Frap, FKBP12 rapamycin-associated protein, is a candidate gene for the plasmacytoma resistance locus *Pctr2* and can act as a tumor suppressor gene

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Susceptibility to mouse plasmacytomagenesis is a complex genetic trait controlled by several *Pctr* loci (*Pctr1*, *Pctr2*, etc). Congenic strain analysis narrowed the genetic interval surrounding the *Pctr2* locus, and genes identified in the interval were sequenced from susceptible BALB/c and resistant DBA/2 mice. *Frap* (FKBP12 rapamycin-associated protein, mTOR, RAFT) was the only gene differing in amino acid sequence between alleles that correlated with strain sensitivity to tumor development. The *in vitro* kinase activity of the BALB/c FRAP allele was lower than the DBA/2 allele; phosphorylation of p53 and PHAS1/4EBP1 (properties of heat and acid stability/eukaryotic initiation factor 4E-binding protein) and autophosphorylation of FRAP were less efficient with the BALB/c allele. FRAP also suppressed transformation of NIH 3T3 cells by ras, with DBA/2 FRAP being more efficient than BALB/c FRAP. Rapamycin, a specific inhibitor of FRAP, did not inhibit growth of plasmacytoma cell lines. These studies identify *Frap* as a candidate tumor suppressor gene, in contrast to many reports that have focused on its prooncogenic properties. *Frap* may be similar to *Tgfb* and *E2f* in exerting both positive and negative growth-regulatory signals, depending on the timing, pathway, or tumor system involved. The failure of rapamycin to inhibit plasma cell tumor growth suggests that FRAP antagonists may not be appropriate for the treatment of plasma cell tumors. *Pctr2* joins *Pctr1* in possessing alleles that modify susceptibility to plasmacytomagenesis by encoding differences in efficiency of function (efficiency alleles), rather than all-or-none, gain-of-function, or loss-of-function alleles. By analogy, human cancer may also result from the combined effects of several inefficient alleles.

Mouse plasmacytomas provide a model for the dissection of complex genetic traits associated with cancer. Plasma cell tumors arise as a result of a chronic inflammatory response to an inducing agent, the oil pristane (1), and require IL-6 for establishment and proliferation *in vivo* (2) and *in vitro* (3). Unlike many single gene disorders, where a mutation in the affected gene frequently abolishes activity of the gene or renders it constitutively active, the pattern emerging for mouse plasma cell tumors raises the possibility that an accumulation of several efficiency allele defects eventually results in tipping the balance toward tumorigenesis.

BALB/cAnPt and NZB/BlNJ mice are the only known inbred strains to develop pristane-induced plasma cell tumors, with an incidence of 60% and 30%, respectively (4). Most other strains of mice either do not develop tumors or have a relatively low (10%) incidence over the course of a year after treatment with pristane. Several years ago, we took advantage of the high (60%) incidence in BALB/cAn mice compared with the resistance (0%) seen in DBA/2N mice to perform linkage analysis of resistance/susceptibility phenotypes (cytological identification of neoplastic plasma cells in Giemsa-stained ascites smears) with genotypes (RFLPs, SSLPs) in backcross mice to identify several plasmacytoma resistance/susceptibility (*Pctr*) loci (5).

Results from these backcross experiments were confirmed with data from congenic strains (6–9), leading to the identification of several *Pctr* loci involved in the genetic susceptibility of BALB/cAn mice and the resistance of DBA/2N mice. We have identified the cyclin-dependent kinase inhibitor *Cdkn2a* as a likely candidate for the *Pctr1* locus located in the middle of mouse chromosome (Chr) 4 (8, 9). A single nucleotide variant in the p16 gene product encoded by the *Cdkn2a* locus led to a difference in the efficiency with which the p16 protein functions in resistant vs. susceptible mice (8–10). Furthermore, p16 expression was found to be lower in susceptible BALB/c mice than in resistant DBA/2 mice after pristane treatment; this difference may be linked to allelic sequence differences in the promoter, which lead to differential binding efficiency of the transcription factor RREB (11).

The *Pctr2* locus resides in the telomeric region of mouse Chr 4, and the resistant DBA/2 allele was found to delay the onset of tumorigenesis (7). In this study, we report the identification of *Frap* (FKBP12 rapamycin-associated protein) as the most likely candidate gene for the *Pctr2* locus. The *Frap* gene encodes a kinase capable of phosphorylating many substrates and interacting with a large number of proteins involved in the phosphatidylinositol 3-kinase signal transduction pathway (12–19). This pathway can be activated by IL-6 receptor signaling (20), known to be required for both plasma cell tumor (2, 3) and multiple myeloma cell (21, 22) growth.

In this study, we present evidence that *Frap* may act as a tumor suppressor locus for pristane-induced plasmacytomas. In contrast, studies with other systems have suggested that *Frap* is more likely to promote cell growth and proliferation (18, 23). We provide evidence that allelic variation in a single amino acid at the *Frap* locus leads to quantitative differences in protein activity in biochemical and biological assays: phosphorylation of both p53 and PHAS1, and suppression of ras transformation, respectively. In both assays, BALB/c FRAP, which differs from DBA/2 FRAP by a single amino acid in its coding sequence, was less efficient than DBA/2 FRAP in its activity. These functional assays further implicate *Frap* as a candidate for the *Pctr2* tumor susceptibility locus. The identification of a second locus (*Pctr2*, *Frap*) with yet another defect resulting in inefficient protein function suggests that an accumulation of partial defects in multiple effector pathways leads to plasma cell tumorigenesis in BALB/c mice. Our FRAP data, combined

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Abbreviations: FRAP, FKBP12 rapamycin-associated protein; Chr, chromosome; BAC, bacterial artificial chromosome; FRB, FKBP12 rapamycin-binding domain; SNP, single-nucleotide polymorphism; PHAS1/4EBP1, properties of heat and acid stability/eukaryotic initiation factor 4E-binding protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF091392, AF091505, AF091506, AF092074–AF092084, AF097512, AF107563, AF107564, AF152839–AF152841, AH008266, AH008346, B07891–B07948, B18208–B18224, NM_016699, NM_019393, and NM_019781).

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with studies suggesting that FRAP can stimulate growth in some situations, suggest that FRAP may inhibit tumorigenesis in some settings and foster it in others.

Methods

Development and Pheno-/Genotyping of the C.D2-Fv1B Strain. The C.D2-Fv1$^{n/n}$ B strain was derived by backcrossing C.D2-Fv1$^{n/n}$ N22 (6, 7) mice to BALB/cAnPt to create F_1 hybrids that were intercrossed and resulted in C.D2-Fv1$^{n/n}$ B progeny that were homozygous D/D at *Nppa*, *D4Mit129*, *D4Mit33*, and *D4Mit42*; they were homozygous C/C at *Xmv8* and *Xmv44*. Mice were phenotyped for tumor susceptibility as described (5). Briefly, mice were given three 0.5-ml injections of pristane on days 0, 60, and 120. The development of tumors was monitored biweekly with ascites slides. Induction studies were performed in accordance with Animal Care and Use Committee-approved protocols. Mouse DNAs were genotyped by PCR amplification with specific primers and analysis on agarose gels as described (5).

Identification and Comparative Sequencing of Genes in the *Pctr2* Interval. Down-to-the-well DNA pools (Genome Systems, St. Louis) were used for bacterial artificial chromosome (BAC) library screening by PCR. BAC libraries were generated from the 129Sv/J mouse strain. BAC purifications were done with kb-100 magnum columns (Genome Systems). PCR primers for BAC library screening were designed from Massachusetts Institute of Technology marker sequences D4Mit129 and D4Mit310. PCR primers for cDNAs and genomic DNA were originally designed from available cDNA and/or genomic sequences as well as ESTs.

Primers (v499: 5′-GACACTTGGTTACAGGTTATACCT-CAGCTC-3′ and v481:5′-GGCTGGTTCTCCAAGTTCTA-CACCG-3′) designed to encompass 269 bases surrounding the FKBP12-rapamycin binding domain (FRB) of *Frap* were used to generate RT-PCR products from the cDNAs of the following plasmacytoma cell lines: TEPC1165, TEPC 2027, MOPC460, D81–288, and D107–401.

Sequencing was done via Big Dye Terminators (Perkin-Elmer) types 1–3 on an Applied Biosystems 377 DNA Sequencer. Sequence analysis was performed with SEQUENCHER 3.0 (Gene Codes, Ann Arbor, MI) and GENEWORKS (IntelliGenetics) software, as well as BLAST, SPIDEY (National Center for Biotechnology Information), and BLAST at Celera Genomics Discovery System. GenBank, University of California at Santa Cruz, Ensembl, and Celera genomic databases (Rockville, MD). See Table 1, which is published as supporting information on the PNAS web site, for additional sequence data.

Allele-Specific PCR. To determine which nucleotide (C or T) is present at the position corresponding to 1977 of *Frap* cDNA in different strains of mice, genomic DNAs were amplified by PCR with the following primers: (011499F), AGCACAAGGAGATC-CGCATGGAAG; and (v506R), CACCACCTGCACTG-CAGTCTGG. Ten microliters of each 50-μl PCR was restricted with 2.5 units of *Aci*I restriction endonuclease. Restricted products were analyzed by PAGE on 20% acrylamide TBE gel, 200 V, overnight. Distinctive patterns were observed for the two alleles at this position.

Functional Assays of FRAP Kinase Activity. All kinase steps were performed at 4°C unless noted. For functional assay, both variants of *Frap* cDNA, with FLAG sequence attached to the 5′ end, were cloned into the pcDNA3 expression vector (Invitrogen). Plasmids (BALB/c, DBA, or pcDNA3 alone) were transfected into HEK293 cells for 5 h by using the Lipofectamine Plus kit (Life Technologies/Invitrogen). Cells were harvested 48 h later. Adherent tissue culture cells (100-mm round dishes) were rinsed twice with 5 ml of PBS and then overlaid with 1 ml of lysis buffer [50 mM Tris·HCl, pH 7.4/100 mM NaCl/50 mM β-glycerophosphate/10% (wt/vol) glycerol/1% (wt/vol) Tween 20/1 mM DTT/1 mM $MgCl_2$/10 μg/ml leupeptin/5 μg/ml aprotinin/1 mM AEBSF/1 mM sodium orthovanadate] and rocked for 30 min. Cells were scraped, collected into 1.7-ml Eppendorf tubes, and spun at 16,000 $\times$ *g* for 10 min. The supernatants were retained, and the protein concentration of the lysates was determined via bicinchoninic acid (BCA) assay (Pierce).

Before binding, 30 μl of 50% (vol/vol) of anti-FLAG bead suspension (A2220, Sigma) was washed in 1 ml of wash buffer [50 mM Tris·HCl, pH 7.4/100 mM NaCl/50 mM β-glycerophosphate/10% (wt/vol) glycerol/1% (wt/vol) Tween 20] and spun down (9,000 $\times$ *g* for 30 s), and all excess liquid was removed. Varied amounts of lysate were added to the rinsed beads, and the mixture was brought to 1 ml in lysis buffer. The mixture was rotated for 2 h at 4°C. The complex was then washed twice with wash buffer, once with high salt buffer (100 mM Tris·HCl, pH 7.4/500 mM LiCl), and finally, twice with kinase buffer (10 mM Hepes, pH 7.4/50 mM NaCl/1 mM DTT/5 mM β-glycerophosphate/2 mM EDTA).

Washed complexes were reacted in 50 μl of reaction mixture [kinase buffer plus 50 μM ATP, 10 mM $MnCl_2$, 1 μg of recombinant GST-p53 (Sc-4246, 80 kDa, Santa Cruz Biotechnology), 0.5 μg of recombinant PHAS1 (P-1021, 14.5 kDa, A. G. Scientific, San Diego), and 5 μCi (1 Ci = 37 GBq) [γ-^{32}P]ATP] for 30 min at 30°C. Substrate specificity was evident when substrates were used, singly and in combination.

After reaction, SDS/PAGE sample buffer was added, and samples were heat shocked at 95°C for 5 min and run on large 10–20% Tris glycine gels. Electrophoresis, drying, and autoradiography followed standard methods.

Western Blots. The same initial lysates were run on a small control Tris glycine Western blot (Novex/Invitrogen). Frap was resolved on 6% gel, and actin on 10% gel. Frap conditions were as follows: primary antibody (F4042, Sigma), 1:400, 15 h, 4°C; secondary (NA931, Amersham Pharmacia), 1:1000, 30 min, room temperature. Actin conditions (kit CP01, Oncogene) were as follows: primary antibody, 1:10,000, 2 h; secondary (anti-IgM), 1:2000, 30 min.

Functional Assays for Inhibition of ras Transformation. NIH 3T3 cells were maintained in DMEM containing 10% FCS, and fresh medium was supplemented twice per week. For transfection, cells were plated in 35-mm dishes at a density of 1×10^5 cells per plate, and transfection was performed by using the standard calcium phosphate method (24). NIH 3T3 cells were transfected by using a constant amount of v-*ras* (150 ng) expression plasmid (p1423) and an increasing amount of DBA/2 or BALB/c FRAP plasmid. Both plasmids contain Neo^R. Total plasmid DNA was kept constant by including compensatory amounts of vector DNA. Cells were trypsinized on the day after transfection and seeded in multiple tissue culture plates. Triplicate dishes were grown to confluence, and ras foci were counted 7 days after transfection. The remaining dishes were treated with G418, and the number of resistant colonies was counted.

Treatment of Plasmacytoma Cells with Rapamycin. MOPC460, TEPC1165, BPC4, D81–288, and D107–403 cells were cultured in RPMI medium 1640/2 mM L-glutamine/10% FCS/50 μM 2-mercaptoethanol with 3.6 ng/ml IL-6 (40 units/ml) at 37°C in 5% CO_2 to a maximum density of 5×10^5 cells per ml. The OVCAR and PC3 cells were cultured in the same media without IL-6 and 2-mercaptoethanol. All cells were split into 10-cm^2 dishes and treated with either 10, 100, or 1,000 ng/ml for 24 h. At that point, they were collected and washed twice with PBS (containing 0.02% BSA and 0.01% EDTA). All cells were fixed in 75% ethanol at 4°C for 1 h and then stained with propidium iodide and analyzed by FACScan (Becton Dickinson) for cell cycle profiles (9).

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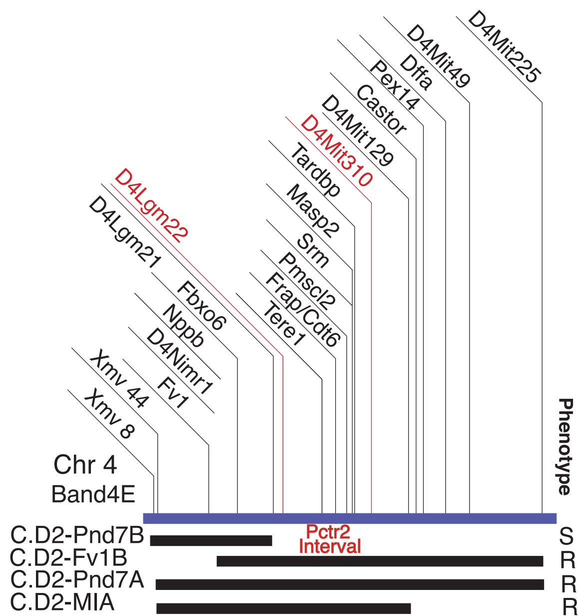

Fig. 1. Definition of the *Pctr2* interval by congenic strain and sequence analysis. The blue bar represents a portion of band 4E on mouse Chr 4. The intervals of several C.D2 congenic strains are shown under the chromosome with the associated DBA/2 alleles represented by the horizontal black bars. The tumor phenotype is indicated as "S" for susceptible and "R" for resistant. The red vertical lines represent the proximal and distal boundaries of the *Pctr2* interval. With respect to gene order, *Xmv8* is more centromeric and *D4Mit225* is telomeric. Results for the C.D2-Fv1B strain are shown in Fig. 2. The other strains are described in ref. 7.

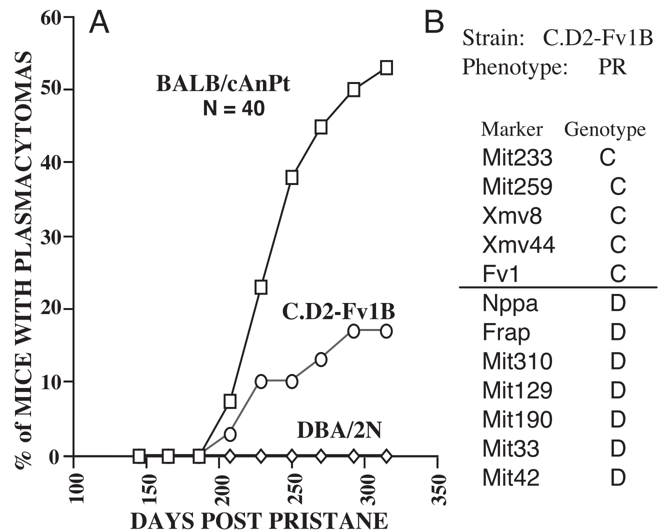

Fig. 2. A congenic strain further defines the genetic interval surrounding the *Pctr2* locus. The *Xmv8–Xmv44* interval was ruled out as a potential location for *Pctr2*. These studies, coupled with earlier backcross studies, confine the *Pctr2* interval to a region of distal mouse Chr 4 surrounding the microsatellite marker *D4Mit310*.

Results

Establishing the Genetic Interval Surrounding the *Pctr2* Locus. In our earlier studies using the congenic strains of mice C.D2-MIA, C.D2-Pnd7A, and C.D2-Pnd7B (7), we localized the *Pctr2* locus to two possible intervals: one 500-kb region between *Xmv8* and *Xmv44*, and the other distal to *Fv1* in a 1-Mb region surrounding *D4Mit310* and *D4Mit129* in the telomeric portion of mouse Chr 4 (see Fig. 1). The C.D2-MIA congenic carries DBA/2 alleles of genes across the distal half of mouse Chr 4; this congenic was solidly resistant to development of mouse plasma cell tumors and is likely to carry three separate loci that determine the resistance of DBA/2 mice (7). Genotypic analysis of this strain serves to delineate the distal border of the *Pctr2* interval. Similarly, two other strains were used to establish the proximal border of the interval. Resistant C.D2-Pnd7A and susceptible C.D2-Pnd7B mice have directly overlapping segments of DBA/2 alleles of genes (7) (see Fig. 1); however, the C.D2-Pnd7B mouse carries a shorter segment of DBA/2N chromatin. Both of these strains carried the BALB/c allele of *Xmv8* and the DBA/2 allele of *Xmv44*; therefore, theoretically, the *Pctr2* locus could reside in the *Xmv8-Xmv44* interval or in the distal interval near *D4Mit310* and *D4Mit129*, where the susceptible C.D2-Pnd7B strain carries BALB/cAn alleles of several genes.

In this study, we created a congenic strain that served to exclude the *Xmv8* to *Xmv44* interval from consideration as the site of the *Pctr2* locus. The C.D2-Fv1B congenic was constructed to carry BALB/cAn alleles at *Xmv8* and *Xmv44*. This strain, which carries DBA alleles across the interval *Nppa* to *D4Mit129* (Fig. 2*B*), was as resistant to developing plasma cell tumors (Fig. 2*A*) as the C.D2-Pnd7A strain used to define the *Pctr2* locus originally (7), confirming that the *Pctr2* locus resides near *D4Mit310*. In our earlier backcross studies (5), there were three more recombinants/discordances between resistant/susceptible phenotypes and *D4Mit129* genotypes than with *D4Mit310* genotypes; higher linkage (LOD) scores for the tumor susceptibility/resistance phenotype were obtained with *D4Mit310* than with *D4Mit129*. Thus, the focus of our detailed sequencing efforts concentrated on refining the borders of the *Pctr2* interval surrounding *D4Mit310*.

Identification/Comparative Sequencing and Mapping of Genes Near *D4Mit310* Narrow the Genetic Interval for the *Pctr2* Locus. BAC clones containing the MIT microsatellite markers *D4Mit310* and *D4Mit129* were isolated from a 129 ES cell BAC library (Genome System). These were used as a start point for cloning and sequencing of genes within the *Pctr2* interval. Genes were identified by direct sequencing and comparison with EST databases and subsequent data mining of public databases (GenBank, National Center for Biotechnology Information, University of California at Santa Cruz, Ensembl) and private databases (Celera)(Fig. 1). Through our efforts, those of the public sequencing projects, and Celera, this region has been sequenced and assembled. Sequences of the genes were compared between the plasmacytoma-susceptible BALB/cAnPt and resistant DBA/2NPt strains of mice; polymorphisms were not found in the coding regions of the following genes: *Tere1*, *Cdtf6*, *Catbnip1*, and *Castor/Ming*. Polymorphisms were found in *Pmscl2*, *Srm*, *Tardbp*, and *Pex14* [see single-nucleotide polymorphisms (SNPs) in Table 1]; however, they did not result in amino acid changes.

Coding region polymorphisms that result in amino acid differences between susceptible and resistant strains of mice were found in only three of the genes. One gene, *Fbxo6b* (F box only protein 6b), is widely expressed in several tissues and is a component of modular E3 ubiquitin ligases, which function in phosphorylation-dependent ubiquitination (25). Several polymorphisms were noted between DBA/2 and BALB/c in *Fbxo6b* (Table 1); however, when we mapped this gene in our resistant C.D2-Pnd7A and susceptible C.D2-Pnd7B congenics (Fig. 1), we were able to rule it out as a candidate for the *Pctr2* locus, in that both the resistant and susceptible congenics carried the DBA/2 allele of *Fbxo6b*. This established *Fbxo6b* as a new proximal limit to the *Pctr2* interval.

Subsequent sequencing of regions ≈120 kb distal to *Fbxo6b* identified four polymorphisms (designated here as *D4Lgm21*,*D4Lgm22*, *D4Lgm23*, and *D4Lgm24*) in noncoding regions. *D4Lgm21* (DBA = TA, BALB/c = AT) resides at a position 58 kb from the 3′ portion of *Fbxo6b*; once again, both the resistant

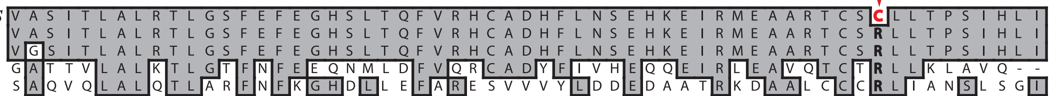

Fig. 3. Evolutionary conservation of FRAP. The polymorphism found between BALB/c and DBA/2 mice at residue 628 represents a rare allele in the BALB/c strain. Arginine has been conserved at this residue over the course of 1.2 billion years.

and susceptible congenics carried the same (DBA) allele, thereby further redefining the proximal boundary of the *Pctr2* interval. Data mining revealed two other F-box genes, F-box protein FBG2 (AF233223) and neural F-box protein (AF098301), in the interval between *Fbxo6b* and *D4Lgm21;* these were also excluded as candidates because C.D2-Pnd7A and -Pnd7B mice have the same (DBA) alleles.

D4Lgm22, *-23*, *and -24* were found ≈25, 37, and 60 kb distal to *D4Lgm21*, respectively. By contrast, the susceptible C.D2-Pnd7B congenic carried the BALB/c alleles of these three polymorphic markers whereas the resistant C.D2-Pnd7A mouse carried the DBA/2 alleles. This result established a new proximal boundary of the *Pctr2* interval to a position ≈83 kb distal to *Fbxo6b*, between *D4Lgm21* and *D4Lgm22*, and confined the interval to <0.8 Mb.

SNPs in Coding Regions. The other two genes in the *Pctr2* interval that carried polymorphisms resulting in amino acid changes within coding regions were *Masp2* (mannan-binding lectin serine protease 2), which is predominantly expressed in liver (26, 27), and *Frap*, which is widely expressed (28). Both of the genes mapped within the *Pctr2* interval (Fig. 1); resistant C.D2-Pnd7A and C.D2-MIA congenics carried the DBA/2 allele of each locus whereas the susceptible C.D2-Pnd7B congenic carried the BALB/c allele at both *Masp2* and *Frap*.

Masp2 was not considered a likely candidate due to the relatively limited tissue expression noted above and the lack of evolutionary conservation in the residue that was polymorphic between BALB/c and DBA/2. Silent substitutions were seen at positions 1043 and 2073 (DBA = "A" and BALB/c = "G" at both locations). The only SNP that resulted in an amino acid change from arginine (Arg) in DBA/2 to glycine (Gly) in BALB/c occurred at nucleotide position 2073; the arginine seen in DBA/2 was conserved in rat, but in human this residue coded for serine (Ser). In addition, the plasmacytoma-resistant strain 129/J carried the BALB/c allele coding for glycine at this residue. These observations strongly suggest that *Masp2* is not a likely candidate for the *Pctr2* locus.

Allelic Variation at the *Frap* Locus. *Frap*, which is expressed in lymphocytes and many other cell types, is 110 kb long and contains 58 exons that encode a 2,549-aa protein. It has SNPs at nucleotide positions 1977, 3668, and 5441 (all DBA = "C" and BALB = "T"). Only one of these polymorphisms resulted in an amino acid change, from arginine (Arg) in DBA/2N mice to cysteine (Cys) in BALB/cAn mice at position 1977 (R628C). Arginine is found at this position of *Frap* in mouse, human, rat, *Drosophila*, and *Arabidopsis* (Fig. 3), which implies that the arginine at this position has been conserved for at least 1.2 billion years. Most inbred strains of mice (AKR/N, 129/J, CBA/N, C57BL/6N, C57BL/10SnJ, C57L/J, DBA/1J, P/J, 020/A, and STS/A) and several wild strains (including *Mus spretus*, *Mus booduga*, *Mus brevirostris*, *Mus platythrix*, and *Mus gradsko*) that were tested carried the DBA/2 allele, which coded for Arg at residue 628. Only a few strains of mice (BALB/cAnPt, NZB/BlNJ, A/J, ABP/Le, and SENCAR) carried the allele that coded for Cys at residue 628.

DBA/2 FRAP Is a More Active Kinase than BALB/c FRAP. To evaluate the possible functional significance of the *Frap* alleles at nucleotide position 1977, biochemical and biological assays have been performed with DBA/2 vs. BALB/cAn versions of *Frap* cloned into FLAG-tagged mammalian expression vectors. For biochemical analysis, the constructs were transiently transfected into HEK293 cells, and *in vitro* kinase assays were performed with BALB/cAn and DBA/2 FRAP that had been immunoprecipitated by FLAG antibody. To verify that similar amounts of BALB and DBA FRAP would be added to the kinase assay, 2-fold serial dilutions of protein lysates from HEK293 cells transfected with the FLAG-tagged FRAP alleles were subjected to SDS/PAGE and Western blotted with an anti-FLAG antibody (Fig. 4 *Right*). We then examined the ability of BALB/c vs. DBA/2 FRAP to be autophosphorylated, and to phosphorylate its well known substrate PHAS1 (15, 29–31) and p53, a more recently identified substrate (32). For each substrate, the activities of both alleles were dose-dependent, and the DBA allele was more active than the BALB allele (Fig. 4 *Left*).

DBA/2 FRAP Can Suppress ras Transformation in NIH 3T3 Cells More Efficiently than BALB/cAn FRAP. To test the relative biological activities of the BALB/cAn and DBA/2N FRAP proteins, their abilities to influence focal transformation induced by an oncogenic *ras* were compared. NIH 3T3 cells were cotransfected with a constant amount of v-ras and increasing amounts of DBA/2N or BALB/cAn FRAP in accordance with previously described methods (24). Similar numbers of G418 resistant colonies were obtained in the presence or absence of either FRAP allele (data not shown). Under these conditions, FRAP from either mouse strain inhibited the formation of ras-transformed foci in a dose-dependent manner (Fig. 5). However, the BALB/cAn FRAP was less efficient than its DBA/2 counterpart in suppressing *ras* transformation. Thus, FRAP was a suppressor of transformation in this bio-assay. As with the biochemical assay, the activity of the BALB/cAn allele was lower than the DBA/2N allele.

Mouse Plasma Cell Tumors Are Resistant to Treatment with Rapamycin. Five plasmacytoma cell lines were treated with increasing amounts of the FRAP-specific inhibitor rapamycin to determine

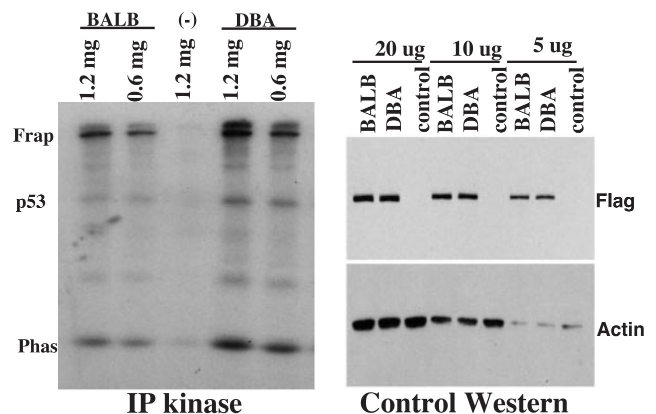

Fig. 4. *In vitro* kinase activity of FRAP. Anti-FLAG immunoprecipitates of HEK293 cells transiently transfected with plasmid encoding FLAG-tagged BALB/c FRAP or DBA/2 FRAP (*Right*) were tested in an *in vitro* kinase reaction with PHAS1 and p53 added as exogenous substrate (*Left*). DBA/2 FRAP kinase was more active in phosphorylating FRAP, itself, PHAS1, and p53. Kinase assays were repeated three times with similar results.

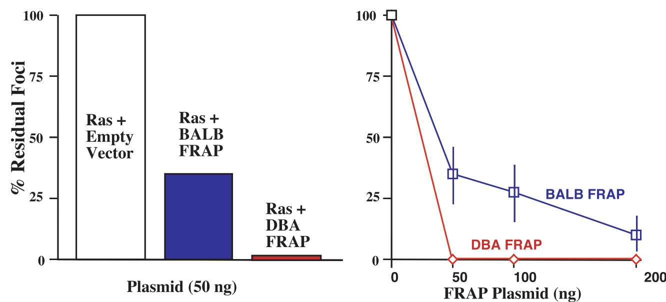

Fig. 5. Inhibition of ras-mediated focus formation by FRAP. NIH 3T3 cells were cotransfected with BALB/c and DBA/2 variants of FRAP along with v-ras. The percentages of residual foci formed relative to the foci detected on the plates cotransfected with pcDNA vector control and v-ras are shown. Transfection assays were repeated three times with similar results. DBA FRAP was more efficient at suppressing ras transformation than BALB/c FRAP.

whether they were sensitive or resistant to rapamycin treatment. These cell lines were compared with OVCAR and PC3, two cell lines that have been well characterized as rapamycin sensitive (33, 34). After 24 h of treatment, cells were analyzed by FACScan, and the percentage of cells in G_1 was determined. OVCAR and PC3 cells responded in a dose-dependent manner to rapamycin, as shown previously. In contrast, growth of plasmacytoma cells was not affected by even the highest dose (1,000 ng/ml) of rapamycin (Fig. 6). No mutations were found in the FRB of *Frap* in the plasmacytoma cell lines that were sequenced across this region (data not shown). Therefore, FRB domain mutations do not account for rapamycin resistance of the plasmacytoma cell lines.

Discussion

The majority of the literature on *Frap* has suggested that it acts as an oncogene, by increasing the translation of mRNAs such as myc, which have highly structured 5′ UTRs (35), and that the specific pharmocologic inhibitor rapamycin and its analogs can often inhibit FRAP's growth-promoting activities (14, 36). Inhibitors of FRAP, such as the rapamycin analog CC1–779, are in widespread experimental use as therapeutic agents against a variety of tumors (37, 38). *Frap* mutations have not been found in cancers, and, before our studies, *Frap* had not been implicated as a locus involved in predisposition to cancer (18).

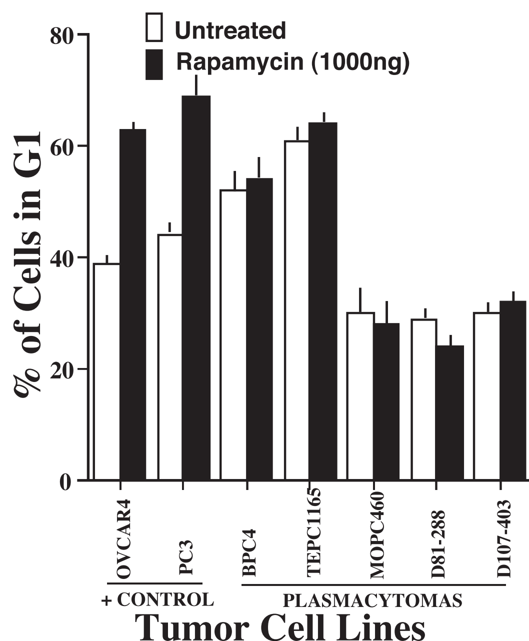

Fig. 6. Resistance of plasmacytoma cell lines to rapamycin. OVCAR4 and PC3 cells were used as control cell lines that are known to respond to rapamycin treatment with an increase in the number of cells arresting in the G_1 phase of the cell cycle. Treatment of plasmacytoma cell lines with rapamycin did not induce an increase in cells in G_1. Mutations were not detected in the region surrounding the rapamycin-binding FRB domain of *Frap* in the plasmacytoma cell lines examined (see *Methods*).

In contrast, our data provide support for a role for *Frap* as a tumor suppressor. First, *Frap* was identified as a candidate gene for the plasmacytoma susceptibility/resistance locus *Pctr2*, whose resistant form has been shown to delay the onset of tumorigenesis in BALB/c mice. *Frap* was identified as having a coding region SNP and became the most likely candidate to encode the *Pctr2* gene. The amino acid (Arg) in the wild-type DBA/2 mouse was evolutionarily conserved in both human and rat; both strains of mice that are susceptible to plasmacytomagenesis carried a rare variant, a Cys at residue 628. This codon resides near a HEAT (Huntington, Elongation factor 3, protein phosphatase 2A, TOR1) domain (no. 2) of FRAP (39) thought to be important for the binding of FRAP to gephyrin (40), and which may affect FRAP's subsequent subcellular localization. More recently, FRAP has been shown to bind to RAPTOR (regulatory-associated protein of MTOR) (41, 42) and CLIP170 (cytoplasmic linker protein 170/restin) (43), both of which may regulate its kinase activity under certain conditions.

Second, when the allelic variants of FRAP were tested for their kinase activity *in vitro*, the variant of FRAP associated with the susceptible phenotype (BALB/c) had less activity, rather than more, which would have been associated with prooncogenic properties. Phosphorylation of the tumor suppressor p53 was less with the BALB/c version of FRAP kinase compared with the DBA/2 version. FRAP's tumor suppressor activities may be a direct function of p53 activation by phosphorylation. FRAP can activate the tumor suppressor p53 (32), which has been shown to mediate G_1 growth arrest, the induction of apoptosis, and DNA repair (44).

Third, when allelic variants of FRAP were cotransfected with ras into NIH 3T3 cells, both BALB/c and DBA/2 FRAP were able to suppress ras-induced focal transformation. In our experiments, the BALB/c allele of Frap, associated with tumor susceptibility, was once again less efficient in its activity to inhibit transformation. Although these results are the opposite of the prevalent view that FRAP stimulates growth, they are actually consistent with those reported by Aoki *et al*. (45), who tested the effects of rapamycin on the transforming activities of 12 oncogenes. The focus of that report was that cell transformation by phosphatidylinositol 3-kinase and AKT was inhibited by rapamycin. However, rapamycin did not inhibit transformation by the 10 other oncogenes, including ras, where rapamycin actually potentiated its transforming activity.

Fourth, when we treated plasmacytoma cells with rapamycin, their growth was not inhibited. This result is also consistent with the published observation that rapamycin actually enhanced the survival of hybridoma cell cultures *in vitro* (46). Recently, it was shown that rapamycin did not inhibit the growth of the two multiple myeloma cell lines OPM2 and RPMI8226 (47).

Last, *Frap* is a PIK (phosphoinositide-3-kinase)-like protein, and other PIK superfamily members, such as ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and rad3-related) are tumor suppressor genes. It is of interest to note that the *abl* (Abelson) oncogene can cooperate with *myc* to induce plasmacytomas, independent of pristane priming (48, 49), and ABL can directly bind to FRAP and inhibit its kinase activity (50), thereby abrogating its effects on p53.

These observations suggest that the growth-promoting functions of FRAP (increased translation of growth-promoting genes, such as c-myc and cyclinD1, with highly structured 5′ UTRs by the phosphorylation of p70 S6 kinase and PHAS1 to increase translation) either may not be essential in plasmacytomas/B cells or may be carried out by other genes/mechanisms. The Ig-myc translocations

resulting in overexpression of c-myc observed in most plasmacytomas may bypass or supercede translational up-regulation by FRAP. These arguments are consistent with a tumor suppressor role for FRAP in the plasmacytoma system, but they do not preclude an oncogenic role for FRAP in other tumor settings or induction protocols.

In this context, FRAP may join the ranks of TGF-β or E2F1 in its dual role as a tumor inhibitor or promoter. Mouse models with targeted loss of either *Tgf*β (51, 52) or *E2f1* (53) have more tumors than their littermate controls. *Tgf*β has tumor suppressor activity early during carcinogenesis, but, in later stages, suppressor activity is frequently lost and prooncogenic activities dominate (51, 54). Elevated levels of E2F1 in transgenic mice have been shown to be both more predisposing to development of certain tumors and less disposing to others (55–57). A determination of the conditions necessary to elicit growth-promoting or growth-inhibiting functions of FRAP are needed to better understand when to inhibit or augment its activity therapeutically.

The mouse plasmacytoma model for susceptibility to cancer as a complex genetic trait has now been used to identify two genes, *Cdkn2a* (p16) and *Frap*, as candidates for the *Pctr1* and *Pctr2* tumor resistance loci, respectively. It is striking that the allelic variants found in susceptible mice for both genes have lower, less efficient biological and biochemical activity than the alleles found in resistant mice. In human cancer, an analogous accumulation of such efficiency alleles is likely to account for susceptibility more frequently than are single all-or-none, gain-of-function, or loss-of-function alleles.

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