(1988)]. The spheroplasts were stabilized with 20 mM $MgSO_4$, the periplasm was removed, the pelleted spheroplasts were lysed by suspension in 10 mM tris-HCl (*p*H 7.6) and 5 mM Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and sonication. The lysate was centrifuged at 27,000*g* for 10 min, and the pellet was suspended in 10 mM tris-HCl (*p*H 7.6), 3% Triton X-100, and 5 mM EDTA to solubilize membranes. The insoluble material was collected by centrifugation as before and dissolved in 25 mM bis-tris-HCl, and 6 M urea, (*p*H 6.3), for chromatofocusing. Chromatofocusing was performed with a Pharmacia Mono P HR 5/20 column and a gradient of *p*H 6.0 to *p*H 4.0 generated by Polybuffer 74–HCl diluted 1/10 in 6 M urea, *p*H 4.0. One-milliliter fractions were collected and the fraction containing the most precursor maltose-binding protein (*p*H ~5.5) was dialyzed against 10 mM Hepes and 2 M GuHCl (*p*H 7.6). The precursor maltose-binding protein was stored in this solution at −20°C. SecB was purified as described (*2*) except that the second gel-filtration chromatography was replaced by ion-exchange chromatography with a Pharmacia Mono Q HR 5/5 column in 10 mM tris-HCl (*p*H 7.6) with a linear 150-ml gradient of 0 to 0.6 M NaCl.

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Inhibition of FKBP Rotamase Activity by Immunosuppressant FK506: Twisted Amide Surrogate

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The immunosuppressive agents cyclosporin A and FK506 inhibit the transcription of early T cell activation genes. The binding proteins for cyclosporin A and FK506, cyclophilin and FKBP, respectively, are peptidyl-prolyl-*cis-trans* isomerases, or rotamases. One proposed mechanism for rotamase catalysis by cyclophilin involves a tetrahedral adduct of an amide carbonyl and an enzyme-bound nucleophile. The potent FKBP rotamase inhibitor FK506 has a highly electrophilic carbonyl that is adjacent to an acyl-pipicolinyl (homoprolyl) amide bond. Such a functional group would be expected to form a stabilized, enzyme-bound tetrahedral adduct. Spectroscopic and chemical evidence reveals that the drug interacts noncovalently with its receptor, suggesting that the α-keto amide of FK506 serves as a surrogate for the twisted amide of a bound peptide substrate.

CYCLOSPORIN A **1** (CsA, FIG. 1) (*1*) is an immunosuppressive agent that has found widespread clinical use in organ transplantation. FK506 **2** (*2, 3*) is a structurally unrelated and potent immunosuppressant (*4*) shown to be effective in human organ recipients at doses significantly lower than those necessary with CsA (*5*). These compounds, especially FK506 (*6*), have potential for the treatment of autoimmune disorders (*7*) and for the prevention of graft rejection following organ transplantation.

Both CsA and FK506 suppress immune response by inhibiting the transcription of early T cell activation genes [interleukins-2, -3, and -4, granulocyte-macrophage colony stimulating factor, and interferon-γ (*8*)], apparently by controlling the synthesis of or modifying transcriptional regulators such as nuclear factor of activated T cells (NF-AT) (*9*). A possibly related finding is that the binding proteins for CsA and FK506, cyclophilin (*10*) and FKBP (immunophilins) (*11, 12*), respectively, are peptidyl-prolyl-*cis-trans*-isomerases (rotamases, Fig. 2), which are potently inhibited by their respective ligands. Furthermore, the inhibition is highly selective: FK506 does not inhibit the rotamase activity of cyclophilin, and CsA does not inhibit that of FKBP. This information, together with recent reports of related proteins implicated in signaling pathways (*13, 14*), led to the suggestion that the immunophilins are involved in signaling processes that lead to T cell activation (*11*); the biological importance of cyclophilin has been firmly established, in fact, by a study demonstrating that the protein mediates the effects of CsA on the lower eukaryotes *Neurospora crassa* and *Saccharomyces cerevisiae* (*15*).

Elucidation of the mechanism of catalysis by rotamases and the mode of binding and inhibition by immunosuppressive agents is prerequisite to understanding the role of the immunophilins in T cell activation. Herein, we report on a ^{13}C nuclear magnetic resonance (NMR)–based investigation of the complex between recombinant human FKBP (rFKBP) and FK506 that provides new insights into these issues.

The ability of the immunophilins to catalyze the interconversion of *cis* and *trans* proline amide rotamers implies that they recognize proline-containing epitopes. [In support of this suggestion, we note that the naturally occurring immunosuppressant FR900525 contains proline in place of pipicolinic acid but is otherwise identical to FK506 (*16*).] Recognition could involve either an enzyme-substrate tetrahedral adduct (Fig. 2B, tetrahedral adduct mechanism) or a twisted peptidyl-prolyl amide bond (Fig. 2C, twisted amide mechanism). Either mechanism would explain the catalytic properties of the immunophilins, as the loss of amide resonance should lower the barrier to rotation.

Early support for a tetrahedral hemithioorthoamide adduct mechanism in the case of cyclophilin came from the studies of Fischer *et al.*, who found that *p*-hydroxymercuribenzoic acid modifies an active-site cysteine residue with concommitant loss of rotamase activity (*17*) and observed an inverse secondary isotope effect ($k_H/k_D < 1$) with a deuterated substrate containing an [α,α-^{2}H]Gly-Pro fragment (*18*). However, the latter result has been challenged (*19*), and recent studies have shown that any of the four cysteines of cyclophilin can be mutated to alanine without affecting either affinity for CsA or rotamase activity (*20*).

The structures of FK506 **2** and a structurally related immunosuppressant rapamycin **4** (*21, 22*), which also binds FKBP (*11*) and

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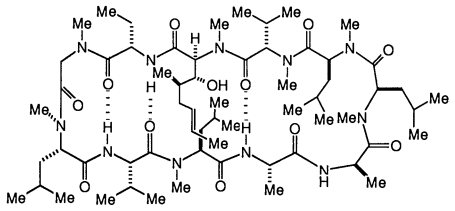
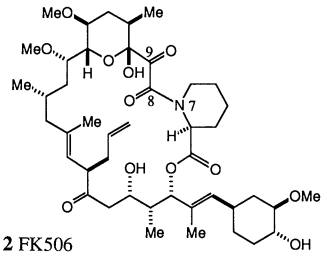
inhibits its rotamase activity (*23*), suggested a basis for their enzyme inhibitory properties. The acyl-homoprolyl amide moiety in these drugs is flanked by an α-keto (and β-hemiketal) group that should increase the electrophilicity of the two carbonyl carbons C-8 and C-9. Thus, according to this scheme, the inhibitory properties of FK506 would result from the formation of a long-lived, tetrahedral adduct of a nucleophile at C-8 or C-9 (*24*) in the FKBP active site (Fig. 3A). This nucleophile could be either a residue on the enzyme or a bound water molecule.

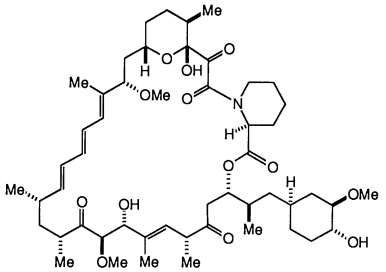


1 Cyclosporin A (CsA)



2 FK506

3 [8,9-^{13}C]FK506



4 Rapamycin

Fig. 1. Structures of the immunosuppressive agents cyclosporin A, FK506, and rapamycin.

In order to examine this possibility, we have undertaken the asymmetric total synthesis of [8,9-^{13}C]FK506 **3** (*25*) and the isolation (*11*), molecular cloning, and overexpression in *Escherichia coli* of human FKBP (*26*) by the expression cassette polymerase chain reaction (ECPCR) method (*27*). The ^{13}C-labeled analogue **3** was chosen as a reagent for use in NMR studies to determine the tendency of the C-8 and C-9 trigonal carbons of FK506 to convert to tetrahedral centers in nucleophilic solvents and in the presence of FKBP. The ^{13}C NMR spectrum in $CDCl_3$ of **3** (Fig. 4A), prepared by total synthesis (*25*), reveals the existence of major (*cis*) and minor (*trans*) amide rotamers of FK506. The assignment of rotamer configuration follows an analysis of the ^{1}H NMR spectra of FK506. Two rotamers were also observed in protic solvents, including methanol and methanol-water mixtures, without any indication of the formation of a hydrate or hemiketal. The insolubility of **3** in solutions containing more than ~40% water prevented NMR analysis under such conditions, and no ^{13}C NMR signal whatsoever could be detected in a saturated D_2O solution.

However, in the presence of a slight excess of rFKBP (1 μmol), a 0.6-μmol sample of the drug is completely soluble in 0.4 ml of 10 m*M* phosphate buffer. Two striking features of the ^{13}C NMR spectrum of this solution (Fig. 4, B and C) are immediately apparent. First, no new resonances are observed in the chemical shift range of 90 to 130 ppm, which is that expected for a tetrahedral adduct at either the C-8 or C-9 carbonyl. Second, only one set of signals arises from the bound form of the drug, which is consistent with the complexation of a single rotamer of FK506 by FKBP. The line broadening seen in the ^{13}C spectrum is consistent with the molecular weight of FKBP (11,819 dalton) (*28*). In addition, the chemical shifts of the carbonyl carbons are consistent with preserved (planar) ketone and amide functionalities and not with the amide and carboxylic acid carbonyls that would exist if the benzilic acid rearrangement, known to occur with FK506 derivatives (*29*), were to have taken place in the active site. Likewise, the formation of an imine at C-9 is not consistent with the data.

The integrity of the bound ligand was confirmed by denaturing the complex with guanidinium chloride and recovering FK506 by repeated extraction with chloroform. This procedure gave rise to a near quantitative yield of recovered [8,9-^{13}C]-FK506 **3** (and FK506 **2** when performed on the nonlabeled material) that was shown to be identical to the original sample by spectroscopic and chromatographic comparisons. We conclude from these experiments that the complexation of FK506 by its receptor, FKBP, is fully reversible and does

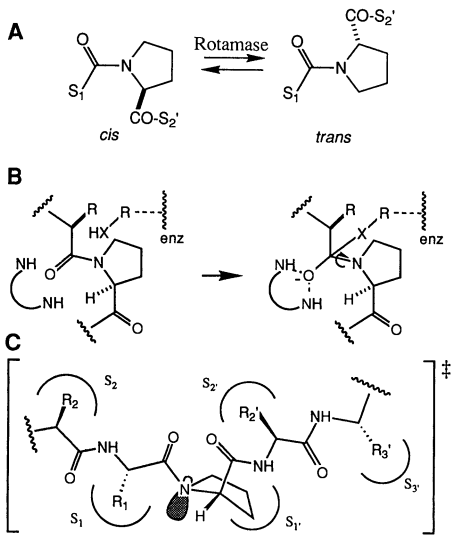


Fig. 2. (**A**) Catalysis of isomerization by rotamase enzymes. (**B**) Hypothetical tetrahedral-adduct mechanism of rotamase action. The HN–NH group symbolizes an array that can stabilize a tetrahedral intermediate. (**C**) Hypothetical twisted-amide mechanism of rotamase action.

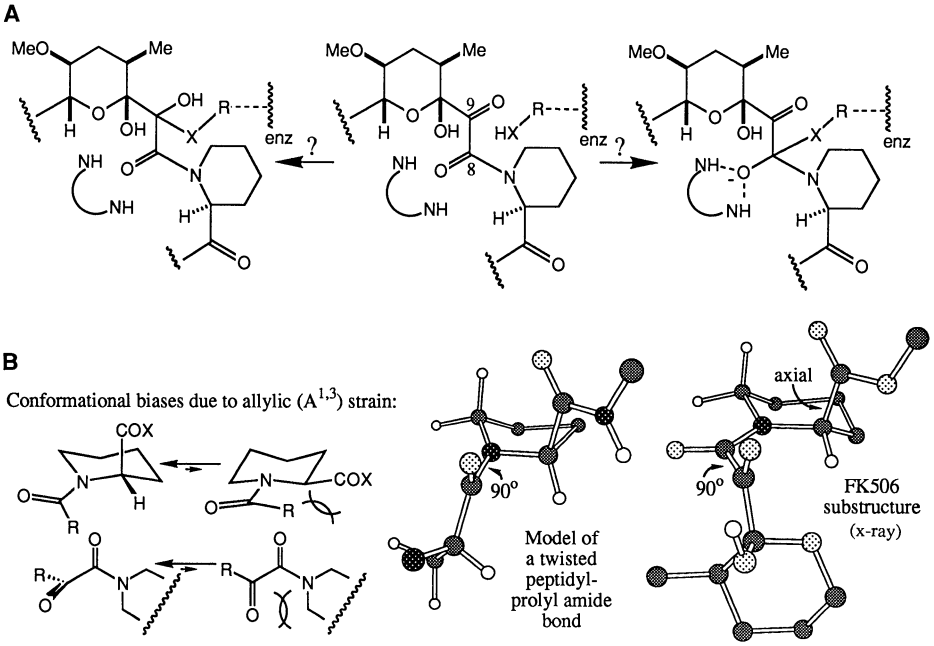


Fig. 3. Possible mechanisms of inhibition of FKBP by FK506 for (**A**) a tetrahedral adduct and (**B**) a twisted-amide surrogate.

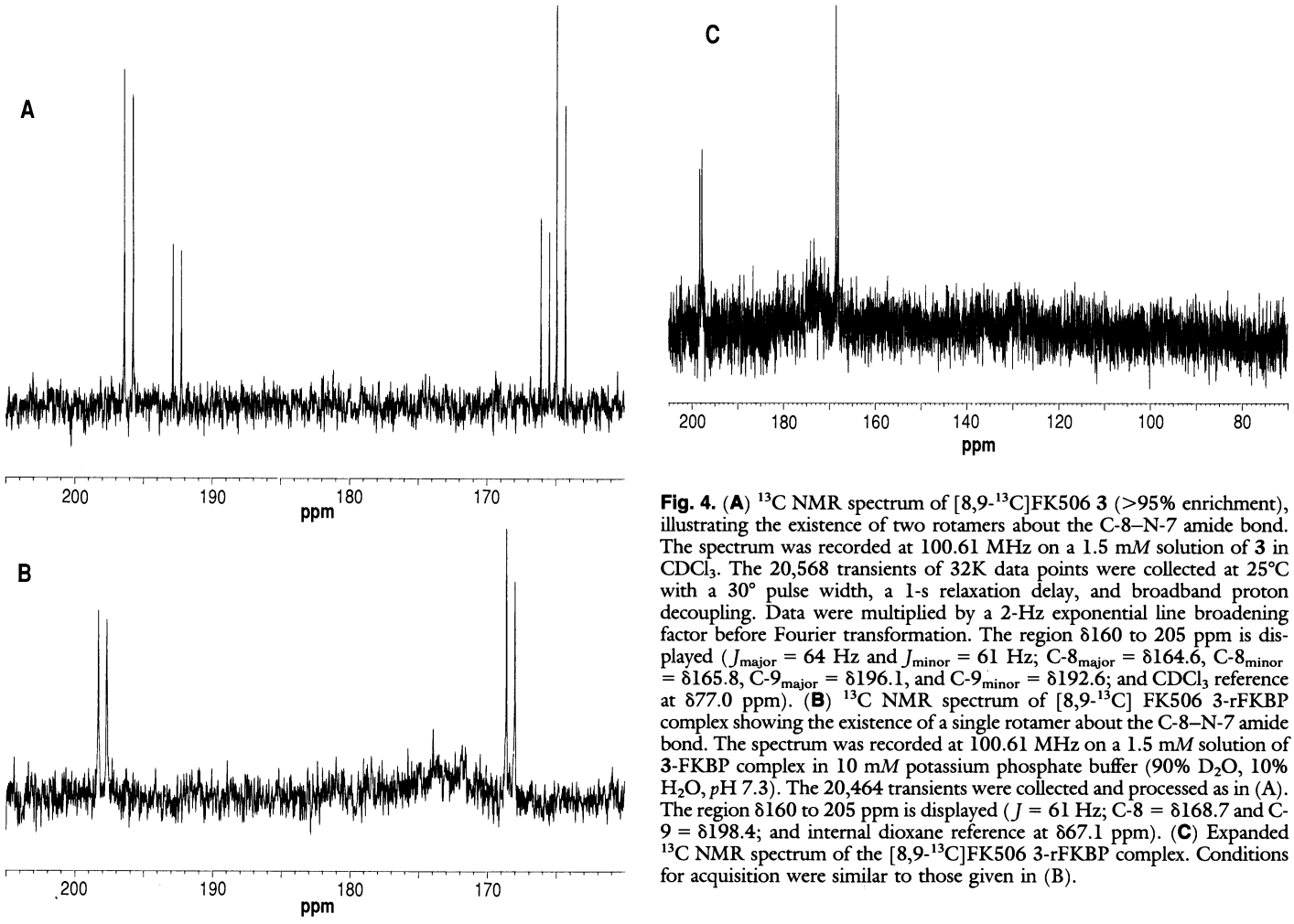


Fig. 4. (**A**) ^{13}C NMR spectrum of [8,9-^{13}C]FK506 **3** (>95% enrichment), illustrating the existence of two rotamers about the C-8–N-7 amide bond. The spectrum was recorded at 100.61 MHz on a 1.5 m*M* solution of **3** in $CDCl_3$. The 20,568 transients of 32K data points were collected at 25°C with a 30° pulse width, a 1-s relaxation delay, and broadband proton decoupling. Data were multiplied by a 2-Hz exponential line broadening factor before Fourier transformation. The region δ160 to 205 ppm is displayed (J_{major} = 64 Hz and J_{minor} = 61 Hz; C-8_{major} = δ164.6, C-8_{minor} = δ165.8, C-9_{major} = δ196.1, and C-9_{minor} = δ192.6; and $CDCl_3$ reference at δ77.0 ppm). (**B**) ^{13}C NMR spectrum of [8,9-^{13}C] FK506 3-rFKBP complex showing the existence of a single rotamer about the C-8–N-7 amide bond. The spectrum was recorded at 100.61 MHz on a 1.5 m*M* solution of 3-FKBP complex in 10 m*M* potassium phosphate buffer (90% D_2O, 10% H_2O, *p*H 7.3). The 20,464 transients were collected and processed as in (A). The region δ160 to 205 ppm is displayed (J = 61 Hz; C-8 = δ168.7 and C-9 = δ198.4; and internal dioxane reference at δ67.1 ppm). (**C**) Expanded ^{13}C NMR spectrum of the [8,9-^{13}C]FK506 3-rFKBP complex. Conditions for acquisition were similar to those given in (B).

not involve a covalent interaction at either of the electrophilic carbonyl moieties of the drug. These results remove from further consideration the hypothesis that FKBP inhibition involves a tetrahedral adduct with FK506 at either C-8 or C-9. The absence of a tetrahedral entity at the α-dicarbonyl moiety of the drug is fully consistent with the selective stabilization of twisted (~90°) peptidyl-prolyl amide bonds of substrates in the FKBP enzyme active site (Fig. 3B), a mechanism that appears to be operative in the rotamase enzyme cyclophilin (*19, 20*).

These experiments suggest a rationale for the inhibitory properties of FK506 and rapamycin. In the solid state, both compounds have a 95° dihedral angle (O=C–C=O) about the bond between C-8 and C-9 (*3, 21*), as expected for an *N*, *N*-disubstituted α-keto amide; in such systems, the normally preferred *anti*-α-diketo rotamer (dihedral angle = 180°) is destabilized by allylic [$A^{(1,3)}$] strain (*30*). Thus the ketocarbonyl of FK506 lies in a plane orthogonal to that of the adjacent amide and, as a consequence, orthogonal to the best plane of the homoprolyl ring, much as the carbonyl of a twisted peptidyl-prolyl amide lies in a plane orthogonal to the best plane of the prolyl ring. The essential difference is that for the peptide, this arrangement is 15 to 20 kcal/mol less stable than the ground state (*31*), whereas for FK506, it *is* the ground state. We suggest that the α-keto carbonyl of FK506 is appropriately oriented to occupy the site of the twisted carbonyl of a bound peptide substrate and, thus, the α-keto amide of FK506 serves as a surrogate of a twisted amide bond.

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Enhancement of the GDP-GTP Exchange of RAS Proteins by the Carboxyl-Terminal Domain of SCD25

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In *Saccharomyces cerevisiae*, the product of the *CDC25* gene controls the *RAS*-mediated production of adenosine 3′,5′-monophosphate (cAMP). In vivo the carboxyl-terminal third of the *CDC25* gene product is sufficient for the activation of adenylate cyclase. The 3′-terminal part of *SCD25*, a gene of *S. cerevisiae* structurally related to *CDC25*, can suppress the requirement for *CDC25*. Partially purified preparations of the carboxyl-terminal domain of the *SCD25* gene product enhanced the exchange rate of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) of pure RAS2 protein by stimulating the release of GDP. This protein fragment had a similar effect on the human c-H-*ras*–encoded p21 protein. Thus, the SCD25 carboxyl-terminal domain can enhance the regeneration of the active form of RAS proteins.

IN *Saccharomyces cerevisiae* THE RAS PROTEINS are components of the enzymatic cascade that activates adenylate cyclase and plays a key role in cell cycle progression. They belong to a family of proteins that bind GTP and GDP tightly and display a low-turnover guanosine triphosphatase (GTPase) activity that changes the active form induced by GTP, needed for interaction with effectors, into the inactive form bound to GDP (*1–3*). The conversion of RAS·GDP to RAS·GTP therefore represents the mechanism of activation of these proteins (*1*). In *S. cerevisiae*, the *CDC25* gene product controls the RAS-mediated production of cAMP, probably by enhancing the rate of GDP-GTP exchange of RAS proteins (*4, 5*). This mechanism is implied by indirect observations that CDC25 is dispensable in cells with either a permanently activated RAS protein, such as the product of the RAS2($Gly^{19} \rightarrow Val$) allele (*5*), or a RAS protein displaying an enhanced rate of GDP-GTP exchange, such as the product of the RAS2($Thr^{152} \rightarrow Ile$) allele (*6*). In vivo the 3′-terminal third of *SCD25* is sufficient for the complementation of the *cdc25-5* mutant (*5*). The 3′-terminal part of *SDC25*, a gene of *S. cerevisiae* structurally related to *CDC25*, can suppress the requirement for *CDC25*, most likely by activation of RAS proteins (*7, 8*). Therefore, we have tested the effect of the SCD25 carboxyl-terminal domain on the RAS2–guanine nucleotide interaction in vitro.

The SCD25 COOH-terminal domain was produced in *Escherichia coli*. For this purpose a 1878-bp fragment from pLA2 starting at the Xba I site and containing the 3′-terminal part of *SCD25* was inserted into the pUC19 polylinker (Appligene). The resulting plasmid pJEL11 drives the expression of a chimeric protein composed of the first 13 amino acid residues of β-galactosidase encoded by pUC19 followed by 550 amino acids of SCD25, under control of the lacZ promoter. The plasmid was introduced in *E. coli* JM101 cells and expression of the fused protein was induced by isopropyl β-D-thiogalactoside. As a control, pJEL11ΔBam H1 was constructed by a 741-bp deletion between the two Bam H1 sites of the *SCD25* portion (*7, 8*). This deletion leads to the loss of the 247 amino acids in the SCD25 COOH-terminal domain essential for complementation; the open reading frame upstream and downstream of this fragment are fused in frame.

To test the effect of the SCD25 COOH-terminal domain on RAS2 functions, we used a pure 30-kD form of RAS2 isolated from *E. coli* (*6*). This truncated form results from the removal of the COOH-terminal end of the protein by proteolytic cleavage, a deletion that affects neither the 1:1 stoichiometric binding of GDP or GTP nor the ability to activate the adenylate cyclase in vitro. The supernatant from sonicated, pJEL11-transformed cells dramatically enhanced the release of [^{3}H]GDP from the RAS2·[^{3}H]GDP complex, whereas the supernatant from cells transformed with pUC19 or pJEL11ΔBam H1 was inactive (Table 1). The releasing activity in the supernatant could only be detected by using a filtration method on short columns (*9*). In fact, without purification, the large amount of contaminating protein in the supernatant hindered binding of the complexes of GTP or GDP and RAS2 to the nitrocellulose filters. By contrast, partial purification of the SCD25 COOH-terminal domain (*10*) allowed us to use filtration on nitrocellulose to measure the dissociation kinetics of the RAS2 complexes. The SCD25 COOH-terminal domain had neither guanine nucleotide binding activity nor GTPase activity and did not have any proteolytic effects on RAS2, as verified by immunoblots after SDS–polyacrylamide gel electrophoresis. The

Table 1. Effect on the RAS2·[^{3}H]GDP dissociation rate of the SCD25 COOH-terminal domain. GDP-free (*6*) RAS2 protein (30 pmol) in a standard buffer containing 25 mM tris-HCl, pH 7.5, 1 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM dithiothreitol (DTT), and 0.1 mg/ml bovine serum albumin (BSA) was incubated in 80 μl with 35 pmol [^{3}H]GDP (10 Ci/mmol) for 30 min at 30°C to achieve equilibrium conditions. The dissociation reaction was started by adding a 1000-fold excess (35 nmol) of unlabeled GDP in 30 μl of storage buffer (50% glycerol in 50 mM tris-HCl, pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, and 7 mM 2-mercaptoethanol or 30 μl of a mixture containing 35 nmol of unlabeled GDP and 200 μg of each of the different extracts in storage buffer. After a 15-min incubation at 30°C, 90 μl of each sample were loaded onto a Sephadex G25 column (18 by 0.4 cm) equilibrated with standard buffer and eluted in 100-μl fractions, and the radioactivity was measured. To obtain the cell extracts, *E. coli* cells (9 g) were sonicated in a threefold volume of 50 mM tris-HCl, pH 7.5, 1 mM $MgCl_2$, 100 mM KCl, 0.25 M sucrose, 7 mM 2-mercaptoethanol, 2 mM phenylmethylsulphonylfluoride (PMSF), and 1 mM EDTA, and centrifuged at 100,000*g* for 90 min. The supernatant was dialyzed against the storage buffer and kept at −30°C. The mean ± SEM was calculated from four experiments for each condition.

Conditions	RAS2·[^{3}H]GDP (pmol)
No addition	22 ± 2
+ pJEL11 transformed cell extract	2.6 ± 0.3
+ pUC19 transformed cell extract	23 ± 1
+ JEL11ΔBam H1 transformed cell extract	22 ± 2.5

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