common ovarian abnormalities and high plasma A concentrations suggest that PCOS may be the consequence of placental T synthesis and androgen effects on the hypothalamic-pituitary-ovarian axis late in fetal life that result in excessive LH and A secretion following puberty.

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15. The spotted hyenas were collected as infants in southwest Kenya and reared in social groups maintained at the Field Station for Behavioral Research adjacent to the University of California, Berkeley campus. Hyena ovaries and placentas were obtained from pregnancies terminated within the last 30 days of the 110-day gestational period according to University of California institutional guidelines. Details of these operations and results of experiments on plasma steroid hormone levels are described elsewhere (*14*). Human placentas were obtained from cesarean sections at term of uncomplicated pregnancies. Placentas were removed from the uterus, dissected free from fetal membranes, and washed in ice-cold phosphate-buffered saline (pH 7.4). All samples were transferred to the laboratory on ice within 2 hours of surgery.
16. Minced fragments of corpora lutea and stromal tissue were incubated with 1 × 10^6 cpm of [^{3}H]progesterone (New England Nuclear, Boston; 30 mCi/mmol) for 4 hours in 0.5 ml of DME as described in Fig. 1. Radiolabeled products were extracted twice with 1 ml of ethyl acetate. The extracts were combined, evaporated, and analyzed on a Waters high-performance liquid chromatography unit fitted with a reversed-phase column and gradient elution device. We quantified radiolabeled products by counting fractions associated with nonradioactive steroid standards. In three separate incubations, [^{3}H]A accounted for more than 85% of the products formed by stromal tissue.
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The Role of T_H1 and T_H2 Cells in a Rodent Malaria Infection

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CD4$^+$ T cells play a major role in protective immunity against the blood stage of malaria, but the mechanism of protection is unclear. By adoptive transfer of cloned T cell lines, direct evidence is provided that both T_H1 and T_H2 subsets of CD4$^+$ T cells can protect mice against *Plasmodium chabaudi chabaudi* infection. T_H1 cells protect by a nitric oxide–dependent mechanism, whereas T_H2 cells protect by the enhancement and accelerated production of specific immunoglobulin G1 antibody.

Malaria, principally *Plasmodium falciparum* malaria, affects 250 to 400 million people and causes more than 1 million deaths annually (*1*). Vaccine development would be facilitated by a better understanding of the host immune mechanisms (*2*).

P. c. chabaudi AS strain infection in the laboratory mouse is a recognized model for *P. falciparum* infection of humans. NIH mice infected with *P. c. chabaudi* (1 × 10^5 parasitized red blood cells, pRBC) develop an acute primary parasitemia that peaks on day 10 and lasts 14 to 18 days, usually followed by one more patent parasitemias (recrudescence) between days 25 and 35 after infection. Further infections are resolved more rapidly. CD4$^+$ T cells are involved in immunity to the asexual erythrocytic stages of *P. c. chabaudi*, and both T_H1 and T_H2 subsets of CD4$^+$ T cells may mediate protection (*3*).

We investigated the effect of injecting L-N^γ-monomethylarginine (L-NMMA), a specific inhibitor of nitric oxide (NO) synthase, on the course of infection (*4*). Normal mice produced significant levels of nitrate in the serum (630 ± 85 nmol/ml), the peak of which coincided with the peak of the primary parasitemia (*5*) and declined rapidly thereafter. Mice receiving L-NMMA developed a markedly increased primary parasitemia [peak of 4.8 log(pRBC/10^5 RBC)] lasting 22 days, and sera from these mice contained little or no detectable nitrate.

A panel of eight CD4$^+$ T cell clones specific for trophozoite-schizont antigens and restricted by major histocompatibility complex class II molecules was derived from mice infected with *P. c. chabaudi* (*6*). Four were T_H1 clones, secreting interleukin-2 (IL-2) and interferon-gamma (IFN-γ); the other four were T_H2 clones in that they secreted IL-4 and provided help for antibody production. Two representative clones, WEP 999 (T_H1, from day 16 of infection) and WEP 988 (T_H2, derived from a reinfected donor), were selected for functional study; both were CD3$^+$, CD4$^+$, CD8$^-$, T cell receptor (TCR) αβ$^+$ and TCRγδ$^-$.

Female adult inbred NIH mice (*7*) were thymectomized and injected intraperitoneally with rat monoclonal antibody (mAb) specific for mouse CD4 or CD8 or with normal rat immunoglobulin G (IgG) (*8*) and infected with *P. c. chabaudi*. This protocol depleted ~98% of CD4$^+$ or CD8$^+$ T cells for more than 60 days (*9*). Mice depleted of CD4$^+$ T cells suffered 75 to 90% mortality within 20 days of infection, and those that survived maintained a constant and high level of parasitemia [3.4 to 3.8 log(pRBC/10^5 RBC)] for more than 60 days. In contrast, mice depleted of CD8$^+$ T cells had a parasitemia profile indistinguishable from that of mice treated with normal rat IgG, and all mice recovered from infec-

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tion. Thus, CD4+ but not CD8+ T cells protect against asexual blood stage malaria infection in this system.

Mice depleted of CD4+ T cells were injected intravenously with 4×10^7 viable WEP 999 (T_H1) or WEP 988 (T_H2) cells, or with splenic T cells from normal mice that had been enriched (to 98% purity) for CD4+ T cells, and infected immediately with *P. c. chabaudi*. Some mice were treated during the ascending phase of the primary parasitemia with L-NMMA to block NO production or with its biologically inert enantiomer D-NMMA or with the NO synthase substrate L-arginine. Mice depleted of CD4+ T cells and given no T cell replacement developed high levels of parasitemia, with 80 to 100% mortality within 20 days of infection; nitrate levels in the sera were low and not distinguishable from those of uninfected normal mice (15.1 ± 6.7 nmol/ml) (Fig. 1A). In mice reconstituted with naïve CD4+ T cells, the 60% of mice that survived infection suffered a chronic patent parasitemia; nitrate levels at peak parasitemia were raised but were much lower than those of the immune T_H1 recipients (Fig. 1B). Mice reconstituted with WEP 999 (T_H1) cells showed courses typical of a fully competent immune system (Fig. 1C) unless treated with L-NMMA, when they developed severe infection (with 75 to 100% mortality within 20 days of infection) and had serum nitrate levels similar to those of nonreconstituted mice (Fig. 1D). D-NMMA or L-arginine had no effect on the course of infection or nitrate levels.

Mice depleted of CD4+ T cells reconstituted with WEP 988 (T_H2) cells also showed courses of infection typical of fully immunocompetent mice (Fig. 2A). However, there was no elevation of serum nitrate levels, and the course of infection was not affected by treatment with L-NMMA (Fig. 2B).

Mice reconstituted with WEP 999 (T_H1) cells produced a moderate amount of malaria-specific IgG2a. This was detectable 8 days after infection and peaked at 16 days and again between days 32 and 36 (Fig. 3A). In contrast, mice reconstituted with WEP 988 (T_H2) cells produced high levels of IgG1, detectable 4 days after infection, which plateaued between days 12 and 16 (Fig. 3B); in these animals, parasitemias peaked on day 10 (Fig. 2B). A second peak of IgG1 appeared on days 32 to 36, which paralleled the peak of recrudescence. L-NMMA markedly reduced production of IgG2a in the T_H1-reconstituted mice but had no effect on the response in the T_H2-reconstituted mice. The IgM response was enhanced by T_H1 cells (peak titer, 1:1024 on day 8) but not by T_H2 cells and was not affected by L-NMMA treatment. Other antibody isotypes were either detected at very low levels (IgG2b and IgG3) or were not present (IgA and IgE) and were not different between reconstituted and nonreconstituted mice.

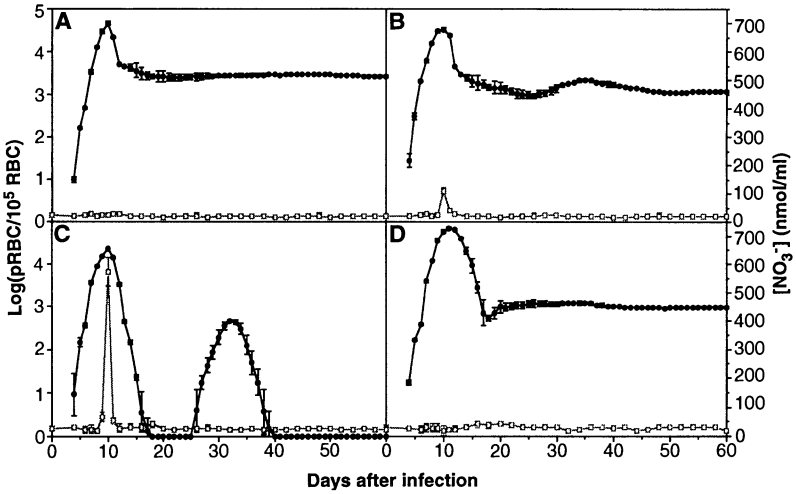

Fig. 1. Protection of mice depleted of CD4+ T cells by a T_H1 cell line, and the effect of L-NMMA (*22*). (**A**) Control mice, given no cells or drugs. (**B**) Mice given CD4+ T cell–enriched normal spleen cells. (**C**) Mice given WEP 999 cells. (**D**) Mice given WEP 999 cells and treated with L-NMMA on days 5 to 10 after infection. Differences in parasitemias (●) between T_H1-reconstituted mice (C) and similar L-NMMA–treated mice (D), unreconstituted controls (A), or mice reconstituted with CD4-enriched normal spleen cells (B) were significant from days 8, 13, and 15, respectively ($P < 0.0025$). For mice in (A), (B), and (D), 90%, 40%, and 85%, respectively, of mice died, all within 20 days of infection. Nitrate levels (□) in T_H1-reconstituted mice (C) on day 10 were significantly raised [$P < 0.001$ as compared with (A) and (D) and $P < 0.005$ as compared with (B)].

Mice depleted of CD4+ T cells and reconstituted with either WEP 999 or WEP 988 cells were treated with a goat antibody to mouse IgG1 (anti-IgG1), IgG2a (anti-IgG2a), or whole molecule IgG (Sigma) (*10*) and infected with *P. c. chabaudi*. Mice reconstituted with WEP 988 cells and treated with anti-IgG1 all died within 14 days of infection, as did those treated with anti-IgG whole molecule, whereas animals given anti-IgG2a controlled infection as for untreated animals. In contrast, specific Ig isotype depletion of mice reconstituted with WEP 999 cells had no apparent effect on the protection transferred by these cells, whereas complete IgG class (IgG1, IgG2a, IgG2b, and IgG3) depletion allowed a slight but significant increase in parasitemia [3.1 log(pRBC/10^5 RBC)] ($P < 0.05$) during the recrudescent phase of infection (days 31 to 40).

Nitric oxide inhibits the development of exoerythrocytic stages of *Plasmodium berghei*

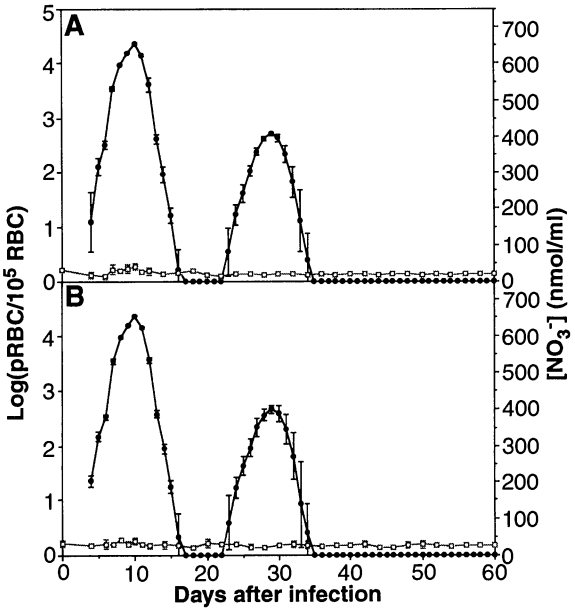

Fig. 2. Protection of mice depleted of CD4+ T cells by a T_H2 cell line, WEP 988, and the effect of L-NMMA (*22*). (**A**) Mice given WEP 988 cells. (**B**) Mice given WEP 988 cells and treated with L-NMMA on days 5 to 10 after infection. Parasitemias (●) and day 10 nitrate levels (□) in T_H2-reconstituted mice (A) and those in similar mice treated with L-NMMA (B) were not significantly different ($P > 0.05$).

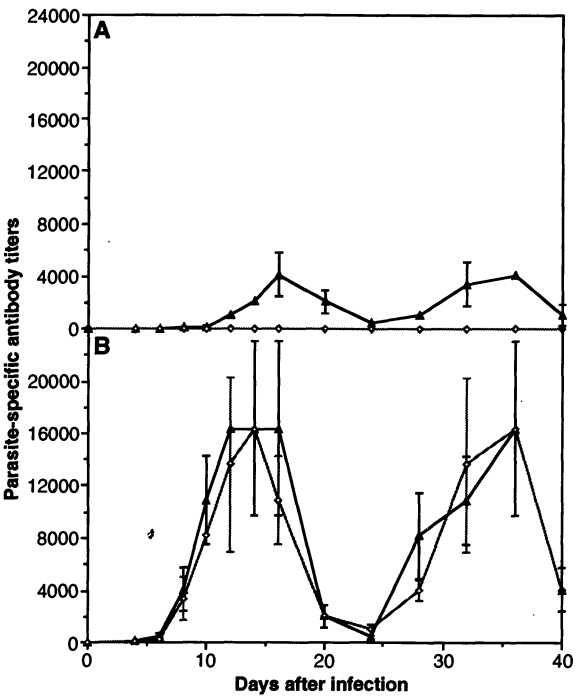

Fig. 3. Antibody responses of mice depleted of CD4+ T cells, reconstituted with a T_H1 (WEP 999) or T_H2 (WEP 988) cell line and infected with *P. c. chabaudi* (*23*). Results are expressed as the mean reciprocal antibody titer ± 1 SD for triplicate serum samples from either untreated mice (▲) or those receiving L-NMMA (◇). (**A**) Immunoglobulin G2a; only WEP 999–reconstituted mice ± L-NMMA treatment are shown. All other groups (WEP 988 cells, CD4+ T cell–enriched normal spleen cells, no adoptive transfer, each ± L-NMMA) gave negligible titers. Differences in IgG2a levels between WEP 999–reconstituted mice not receiving L-NMMA and those either receiving L-NMMA or in other groups were significant (*t* test, $P < 0.005$). (**B**) Immunoglobulin G1; only WEP 988–reconstituted mice ± L-NMMA treatment are shown. All other groups gave negligible titers. Differences in IgG1 levels between T_H2-reconstituted mice ± L-NMMA treatment and those in other groups were significant ($P < 0.005$) but were not significant between each other ($P > 0.05$).

(*11*) and *Plasmodium yoelii* (*12*) in vitro, and NO derivatives kill asexual erythrocytic stages of *P. falciparum* (*13*) in vitro. Our results demonstrate that NO plays a significant role in host control of the primary patent parasitemia, but not in the recrudescent parasitemia, in malaria infection in vivo. NO involvement may be mediated by T_H1 secretion of IFN-γ, which activates macrophages to produce large amounts of NO (*14*) to kill the parasites directly. Alternatively, NO may have an indirect effect by causing blood vessel vasodilation (*15*). The effect would lead to less efficient parasite sequestration in deep tissue capillaries, allowing removal of the parasites by macrophages.

T_H2 cells secrete IL-4, which does not activate macrophages to produce NO (*16*) but helps B cells in the production of IgG1 and IgE antibodies (*17*). The capacity of the T_H2 cells to protect against *P. c. chabaudi* infection was abrogated by depletion of IgG1, an isotype that is produced only after secondary exposure to antigen (*18*). Because the T_H2 clone we used was derived after reinfection, the isotype expression pattern of the T_H2-reconstituted CD4-depleted mice reflected this. T_H1 cells enhanced the IgG2a response (*17*), which was markedly reduced by treatment with L-NMMA. However, the late appearance of IgG2a did not contribute to the T_H1-mediated protection, because in its absence the capacity of WEP 999 cells to protect was unaffected. The mechanism underlying the reduction of IgG2a levels by L-NMMA is at present unclear. It may be that NO has a direct effect on T_H1 cells.

Neither NO nor antibody production after the adoptive transfer of T_H1 or T_H2 cells, respectively, had any apparent effect on the appearance of the recrudescence of malaria infection. *Plasmodium c. chabaudi* can undergo antigenic variation, and therefore the emergence of antigenically distinct variant parasites during recrudescence (*19*) is considered to be likely.

These data provide direct evidence that both T_H1 and T_H2 responses protect the host from malaria infection. Furthermore, they do so by distinct mechanisms. We do not yet know whether the same or different antigens stimulate the protective T_H1 and T_H2 clones, and our results do not exclude the possibility that other T_H1 and T_H2 clones could contribute to the immunosuppression (*20*) or immunopathology (*21*) seen in some cases of acute malaria and in some experimental models. However, this work does suggest that a vaccine to *P. falciparum* should include antigens that can sensitize both T_H1 and T_H2 CD4+ T cells.

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4. L-NMMA (250 mg/kg) was injected intraperitoneally for six consecutive days during the ascending phase of the primary (days 5 to 10) and secondary (days 27 to 32) parasitemia. Sera were collected on consecutive days by bleeding of the mice from the tail, and nitrate in the sera was assayed by chemiluminescence as described [R. M. J. Palmer, A. G. Ferrige, S. Moncada, *Nature* **327**, 524 (1987)]. For each group, individual mice were bled on alternate days. Nitrate levels given represent the mean ± 1 SD of individual values (nanomoles per milliliter) from three mice within each group on each day of three separate experiments, and these were compared by Student's *t* test. Nitrate levels reflect the NO levels in the serum [S. Moncada, R. M. J. Palmer, E. A. Higgs, *Pharmacol. Rev.* **43**, 109 (1991)].
5. Parasitemias were followed daily by examination of Giemsa's-stained thin blood films, expressed as the logarithm of the mean parasitemia ± 1 SD of each group for three separate experiments, and compared with the use of Kruskal-Wallis one-way analysis of variance.
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7. Animal protocols used in this work were in accordance with Project and Personal Licenses issued by the British Home Office and were carried out in Approved Premises and following Institutional Guidelines.
8. Mice were surgically thymectomized at 5 weeks of age as previously described [A. P. Monaco, M. L. Wood, P. S. Russel, *Ann. N.Y. Acad. Sci.* **129**, 190 (1966)]. Before we operated, mice were anesthetized by intraperitoneal injection of a 1:10 dilution of Hypnorm (Janssen Animal Health) (0.1 ml per 30 g of body weight) and valium (Roche) (5 mg/kg). At the end of each of three separate experiments, mice were checked for thymic remnants; none were found. Four weeks after thymectomy, mice were given two intraperitoneal injections of 250 μg of purified mAb 4 days apart and infected with *P. c. chabaudi* 12 days after the second injection {modified from a method described by T. P. Leist, S. P. Cobbold, H. Waldmann, M. Aguet and R. M. Zinkernagel [*J. Immunol.* **138**, 548 (1984)]}. Monoclonal antibodies from the hybridomas YTS 191.1 (antibody to CD4) and YTS 169.4 (antibody to CD8) were purified from ascitic fluids obtained from pristane-primed (LOU × DA) F_1 rats as described [S. P. Cobbold, A. Jayasuriya, A. Nash, T. D. Prospero, H. Waldmann, *Nature* **312**, 548 (1984)].
9. For analysis, lymphocytes were collected from peripheral blood or spleen cell preparations by Ficoll gradient centrifugation (Lympholyte-M; Cedarlane). Each preparation was tested against rat antibody to mouse (anti-mouse) primary mAbs specific for Thy 1.2, CD4, and CD8 (Sera-Lab) at a predetermined optimal dilution of 1:100 in phosphate-buffered saline (PBS). Normal rat serum was used as a negative control. A 1:200 dilution in PBS of fluoroscein isothiocyanate (FITC)–conjugated goat antibody to rat IgG (Sigma) was used as the secondary mAb. Labeled cells were analyzed by flow cytometry (FACScan; Becton Dickinson).
10. Effective depletion of specific serum Ig isotypes was attained in CD4-depleted mice by thrice weekly intraperitoneal injections of goat antibody (250 μg; Sigma) beginning 6 weeks before, and continuing after, infection. This treatment gave ~96% depletion of splenic B cells of the appropriate Ig isotype throughout infection as compared with undepleted infected control mice. Lymphocytes were prepared for flow cytometry as described (*9*) and stained with goat anti-mouse primary mAbs specific for IgG1, IgG2a, and whole molecule IgG (Sigma) at a 1:100 dilution. FITC-conjugated rabbit antibody to goat IgG (1:200; Sigma) was used as the secondary antibody. In infected animals, liver and spleen sizes increased commensurately with malaria infection; however, in uninfected depleted control mice, spleen and liver were not enlarged, showing that there had been no significant accumulation of immune complexes in these organs. There was no apparent toxic effect of anti-Ig injections, and there was no indication that the goat Ig by itself

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22. Mice (six per group) were CD4-depleted (*8*). Twelve days after the last antibody treatment, they were injected intravenously with 4×10^7 cells per mouse, or not reconstituted, and then infected intravenously immediately with 10^5 *P. c. chabaudi* pRBC. The preparation of the T cell clone has been described in detail (*6*). We enriched naïve splenic T cells for $CD4^+$ cells using a mouse T cell separation kit (Pierce). Some of the mice reconstituted with T_H1 or T_H2 were injected intraperitoneally with L-NMMA (250 mg/kg per day) dissolved in sterile PBS. Courses of infection and nitrate levels in the sera were monitored as described (*4, 5*). Data shown are pooled from three separate experiments.
23. We assayed parasite-specific antibody in the serum by an indirect fluorescent antibody test [S. A. McLean, C. D. Pearson, R. S. Phillips, *Exp. Parasitol.* **54**, 213 (1982)], using trophozoite-schizont–infected RBC as the target antigen. Isotype-specific antibodies were obtained from serum separated by affinity chromatography on protein A–Sepharose CL-4B into fractions containing IgG1, IgG2a, IgG2b, or IgG3 [I. Seppälä, H. Sarvar, F. Peterfy, O. Mäkelä, *Scand. J. Immunol.* **14**, 335 (1981)]. Immunoglobulin M was isolated by Sepharose 6B gel filtration from nonbinding material that contained a pool of IgA and IgE. We confirmed the purity of each fraction by double radial immunodiffusion, using antibodies specific to each Ig isotype.
24. We thank H. Hodson for L-NMMA and D-NMMA and E. Galloway and D. McLaughlin for technical support. We thank The Wellcome Trust for financial support.

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Selective Inhibition of *ras*-Dependent Transformation by a Farnesyltransferase Inhibitor

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To acquire transforming potential, the precursor of the Ras oncoprotein must undergo farnesylation of the cysteine residue located in a carboxyl-terminal tetrapeptide. Inhibitors of the enzyme that catalyzes this modification, farnesyl protein transferase (FPTase), have therefore been suggested as anticancer agents for tumors in which Ras contributes to transformation. The tetrapeptide analog L-731,735 is a potent and selective inhibitor of FPTase in vitro. A prodrug of this compound, L-731,734, inhibited Ras processing in cells transformed with v-*ras*. L-731,734 decreased the ability of v-*ras*–transformed cells to form colonies in soft agar but had no effect on the efficiency of colony formation of cells transformed by either the v-*raf* or v-*mos* oncogenes. The results demonstrate selective inhibition of *ras*-dependent cell transformation with a synthetic organic inhibitor of FPTase.

The mammalian *ras* genes encode guanosine triphosphate (GTP)–binding proteins that can acquire the potential to transform mammalian cells as a result of point mutations in codons 12, 13, or 61 (*1*). Mutated, oncogenic forms of *ras* are frequently found in many human cancers, most notably in more than 50% of colon and pancreatic carcinomas (*1, 2*). These observations indicate that Ras functions in the pathogenesis of human cancers and emphasize the potential broad utility of anticancer agents directed against *ras*-induced cell transformation.

Ras is synthesized as a cytosolic precursor that ultimately localizes to the cytoplasmic face of the plasma membrane after a series of posttranslational modifications (*3*). The first and obligatory step in this series is the addition of a farnesyl moiety to the cysteine residue of the COOH-terminal CAAX motif (C, cysteine; A, usually aliphatic residue; X, any other amino acid) in a reaction catalyzed by farnesyl protein transferase (FPTase). This modification is essential for Ras function, as demonstrated by the inability of Ras mutants lacking the COOH-terminal cysteine to be farnesylated, to localize to the plasma membrane, and to transform mammalian cells in culture (*4, 5*). Moreover, strains of *Saccharomyces cerevisiae* having a mutation in *RAM1*, a gene that encodes one of the structural polypeptides of the yeast FPTase, are resistant to the biological effects of oncogenic *ras* (*6*). The subsequent posttranslational modifications—cleavage of the AAX residues, carboxyl methylation of the farnesylated cysteine, and palmitoylation of cysteines located upstream of the CAAX motif—are not obligatory for Ras membrane association or cell-transforming activity (*5, 7*). Thus, FPTase appears to be an appropriate biochemical target for the development of inhibitors of posttranslational processing of Ras that might be expected to interfere with Ras-mediated cellular transformation.

The substrates of the farnesylation reaction, farnesyl diphosphate (FPP) and polypeptides containing a CAAX motif, can be used as a starting point for the design of FPTase inhibitors. Several analogs of FPP are potent and selective inhibitors of FPTase in vitro (*8, 9*), and one has shown activity in cells (*9*). The CAAX tetrapeptide is the minimal sequence required for the interaction of Ras with FPTase (*10, 11*). Thus, tetrapeptides with amino acid sequences identical to the COOH-terminal sequences of protein substrates for FPTase compete with Ras for farnesylation by acting as alternative substrates (*12, 13*). CAAX derivatives have also been identified that are not substrates for farnesylation and therefore behave as pure inhibitors of FPTase (*8, 13, 14*).

Although other cellular proteins besides Ras are in vivo substrates for farnesylation, most isoprenylated proteins are modified by the 20-carbon geranylgeranyl moiety (*15*). Two classes of enzymes that catalyze the addition of a geranylgeranyl group to proteins have been identified in mammalian cells. One class consists of geranylgeranyl protein transferase–I (GGPTase-I), which modifies proteins having a COOH-terminal CAAX sequence where X is a Leu residue (*12, 16*). The other class consists of one or more enzymes (for example, GGPTase-II) that catalyze the modification of proteins terminating in Cys-Cys or Cys-X-Cys (*12, 17*). Comparison of the activity of various CAAX tetrapeptides suggests that it may be possible to design a specific inhibitor of FPTase that does not affect the GGPTases. For example, CAAX tetrapeptides terminating in Ser or Met are potent inhibitors of FPTase but are less active as inhibitors of GGPTase-I or GGPTase-II (*12*). Conversely, CAAX tetrapeptides terminating in Leu, which act as effective inhibitors of

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