Combined Inhibition of MEK and mTOR Prevents Proliferation of Glioblastoma Cell Lines by Abolishing Phosphorylation of Cyclin-Dependent Kinase 4

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Abstract

Ras-Raf-MEK-Erk and PI3K-Akt-mTOR signalling 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 the MEK/Erk and mTOR pathways are currently tested as anticancer agents. These drugs are generally considered to induce a G1 cell cycle arrest through down-regulation of D-type cyclins and up-regulation of the CDK inhibitor $p27^{kip1}$. In the present study, we examined the effect of targeting mTOR by rapamycin and/or MEK by PD184352 in human glioblastoma cell lines including T98G cells. In combination, rapamycin and PD184352 cooperatively and very potently inhibited the G1-S transition and the phosphorylation of pRb. The cooperation of both drugs could not be explained by their partial and differential inhibitory effects on cyclin D1 or cyclin D3 accumulation, but instead by their synergistic inhibition of the activating T172-phosphorylation of CDK4. This appeared independent of p27 and unrelated to weak modulations of the CDK-activating kinase (CAK) 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.

Introduction

The Ras-Raf-MEK-Erk and PI3K-Akt-mTOR signalling pathways are aberrantly activated in many tumors including glioblastomas. This leads 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 (1-4). Rapamycin very specifically inhibits mTOR-raptor complex (mTORC1), resulting in dephosphorylation of p70 S6 kinase and 4EBP1 and inhibition of protein translation (4). Moreover, MEK inhibitors and rapamycin induce G1 cell cycle arrests in various cell systems, which is associated with inhibition of retinoblastoma protein (pRb) phosphorylation through inactivation of cyclin-dependent kinases (CDKs) 4/6 and 2 (5). This inhibition of CDK4 activity is most generally believed to result from down-regulation of D-type cyclins (6-9) and/or accumulation of the CDK inhibitor $p27^{kip1}$ (10).

CDK4 is a master integrator that couples mitogenic/oncogenic signalling pathways with the core cell cycle regulation. Its activation has been recently demonstrated to be essential for many oncogenic transformation processes (8, 11). Various critical features of CDK4 activation remain poorly known or debated, including the regulation of its association with D-type cyclins, its subcellular location, its activating T172-phosphorylation and the roles of Cip/Kip CDK "inhibitors" in these processes (12). We have recently identified the activating T172-phosphorylation of CDK4 as a crucial target for both mitogenic and antimitogenic signals (13, 14). In the present study in three human glioblastoma cell lines including T98G cells, we identify CDK4 T172-phosphorylation as the target of very strong inhibitions of S-phase entry by a combined inhibition of mTORC1 and MEK1,2 by rapamycin and PD184352. This abolition of CDK4 phosphorylation could not be explained by variations in cellular concentrations of D-type cyclins and p27 or inhibition of CDK-activating kinase (CAK) activity. Understanding how both pathways cooperate to induce CDK4 phosphorylation might suggest novel cell cycle-based therapies in malignant glioblastomas, which are resistant to most medical therapies, and other tumors.

Materials and Methods

Cell culture and reagents. T98G, U-87 MG and U-138 MG (American Type Culture Collection) are tumorigenic cell lines originating from human glioblastoma. 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. At the end of the mitogenic stimulation, DNA replicating cells were identified by a 30 min incubation with BrdU. Rapamycin was from Calbiochem and PD184352 (CI-1040, Pfizer) was kindly provided by Dr. Philip Cohen (University of Dundee, UK). 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 on SDS-PAGE and immunodetected after western blotting using the antibodies detailed in the *Supplementary information*.

Immunoprecipitation. Co-immunoprecipitations were performed exactly as described (13, 14) using monoclonal antibodies against cyclin D1 (DCS-11), cyclin D3 (DCS-28) (Neomarkers), a mixture of the K25020 anti-p27 monoclonal antibody from BD-Transduction Laboratories and the C-15 p27 polyclonal antibody (Santa Cruz Biotechnology), or polyclonal antibody against cyclin H (C-18; Santa Cruz Biotechnology).

pRb-kinase assay. Exactly as described previously (13, 14), 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 (using a phosphospecific-pRb antibody from Cell Signaling Technology), cyclin D1, cyclin D3, CDK4 and p27.

Separation of phosphorylated CDK4 by two-dimensional (2D)-gel electrophoresis. Exactly as described previously (13), immunoprecipitated protein complexes were denatured in a buffer containing 7 M urea and 2 M thiourea. Proteins were separated by isoelectric focusing on immobilized linear pH gradient (pH 3 to 10) strips (Amersham Biosciences). After SDS-PAGE separation and blotting, CDK4 was immunodetected using the C-22 polyclonal CDK4 antibody (Santa-Cruz). Enhanced chemiluminescence detections were quantified using a GS-800 densitometer and the Quantity One software (Biorad Laboratories).

CAK activity assay. Inactive cyclin D3 complexes containing non-phosphorylated CDK4 or CDK6 were immunoprecipitated from serum-starved T98G cells as above in NP-40 lysis buffer (13). They were used as a substrate for activation by recombinant CAK (positive control) or CAK complexes immunoprecipitated from the assayed T98G cells. Their *in vitro* activation was then assessed by their pRb-kinase activity. Cyclin D3 complexes from quiescent T98G cells were thus washed three times with NP-40 lysis buffer and then three times with CAK buffer (80 mM β-glycerophosphate [pH 7.3], 15 mM $MgCl_2$, 20 mM EGTA and 5 mM DTT (15)). The beads were resuspended in 30 µl of CAK buffer containing protease and phosphatase inhibitors with or without 1 µg of recombinant CAK (CDK7-cyclin H-MAT1 complex (Upstate)) or mixed with cyclin H-CDK7 complexes (co-immunoprecipitated as above using the C-18 cyclin H antibody) from T98G cells treated or not with FBS and inhibitors. After addition of 2 mM ATP, the suspensions were incubated at 30°C for 30 min. After three washes in CAK buffer and three washes in pRb-kinase buffer, the pRb-kinase activity of the immunoprecipitated proteins was assayed as above.

Transfection. During starvation in 0.2 % FBS, T98G cells were transfected for 12 h using lipofectamine (Invitrogen) with 2 µg/ml of pcDNA3 vectors encoding HA-tagged wt CDK4 or T172ACDK4 (13). Cells were then stimulated by 15 % FBS for 16 h with BrdU

during the last 30 min. After fixation, BrdU incorporation and CDK4-HA were sequentially detected by indirect immunofluorescence as detailed in the *Supplementary information.*

Results and Discussion

Complete inhibition of MEK1/2 or mTOR-raptor only partially inhibits DNA synthesis. Rapamycin and PD184352 are orally bioavailable inhibitors that are, or have been, tested as potential anticancer drugs in various phase I and phase II clinical trials (1, 4), including on patients with recurrent malignant gliomas in the case of rapamycin and derivatives (2). In serum-stimulated T98G glioblastoma cells, inhibition of MEK1/2 by PD184352 resulted in a selective dose-dependent decrease of Erk1/2 phosphorylation, without affecting PI3kinase and mTOR signalling cascades as judged from phosphorylations of Akt, $p70^{S6K}$ and S6 (Fig. 1*A*). On the other hand, rapamycin completely inhibited the phosphorylations of $p70^{S6K}$ and S6 protein, reflecting mTOR-raptor (mTORC1) activity, without affecting the Erk and Akt signalling pathways (Fig. 1*A*). As shown in Fig. 1*B*, PD184352 at concentrations that completely blocked Erk phosphorylations and rapamycin only partly inhibited the serum-induced DNA synthesis in T98G cells.

PD184352 cooperates with rapamycin to completely prevent DNA synthesis and pRb phosphorylation. Although rapamycin or PD184352 alone had only a moderate or weak (respectively) inhibitory effect on DNA synthesis in T98G cells, the combination of both drugs cooperatively blocked serum-stimulated S-phase entry (Fig. 2*A*) and proliferation (Fig. 2*B*). In two other glioblastoma cell lines (U-87 MG and U-138 MG) that maintained a somewhat higher rate of DNA synthesis upon serum deprivation, combined rapamycin and PD184352 treatment in the presence of serum similarly cooperated to very strongly inhibit DNA replication, though the inhibition by PD184352 alone was more pronounced in U-87

MG cells (Supplemental Fig. S1*A,D*). On the other hand, rapamycin and PD184352, either alone or in combination, did not increase the small proportion of apoptotic cells, as detected by immunofluorescent detection of active caspase 3 and cleaved poly ADP-ribose polymerase (PARP) (not shown) and western blotting detection of PARP and its cleaved product (Fig. 2*C*).

In the three glioblastoma cell lines, the respective inhibitory effects of rapamycin and PD184352 and their combination on DNA synthesis correlated with similar inhibitions of pRb phosphorylation, including at T826 which is specifically targeted by CDK4 (Fig. 2*C*; Supplemental Fig. S1*B,E*). We thus analysed the expression of CDK4 and its regulatory proteins (Fig. 2*C*; Supplemental Fig. S1*B,E*). CDK4 levels were almost not affected. Serum-induction of cyclin D1 in T98G cells was very partially inhibited by PD184352 and slightly more by combined drugs (Fig. 2*C*). In the two other cell lines, cyclin D1 accumulation was constitutive and only slightly reduced by PD184352 in U-87 MG cells (Supplemental Fig. S1*B,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*; Supplemental Fig. S1*B,E*). In T98G cells, PD184352 and more weakly rapamycin prevented the serum-induced down-regulation of p27, which was completely inhibited when both drugs were combined (Fig. 2*C*). This was less apparent in the other cell lines in which serum had a weaker impact on p27 levels (Supplemental Fig. S1*B,E*).

Rapamycin and PD184352 cooperatively inhibit CDK4 activity. We next analyzed the formation and pRb-kinase activity of CDK4 complexes co-immunoprecipitated using cyclin D1 or cyclin D3 antibodies. Serum induced the pRb-kinase activity associated with cyclin D1 and cyclin D3 in T98G cells (Fig. 2*D*). In cyclin D1 complexes, this was at least in part explained by increased cyclin D1 expression. By contrast, cyclin D3-CDK4 complexes were already present in serum-starved cells but their activity absolutely depended on serum

stimulation as previously described (13). Rapamycin alone inhibited the activity of cyclin D3-CDK4 complexes. This could be partly due to their reduced concentration, resulting from cyclin D3 down-regulation. Also likely resulting from the reduction of cyclin D3 presence, rapamycin appeared to reorient CDK4 to cyclin D1 complexes, thus slightly increasing cyclin D1-CDK4 concentration, and it did not reduce the activity of cyclin D1 complexes (Fig. 2*D*). Conversely, PD184352 weakly decreased the amount of cyclin D1 and cyclin D1-CDK4 complexes, resulting in increased CDK4 association to cyclin D3. As compared to the respective detections of CDK4 in both complexes, PD184352 inhibited in part the pRb-kinase activity of both cyclin D1-CDK4 and cyclin D3-CDK4 (Fig. 2*D*). An almost complete inhibition of pRb-kinase activity was achieved in both cyclin D1 and cyclin D3 complexes only when rapamycin and PD184352 were combined. This inhibition exceeded by far the reduction of the concentration of cyclin D1- and cyclin D3-CDK4 complexes (Fig. 2*D*). Noteworthy, in both cyclin D1 and cyclin D3 immunoprecipitations and all the different cell treatments, the association of p27 closely correlated with CDK4 presence (Fig. 2*D*). As previously shown (13), this indicates that even at its reduced levels in serum-stimulated T98G cells (as seen in Fig. 2*C*) p27 was not limiting for its association with cyclin D1/3-CDK4 complexes. This explains that the observed modulations of p27 accumulation in response to rapamycin and/or PD184352 (Fig. 2*C*) had no impact on p27 association to CDK4 complexes (Fig. 2*D*). Therefore, p27 binding did not appear to explain the inhibition of the activity of cyclin D1/3-CDK4 complexes in response to rapamycin and/or PD184352.

Rapamycin and PD184352 cooperatively inhibit CDK4 activating phosphorylation. In several systems including serum-stimulated T98G cells, we have recently identified the activating T172-phosphorylation of CDK4 as a crucial target for regulation of cyclin D3-CDK4 activity, pRb phosphorylation and cell cycle progression (12-14). The relative presence of phosphorylated and non-phosphorylated CDK4 forms associated

with cyclin D1, cyclin D3 and p27 was assessed as previously demonstrated (13) using 2D-gel electrophoresis (Fig. 3*A*). In these separations, we have previously identified the most negatively charged form as the T172-phosphorylated CDK4 using ^{32}P-phosphate incorporation, a new phosphospecific antibody, *in vitro* phosphorylation by recombinant CAK and analysis of T172A-mutated CDK4 (13).

As previously observed (13), serum stimulation of T98G cells strongly induced the T172-phosphorylation of CDK4 associated not only with cyclins D1 and D3 but also with p27 (Fig. 3*A*). At variance with initial claims (16), p27 thus unlikely prevented the activating phosphorylation of CDK4 (13). In all the conditions, the amount of T172-phosphorylated CDK4 (Fig. 3*A*) closely correlated with the pRb-kinase activity (Fig. 2*C*). Most importantly, combined treatment with rapamycin and PD184352 totally inhibited the phosphorylation of CDK4 co-immunoprecipitated with cyclin D1, cyclin D3 or p27 (Fig. 3*A*), thus explaining the total inhibition of pRb-kinase activity (Fig. 2*D*). In response to both drugs used alone, very partial inhibitions of CDK4 phosphorylation were achieved, as judged from the proportion of its phosphorylated and non-phosphorylated forms (Fig. 3*A*). In U-87 MG and U-138 MG cells, the phosphorylation of CDK4 associated to cyclin D1 or cyclin D3 was also strongly inhibited only when rapamycin and PD184352 were combined (Supplemental Fig. S1*C*,*F*). Ectopically expressed non-phosphorylatable T172ACDK4 but not wild type CDK4 prevented serum-induced DNA synthesis in T98G cells (Fig. 3*C*), directly showing the crucial role of T172-phosphorylation of CDK4 in cell cycle progression. To conclude, the complete inhibition of DNA synthesis and pRb phosphorylation by combined rapamycin and PD184352 could not be explained by their partial and differential inhibitory effects on cyclin D1 or cyclin D3 accumulation, but instead by their cooperative inhibition of the activating T172-phosphorylation of CDK4, which appears independent of p27. In different glioblastoma

cell lines, the T172-phosphorylation of CDK4 thus appears as a crucial node integrating the activity of both MEK/Erk and mTOR pathways.

Combined rapamycin and PD184352 do not block CAK activity. The activating T172-phosphorylation of CDK4, like the analogous T-loop phosphorylations of the other CDKs, is considered to be performed only by CAK, the cyclin H-CDK7-Mat1 complex (15, 17). Nevertheless, the activity of CAK has been generally found to be constitutive and non-regulated during stimulations or inhibitions of proliferation (12, 13, 15). We thus decide to directly assess the activity of cyclin H-CDK7 complexes from cells treated or not with rapamycin and/or PD184352. As a substrate, we used inactive CDK4/6 complexes co-immunoprecipitated by the cyclin D3 antibody from quiescent T98G cells, as detailed in *Materials and Methods*. As shown in Fig. 3*C*, cyclin H-CDK7 complexes from serum-stimulated T98G cells had only a very weak intrinsic pRb-kinase activity when they were assayed on a mock immunoprecipitation from quiescent T98G cells (*lane 9*). However, these cyclin H-CDK7 complexes strongly increased the pRb-kinase activity of cyclin D3 complexes from quiescent cells (*lanes 2,8*). Unstimulated cells (*lane 1*) also contained active cyclin H-CDK7 complexes though they were somewhat less abundant than in serum-stimulated cells (*lane 2*). Rapamycin (*lane 3*) but not PD184352 (*lane 4*) very slightly inhibited cyclin H expression and CAK activity, and PD184352 did not amplify this weak inhibition by rapamycin (*lane 5*). Overall, the modulations of CDK4 phosphorylation (Fig. 3*A*) did not correlate with the presence and activity of CAK (Fig. 2*C*; Fig. 3*C*).

The present study provides a first evidence that MEK and mTOR-dependent cascades might signal cell cycle progression mainly through T172-phosphorylation of CDK4. It thus reinforces the emerging concept that CDK4 phosphorylation constitutes a major regulatory target determining pRb phosphorylation and cell cycle progression (12). Together with other unexpected observations, such as our findings that the analogous T-loop phosphorylation of

cyclin D3-bound CDK6 is not induced by serum in T98G cells (13) or that CDK4 phosphorylation is not precluded by p27 binding in various intact cells (13), the present results support our hypothesis that CAK might not be the regulated CDK4-activating kinase (12). One simple hypothesis compatible with the cooperative inhibitory effects of rapamycin and PD184352 on CDK4 phosphorylation would be that Ras-Raf-MEK-Erk and mTOR pathways could activate 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 promising targets for therapeutic intervention.

Rapamycin and derivatives 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). Clinical studies with MEK inhibitors have been similarly disappointing (1, 3). On the other hand, a few *in vitro* studies recently reported strong cooperative antiproliferative effects of combined suppression of MEK and mTOR activities in tumor cells of various origins (18-20). Whether CDK4 activity and phosphorylation could be similarly targeted in these different models, as found here in glioblastoma cell lines, remains to be defined. Our results suggest that a combined inhibition of mTOR and ERK pathways should be considered, in preclinical animal models as well as in clinical settings, as a promising bitherapy to treat not only glioblastomas but also other tumors harboring a deregulated CDK4 activity.

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References

1. Rinehart J, Adjei AA, Lorusso PM, Waterhouse D, Hecht JR, Natale RB, et al. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. J Clin Oncol 2004;22:4456-62.

2. Reardon DA, Rich JN, Friedman HS, Bigner DD. Recent advances in the treatment of malignant astrocytoma. J Clin Oncol 2006;24:1253-65.

3. Kohno M, Pouyssegur J. Targeting the ERK signaling pathway in cancer therapy. Ann Med 2006;38:200-11.

4. Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer 2006;6:729-34.

5. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell 2002;2:103-12.

6. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 1996;271:20608-16.

7. Squires MS, Nixon PM, Cook SJ. Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1. Biochem J 2002;366:673-80.

8. Tetsu O, McCormick F. Proliferation of cancer cells despite CDK2 inhibition. Cancer Cell 2003;3:233-45.

9. Hleb M, Murphy S, Wagner EF, Hanna NN, Sharma N, Park J, et al. Evidence for cyclin D3 as a novel target of rapamycin in human T lymphocytes. J Biol Chem 2004;279:31948-55.

10. Motti ML, De Marco C, Califano D, De Gisi S, Malanga D, Troncone G, et al. Loss of p27 expression through RAS-->BRAF-->MAP kinase-dependent pathway in human thyroid carcinomas. Cell Cycle 2007;6:2817-25.

11. Lee YM, Sicinski P. Targeting cyclins and cyclin-dependent kinases in cancer: lessons from mice, hopes for therapeutic applications in human. Cell Cycle 2006;5:2110-4.

12. Bockstaele L, Coulonval K, Kooken H, Paternot S, Roger PP. Regulation of CDK4. Cell Division 2006;1:25.

13. Bockstaele L, Kooken H, Libert F, Paternot S, Dumont JE, de Launoit Y, et al. Regulated activating Thr172 phosphorylation of cyclin-dependent kinase 4(CDK4): its relationship with cyclins and CDK "inhibitors". Mol Cell Biol 2006;26:5070-85.

14. Paternot S, Coulonval K, Dumont JE, Roger PP. Cyclic AMP-dependent phosphorylation of cyclin D3-bound CDK4 determines the passage through the cell cycle restriction point in thyroid epithelial cells. J Biol Chem 2003;278:26533-40.

15. Matsuoka M, Kato JY, Fisher RP, Morgan DO, Sherr CJ. Activation of cyclin-dependent kinase 4 (cdk4) by mouse MO15-associated kinase. Mol Cell Biol 1994;14:7265-75.

16. Kato JY, Matsuoka M, Polyak K, Massague J, Sherr CJ. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. Cell 1994;79:487-96.

17. Fisher RP. Secrets of a double agent: CDK7 in cell-cycle control and transcription. J Cell Sci 2005;118:5171-80.

18. Costa LJ, Gemmill RM, Drabkin HA. Upstream signaling inhibition enhances rapamycin effect on growth of kidney cancer cells. Urology 2007;69:596-602.

19. Legrier ME, Yang CP, Yan HG, Lopez-Barcons L, Keller SM, Perez-Soler R, et al. Targeting protein translation in human non small cell lung cancer via combined MEK and mammalian target of rapamycin suppression. Cancer Res 2007;67:11300-8.

20. Lasithiotakis KG, Sinnberg TW, Schittek B, Flaherty KT, Kulms D, Maczey E, et al. Combined inhibition of MAPK and mTOR signaling inhibits growth, induces cell death, and abrogates invasive growth of melanoma cells. J Invest Dermatol 2008;128:2013-23.

Figures Legends

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

Figure 2. PD184352 cooperates with rapamycin to completely prevent DNA synthesis (*A*), cell proliferation (*B*), pRb phosphorylation (*C*) and pRb-kinase activity (*D*). Quiescent T98G cells were stimulated or not with 15 % FBS for 18 h (*A*) or 10 h (*C*,*D*) and treated or not with rapamycin (40 nM), PD184352 (5 µM) or a combination of both drugs. *A*, DNA synthesis was evaluated from duplicate dishes by counting the percentage of nuclei having incorporated BrdU 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*), cyclins D1 and D3, CDK4, p27, cyclin H and CDK7 were detected by Western Blotting from whole cell lysates. Hyperphosphorylated forms of pRb are indicated by an arrow. The cleaved form of PARP (*arrowhead*) was detected as a marker of apoptosis, and α-tubulin was detected as a loading control. *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

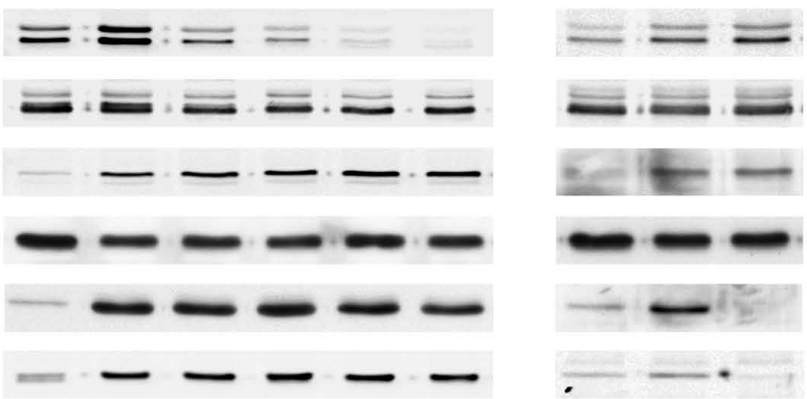
immunoblotted. Cyclin D1, cyclin D3, CDK4, p27 and the pRb fragment phosphorylated *in vitro* at S780 (*pRb-kinase*) were detected using specific antibodies.

Figure 3. PD184352 cooperates with rapamycin to completely 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*, quiescent T98G cells were stimulated with 15 % FBS for 10 h and treated with rapamycin (40 nM), PD184352 (5 µM) or a combination of both drugs. Cell lysates were immunoprecipitated (*IP*) with anti-cyclin D1 (*cyc D1*), anti-cyclin D3 (*cyc D3*) or p27 antibodies, separated by 2D-gel electrophoresis and CDK4 was immunodetected. Arrows indicate the T172-phosphorylated form of CDK4. Values indicate the proportion (%) of the phosphorylated form relative to total CDK4, as quantified by scanning densitometry of CDK4 immunodetections. *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 BrdU during the last 30 min. In duplicate dishes, DNA synthesis was evaluated in productively transfected cells by counting the proportion of nuclei having incorporated BrdU in 100 cells/dish displaying the HA-epitope. The upper panel illustrates the double immunofluorescent detection of the HA-epitope (red) and BrdU (green). *C*, the activity of co-immunoprecipitated cyclin H-CDK7 complexes (*IP cyc H*) was evaluated from T98G treated as in *A*. In this assay, these cyclin H-CDK7 complexes (*lanes 2-5 & 8*) or a recombinant cyclin H-CDK7-Mat 1 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; lane9*)) used as a substrate. The resulting activation of the cyclin D3-CDK4/6 complexes was then assayed by their pRb-kinase activity (second incubation in the presence of the pRb fragment and ATP). The mixtures were separated by SDS-PAGE and immunoblotted. We then detected the

presence of cyclin H (*cyc H*) and CDK7 co-immunoprecipitated by the cyclin H antibody from T98G cells or recombinant CAK complex, the presence of the substrate, i.e. cyclin D3-CDK4 complexes from quiescent T98G cells (*CDK4*), and the *in vitro* activation of these cyclin D3 complexes reflected by the phosphorylation at S780 of the pRb fragment (*pRb-kinase*).

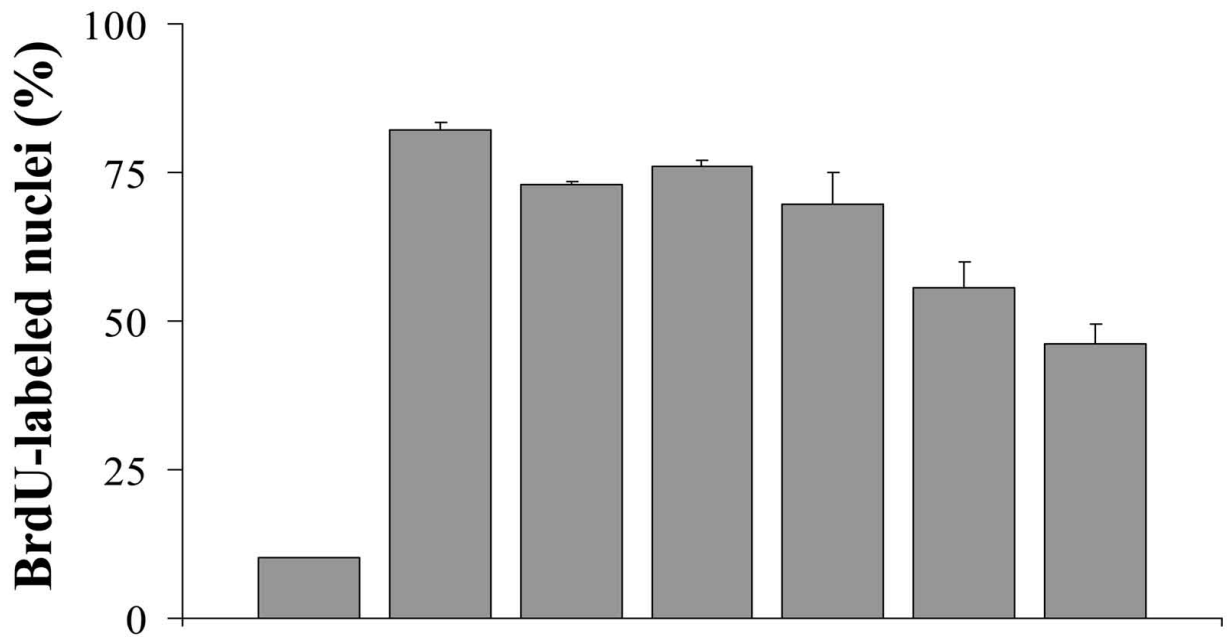
Paternot Figure 1

A



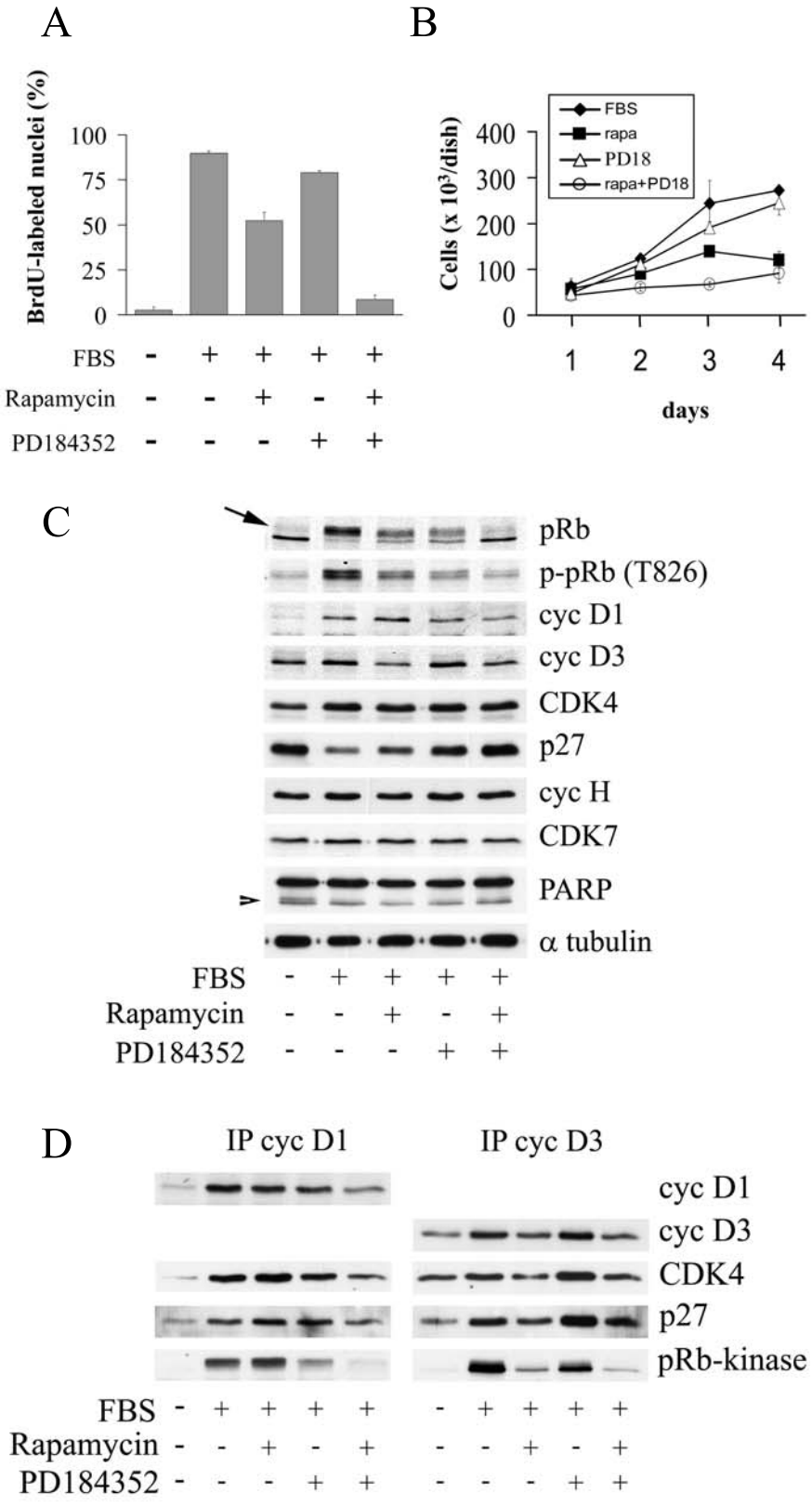
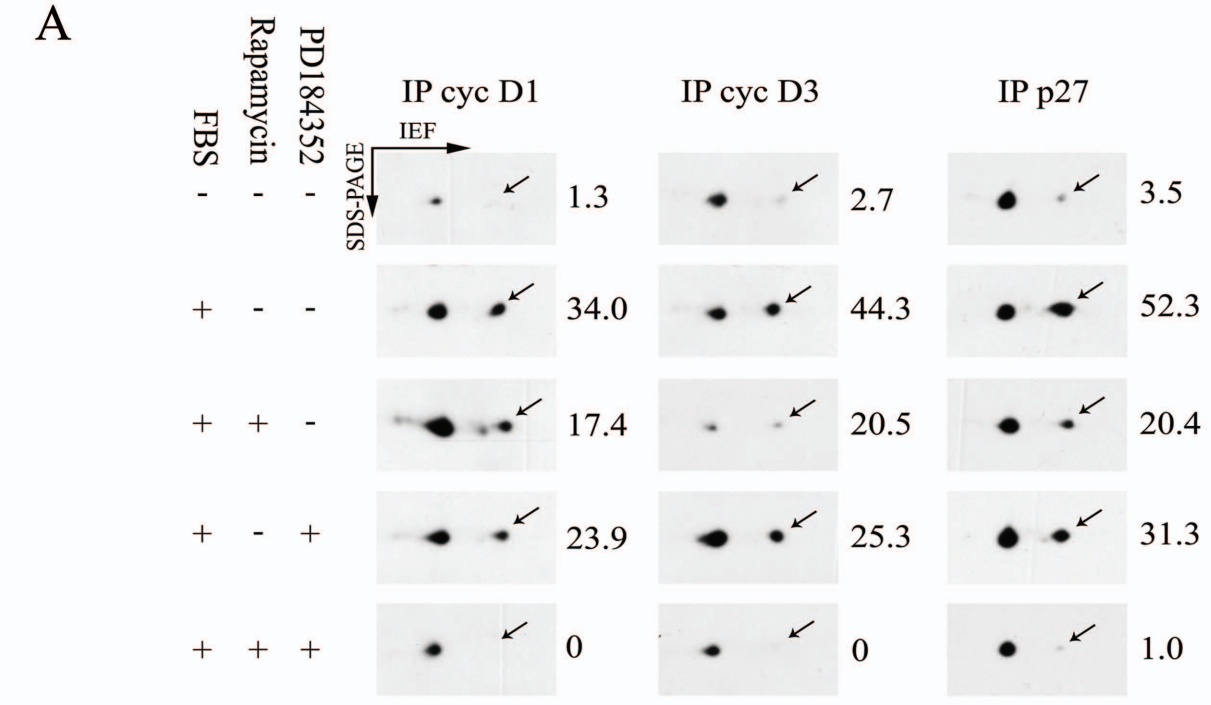
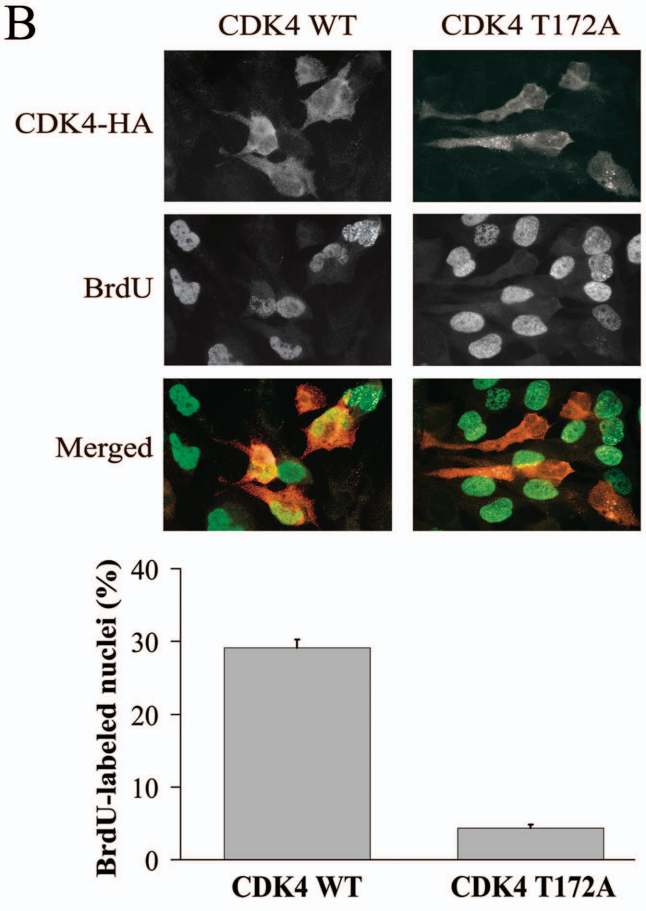
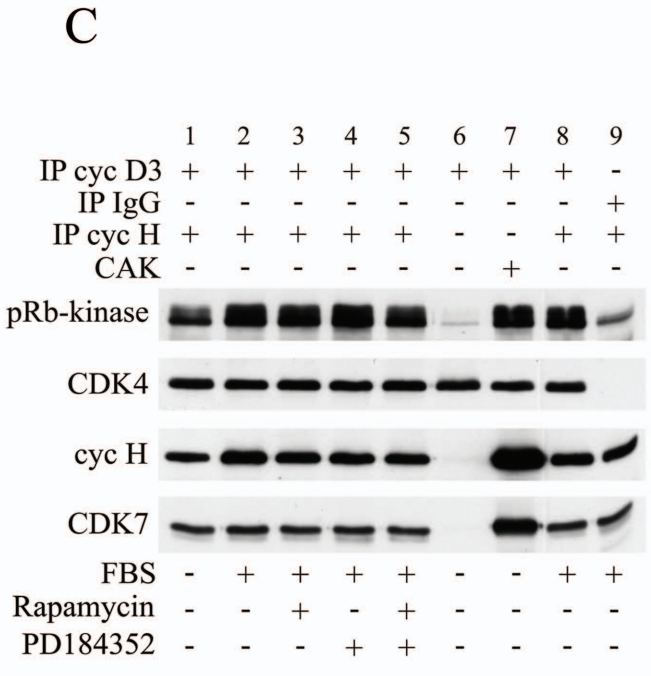
FBS	-	+	+	+	+	+	-	+	+
Rapamycin (nM)	-	-	-	-	-	-	-	-	40
PD184352 (μM)	-	-	1	2	5	10	-	-	-

B



FBS	-	+	+	+	+	+	+
Rapamycin (nM)	-	-	-	-	-	-	**40**
PD184352 (μM)	-	-	**1**	**2**	**5**	**10**	-

Paternot Figure 2



Paternot Figure 3