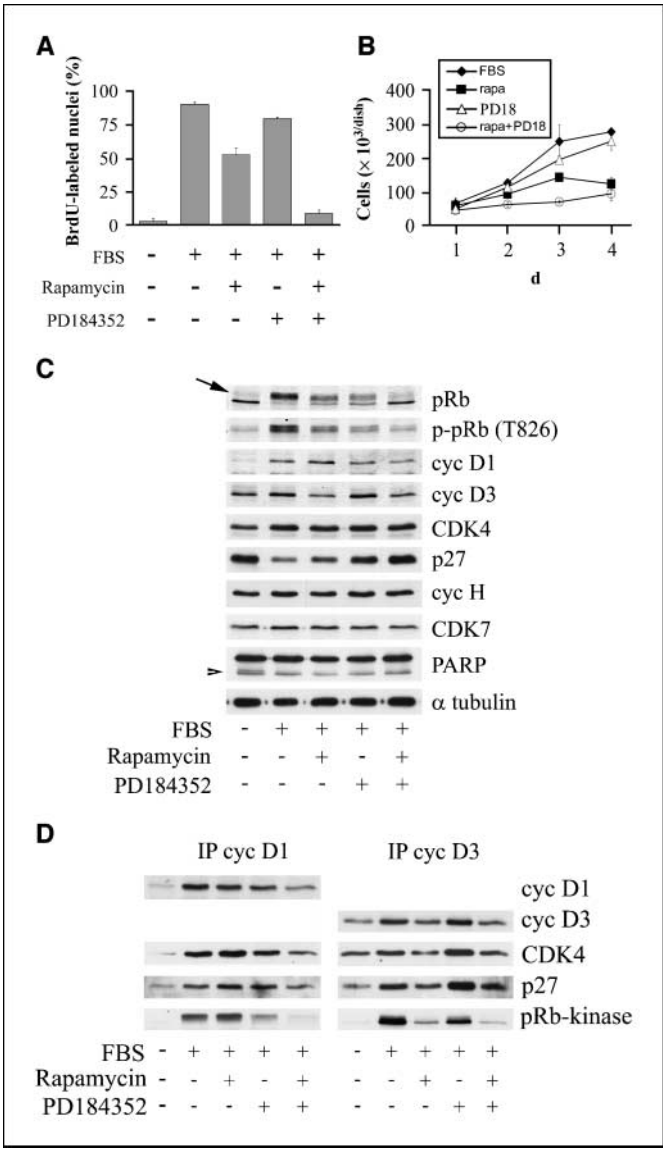

Figure 2. PD184352 cooperates with rapamycin to completely prevent DNA synthesis (*A*), cell proliferation (*B*), pRb phosphorylation (*C*), and pRb kinase activity (*D*). T98G cells were stimulated or not with 15% FBS for 18 h (*A*) or 10 h (*C* and *D*) and treated or not with rapamycin (*rapa*; 40 nmol/L) and/or PD184352 (5 μmol/L). *A,* DNA synthesis was evaluated from duplicate dishes by counting the percentage of nuclei having incorporated BrdUrd during the last 30 min of stimulation. *B,* cell proliferation in the continuous presence of FBS and inhibitors as indicated was evaluated by cell counting from duplicate 30 mm Petri dishes. *C,* pRb, T826 phosphorylation of pRb (*p-pRb*), cyclin D1 and D3, CDK4, p27, cyclin H, and CDK7 were immunodetected from whole-cell lysates. *Arrow*, hyperphosphorylated forms of pRb. The cleaved form of poly(ADP-ribose) (*PARP*) polymerase (*arrowhead*) was detected as a marker of apoptosis. *D,* cell lysates were immunoprecipitated (*IP*) with anti-cyclin D1 (*cyc D1*) or anti-cyclin D3 (*cyc D3*) antibodies, assayed for pRb kinase activity, separated by SDS-PAGE, and immunoblotted. Cyclin D1, cyclin D3, CDK4, p27, and the in vitro S780 phosphorylation of the pRb fragment (*pRb-kinase*) were detected using specific antibodies.

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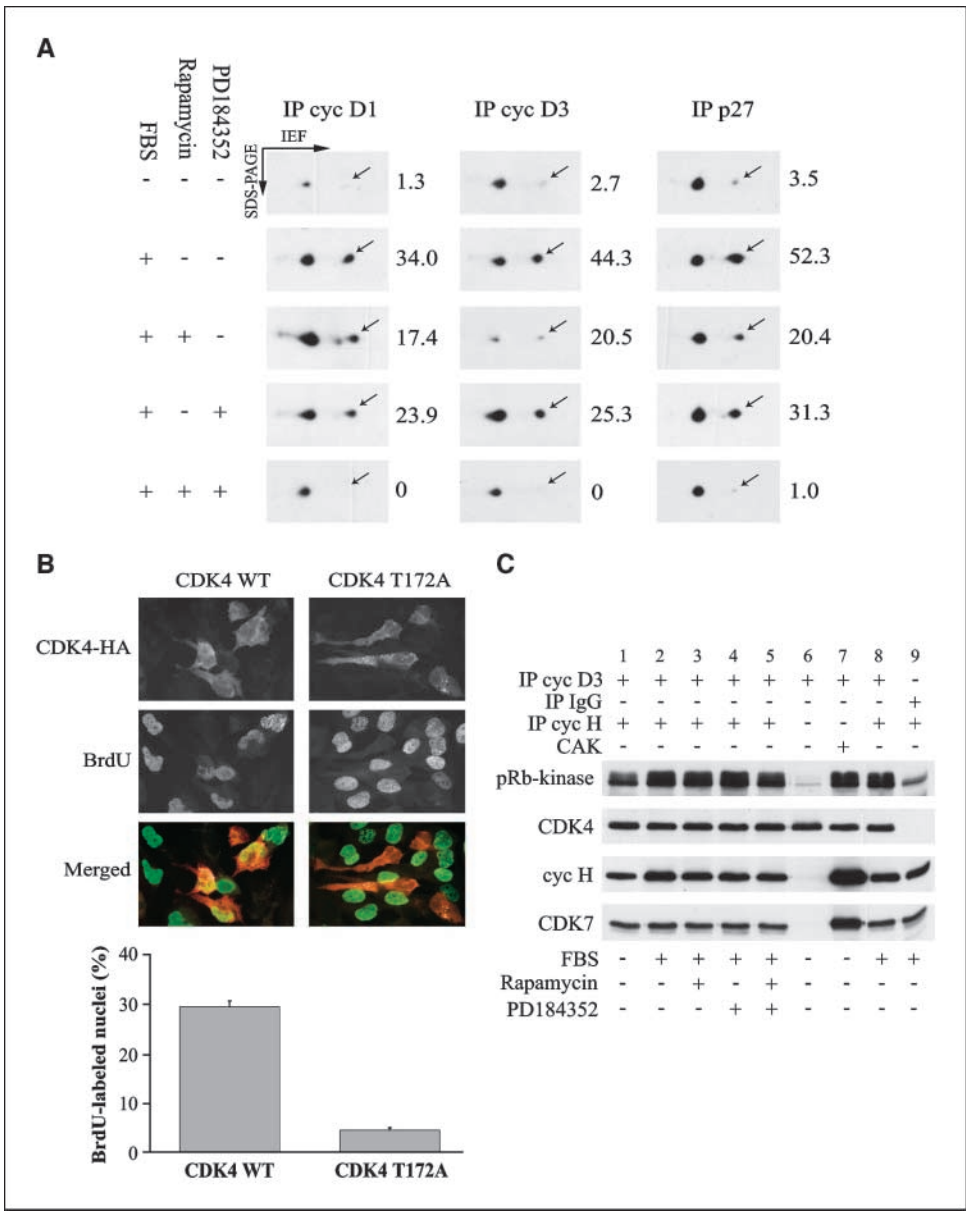

Figure 3. PD184352 cooperates with rapamycin to prevent CDK4 activating phosphorylation (*A*), which is crucial for DNA synthesis induction (*B*), but CAK activity is not markedly affected by combined drugs (*C*). *A,* T98G cells were stimulated with 15% FBS for 10 h and treated with rapamycin (40 nmol/L) and/or PD184352 (5 μmol/L). Cell lysates were immunoprecipitated with anti-cyclin D1, anti-cyclin D3, or p27 antibodies and separated by two-dimensional gel electrophoresis and CDK4 was immunodetected. *Arrows*, T172 phosphorylated form of CDK4. Proportion (%) of the phosphorylated form relative to total CDK4. *B,* quiescent T98G cells were transfected with plasmids encoding wild type (wt) CDK4-HA or T172ACDK4-HA and stimulated with 15% FBS for 16 h with BrdUrd during the last 30 min. In duplicate dishes, DNA synthesis was evaluated in 100 cells per dish displaying the HA epitope by counting the proportion of nuclei having incorporated BrdUrd. Double immunofluorescent detection of the HA epitope (*red*) and BrdUrd (*green*). *C,* activity of coimmunoprecipitated cyclin H-CDK7 complexes (*IP cyc H*) was evaluated from T98G cells treated as in *A*. In this assay, these cyclin H-CDK7 complexes (*lanes 2–5* and *8*) or a recombinant cyclin H-CDK7-Mat1 complex (CAK; *lane 7*) were mixed and incubated with ATP and inactive cyclin D3-CDK4/6 complexes immunoprecipitated from quiescent T98G cells (*IP cyc D3*; *lane 6*), or a similar mock immunoprecipitation (*IP IgG*; *lane 9*), used as a substrate. The resulting activation of the cyclin D3-CDK4/6 complexes was then assayed by their pRb kinase activity. The mixtures were separated by SDS-PAGE and immunoblotted. We detected cyclin H (*cyc H*) and CDK7 coimmunoprecipitated by the cyclin H antibody from T98G cells or recombinant CAK complex, the presence of the substrate, that is, cyclin D3-CDK4 complexes from quiescent T98G cells (*CDK4*), and its *in vitro* activation reflected by the S780 phosphorylation of the pRb fragment.

very slightly inhibited cyclin H expression and CAK activity, and PD184352 did not amplify this weak inhibition (*lane 5*). Overall, the modulations of CDK4 phosphorylation (Fig. 3*A*) did not correlate with the presence and activity of CAK (Figs. 2*C* and 3*C*). Of note, the inactive yet activatable cyclin D3-CDK4/6 complexes from quiescent T98G cells did not autoactivate (and thus did not autophosphorylate) during incubation with 2 mmol/L ATP (Fig. 3*C, lane 6*), consistent with the normal T172 phosphorylation of inactive K35RCDK4-cyclin D3 in serum-stimulated cells.[1]

Discussion

In this study, we have observed in several glioblastoma cell lines that an almost complete inhibition of DNA synthesis, pRb phosphorylation, and CDK4 activity could not be achieved using rapamycin or PD184352 alone but with the combination of both drugs. The synergy of rapamycin and PD184352 on CDK4 activity could not be explained by their partial and differential inhibitory effects on cyclin D1 or D3 accumulation but instead by their cooperative inhibition of CDK4 T172 phosphorylation, which appears independent of p27 or modulation of CAK activity. Although it remains possible that combined rapamycin and PD184352 could also impair cell cycle progression by other mechanisms unrelated to CDK4, the observed inhibition of CDK4 phosphorylation and activity is fully sufficient to explain the G_1-phase arrest. The present study thus provides first evidence that MEK and mTOR-dependent cascades might signal cell cycle progression through T172 phosphorylation of CDK4, which appears as a crucial node integrating these pathways.

The mechanisms of regulation of CDK4 activating T172 phosphorylation are unknown. Together with other unexpected observations, such as our findings that the analogous T-loop

[1] H. Kooken, L. Bockstaele, P.P. Roger, unpublished data.

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phosphorylation of cyclin D3-bound CDK6 is not induced by serum in T98G cells (14) or that CDK4 phosphorylation is not precluded by p27 binding in various cells (14), the present results support our hypothesis that CAK might not be the regulated CDK4-activating kinase (13). Ras/Raf/MEK/ERK and mTOR pathways might cooperate by activating different CDK4 kinases (and/or inactivate different CDK4 phosphatases), separately concurring to the crucial activation of D-type cyclin-CDK4 complexes. Alternatively, they could converge at an upstream step regulating such unknown CDK4 kinase(s). The major challenge ahead will be to elucidate such intermediary mechanisms, which may lead to identification of novel therapeutic targets and suggest novel cell cycle-based therapies in malignant glioblastomas and other tumors.

Rapamycin and derivatives, as well as MEK inhibitors, have been tested extensively in patients with highly proliferative tumors such as recurrent glioblastomas, but only a minority of patients showed an anticancer activity and the molecular determinants of drug response are generally unknown (2, 4, 5). On the other hand, a few *in vitro* studies recently reported cooperative antiproliferative effects of combined suppression of MEK and mTOR activities in tumor cells of various origins (20–22). A recent study by Rich et al. has shown the combinatorial benefits of RAD001 mTORC1 inhibitor and LBT613, a new Raf inhibitor, in blocking proliferation and invasion of glioma cell lines (23). Together with this prior study, our results suggest that the combined inhibition of mTOR and ERK pathways should be considered, in preclinical animal models as well as in clinical settings, to treat not only glioblastomas, which are resistant to most therapies, but also other tumors harboring a deregulated CDK4 activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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S. Paternot and P.P. Roger are postdoctoral researcher and senior research associate of the Belgian Fonds de la Recherche Scientifique-FNRS, respectively.

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