tryptophan in human fibroblasts, which results in inhibition of growth of intracellular *T. gondii* (*19*). In contrast, IFN-γ did not induce tryptophan degradation in some mouse fibroblast cell lines and primary cultures of embryo fibroblasts of BALB/c mice (*20*). Therefore, in resistance to *T. gondii* in the mouse model, the primary effect of IFN-γ in vivo appears to be its ability to activate macrophages. Critical to this point is that rIFN-γ can activate macrophages to inhibit or kill *T. gondii* without collaboration of any other lymphokine (*7, 8, 10*). Our results along with those reported by Murray and his colleagues on the importance of IFN-γ in resistance to *T. gondii* (*21*) and the impaired production of this lymphokine in patients with AIDS (*22*) support the suggestion that the IFN-γ should be evaluated for treatment of toxoplasmosis in patients with AIDS (*22*).

Note added in proof: Since submission of this report, Schofield *et al.* (*23*) demonstrated that anti–IFN-γ reversed immunity to sporozoite (malaria) challenge in mice and rats.

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Guanosine Triphosphatase Activating Protein (GAP) Interacts with the p21 *ras* Effector Binding Domain

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A cytoplasmic protein that greatly enhances the guanosine triphosphatase (GTPase) activity of N-*ras* protein but does not affect the activity of oncogenic *ras* mutants has been recently described. This protein (GAP) is shown here to be ubiquitous in higher eukaryotes and to interact with H-*ras* as well as with N-*ras* proteins. To identify the region of *ras* p21 with which GAP interacts, 21 H-*ras* mutant proteins were purified and tested for their ability to undergo stimulation of GTPase activity by GAP. Mutations in nonessential regions of H-*ras* p21 as well as mutations in its carboxyl-terminal domain (residues 165–185) and purine binding region (residues 117 and 119) did not decrease the ability of the protein to respond to GAP. In addition, an antibody against the carboxyl-terminal domain did not block GAP activity, supporting the conclusion that GAP does not interact with this region. Transforming mutations at positions 12, 59, and 61 (the phosphoryl binding region) abolished GTPase stimulation by GAP. Point mutations in the putative effector region of *ras* p21 (amino acids 35, 36, and 38) were also insensitive to GAP. However, a point mutation at position 39, shown previously not to impair effector function, did not alter GAP-p21 interaction. These results indicate that GAP interaction may be essential for *ras* p21 biological activity and that it may be a *ras* effector protein.

Ras GENES ENCODE MEMBRANE-ASsociated proteins that are involved in the control of cell proliferation (*1*). Members of this gene family are widely expressed in the cells of both lower and higher eukaryotes. In *Saccharomyces cerevisiae*, *ras* genes have been shown to regulate adenylate cyclase activity (*2*), however, the function of mammalian *ras* gene products remains unclear. All *ras* proteins bind guanine nucleotides with high affinity and possess an intrinsic GTPase activity (*1, 3*). They resemble other guanine binding proteins (such as the signal-transducing G proteins) in that they are biologically active when in the guanosine triphosphate (GTP)–bound form and inactive when bound to guanosine diphosphate (GDP) (*4, 5*).

Each of the three closely related mammalian *ras* genes (H-*ras*, K-*ras*, and N-*ras*) encode a 21-kD protein (p21) possessing a low intrinsic GTPase activity. This activity is reduced by three- to tenfold in many oncogenic mutants of p21 (*1, 3, 4*). However, these differences in intrinsic GTPase activity are insufficient to explain the greatly increased biological potency of the oncogenic mutants of p21. A cytoplasmic protein has recently been detected that can accelerate the GTPase activity of the wild-type human N-*ras* protein more than 100-fold (*4*). As a result of interaction with this protein (referred to as GAP, GTPase activating protein), N-*ras* p21 injected into *Xenopus* oocytes was shown to be maintained in the inactive, GDP-bound state. By contrast, two N-*ras* oncogenic mutants (Val 12 and Asp 12) were not responsive to GAP, remaining in the active (GTP-bound) form.

GAP appears to be widely expressed in the cells of higher eukaryotes. We have detected GAP-like activity (ability to stimulate intrinsic N-*ras* p21 GTPase activity) in cell extracts from human and mouse (i) normal tissues (brain, liver, placenta, B cells,

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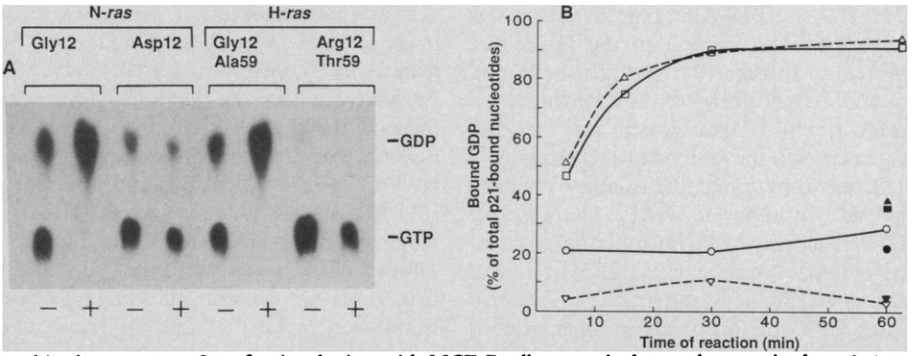

Fig. 1. GAP effects on the intrinsic GTPase activities of the wild-type and the oncogenic mutants of N-*ras* and H-*ras* proteins. (**A**) *Ras* proteins were bound to α-^{32}P–GTP and assayed for their sensitivity to GAP activity in MCF-7 cell extract. (+) Samples incubated in the presence of MCF-7 extract; (−) control samples incubated in the presence of TNMN buffer alone. MCF-7 cells were lysed in TNMN buffer [20 m*M* tris-HCl (*p*H 8.0), 100 m*M* NaCl, 5 m*M* $MgCl_2$, and 0.5% NP40]. The lysate was spun to remove cell debris and the protein concentration in the supernatant was adjusted to 1.5 mg/ml. The procedure used for GAP activity assay was a modification of that described (*4*). Purified N-*ras* proteins (200 ng) in 350 m*M* guanidine hydrochloride were incubated in the presence of α-^{32}P–GTP (1.3 mCi/ml; 1.7 μ*M*) for 20 minutes at 37°C. MCF-7 cell extract (10 μl) was then mixed with 1.5-μl aliquots of the reaction mixtures and incubation continued for additional 60 minutes at room temperature. Control samples received TNMN buffer instead of cell extract. The p21/guanine nucleotide complex in each sample was immunoprecipitated with 1 μg of Y13-259 monoclonal antibody, 2 μg of goat anti-rat IgG and 10 μl of protein A–Sepharose beads. To dissociate the bound nucleotide, each pellet was heated to 60°C in the presence of 10 μl of buffer containing 1% SDS and 20 m*M* EDTA. The dissociated nucleotides were analyzed by thin-layer chromatography (with 1*M* LiCl as solvent) followed by autoradiography. The observed decrease in total nucleotides bound to v–H-*ras* p21 after incubation with MCF-7 cell extract is due to the protein degradation. Western blot analysis has indicated that v–H-*ras* is less stable than c–H-*ras* p21 under these conditions (*6*). (**B**) Kinetics of GAP stimulation of N-*ras* and H-*ras* proteins. At various times after the addition of the MCF-7 cell extracts, samples were removed from the reaction mixtures and the extent of GTP hydrolysis by *ras* was determined. The labeled nucleotides were scraped off the chromatography plate and counted in scintillation fluid. N-*ras* Gly 12 (squares); Asp 12 N-*ras* (circles); Gly 12, Ala 59 H-*ras* (upright triangles); and Arg 12, Thr 59 H-*ras* (inverted triangles). Open symbols represent samples that were incubated in the presence of the cell extract, and closed symbols represent TNMN buffer controls.

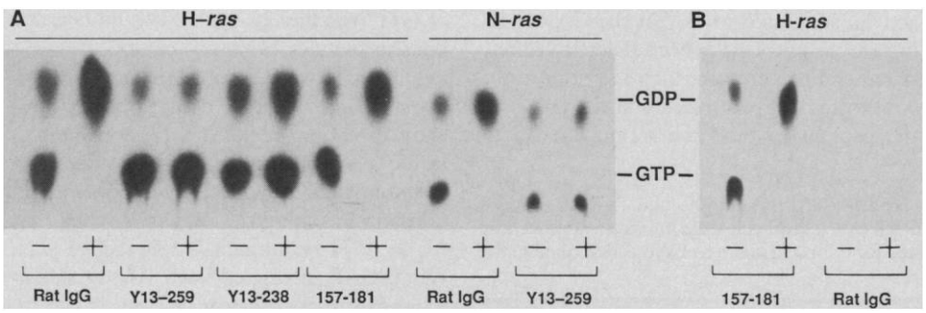

Fig. 2. The ability of *ras*-specific monoclonal antibodies to block GAP stimulation of N-*ras* and H-*ras* p21 GTPase. (**A**) Effects of antibody binding in solution on stimulation by GAP. H-*ras* and N-*ras* proteins bound to α-^{32}P–GTP [0.8 μg of each protein in 15 μl of buffer A (20 m*M* tris-HCl, *p*H 7.5, 1 m*M* $MgCl_2$, 1 m*M* DTT, and 50 μg/ml bovine serum albumin)] were incubated in the presence of *ras*-specific antibodies (13 μg) for 30 minutes at 0°C. Rat IgG was used in control samples. Ten microliters of MCF-7 cell extract (+) or TNMN buffer (−) were added to 4-μl aliquots of each sample and the reaction mixtures were incubated for 60 minutes at room temperature. Thirteen microliters of Y13-259 were then added to the control sample and the immune complexes were precipitated with either 10 μg of sheep anti-mouse IgG (when 157–181 was the primary antibody) or 10 μg of goat anti-rat IgG (with the rest of the samples) and 10 μl of protein A–Sepharose beads. The bound nucleotides were analyzed by thin-layer chromatography. (**B**) Stimulation of immunoprecipitated H-*ras* by GAP. H-*ras* protein (0.8 μg) bound to α-^{32}P–GTP was precipitated with 13 μg of 157–181 antibody, 10 μg of sheep anti-mouse IgG, and 10 μl of protein A–Sepharose beads. (The control received rat IgG and goat anti-rat IgG.) The pellet was washed with buffer B [20 m*M* tris-HCl (*p*H 7.5), 20 m*M* NaCl, 1m*M* $MgCl_2$ and 1m*M* DTT] and 4-μl aliquots of the immune-complex were then mixed with 10-μl of MCF-7 cell extract (+) or TNMN buffer (−). After 60 minutes incubation at room temperature, the beads were washed again and the bound nucleotides were analyzed as before.

or platelets), (ii) nontransformed continuous cultures (NIH 3T3 and Sf9), (iii) tumor cell cultures [human mammary cancer (MCF-7), retinoblastoma (Y79), and Wilm's tumor (G401)], and (iv) transformed cultured cells [human mammary epithelial cells (A1) transformed with either SV40 T-antigen, v–H-*ras*, or v-*mos* (*11*); NIH 3T3 transformed with v-*src*]. GAP-like activity was also detected in extracts made from insect cells (*Spodoptera frugipedra*), but not from cells of two yeast species, *S. cerevisiae* and *S. pombe* (*6*).

The ubiquitous presence of GAP in metazoan cells suggested to us that GAP may interact not only with N-*ras* p21 but also with other p21 species. Indeed, the GTPase activities of both H-*ras* and N-*ras* proteins were greatly increased in the presence of an extract of MCF-7 cells (Fig. 1A). The stimulated rate of GTP hydrolysis was identical for the two *ras* species, resulting in the complete conversion of bound GTP to GDP within 30 minutes of incubation at room temperature (Fig. 1B). The rate of hydrolysis of bound GTP was negligible in the absence of cell extract. The viral form of the H-*ras* p21 protein (which is encoded by Harvey murine sarcoma virus and carries transforming mutations at positions 12 and 59) was completely insensitive to GAP action. Other oncogenic variants of H-*ras* were also found to be unresponsive (see below), as were oncogenic mutants of N-*ras* p21 (Fig. 1) (*4*). Thus GAP seems to interact with both *ras* p21 proteins in the same fashion. Nevertheless, it is not yet clear whether each *ras* protein interacts with the same GAP species or with distinct versions of GAP that may co-exist within the cell.

It is likely that GAP interacts with the same structural feature in both *ras* proteins. We therefore suspected the GAP association does not involve the variable COOH-terminal domain of *ras* p21 proteins (amino acid residues 164–187), but that its binding site must be located within the first 163 residues, which are highly conserved among all *ras* proteins. This hypothesis is consistent with the observation (*4*) that a monoclonal antibody that binds to an epitope located within the NH_2-terminal region of all *ras* proteins (Y13-259) (*7*) can block GAP stimulation of N-*ras* p21 GTPase activity. As expected, we found that this antibody also prevented GAP activation of H-*ras* p21 (Fig. 2A). Another monoclonal antibody (Y13-238) (*7*) had the same inhibitory effects on H-*ras* p21. The epitope recognized by this H-*ras*–specific antibody, which apparently includes some amino acids between 93–139, is still incompletely defined (*8*).

A monoclonal antibody directed against the COOH-terminal residues 157–181 had no effect on the ability of GAP to stimulate the GTPase activity of H-*ras* protein, unlike

Y13-259 and Y13-238 (Fig. 2A). As it was not clear what fraction of the H-*ras* p21 protein in this reaction was actually bound to antibody, we carried out another experiment in which H-*ras* protein was first precipitated with the antibody to residues 157–181 and then tested the immune complex for its sensitivity to GAP. The intrinsic GTPase activity of H-*ras* could be stimulated by GAP even though its COOH-terminal region was bound to the antibody (Fig. 2B). Thus GAP interaction does not appear to involve the COOH-terminal domain of p21.

To localize more precisely the GAP interaction site on the p21 molecule, a series of deletion and substitution mutations were made in c–H-*ras* and their ability to be stimulated by GAP was tested (Table 1). Previous studies have identified four segments of the viral H-*ras* protein that are not essential for transformation (amino acids 64–76, 93–108, 120–138, and 166–183) (*8*). Amino acid residues in all of these regions (with the exception of residues 97–108) could be either substituted or deleted from c–H-*ras* without affecting GTPase stimulation by GAP (Table 1). The results with mutants carrying deletions within region 166–183 of c–H-*ras* reinforces the conclusion (Fig. 2) that the p21 COOH-terminal end is not involved in GAP interaction. Further support for this comes from the observation (*9*) that the substitution of Cys 186 by Ser 186 does not alter p21 sensitivity to GAP.

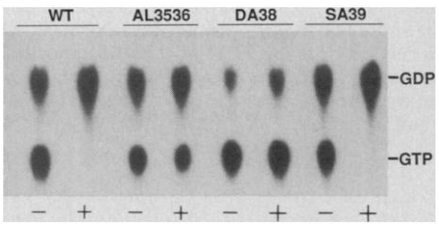

Fig. 3. Sensitivity of c–H-*ras* effector binding site mutants to GAP activity. GAP activity assays were performed exactly as described in Fig. 1. The three mutants are described in Table 1. WT, wild-type c–H-*ras* protein.

Deletion of residues 97–103 or 102–108 resulted in a partial loss of sensitivity to GAP. The relatively large sizes of these deletions and their retention of some GAP activity suggested to us that the GAP binding site may not have been deleted in these mutants. Furthermore, such deletions in the viral form of H-*ras* resulted in the failure of the protein to bind the monoclonal antibody Y13-238 and to autophosphorylate the threonine residue at position 59 (*8*), suggesting that the deletions caused conformational changes in the *ras* protein. To determine whether the reduced GAP activity had resulted from an altered conformation or was sequence specific, the ability of GAP to act on three less drastic mutants in this region was tested. These mutants, each carrying three novel amino acids replacing residues 93–95, 101–103, or 107–108, exhibited normal sensitivity to GAP. It was therefore concluded that nonessential p21 sequences, including amino acids 93–108, do not participate in GAP binding.

The ability of H-*ras* mutants carrying substitutions within the GTP binding site to respond to GAP was then tested. The results indicated that lesions involving residues that are in close contact with the phosphoryl moiety of GTP (residues 12, 59, and 61) (*1, 10*) abolished H-*ras* sensitivity to GAP (Table 1 and Fig. 1). In contrast, substitutions of residues that interact with the purine moiety of GTP (residues 117 and 119) did not alter the sensitivity of H-*ras* protein to GAP.

Finally, H-*ras* p21 mutants carrying amino acid substitutions within the putative effector-binding region (*8, 11*) were tested. It has been shown that v–H-*ras* mutants carrying Asp 38 or Ala 35/Leu 36 substitutions lack biological activity, while the Ala 39 v–H-*ras* mutant is fully active (*11*). While GAP interacted with Ala 39 substituted H-*ras* in a normal fashion, it had no effect on Ala 35/Leu 36 or Asp 38 mutants (Fig. 3). Thus, p21 residues that are required for effector binding also appear to be required for GAP interaction.

In summary, a number of H-*ras* p21 mutants were tested for their ability to interact with GAP. Only those that carried lesions either in the phosphoryl binding site or in the putative effector binding domain lost the ability to respond to GAP. These results suggest that GAP binds to the effector binding site of mammalian *ras* proteins. This conclusion is consistent with a recent x-ray crystallography study which showed that this region is present on the surface of the H-*ras* p21 molecule and, therefore, may associate with other proteins (*10*). The same region in *S. cerevisiae RAS2* protein may interact directly with adenylate cyclase, which is thought to be a principal effector of *RAS2* (*2*). We speculate that mutations within the phosphoryl-binding pocket (residues 12, 59, and 61) result in a conformational change that does not allow the stimulation of the intrinsic GTPase activity, but probably does not interfere with GAP binding to the effector region of the p21 molecule.

It has been suggested previously that the

Table 1. Stimulation of c–H-*ras* mutant proteins (*15*) by GAP. Wild-type and mutant proteins produced in *Escherichia coli* were extracted from lysed cell pellets by small-scale purification procedure modified from Manne *et al.* (*3*). The proteins were tested for their sensitivity to GAP as described in the legend to Fig. 1.

Residue	Change		Stimulation by GAP	Biological activity‡
	From	To		
	Mutations in non-essential regions			
WT	—	—	+	+
65	S	A	+	+
75, 76	GE	LIR	+	+
93–95	IHQ	LIR	+	+
101–103	KRV	LIR	+	+
107–108	DD	ADE	+	+
97–103	REQIKRV	LIR	+/−*	+
102–108	RVKDSDD	PDQ	+/−*	+
124–129	TVESRQ	LIR	+	+
166–179	HKLRKLNPPDESGP	PDQ	+	+
166–183	HKLRKLNPPDESGPGCMS	PDQ	+	+
	Oncogenic mutations in the GTP-binding site†			
12, 59	GA	RT	−	
12	G	R	−	
59	A	T	−	
61	Q	L	−	
61	Q	R	−	
61	Q	P	−	
117	K	R	+	
119	D	E	+	
	Mutations in the effector binding site			
35, 36	TI	AL	−	−
38	D	A	−	−
39	S	A	+	+

*In the presence of GAP, these mutants required 150 minutes of incubation at room temperature to completely hydrolyze the bound GTP. Under the same conditions, wild-type and p21 mutants that are fully responsive to GAP completed the reaction within only 30 minutes. †Transforming capacity of these mutants has previously been determined (*1, 14*). Recent work by Der and McDonald (*16*) has shown that, in contrast to previous observations, Pro 61 mutant of H-*ras* p21 has a weak transforming phenotype. ‡Biological activity is defined as the ability of v–H-*ras* proteins carrying the same mutations to transform NIH 3T3 cells and to complement *RAS2* mutations in *S. cerevisiae* (*11*).

physiological role of GAP is to down-regulate *ras* p21 and that GAP, therefore, acts upstream from *ras* p21 in a signal transduction pathway (*4*). However, the results presented here, which suggest that GAP and the *ras* effector bind to the same site on p21, raise the possibility that GAP is the effector protein itself. If so, it would be expected that the interaction between GAP and p21 occurs on the plasma membrane, since membrane association is known to be required for *ras* function (*12*). We therefore propose that GAP, which has been shown previously to be a cytosolic protein (*4*), translocates to the membrane through association with the GTP-bound form of p21. We speculate that this translocation is necessary to bring GAP in close proximity to its own target (or targets) and that the formation of this complex generates a signal for cellular proliferation. Thus, GAP activation may be analogous to that of protein kinase C, which has been shown to involve translocation to the plasma membrane in response to mitogenic stimulation (*13*). An interesting aspect of the model proposed here is that it provides a means for down-regulating the active complex through the stimulation of GTP hydrolysis by GAP, resulting in the dissociation of GAP from the complex. Since the GTPase activity of oncogenic p21 mutants cannot be stimulated by GAP, constitutive association between GAP and its putative target (or targets) takes place, which in turn results in loss of growth control.

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15. The following abbreviations were used for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
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Evidence from Cassette Mutagenesis for a Structure-Function Motif in a Protein of Unknown Structure

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The three-dimensional structure of most enzymes is unknown; however, many enzymes may have structural motifs similar to those in the known structures of functionally related enzymes. Evidence is presented that an enzyme of unknown structure [Ile-transfer RNA (tRNA) synthetase] may share a functionally important structural motif with an enzyme of related function (Tyr-tRNA synthetase). This approach involves (i) identifying segments of Ile-tRNA synthetase that have been unusually conserved during evolution, (ii) predicting the function of one such segment by assuming a structural relation between Ile-tRNA synthetase and Tyr-tRNA synthetase, and (iii) testing the predicted function by mutagenesis and subsequent biochemical analysis. Random mutations were introduced by cassette mutagenesis into a ten-amino-acid segment of Ile-tRNA synthetase that was predicted to be involved in the formation of the binding site for isoleucine. Few amino acid substitutions appear to be tolerated in this region. However, one substitution (independently isolated twice) increased the Michaelis constant K_m for isoleucine in the adenylate synthesis reaction by greater than 6000-fold, but had little effect on the K_m for adenosine triphosphate, the apparent K_m for tRNA, or the rate constant k_{cat}.

ALTHOUGH FUNCTIONALLY RELATED, the members of the aminoacyl–transfer RNA (tRNA) synthetase class of enzymes are heterogeneous in quaternary structure and subunit size and in general show little sequence similarity. However, a subset of these enzymes (including Ile-tRNA synthetase) share a similar 11-amino-acid segment known as the signature sequence (*1*). In the two aminoacyl-tRNA synthetase structures that have been partially solved (Tyr- and Met-tRNA synthetase), this sequence occupies the same position in a mononucleotide binding fold (*2*) (Fig. 1). In the case of *Bacillus stearothermophilus* Tyr-tRNA synthetase, residues in this sequence are known to be involved in the binding of adenosine triphosphate (ATP) (*3*) and in the catalytic formation of the aminoacyl-adenylate intermediate (*4*). We present evi-

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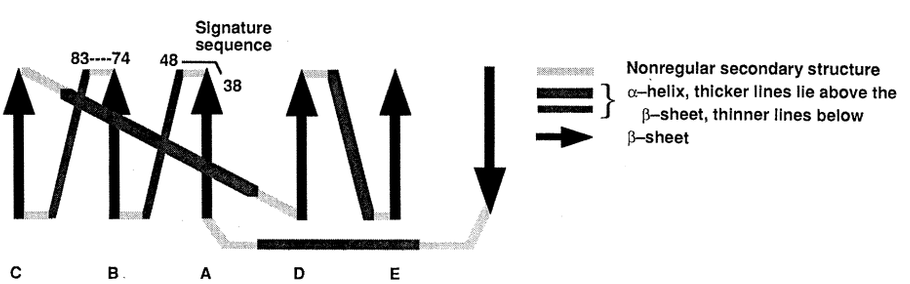

Fig. 1. Schematic of the dinucleotide binding fold type structure of *B. stearothermophilus* Tyr-tRNA synthetase. This drawing (not to scale) represents one selected part of the solved structure of Tyr-tRNA synthetase based on published drawings and descriptions (*2, 8, 9*). The letters below the schematic indicate the β-strands according to the convention of Blow *et al.* (*2*). The approximate location of the signature sequence found in a subset of aminoacyl-tRNA synthetase is shown, as is the location of a segment that starts 26 amino acids beyond the end of the signature sequence. This segment may be analogous to the segment of Ile-tRNA synthetase that we have subjected to mutagenesis in this work.