14. Adamczewski, M., Köhler, G. & Lamers, M. C. *Eur. J. Immun.* **21,** 617–626 (1991).
15. Liu, Y.-J., Johnson, G. D., Gordon, J. & MacLennan, I. C. M. *Immun. Today* **13,** 17–21 (1992).
16. Maeda, K. *et al. J. Immun.* **148,** 2340–2347 (1992).
17. Heyman, B., Tianmin, L. & Gustavsson, S. *Eur. J. Immun.* **23,** 1739–1742 (1993).
18. Lebrun, P. & Spiegelberg, H. L. *J. Immun.* **139,** 1459–1465 (1987).
19. Kopf, M. *et al. Nature* **362,** 245–247 (1993).
20. Keegan, A. D., Snapper, C. M., Van Dusen, R., Paul, W. E. & Conrad, D. H. *J. Immun.* **142,** 3868–3874 (1989).
21. Luo, H., Hofstetter, H., Banchereau, J. & Delespesse, G. *J. Immun.* **146,** 2122–2129 (1991).
22. Sherr, E., Macy, E., Kimata, H., Gilly, M. & Saxon, A. *J. Immun.* **142,** 481–489 (1989).
23. Saxon, A., Kurbe-Leamer, M., Behle, K., Max, E. E. & Zhang, K. *J. Immun.* **147,** 4000–4006 (1991).
24. Street, N. E. *et al. J. Immun.* **144,** 1629–1639 (1990).
25. Campbell, K. A., Lees, A., Finkelman, F. D. & Conrad, D. H. *Eur. J. Immun.* **22,** 2107–2112 (1992).
26. Sinclair, N. R. StC. *Immun. Today* **4,** 35–36 (1983).
27. Nitschke, L., Kopf, M. & Lamers, M. C. *BioTechniques* **14,** 914–916 (1993).
28. Ayane, M. P., Neilsen, P. & Köhler, G. *Nucleic Acids Res.* **17,** 6722 (1990).
29. Rao, M., Lee, W. T. & Conrad, D. H. *J. Immun.* **138,** 1845–1851 (1987).

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A mammalian protein targeted by G1-arresting rapamycin–receptor complex

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THE structurally related natural products rapamycin and FK506 bind to the same intracellular receptor, FKBP12, yet the resulting complexes interfere with distinct signalling pathways[1,2]. FKBP12–rapamycin inhibits progression through the G1 phase of the cell cycle in osteosarcoma[3], liver[4,5] and T cells[6,7] as well as in yeast[8], and interferes with mitogenic signalling pathways that are involved in G1 progression[9,10], namely with activation of the protein $p70^{S6k}$ (refs 5, 11–13) and cyclin-dependent kinases[3,14–16]. Here we isolate a mammalian FKBP–rapamycin-associated protein (FRAP) whose binding to structural variants of rapamycin complexed to FKBP12 correlates with the ability of these ligands to inhibit cell-cycle progression. Peptide sequences from purified bovine FRAP were used to isolate a human cDNA clone that is highly related to the *DRR1/TOR1* and *DRR2/TOR2* gene products from *Saccharomyces cerevisiae*[8,17,18]. Although it has not been previously demonstrated that either of the *DRR/TOR* gene products can bind the FKBP–rapamycin complex directly[17,19], these yeast genes have been genetically linked to a rapamycin-sensitive pathway and are thought to encode lipid kinases[17–20].

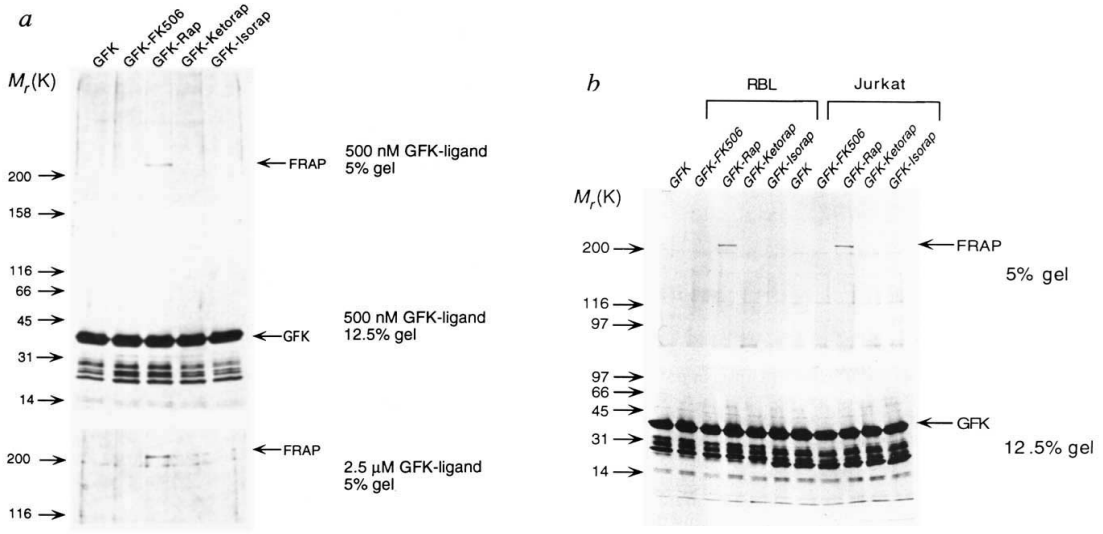
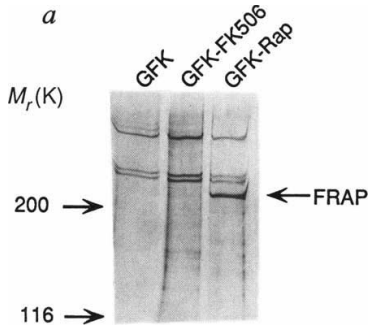


FIG. 1 Identification of FRAP protein in three mammalian cell lines. *a*, GFK alone or individual GFK–ligand complexes were added to MG-63 cell lysates (2×10^7 cells per condition) to a final concentration of either 500 nM or 2.5 μM and the mixtures incubated for 10 min at 4 C. Fusion protein complexes were recovered by glutathione-affinity chromatography, and the proteins detected by silver staining after 5% SDS–PAGE. Because of compression, FRAP is not resolved by 12.5% SDS–PAGE, so both 5% and 12.5% gels are shown. The amount of FRAP that was retained by affinity chromatography saturated at concentrations of GFK–Rap greater than 500 nM in these experiments and in others using concentrations of GFK–Rap ranging from 100 nM to 5 μM (data not shown). *b*, GFK alone or individual GFK–ligand complexes were added to a final concentration of 500 nM to lysates prepared from either 2×10^8 Jurkat T lymphocytes or 10^8 rat basophilic leukaemia (RBL) cells per condition. Lysates were treated as in *a*. FKBP12, but not FKBP13 or FKBP25 (ref. 23) is able to mediate the actions of rapamycin in *S. cerevisiae*. In addition, we found that YFK188 (ref. 24), an FKBP12 null strain, could be complemented with GFK (P. K. Martin, B. Gladstone, G. Weiss, D. T. Hung, S.L.S., in preparation). Thus the GST appendage of the fusion protein does not preclude binding of the biologically relevant target to the GFK–rapamycin complex in yeast.

METHODS. MG-63, Jurkat and RBL cells were grown in media containing 10% FBS and lysed at 4 °C in PINT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5. 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μM Na_3VO_4, 25 mM 2-glycerophosphate, 0.2 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A and 2 mM DTT) containing 0.5% Triton X-100. Lysates were clarified by centrifugation at 25,000*g*, and the Triton X-100 in the supernatant was diluted to 0.33% by adding 0.5 vol PINT buffer. GFK prebound to stoichiometric quantities of FK506, keto- iso- or unmodified rapamycin was added to lysates as described. Each condition was then passed through a 250-μl glutathione-Sepharose column, which was washed with PINT buffer containing 0.5 M NaCl and 0.3% Triton X-100.



b

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 401 AFRPSAFTDT QYLQDTMNHV LSCVKKEKER TAAFQALGLL SVAVRSEFKV  450
 451 YLPRVLDIIR AALPPKDFAH KRQKAMQVDA TVFTCISMLA RAMGPGIQQD  500
 501 IKELLEPMLA VGLSPALTAV LYDLSRQIPQ LKKDIQDGLL KMLSLVLMHK  550
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1001 IRVCDGAIRE FLFQQLGMLV SFVKSHIRPY MDEIVTLMRE FWVMNTSIQS 1050
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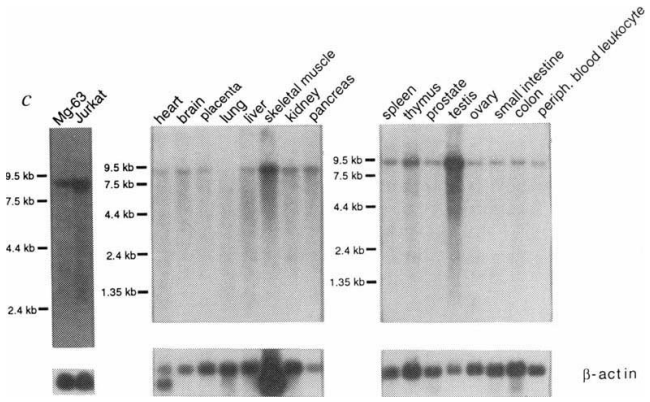

FIG. 2 Purification of FRAP from bovine brain and cDNA cloning of human FRAP. *a*, Fivefold-enriched bovine FRAP (S-column eluate; see below) was conditioned with [100 nM]$_f$ glutathione-S-transferase-FKBP12 fusion protein (GFK), GFK-FK506 or GFK–Rap. Complexes with fusion proteins were recovered by glutathione-affinity chromatography and detected as described in Fig 1 legend. We also found FRAP in bovine liver and thymus. *b*, Predicted translational product of the human FRAP cDNA clone. Bovine FRAP peptide sequences aligned to human FRAP are indicated by underlined segments. In the reading frame shown translational stop codons were not encountered upstream of the initiating methionine. *c*, Northern blot analysis of human tissue, Jurkat T cell and MG-63 cell poly (A)$^+$ RNA. The Jurkat/MG-63 and multiple tissue Northern blots (Clontech) were hybridized with ^{32}P-labelled probes derived from the 182 bp PCR fragment and the 5.5 kb clone (text), respectively. Hybridization to human β-actin probe is shown as an internal control for loading.

METHODS. Bovine FRAP was purified by grinding 900 g of bovine brain in blender with 1 litre of PIP (0.3% Triton X-100, 50 mM sodium phosphate, pH 7.2, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μM Na_3VO_4, 25 mM 2-glycerophosphate, 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine and 2 mM DTT). The homogenate was centrifuged at 25,000*g* and the supernatant (20 g total protein) was loaded onto a 1 litre S-Sepharose (Pharmacia) column. The column was then washed with PIP and eluted with PINP (PIP with 1M NaCl). GFK–rapamycin was added to the pooled eluate to a final concentration of 100 nM and recovered by glutathione-affinity chromatography. FRAP was resolved by SDS–PAGE and transferred to PVDF. Following digestion with trypsin or endoproteinase Lys-C (Boehringer Mannheim) bFRAP peptides were microsequenced[23]. The Jurkat T cell cDNA library (Stratagene) was constructed through random and oligo dT priming of cytoplasmic oligo dT purified RNA (ref. 25). cDNA screening, Jurkat and MG-63 RNA isolation and northern blotting and were performed by procedures similar to those previously described[25]. A 182 bp fragment was amplified from a human brain stem library (Stratagene) and labelled by incorporation of ^{32}P-dCTP in the course of reamplification by PCR. The sequences were analysed using BLAST (ref. 26) and the University of Wisconsin GCG (ref. 27) software. The human FRAP cDNA sequence has been submitted to Genbank.

We used two structural variants of rapamycin, 16-keto-rapamycin (S. D. Meyer and S.L.S., manuscript in preparation) and 25,26-iso-rapamycin[21], to identify any biologically relevant targets of the FKBP–rapamycin complex. Both variants bind tightly to human FKBP12, as shown by their ability to inhibit rotamase activity of the recombinant protein (K_i values were 0.2 nM for rapamycin[6], 2 nM for keto-rapamycin, and 0.1 nM for iso-rapamycin). But the variants are about two orders of magnitude less potent than rapamycin in preventing the progression through G1 of MG-63 human osteosarcoma cells. The values of IC_{50} (half-maximal inhibitory concentration) estimated from dose–response curves are 0.1 nM, 7.5 nM and 50 nM for rapamycin, keto- and iso-rapamycin, respectively. Thus the complexes of iso- and keto-rapamycin with FKBP12 should bind to the FKBP12–rapamycin target less effectively than FKBP12–rapamycin itself.

A fusion protein of glutathione-*S*-transferase with FKBP12 (GFK) was used to identify candidates for the biologically relevant targets of FKBP12 rapamycin. MG-63 cells were lysed by detergent and complexes of GFK–rapamycin, GFK–FK506 or GFK alone were added individually to clarified lysate at a final concentration of 500 nM or 2.5 μM (Fig. 1*a*). A protein of approximate relative molecular mass 220,000 ($M_r \sim 220$K) was detected in the GFK–rapamycin sample by SDS–PAGE and silver staining (Fig. 1*a*, lane 3). This FKBP–rapamycin-associated protein (FRAP) was not retained with GFK–FK506 or GFK alone (Fig. 1*a*, lanes 1 and 2). No other rapamycin-specific proteins were detected by silver staining (Fig. 1*a*) or by a similar

affinity purification procedure using lysates from [^{35}S] methionine-labelled cells (data not shown). The GFK–ketorapamycin and GFK–isorapamycin complexes bound FRAP less effectively than GFK–rapamycin; at concentrations of 500 nM, the keto- and iso- complexes were unable to retain the 220K protein (Fig. 1*a*, lanes 4, 5), whereas at higher concentrations of the complexes (2.5 μM) detectable quantities of FRAP were retained (Fig. 1*a*, lanes 4, 5). This is consistent with the finding that these compounds are still strong cell-cycle inhibitors, albeit less potent than rapamycin itself. Thus, the binding of GFK–ligand complexes to FRAP correlates with the ability of the ligands to impede G1 progression in MG-63 cells. FRAP was also detected in Jurkat *T*-lymphocyte cells and rat basophilic leukaemia cells (Fig. 1*b*), two mammalian cell lines that are also sensitive to rapamycin[6,22]. No other rapamycin-specific bands were observed in each case.

FRAP purified from bovine brain (bFRAP) had a similar specificity for GFK-ligand (Fig. 2*a*). Microsequencing of bFRAP proteolytic fragments (298 amino acids in total, Fig. 2*b*) led to the design of a pair of degenerate oligonucleotides for use in the polymerase chain reaction (PCR). A 182 bp PCR product allowed for the isolation of overlapping clones from a human Jurkat T cell λZAP II cDNA library, yielding 7.6 kb of contiguous sequence. Using these cDNA sequences as probes, a band migrating at approximately 8.5 kilobases was detected by Northern blot analysis of oligo dT purified RNA isolated from a variety of human tissues and cell lines (Fig. 2*c*). The human cDNA sequence encodes an amino-acid open reading frame (ORF) and aligns with 99% identity to the bFRAP peptides (Fig. 2*b*). As N-terminal peptide sequence from purified bovine FRAP was not obtained, the initiating methionine shown in Fig. 2*b* is unconfirmed. The predicted molecular mass of this ORF (~300K) is greater than that inferred by the mobility of FRAP during SDS–PAGE (above).

Human FRAP is highly related to the *DRR1/TOR1* and *DRR2/TOR2* gene products. Overall it is 44% identical to DRR1/TOR1 and 46% identical to DRR2/TOR2. The region of greatest homology to DRR1/TOR1 and DRR2/TOR2 lies in the C-terminal 660 amino acids of human FRAP (57% and 59% identical, respectively). In addition, this region has homology to several known phosphatidylinositol kinases (21% identity on average), including mammalian phosphatidylinositol 3-kinase[17,18] (PI3K), a yeast PI3K *VPS34* (refs. 17 and 18) and *PIK1* (ref. 20). These similarities indicate that FRAP may also have phosphatidylinositol kinase activity.

Through the introduction of minute structural changes in rapamycin, this study implicates FRAP as a mediator of G1 cell cycle progression in mammalian cells. Identification of FRAP as the target of FKBP12-rapamycin together with the earlier demonstration of calcineurin as the target of FKBP12-FK506 (ref. 2) addresses a fascinating aspect of immunophilin research, namely that the immunophilin FKBP12 can bind two distinct natural products and thereby gain the ability to bind two distinct signalling molecules involved in cell cycle entry and progression. Further biochemical characterization of this unique mammalian protein should elucidate its role in propagating the mitogen-initiated signals that lead to the activation of p70^{S6k} and cyclin–Cdk complexes. □

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1. Schreiber, S. L. *Science* **251,** 283–287 (1991).
2. Schreiber, S. L. *Cell* **70,** 365–368 (1992).
3. Albers, M. W. *et al. Ann. N. Y. Acad. Sci.* **698,** 54–62 (1993).
4. Francavilla, A. *et al. Hepatology* **15,** 871–877 (1992).
5. Price, D. J., Grove, J. R., Calvo, V., Avruch, J. & Bierer, B. E. *Science* **257,** 973–977 (1992).
6. Bierer, B. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **87,** 9231–9235 (1990).
7. Dumont, F. J., Staruch, M. J., Koprak, S. L., Melino, M. R. & Sigal, N. H. *J. Immun.* **144,** 251–258 (1990).
8. Heitman, J., Movva, N. R. & Hall, M. N. *Science* **253,** 905–909 (1991).
9. Lane, H. A., Fernandez, A., Lamb, N. J. C. & Thomas, G. *Nature* **363,** 170–172 (1993).
10. Norbury C. & Nurse, P. *A. Rev. Biochem.* **61,** 441–470 (1992).
11. Chung, J., Kuo, C. J., Crabtree, G. R. & Blenis, J. *Cell* **69,** 1227–1236 (1992).
12. Kuo, C. J. *et al. Nature* **358,** 70–73 (1992).
13. Calvo, V., Crews, C. M., Vik, T. A. & Bierer, B. *Proc. natn. Acad. Sci. U.S.A.* **89,** 7571–7575 (1992).
14. Morice, W. G., Wiederrecht, G., Brunn, G. J., Siekierka, J. J. & Abraham, R. T. *J. biol. Chem.* **268,** 22737–22745 (1993).
15. Morice, W. G., Brunn, G. J., Wiederrecht, G., Siekierka, J. J. & Abraham, R. T. *J. biol. Chem.* **268,** 3734–3738 (1993).
16. Albers, M. W. *et al. J. biol. Chem.* **268,** 22825–22829 (1993).
17. Cafferkey, R. *et al. Molec. cell. Biol.* **13,** 6012–6023 (1993).
18. Kunz, J. *et al. Cell* **73,** 585–596 (1993).
19. Helliwell, S. B. *et al. Molec. Biol. Cell* **5,** 105–118 (1994).
20. Flanagan, C. A. *et al. Science* **262,** 1444–1448 (1993).
21. Hayward, C. M., Yohannes, D. & Danishefsky, S. J. *J. Am. chem. Soc.* **115,** 9345–9346 (1993).
22. Hultsch, T., Martin, R. & Hohman, R. J. *Molec. Biol. Cell.* **3,** 981–987 (1992).
23. Galat, A., Lane, W. S., Standaert, R. F. & Schreiber, S. L. *Biochemistry* **31,** 2427–2434 (1992).
24. Foor, F. *et al. Nature* **360,** 682–684 (1992).
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning A Laboratory Manual*, 2nd edn (Cold Spring Harbor Laboratory Press, New York, 1989).
26. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. *J. molec. Biol.* **215,** 403–410 (1990).
27. Devereux, J., Haeberli, P. & Smithies, O. *Nucleic Acids Res.* **12,** 387–395 (1984).

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Functional dissection of the yeast Cyc8–Tup1 transcriptional co-repressor complex

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DNA-BINDING repressor proteins mediate regulation of yeast genes by cell type (Mcm1/α2 and a1/α2), glucose (Mig1) and oxygen (Rox1) (refs 1–4 respectively). An unusual feature of all these regulatory pathways is that transcriptional repression requires two physically associated proteins[5] that do not bind DNA Cyc8(Ssn6) and Tup1. The Cyc8–Tup1 complex has been proposed to be a co-repressor that is recruited to target promoters by pathway-specific DNA-binding proteins[6], but the specific functions of the individual proteins are unknown. Here we show that when it is bound upstream of a functional promoter through the LexA DNA-binding domain, Tup1 represses transcription in the absence of Cyc8. Deletion analysis indicates that Tup1 contains at least two non-overlapping transcriptional repression regions with minimal primary sequence similarity, and a separable Cyc8-interaction domain. These Tup1 domains, which do not include the β-transducin motifs[7], are necessary and partially sufficient for Tup1 function. We suggest that Tup1 performs the repression function of the Cyc8–Tup1 co-repressor complex, and that Cyc8 serves as a link with the pathway-specific DNA-binding proteins.

It has been previously shown that Cyc8 can repress transcription in a Tup1-dependent manner when bound upstream of the intact *CYC1* promoter through the heterologous LexA DNA-binding domain[6]. Similarly, a LexA–Tup1 hybrid protein confers a 16-fold reduction of expression from a promoter containing four LexA operators upstream of the *CYC1* promoter (Table 1). LexA–Tup1 and LexA–Cyc8 also repress expression of a *his3* gene containing a single LexA operator upstream of the T_R TATA element (Fig. 1*a*), suggesting that they can inhibit basal transcription. Surprisingly, LexA–Tup1 retains almost its entire

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