

budding with Ax11. We have recently identified a mutation (*rax1*) that can convert the budding pattern of *ax11* null strain from bipolar to axial (unpublished data). The putative amino-acid sequence of Rax1 contained several conserved residues of B-chains of the insulin-related hormone superfamily (A.F. *et al.*, unpublished data). Characterization of this gene may provide new insight into the control of bud-site selection. □

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1. Drubin, D. G. *Cell* **65**, 1093–1096 (1991).
2. Chant, J. & Herskowitz, I. *Cell* **65**, 1203–1212 (1991).
3. Affholter, J. A. *et al.* *Science* **242**, 1415–1418 (1988).
4. Kuo, W. L. *et al.* *Molec. Endocrinol.* **4**, 1580–1591 (1990).
5. Finch, P. W. *et al.* *Nucleic Acids Res.* **14**, 7695–7703 (1986).
6. Chant, J. *et al.* *Cell* **65**, 1213–1224 (1991).
7. Powers, S. *et al.* *Cell* **65**, 1225–1231 (1991).
8. Cheng, Y. E. & Zipster, D. J. *biol. Chem.* **254**, 4698–4706 (1979).
9. Kirschner, R. J. & Goldberg, A. L. *J. biol. Chem.* **258**, 967–976 (1983).
10. Butler, G. & Thiele, D. J. *Molec. cell. Biol.* **11**, 476–485 (1991).
11. Stillman, D. J. *Genes Dev.* **6**, 93–104 (1992).
12. Rine, J. & Herskowitz, I. *Genetics* **116**, 9–22 (1987).
13. Flescher, E. G., Madden, K. & Snyder, M. J. *Cell Biol.* **122**, 373–386 (1993).
14. Pringle, J. R. *et al.* *Meth. Cell Biol.* **31**, 357–435 (1989).
15. Fujita, A. *et al.* *Gene* **89**, 93–99 (1990).
16. Siliciano, P. G. & Tatchell, K. *Cell* **37**, 969–978 (1984).
17. Miller, A. M. *et al.* *Nature* **314**, 598–603 (1985).
18. Goutte, C. & Johnson, A. D. *Cell* **55**, 875–882 (1988).
19. Pearson, W. R. & Lipman, D. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2444–2448 (1988).
20. Struhl, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **76**, 1635–1639 (1979).
21. Rothstein, R. J. *Meth. Enzym.* **101**, 202–209 (1983).

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Interleukin-2-mediated elimination of the p27^{Kip1} cyclin-dependent kinase inhibitor prevented by rapamycin

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THE cyclin-dependent kinase (Cdk) enzymes, when associated with the G1 cyclins D and E, are rate-limiting for entry into the S phase of the cell cycle^{1,2}. During T-cell mitogenesis, antigen-receptor signalling promotes synthesis of cyclin E and its catalytic partner, Cdk2, and interleukin-2 (IL-2) signalling activates cyclin E/Cdk2 complexes³. Rapamycin is a potent immunosuppressant which specifically inhibits G1-to-S-phase progression, leading to cell-cycle arrest in yeast and mammals^{4–7}. Here we report that IL-2 allows Cdk activation by causing the elimination of the Cdk inhibitor protein p27^{Kip1}, and that this is prevented by rapamycin. By contrast, the Cdk inhibitor p21 is induced by IL-2 and this induction is blocked by rapamycin. Our results show that p27^{Kip1} governs

Cdk activity during the transition from quiescence to S phase in T lymphocytes and that p21 function may be restricted to cycling cells.

Rapamycin prevents the activation of the late-G1 cyclin E/Cdk2 kinase in the T-cell clones D10.G4.1 (Fig. 1a) and CTLL-2 (ref. 8) and primary T lymphocytes (data not shown). The inactivity of cyclin E/Cdk2 in rapamycin-treated cells is not due to an inhibition of the expression of cyclin E or Cdk2 (Fig. 1b), to an inability of cyclin E to associate with Cdk2 (Fig. 1c), or to an inhibition of Cdk-activating kinase (CAK) activity (Fig. 1d).

We find that extracts from both IL-2-starved and IL-2/rapamycin-treated cells contain an inhibitory activity for cyclin E/Cdk2 kinase whereas IL-2-induced controls do not (Fig. 2a, b). Examination of synchronized IL-2-induced and IL-2/rapamycin-treated cells suggest that an inhibitory activity is present for up to 3 h after IL-2 induction and is gradually removed after longer exposure to IL-2 in the absence of rapamycin (Fig. 2c). This inhibitory activity could be titrated out by the addition of supraphysiological amounts of exogenous cyclin E and cyclin E/Cdk2 but not Cdk2 alone (Fig. 2d).

A heat-stable inhibitory activity is directly associated with cyclin E/Cdk2 complexes (Fig. 2e). Surprisingly, the inhibitory activity is also detected in cyclin E/Cdk2 complexes isolated from IL-2-induced cells. Accurate analyses designed to measure the amount of inhibitor present in cyclin E/Cdk2 complexes under these various conditions show that its levels are lower in IL-2-induced cells than in arrested cells (Figs 3c, 4).

Mammalian Cdk activities are inhibited *in vitro* by the associated proteins p16, p21 and p27^{Kip1} (refs 9–15). A heat-stable doublet corresponding to a relative molecular mass of 27,000 is found associated with cyclin E immunoprecipitates of D10 cell extracts, which, like p27^{Kip1}, binds preferentially to recombinant cyclin E/Cdk2 rather than to cyclin E or Cdk2 alone (Fig. 3a). This doublet comigrates with the doublet in p27^{Kip1} immunoprecipitates (Fig. 3a). Proteolytic mapping of these 27K bands establishes that they are identical to p27^{Kip1} (Fig. 3b). Immuno-

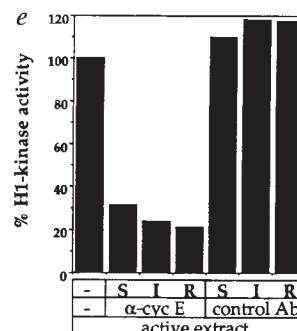
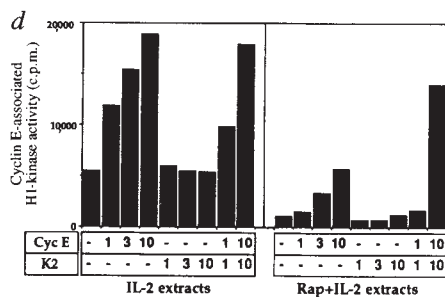
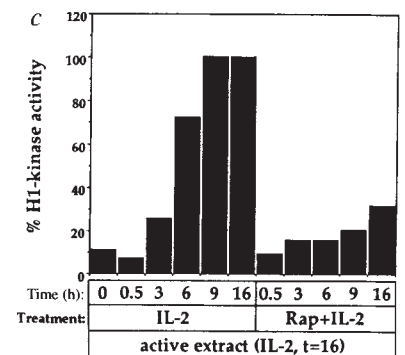
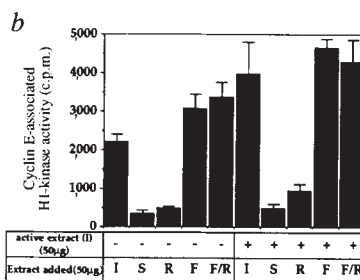
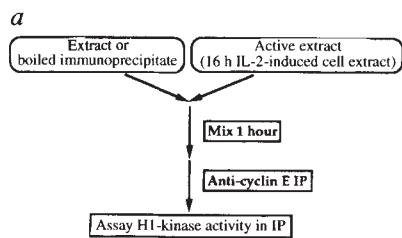
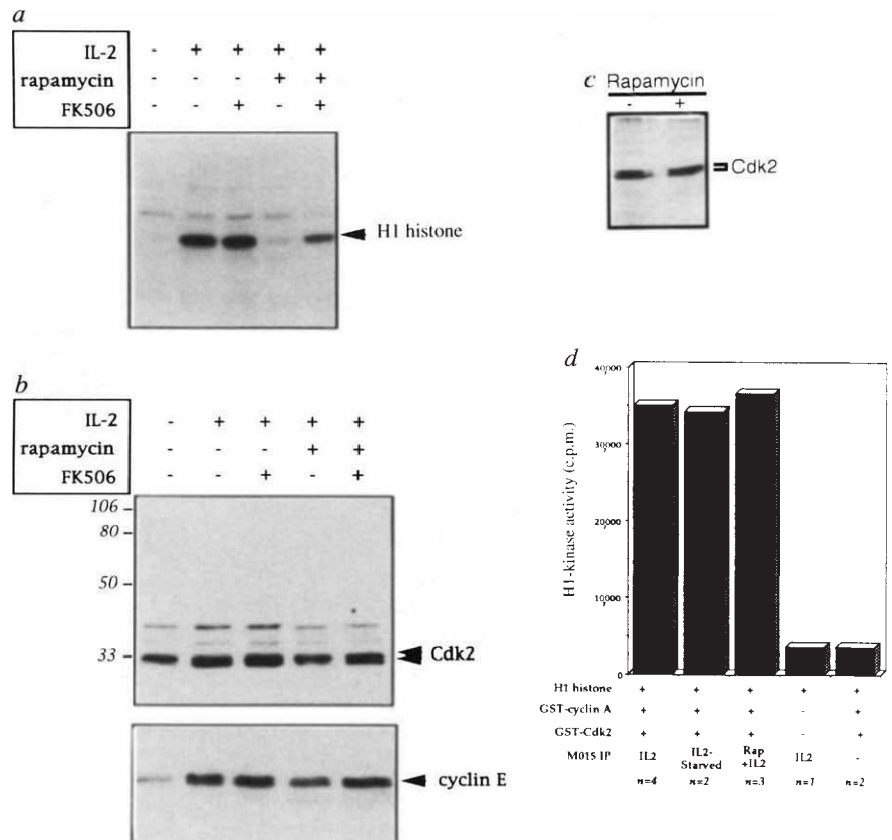
FIG. 2 Rapamycin-treated cells contain a titratable and heat-stable inhibitor which is directly associated with cyclin E/Cdk2 complexes. **a**, Experimental design used in **b**, **c** and **e**. Inhibitory activity is measured in extracts or immunoprecipitates by mixing with active extracts and subsequently measuring cycling E-associated H1 kinase activity. **b**, Kinase activity in active extracts after mixing with extracts from IL-2-starved cells (S), IL-2-induced (I), IL-2/rapamycin-treated (R), IL-2/FK506-treated (F) or IL-2/rapamycin/FK506-treated (F/R). The experiment was performed in triplicate. **c**, Kinase activity in active extracts after incubation with an equal amount of extracts of cells collected at various times (in hours) after treatment. **d**, Cyclin E-associated H1-kinase activity in extracts after incubation with cyclin E and/or Cdk2-HA Sf9 lysates added in multiples (1, 3 or 10 times) of the physiological levels of cyclin E and Cdk2. Data are representative of two independent experiments. **e**, Boiled cyclin E or control immunoprecipitates from the indicated cell extracts are assayed as for **a**. This experiment demonstrates that a Cdk inhibitor is present in cyclin E immune complexes from both growing and arrested cells. This protocol is not designed to quantify the amount of inhibitor present in each condition, because of difficulties in reconstituting the physiological ratio of cyclin E to inhibitor after immunoprecipitation from cell extracts.

METHODS. D10 cell extracts were mixed for 1 h at 37 °C, then cyclin E was immunoprecipitated and assayed for H1-kinase activity as described previously³. ³²P incorporation into H1 histone was quantified using AMBIS Colorprobe software (version 2.12). Sf9 lysates of cyclin E and Cdk2-HA and D10 extracts prepared in NP-40 buffer was described previously³. Physiological concentrations of cyclin E and Cdk2 were determined by comparative western blotting. Washed cyclin E or control immunoprecipitates were resuspended in 50 µl N-P40 buffer containing 1 mg ml⁻¹ aprotinin and heated at 97–100 °C for 3–5 min. After adding 50 µl 2 mg ml⁻¹ BSA in NP-40 buffer, the Sepharose and coagulated proteins were removed by centrifugation and the soluble fraction assayed.

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FIG. 1 Rapamycin inhibits cyclin E/Cdk2 kinase activity without significantly inhibiting cyclin E or Cdk2 expression, cyclin E/Cdk2 association or CAK activity. *a*, Cyclin E-associated H1-kinase activity in IL-2-starved or IL-2-stimulated D10 cells in the absence or presence of the drugs rapamycin and FK506. *b*, Immunoblot analysis of whole D10 cell extracts with anti-Cdk2 (upper panel) or anti-cyclin E antibodies (lower panel). *c*, Anti-Cdk2 immunoblots of α -cyclin E immunoprecipitates from peripheral blood T-lymphocyte extracts. *d*, *In vitro* assay of CAK activity from extracts of IL-2-starved and IL-2-stimulated (IL2) D10 cells in the presence of rapamycin (Rap + IL2).

METHODS. D10 cells were synchronized by washing the cells of IL-2-containing medium and subsequently culturing in IL-2-deficient medium for 20–22 h. Cells were collected (IL-2-starved) or were stimulated with recombinant IL-2 (DNAX) in the presence or absence of either 2 ng ml⁻¹ rapamycin or 2 ng ml⁻¹ FK506 or 2 ng ml⁻¹ rapamycin and 2,000 ng ml⁻¹ FK506 and collected 16 h after stimulation. Rapamycin's inhibition of proliferation is mediated by the intracellular binding protein, FKBP. As FK506 also binds FKBP with similar affinity, it reverses the effects of rapamycin by competitive binding when FK506 is present at 1,000-fold molar excess^{22,23}. Immunoprecipitation, kinase assay and immunoblotting were performed as described³. Peripheral blood T lymphocytes were purified and stimulated as described³. CAK activity was immunoprecipitated from extracts with the anti-M015 antibody¹⁷. Washed immunoprecipitates were mixed with 1 μ g of both recombinant GST-cyclin A and GST-Cdk2 fusion proteins in 30 μ M ATP and 15 mM magnesium acetate in 7.5 μ l. After 30 min at room temperature, 2.5 μ l H1 histone mix containing [γ -³²P] ATP was added and incubated as described¹⁷.



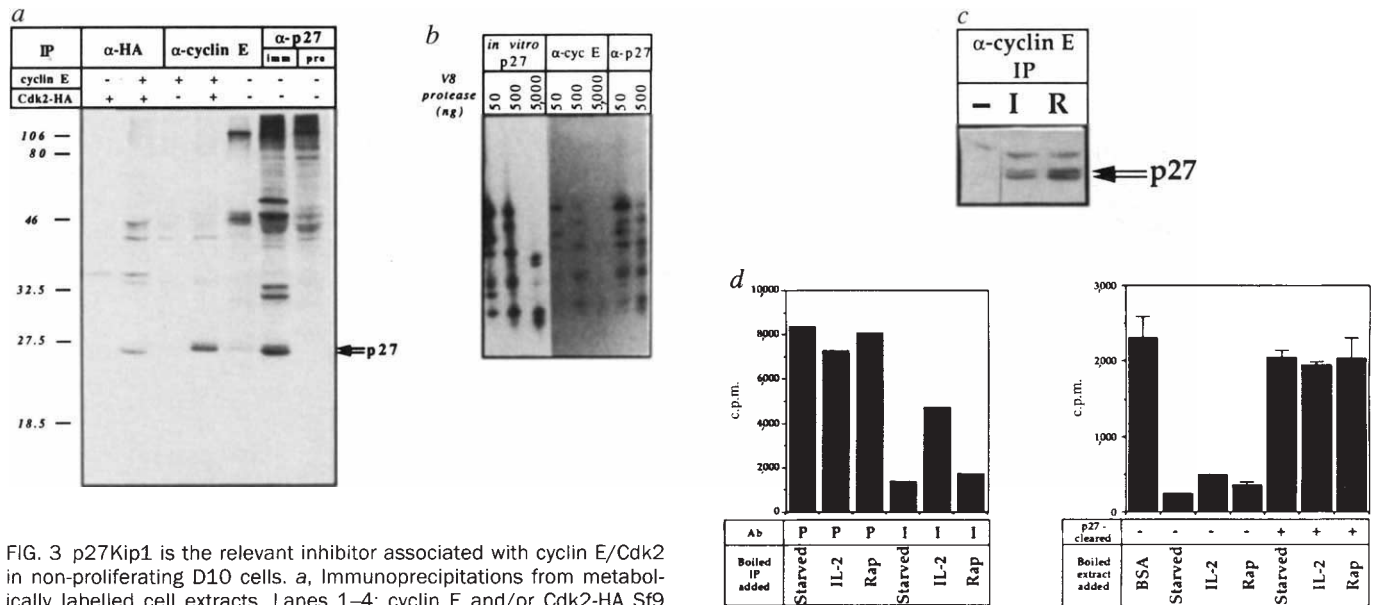


FIG. 3 p27Kip1 is the relevant inhibitor associated with cyclin E/Cdk2 in non-proliferating D10 cells. *a*, Immunoprecipitations from metabolically labelled cell extracts. Lanes 1–4: cyclin E and/or Cdk2-HA Sf9 lysates were added to the uncoagulated fraction of boiled extracts and immunoprecipitated with 12CA5 (anti-HA) or anti-cyclin E antibodies. Lanes 5–7: immunoprecipitations from native extracts with anti-cyclin E, anti-p27Kip1 or preimmune sera. *b*, V8 protease mapping of the p27 bands isolated from cyclin E or p27Kip1 immunoprecipitates and the *in vitro* translated p27Kip1 protein were performed as described²⁴. *c*, Anti-p27Kip1 immunoblot of cyclin E immunoprecipitation from the indicated cell extracts. The control immunoprecipitate labelled as a dash was performed without cell lysate, but with primary (anti-cyclin E) antibody in the immunoprecipitation. *d*, p27Kip1 antibodies remove the inhibitor from rapamycin-treated cell extracts. Boiled preimmune (P) or anti-p27Kip1 (I) immunoprecipitates from cell extracts (left panel) and boiled extracts that had been precleared of p27Kip1 with anti-p27Kip1 or preimmune sera (right panel) were assayed for inhibitory activity as described for Fig. 2a; the experiment was done in triplicate. Anti-p27

antibodies raised against bacterially expressed C-terminal domain of p27 that is divergent from p21 produced the same results. METHODS. Rabbit anti-p27Kip1 antibodies were generated against purified His-tagged, full-length mouse p27Kip1 protein¹⁴. These antibodies are specific for p27Kip1 as p21 is not detected in immunoblots or in p27Kip1 immunoprecipitates of metabolically labelled cells extracts (data not shown). Cells cultured in the absence of IL-2 for 20 h were metabolically labelled for the last 15 h with ³⁵S-promix (Amersham). p27Kip1 was removed from 100 µg extract with affinity-purified anti-p27Kip1 or preimmune serum, and the resulting extract was boiled to remove any remaining cyclin E and to liberate p27Kip1. In a separate experiment (scaled up twofold), immunoprecipitates were assayed as described for Fig. 2d.

blotting cyclin E immunoprecipitates reveals that, by comparison with rapamycin-arrested cells, p27^{Kip1} declines by about 50% in cyclin E complexes from IL-2-induced cells (Fig. 3c); when peripheral blood T-lymphocyte extracts are analysed, p27^{Kip1} is only associated with cyclin E from rapamycin-treated and IL2-starved cells (data not shown).

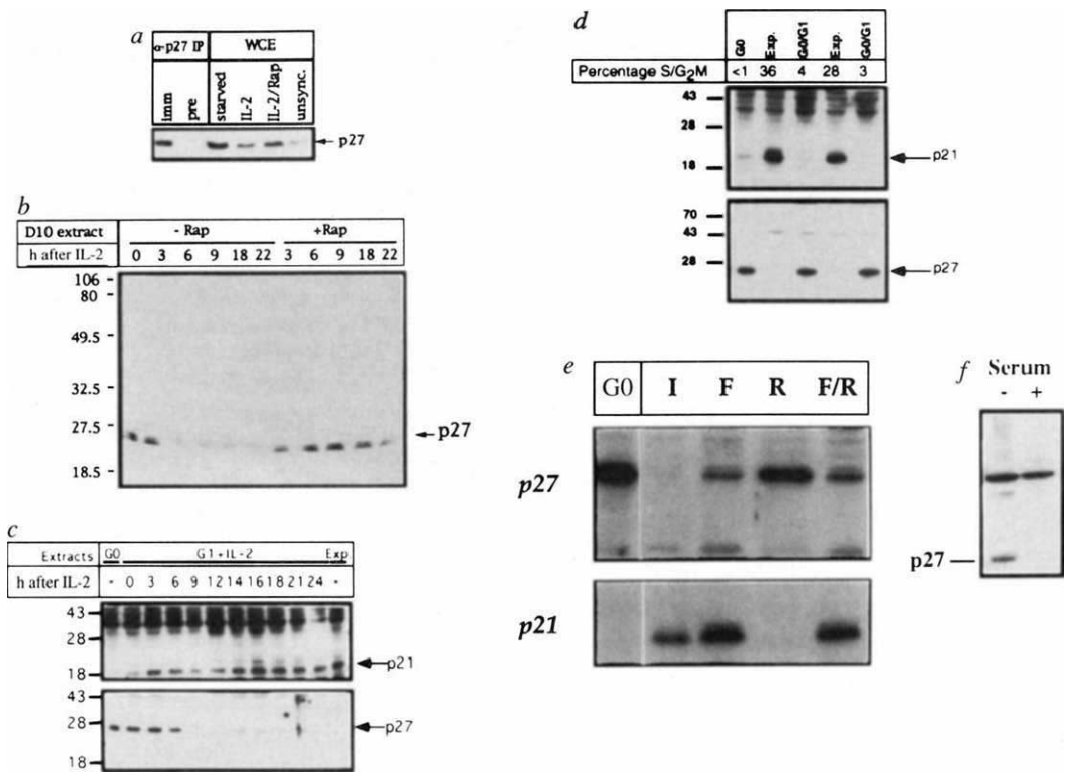
Antibodies specific for p27^{Kip1} clear extracts of all the inhibitory activity in IL2-starved and rapamycin-treated D10 cells (Fig. 3d), suggesting that raised levels of p27 account for essentially all of the inhibitory activity detected in arrested cells. Likewise, RNase protection assays demonstrate that IL-2-starved or rapamycin-treated D10 cells do not have increased levels of p21 messenger RNA compared with IL-2-induced cells (data not shown).

Immunoblotting whole-cell extracts shows that IL-2 induction gradually reduces the amount of p27^{Kip1} and, significantly, that rapamycin prevents removal of p27^{Kip1} (Fig. 4a, b). Some p27^{Kip1} remains in the IL-2-stimulated population, which could be due to contaminating unstimulated cells because 18% of synchronized IL-2-treated cells do not traverse S phase (versus 5% of unsynchronized cells) after 24 h, as monitored by BrdU incorporation (data not shown). Alternatively, IL-2 stimulation may not completely remove p27^{Kip1} in D10 cells. Indeed, p27^{Kip1} is present at low levels in unsynchronized cycling cells (Fig. 4a). The observations that IL-2-induced cells enter S phase and have significant cyclin E/Cdk2 kinase activity, despite the presence of some inhibitor, suggests that p27^{Kip1} levels function as a threshold and that this threshold has been sufficiently lowered in D10 cells to allow cyclin E/Cdk2 function.

Three experiments show that the mechanism of Cdk2 inhibition by p27^{Kip1} is not the inactivation of CAK, which activates Cdk2 by phosphorylation on Thr 160 (refs 16–18). First, the faster-migrating Thr-160-phosphorylated isoform of Cdk2 (ref. 19), is associated with cyclin E in relatively the same abundance in rapamycin-blocked and proliferating cells (Fig. 1c). Second, immunoprecipitation with antibodies specific for the catalytic subunit of CAK (anti-M015)¹⁷ show that CAK activity is equally abundant in extracts of both untreated and rapamycin-treated IL-2-stimulated cells (Fig. 1d). Third, purified p27^{Kip1} does not inhibit purified CAK *in vitro* (R. Sheaff, R. Fisher, D. Morgan and J.M.R., unpublished result). Thus, p27^{Kip1} directly blocks the activity of the Thr-160-phosphorylated cyclin E/Cdk2 complex, as found previously *in vitro*^{13,14}.

The results obtained in D10 cells were extended and confirmed in peripheral blood T lymphocytes. Immunoblot analysis of peripheral blood T lymphocytes at various times after IL-2 stimulation reveals that p21 increases as p27^{Kip1} decreases (Fig. 4c), indicating that the previously described p27 inhibitor in T lymphocytes is indeed p27^{Kip1} (ref. 3). The inverse relationship between p27^{Kip1} and p21 is confirmed by analysis of peripheral blood T lymphocytes undergoing reiterated cycles of proliferation and quiescence. In each cycle proliferation is induced by anti-CD3 antibodies and IL-2, whereas cells are arrested by withdrawal of IL-2 (Fig. 4d). IL-2-mediated removal of p27^{Kip1} is blocked significantly by rapamycin but not by FK506, indicating that it is a TOR/FRAP/RAFT-dependent event^{5,7} and not related to the calcineurin pathway activated by antigen-receptor signalling²⁰ (Fig. 4e). The repression of p27^{Kip1} expression by

FIG. 4 Rapamycin inhibits IL-2-mediated removal of p27^{Kip1} and induction of p21. **a**, p27^{Kip1} immunoblot analysis of preimmune and anti-p27^{Kip1} immunoprecipitates and of whole-cell extracts (WCE). **b**, p27^{Kip1} immunoblot of extracts from synchronized D10 cells stimulated in the absence or presence of rapamycin and collected various times (in hours) after IL-2-induction. **c**, p27^{Kip1} and p21 immunoblots of freshly isolated (G0) and IL-2-induced G1 phase peripheral blood T lymphocytes collected at various times (in hours) after IL-2 treatment and exponentially proliferating (Exp). **d**, p27^{Kip1} and p21 immunoblots of primary T lymphocytes (G0) or long-term cultures of peripheral blood T cells that are resynchronized by IL-2 deprivation (G0/G1) and subsequently restimulated with IL-2 (Exp.). **e**, p27^{Kip1} and p21 immunoblots of G0, IL-2 stimulated (I) or IL-2 stimulated with 4 ng ml⁻¹ FK506 (F), rapamycin (R) or 4 ng ml⁻¹ rapamycin and 4 ng ml⁻¹ FK506 (F/R). **f**, p27^{Kip1} immunoblot of whole-cell extracts of serum-starved (-) or serum-stimulated (+) primary human fibroblasts. METHODS. 10⁸ human primary T lymphocytes were isolated and purified and stimulated as described³. For **d**, stimulation was achieved by plating cells on immobilized anti-CD3 antibodies in complete RPMI medium³ containing 1 µg ml⁻¹ anti-CD28 antibodies. After 2 days at 37 °C, cells were transferred to a new flask with fresh complete RPMI containing



extracellular mitogens is not restricted to lymphocytes: serum growth factors also repress p27^{Kip1} expression in primary human diploid fibroblasts (Fig. 4f).

The inhibition of cyclin E/Cdk2 by p27 is likely to reflect a more general inhibition of G1 cyclin-Cdk complexes in rapamycin-treated cells because (1) rapamycin does not inhibit expression of Cdk4 or Cdk6, or of their partners, cyclins D2 or D3; and (2) p27^{Kip1} associates with Cdk6 as T cells depart from quiescence, and this association persists in rapamycin-treated cells (data not shown).

We have demonstrated that in T cells leaving a quiescent state, IL-2 causes a decrease in p27^{Kip1}, allowing Cdk2 activation and entry into S phase. In contrast, we find that IL-2 induces p21,

and that p21 expression persists in cycling cells. But cycling D10 cells continue to express some p27^{Kip1}, albeit at substantially lower levels than quiescent cells (Fig. 4a). Therefore in normally cycling cells, both p27^{Kip1} and p21 may together establish a threshold and that must be overcome for Cdk activation to occur in G1. Increasing the levels of p21 by intracellular 'checkpoint' signals, such as those resulting from DNA damage, or regulating p27^{Kip1} by extracellular or intracellular signals such as cyclic AMP (ref. 21), can arrest the cell cycle by increasing a threshold that cannot be overcome by physiological concentrations of cyclin-Cdk. Our data suggest that p27^{Kip1} may be the primary regulator of Cdk activity as cells enter and leave the quiescent state, whereas p21 may primarily regulate Cdk activity in cycling cells. □

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- Ohtsubo, M. & Roberts, J. M. *Science* **259**, 1908-1912 (1993).
- Quelle, D. E. et al. *Genes Dev.* **7**, 1559-1571 (1993).
- Firpo, E. J., Koff, A., Solomon, M. J. & Roberts, J. M. *Molec. cell. Biol.* **14**, 4889-4901 (1994).
- Dumont, F. J., Staruch, M. J., Koprak, S. L., Melino, M. R. & Sigal, N. H. *J. Immunol.* **144**, 251-258 (1990).
- Kunz, J. & Hall, M. N. *Trends Biol. Sci.* **18**, 334-338 (1994).
- Brown, E. J. et al. *Nature* **369**, 756-758 (1994).
- Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S. H. *Cell* **78**, 35-43 (1994).
- Morice, W. G., Wiederrecht, G., Brunn, G. J., Siekierka, J. J. & Abraham, R. T. *J. Biol. Chem.* **268**, 22737-22745 (1993).
- Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. *Cell* **75**, 805-816 (1993).
- Xiong, Y. et al. *Nature* **366**, 701-704 (1993).
- Gu, Y., Turck, C. W. & Morgan, D. O. *Nature* **366**, 707-710 (1993).
- Serrano, M., Hannon, G. J. & Beach, D. *Nature* **366**, 704-707 (1993).
- Polyak, K. et al. *Genes Dev.* **8**, 9-22 (1994).
- Polyak, K. et al. *Cell* **78**, 59-66 (1994).

- Toyoshima, H. & Hunter, T. *Cell* **78**, 67-74 (1994).
- Fesquet, D. et al. *EMBO J.* **12**, 3111-3121 (1993).
- Poon, R. Y., Yamashita, K., Adamczewski, J. P., Hunt, T. & Shuttleworth, J. *EMBO J.* **12**, 3123-3132 (1993).
- Solomon, M. J., Harper, J. W. & Shuttleworth, J. *EMBO J.* **12**, 3133-3142 (1993).
- Gu, Y., Rosenblatt, J. & Morgan, D. O. *EMBO J.* **11**, 3995-4005 (1992).
- Crabtree, G. R. & Clipstone, N. A. *Rev. Biochem.* **63**, 1045-1083 (1994).
- Kato, J.-Y., Matsuoka, M., Polyak, K., Massague, J. & Sherr, C. J. *Cell* (in the press).
- Bierer, B. E. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 9231-9235 (1990).
- Schreiber, S. L. & Crabtree, G. R. *Immun. Today* **13**, 136-142 (1992).
- Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988).

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