

LETTERS TO NATURE

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

Eric J. Brown, Mark W. Albers, Tae Bum Shin, Kazuo Ichikawa, Curtis T. Keith, William S. Lane* & Stuart L. Schreiber†

Department of Chemistry, Howard Hughes Medical Institute and *Harvard Microchemistry Facility, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, USA
† To whom correspondence should be addressed

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 osteosarcoma³, liver^{4,5} and T cells^{6,7} as well as in yeast⁸, and interferes with mitogenic signalling pathways that are involved in G1 progression^{9,10}, namely with activation of the protein p70^{56k} (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}.

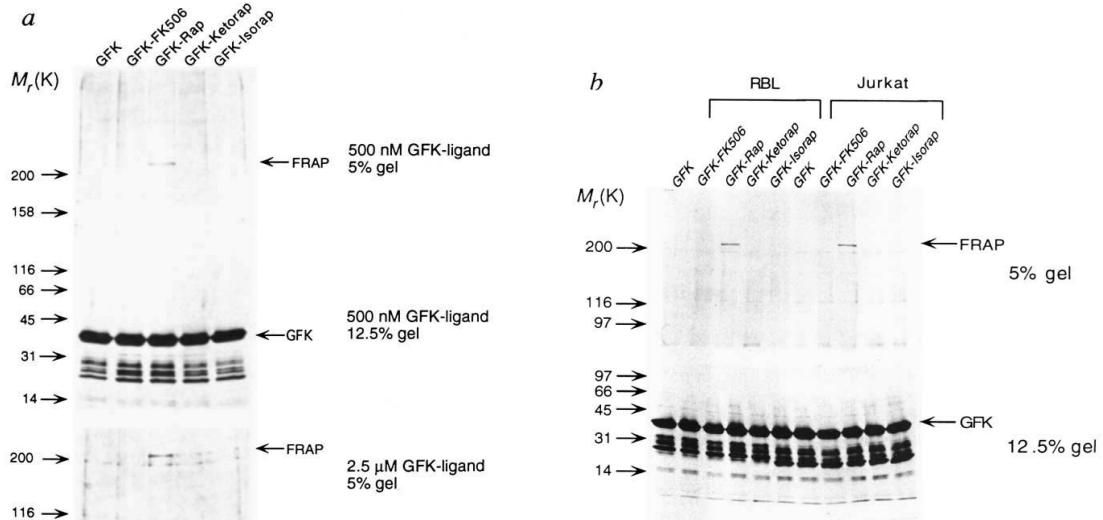
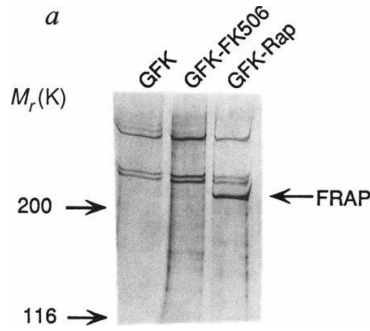


FIG. 1 Identification of FRAP protein in three mammalian cell lines. a, GFK alone or individual GFK-ligand complexes were added to MG-63 cell lysates (2×10^7 cells per condition) to a final concentration of either 500 nM or 2.5 μ M and the mixtures incubated for 10 min at 4 °C. Fusion protein complexes 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 μ M (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 μ M Na₃VO₄, 25 mM 2-glycerophosphate, 0.2 mM PMSF, 1 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ pepstatin A and 2 mM DTT) containing 0.5% Triton X-100. Lysates were clarified by centrifugation at 25,000g, and the Triton X-100 in the supernatant was diluted to 0.33% by adding 0.5 vol PINT buffer. GFK rebound to stoichiometric quantities of FK506, keto- iso- or unmodified rapamycin was added to lysates as described. Each condition was then passed through a 250- μ l glutathione-Sepharose column, which was washed with PINT buffer containing 0.5 M NaCl and 0.3% Triton X-100.



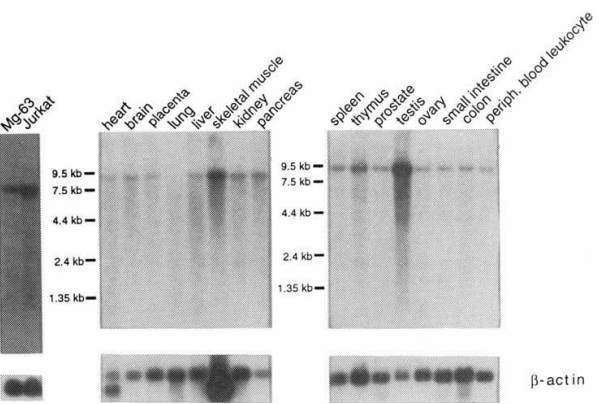
b

1	MLGTGPAAT	TAATTSNV	VLQQFASGLK	SRNEETRKA	AKELQHYVTM	50
51	ELREMSQEF	TREYDOLNHH	IFELYSSDA	NERKGGILLAI	ASLIGVEGNN	100
101	ATRIGRFANY	LRNLLPSNDP	VVEMASKAT	GRLAMAGDTF	TAHYVFEVVK	150
151	RALEWLCADR	NEGRRAHVL	VLRELATISVP	TTFFQVQVFP	FDNLFVAVD	200
201	PKQATREGAV	AALRACLILT	TQREPKMQK	PQWYRHTFEE	AEGFGDTLLA	250
251	KEKGMNRDR	IHGALLILNE	LYRISMEGE	RLREEMEEIT	QQQLVHDKYC	300
301	KDLMGEGTKP	RHITPEISEO	AYOPOQSAL	VGLLGYSSHQ	GLMGFGTSPS	350
351	PAKSTVLVES	CCRDLMEEKF	DQVQWVLC	RNSKNSLIQM	TILNLLPRLA	400
401	AFRPSAFTDT	QYLDQTMNHV	LSCVKKEKER	TAAFQALGLL	SVAVRSEFKV	450
451	YLPVRLDIR	AALPPKDFAH	KRQKAMQVDA	TVFTCSISMLA	RAMGPGIQDD	500
501	IKELLEPMLA	VGLSPALTAV	LYDLSRQIPQ	LKDKIDQDGL	KMLSIVLMHK	550
551	PLRHPGMPKG	LAHQLASPGL	TTLPEASDVG	SITLALRTLQ	SFEFEGHSLT	600
601	QFVRHCADHF	LNSEHKEIRM	EAARTCSRLL	TPSIHLTSGH	AHVVSQTAVQ	650
651	VVADVLSKLL	VVGTDPDPPD	IRYCVLASLD	ERFDHDLAQA	ENLQALFVAL	700
701	NDQVFEIREL	ATCTVGRLSL	MNPAFVMPFL	RKMLTQITLE	LEHSGTGRIK	750
751	EQSARMLGHL	VSNAPRLIRP	YNEPILKALI	LKDKDPPDPP	NPVGINVLLA	800
801	TIGELAQVSG	LEMWKVDELD	FIIMDMLOD	SLLAKRQVA	LWTLGQLVAS	850
851	TGYVVEPYRK	YPTLLEVLN	FLKTEQNGT	RREAIRVGL	LGALDPYKHK	900
901	YNTGMDQSR	DASAVLSSES	KSSQSDSDYS	TSEMLNMGN	LPLDEFYPAV	950
951	SMVALMIRFR	DQSLSHHHTM	VVQAITFIK	SLGLKCVQFL	PQVMPFLNV	1000
1001	IRVCDGAIRE	FLFQQLGMLV	SVFKSHIRPY	MDEIVTLMRE	FVWNTSISQ	1050
1051	TILLIEIQTV	VALGGEFKLY	LPOLIPHLR	VFMHDSNPGR	IVSKLLAAI	1100
1101	QLGFANLDDY	LHLLPPIVK	LFDPAEAPLP	SRKALETVD	RLTESLDEFD	1150
1151	YASBIIHPIV	RTLDSPELBS	STAMDTLSSL	VFQLGKQYQI	F1PMWKNVIV	1200
1201	RHRINHQRVD	VLCIRIVKGY	TLADDEEDPL	TYOHRMLRSG	QGDALASGVE	1250
1251	ETGPMKALHY	STINLOKAWG	AABRVSKDOW	LEMLRLLSLE	LKDKSSPSL	1300
1301	RSCWALQAY	NPMARDLFA	AFVCSKSLER	EDQDELIRS	IELALTSQDI	1350
1351	AEVYOTLNL	AEFHEHSDKG	PLPLRDDNGI	VLLGERAAK	RAYAKALHYK	1400
1401	ELEEQKPTP	AILESLSIN	NKLQPEAAA	GVLEYAMKHF	GELEIQATYI	1450
1451	EKLHEDWAL	VAYDKMOTN	KDDPELMLGR	MRLCEALGEW	QLHQQCEK	1500
1501	WTLVNDQTA	KMARMAAAA	WGLQDQDME	EYTCMIPRD	HDBGFYRVLV	1550
1551	ALHQDLFSLA	QQCIDKARDL	LDDEL TAMAG	ESYSRAYGM	VSCHMLSELE	1600
1601	EVIQYKLVPE	RREIRQIWW	ERLQGCQRYI	EDWQILMVR	SLVSPHEMDE	1650
1651	RTWLKYSALC	GKSGRLALAH	KTLVLLGVD	PSRLDHPILP	TVHPQVITYA	1700
1701	MKNMKSARK	IDAFQHQHF	VQTQQQAQH	AIATEDQQHK	QELHKLMAR	1750
1751	FKLGEQWLN	LQGINESTIP	KVLYQYSAA	EHDRSRYKAW	HAWAVMNEFA	1800
1801	VLHYHQHQA	RDEKCLRRA	SGANITNATT	AATTAATATT	TASTEGNSE	1850
1851	SEASTENSP	TPSPQLKQVY	FDLSKTLMLY	TPVAPQGFRR	S1LSRGNL	1900
1901	QDTRVRLTLW	FYGHWPVNV	EALYEGVKAT	QIDTWLQVTP	QLIARIDTRP	1950
1951	PLVGRILHQL	LTDIGRYHPQ	ALIVPLTVAS	KSTTTRARNA	ANKILKNMCE	2000
2001	HSNTLVQQAM	MVSEELIRVA	ILWHEMHHEG	LEEASRLYFG	ERNKGMFEV	2050
2051	LEPLHAMMER	GPQTLKETSE	NOAQGDOLME	AGEWCRKYM	SGWVKLTOA	2100
2101	WDLVYHFR	ISKQLPQLTS	LELQVSPKL	LMCRDL ELAV	PCTYDQDPT	2150
2151	IBTOSIAEPL	QITISKQRP	KLTLMGCSNGH	EFYFLKGFH	DLRODERWQ	2200
2201	RDYREKTKIL	NDPISLRKLL	SIQRYDHLTL	STMSGLTGW	PHCDTHALI	2250
2251	LFGLVNTLLA	LNI EHRIMLR	MAPPYDHLTL	MQVVEVEEHA	VNNTAGDOLA	2300
2301	KLLWLLSPSS	EVWFDRRTNY	TRSLVMSHW	GYZLLGDRH	PSNMLDRLS	2350
2351	KTLLHIDFG	CEVAVMTRK	FPEKIPFRLT	RMLTNAMEVT	GLDNGRITC	2400
2401	HTMVEVREH	KDSVMVLEA	FVYDPLMNR	LMDTNKGK	RSRTRIDTSY	2450
2451	AGOSVETLDG	VELGEPAAHK	TGTTVPESH	STFGDGLYKP	FALNKKATQI	2500
2501	INRVDRKLTG	RDFSDDTLD	VPTQVELLIK	QATSHENLQC	CYTGWCPFW	2549

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] 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 ³²P-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 μ M Na₃VO₄, 25 mM 2-glycerophosphate, 1 mM PMSF, 1 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ pepstatin A, 1 mM benzamide and 2 mM DTT). The homogenate was centrifuged at 25,000g 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 micro-sequenced²³. 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 described²⁵. A 182 bp fragment was amplified from a human brain stem library (Stratagene) and labelled by incorporation of ³²P-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-rapamycin²¹, 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 rapamycin⁶, 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 IC₅₀ (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 μ M (Fig. 1a). A protein of approximate relative molecular mass 220,000 (*M_r* ~220K) was detected in the GFK-rapamycin sample by SDS-PAGE and silver staining (Fig. 1a, lane 3). This FKBP-rapamycin-associated protein (FRAP) was not retained with GFK-FK506 or GFK alone (Fig. 1a, lanes 1 and 2). No other rapamycin-specific proteins were detected by silver staining (Fig. 1a) or by a similar

affinity purification procedure using lysates from [³⁵S] 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. 1a, lanes 4, 5), whereas at higher concentrations of the complexes (2.5 µM) detectable quantities of FRAP were retained (Fig. 1a, 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. 1b), 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. 2a). Microsequencing of bFRAP proteolytic fragments (298 amino acids in total, Fig. 2b) 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. 2c). The human cDNA sequence encodes an amino-acid open reading frame (ORF) and aligns with 99% identity to the bFRAP peptides (Fig. 2b). As N-terminal peptide sequence from purified bovine FRAP was not obtained, the initiating methionine shown in Fig. 2b 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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Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex

Dimitris Tzamarias & Kevin Struhl*

Department of Biological Chemistry and Molecular Pharmacology, 240 Longwood Avenue, Harvard Medical School, Boston, Massachusetts 02115, USA

DNA-BINDING repressor proteins mediate regulation of yeast genes by cell type (*Mcm1/a2* and *a1/a2*), 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 proteins⁵ 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 proteins⁶, 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 motifs⁷, 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 domain⁶. 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. 1a), suggesting that they can inhibit basal transcription. Surprisingly, LexA-*Tup1* retains almost its entire

* To whom correspondence should be addressed.