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Substitutions of Thr30 provide mechanistic insight into tryptophan-mediated activation of TRAP binding to RNA

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ABSTRACT

TRAP is an 11 subunit RNA binding protein that regulates expression of genes involved in tryptophan biosynthesis and transport in *Bacillus subtilis*. TRAP is activated to bind RNA by binding up to 11 molecules of L-tryptophan in pockets formed by adjacent subunits. The precise mechanism by which tryptophan binding activates TRAP is not known. Thr30 is in the tryptophan binding pocket. A TRAP mutant in which Thr30 is substituted with Val (T30V) does not bind tryptophan but binds RNA constitutively, suggesting that Thr30 plays a key role in the activation mechanism. We have examined the effects of other substitutions of Thr30. TRAP proteins with small β-branched aliphatic side chains at residue 30 bind RNA constitutively, whereas those with a small polar side chain show tryptophan-dependent RNA binding. Several mutant proteins exhibited constitutive RNA binding that was enhanced by tryptophan. Although the tryptophan and RNA binding sites on TRAP are distinct and are separated by ~7.5 Å, several substitutions of residues that interact with the bound RNA restored tryptophan binding to T30V TRAP. These observations support the hypothesis that conformational changes in TRAP relay information between the tryptophan and RNA binding sites of the protein.

INTRODUCTION

In *Bacillus subtilis*, genes involved in tryptophan biosynthesis and transport are regulated by a tryptophan-activated RNA binding protein called TRAP (*trp* RNA binding attenuation protein) (1,2). TRAP regulates both transcription and translation of these genes in response to changes in intracellular levels of L-tryptophan. TRAP is composed of eleven identical subunits arranged in a symmetric ring (3,4). TRAP is activated to bind RNA by binding up to 11 molecules of L-tryptophan in pockets formed by adjacent subunits. The natural RNA targets of TRAP contain 9–11 NAG (where N = G ≈ U > A > C) triplet repeats separated by several non-conserved spacer residues (5–9). As a result of TRAP binding to several RNA targets, expression of the *trp* genes is down regulated. Alternatively, when the tryptophan level is growth limiting, TRAP is not activated to bind RNA, which allows elevated expression of the tryptophan synthesis genes.

Several crystal structures of tryptophan-activated TRAP in complex with RNAs containing 11 GAG or UAG repeats revealed that the RNA binds by wrapping around the outer periphery of the protein ring. The bound RNA interacts primarily with four amino acids of each subunit: Glu36, Lys37, Lys56 and Arg58 (10–12). These residues as well as those involved in binding tryptophan (see below), are completely conserved in all currently available TRAP sequences, which have 64–86% identity to *B.subtilis* TRAP [(3) and P. Gollnick unpublished data].

In comparison to the interaction with RNA, relatively little is known about the mechanism by which tryptophan binding activates TRAP to bind RNA. There is currently no high resolution structure of TRAP in the absence of tryptophan. TRAP is an 11mer in the absence or presence of tryptophan, which rules out a mechanism of activation involving altering oligomerization of the protein (13,14). Although each TRAP 11mer can bind up to 11 molecules of L-tryptophan, studies of hetero-11mers containing varying ratios of WT and mutant subunits (defective in tryptophan binding) have shown that the presence of just one or two bound tryptophans significantly activates TRAP to bind RNA (14). NMR studies have shown that tryptophan binding alters the dynamic properties of TRAP. In particular, tryptophan binding reduces the flexibility of the tryptophan- and RNA-binding regions of TRAP, which is likely an important feature of the activation mechanism (15). The observation that apo-TRAP is quite flexible and contains regions that are dynamically disordered likely explains why it has not been

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possible to obtain good quality crystals of TRAP in the absence of tryptophan.

Each bound tryptophan is completely buried in a pocket between adjacent subunits, and it forms one hydrogen bond with the side chains of four TRAP residues (4). These include Thr49 and Thr52 from one subunit and Thr30 and Ser53 from the adjacent subunit. The interactions with Thr30 and Thr49 have been shown to be essential for activation of TRAP whereas the hydrogen bond with Ser53 is dispensable (16). The interaction of Thr52 with tryptophan has only a limited role in activation of TRAP. In addition, each bound tryptophan hydrogen bonds with the main-chain portions of several TRAP residues, including Thr25, Gly27, Asp29 and Gln47. Of these, only the interactions with Thr25 and Gln47 have been shown to play a role in activation (12,16).

The importance of Thr30 in tryptophan binding and activation of TRAP is demonstrated by the dramatic effects of two substitutions (Ala and Val) at this position. Substituting Ala for Thr30 (T30A) disrupts both the tryptophan- and RNA-binding activities of the protein (16). In contrast, T30V TRAP, with valine at position 30, does not bind tryptophan, but binds RNA constitutively, although with ~100-fold lower affinity than tryptophan-activated WT TRAP (16). Characterization of the interaction of T30V TRAP with RNA showed that the mutant protein recognizes similar features of the RNA as does WT TRAP (17). This observation suggests that the T30V substitution may activate TRAP in a manner similar to how tryptophan binding activates WT TRAP.

To provide a better understanding of the role of Thr30 in activation of TRAP, we introduced several additional substitutions at position 30 and examined the RNA binding and tryptophan binding properties of the resulting mutant proteins. We also determined that in general, the same amino acid residues in WT TRAP and T30V TRAP are important for RNA binding. However, the side chain of Lys37, which plays an important role in the WT complex, does not contribute to the stability of the T30V TRAP–RNA complex. Additionally, we found that altering residues in T30V TRAP that contact the RNA affects the tryptophan binding properties of the resulting double mutant proteins. This finding indicates that information can be transferred from the RNA binding region to the tryptophan binding site even though these sites are distinct and ⩾7.5 Å apart (11).

MATERIALS AND METHODS

Plasmids and strains

For expression of WT or mutant TRAP, the *mtrB* gene, which encodes TRAP, was inserted between the EcoRI and HindIII sites of pBSKS (Stratagene) to create pBSKSmtrB or between the NdeI and NotI sites of pET9a (Novagen) to create pETmtrB. For *in vitro* transcription, pTZ18U(GAGTT)$_{11}$ was used (18). This plasmid has a 55 nt insert consisting of eleven tandem GAGTT repeats ligated between the EcoRI and HindIII restriction sites of pTZ18U (USB). *Escherichia coli* strains SG62052 / pGP1-2 (19) and BL21 Rosetta (Novagen) were used for protein expression and strain K802 was used to propagate plasmids.

Plasmids pVP5WT and pVP5T30V were used to express WT TRAP and T30V TRAP, respectively, in *B.subtilis* BYC1 {Δ*mtrB argC4 amyE*::[P_{trp}-*trp*L-(*trpE′-′lacZ*) Em^r]} for β-galactosidase assays. BYC1 was created by transforming *B.subtilis* BG4233 (Δ*mtrB argC4*) (20) with genomic DNA of CYBS12 {*argC4 amyE*::[P_{trp}-*trp*L-(*trpE′-′lacZ*) Em^r]} (21). pVP5 was constructed from pHB201 (22) by inserting a tetracycline resistance cassette obtained from pDG1513 (23) (*Bacillus* Genetic Stock Center) at a unique ClaI site. pVP5WT was constructed by inserting the 3′ portion of the *mtrA* gene and the full-length *mtrB* gene [809 to 1002, numbering as given by Gollnick *et al*. (24)] between the NotI and BamHI restriction sites in pVP5. pVPT30V was constructed by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) to change codon 30 from Thr to Val (ACA to GTT) using pVP5WT as template.

Site-directed mutagenesis

Two PCR-based site-directed mutagenesis methods were used to generate genes that encode mutant TRAP proteins. The first method, using four oligonucleotide primers, was described previously (12). Briefly, using pBSKSmtrAB (12) as template, two overlapping DNA fragments were generated in two separate PCR. One reaction contained an oligonucleotide with the desired mutation in *mtrB* together with the universal reverse primer. The second PCR contained the universal −40 primer together with an oligonucleotide that annealed to positions 1229 to 1251 of the *mtr* operon [numbering as in Gollnick *et al*. (24)]. The two resulting PCR products were purified, mixed together, denatured by boiling, and annealed (by their partial overlap) by slow cooling to room temperature. The single-stranded 5′ overhangs were filled in using the Klenow enzyme. The resulting product was amplified by PCR using the SK and KS universal primers to generate a full length *mtrB* gene with ~50% mutant and 50% wild-type sequences. These PCR products were digested with EcoRI and HindIII and ligated into similarly digested pBlueScriptKS(−). Alternatively, some of the mutant *mtrB* genes used in this study were created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with pETmtrB as template. In all cases, clones containing the desired mutations were identified by DNA sequencing using Sequenase (USB).

Purification of WT and mutant TRAP

Wild-type TRAP was purified by phenyl agarose chromatography (25). Mutant proteins were purified either by phenyl agarose chromatography, immunoaffinity chromatography or chromatography on Q-Sepharose as described previously (5,25). Each protein preparation was examined by SDS–PAGE and quantified by the BCA protein assay (Pierce) using WT TRAP of known concentration as the standard.

β-Galactosidase assays

Assays of β-galactosidase activity were performed as described previously (21) with *B.subtilis* cells grown in minimal medium with 0.2% acid-hydrolyzed casein in the absence or presence of 50 μg/ml L-tryptophan.

Trypsin cleavage assay

Trypsin cleavage of WT and mutant TRAP proteins was performed as described previously (15). TRAP (37 μM) was

incubated in the absence or presence of equimolar (to TRAP subunits) tryptophan in 50 mM sodium phosphate (pH 8.0) with 100 mM NaCl at room temperature for 5 min. Trypsin (5% w/w) (Sigma) was then added and aliquots were removed at various times, from 0 to 30 min. The reactions were stopped by adding an equal volume of 2× SDS gel loading dye and analyzed by 16% Tris–Tricine SDS–PAGE. Gels were stained with GelCode Blue stain (Pierce). Bands were quantified using ImageQuant (Molecular Dynamics) and the results were plotted as percentage cleavage versus incubation time with trypsin.

RNA synthesis

^{32}P-labeled RNA for binding assays was prepared by *in vitro* transcription of HindIII-linearized pTZ18U(GAGTT)$_{11}$ with T7 RNA polymerase in the presence of [α^{32}P]UTP as described previously (26). The RNA was gel purified on 8 M urea/10% polyacrylamide gels and extracted by a crush and soak procedure (26).

RNA and tryptophan binding assays

RNA binding to TRAP was measured by a nitrocellulose filter binding assay described previously (26). Samples containing ^{32}P-labeled (GAGUU)$_{11}$ RNA (1×10^4 d.p.m.) and increasing amounts of TRAP were incubated at 37°C for 1 h in the absence or presence of 1 mM tryptophan before filtering. The data were analyzed using a nonlinear least squares fitting to single binding site equation (7) (Prizm, Graph-pad Software Inc., San Diego, CA).

Tryptophan binding to TRAP was measured by a fluorescence assay based on competition between tryptophan and ANS (1-anilinonapthalene-8-sulfonic acid) as described previously (17). TRAP (1 μM) was incubated with 5 μM ANS, which is weakly fluorescent by itself but fluoresces strongly at 460 nm (excitation at 372 nm) upon binding to TRAP. Increasing amounts of tryptophan were then added and incubated for 1 min, which was found to be adequate to reach equilibrium. The decrease in fluorescence at 460 nm as a function of tryptophan concentration was determined and fit to the Hill equation [Prizm, Graph-pad Software Inc., San Diego, CA (17)].

RESULTS

T30V TRAP mediated regulation of the *trp* operon *in vivo*

T30V TRAP has been shown to bind *trp* leader RNA constitutively *in vitro* although with lower affinity than tryptophan-activated WT TRAP (16,17). We compared the ability of WT TRAP and T30V TRAP to regulate a *trpE′-′lacZ* translational fusion under control of the *trp* promoter and leader region *in vivo*. This fusion was not regulated in the absence of TRAP (Table 1). As seen previously (16,21), β-galactosidase expression was regulated 200-fold in response to tryptophan in cells containing WT TRAP (Table 1). The level of expression from this fusion in the strain lacking TRAP was nearly 10-fold higher than in WT TRAP containing cells grown in the absence of tryptophan. This difference likely reflects the intracellular levels of tryptophan present in BYC1, which is *trp*$^+$, when these cells are grown in the absence of exogenous tryptophan. In contrast to the results with WT TRAP, cells containing T30V TRAP showed no regulation in response to tryptophan. However, the presence of T30V TRAP (either in the absence or presence of tryptophan) reduced β-galactosidase expression ~15-fold as compared to BYC1 without TRAP, and ~2-fold compared that seen for cells with WT TRAP grown in the absence of tryptophan. These results show that T30V TRAP down-regulated expression of the fusion but did not respond to tryptophan. Hence the ability of T30V TRAP to regulate the *trpE′-′lacZ* fusion closely parallels its activity *in vitro*, i.e. low affinity for RNA that is not affected by the presence of tryptophan correlates with modest regulation that does not respond to tryptophan.

Table 1. Regulation of the *trpE′-′LacZ* by WT and T30V TRAP

Strain[a]	β-Galactosidase activity[b] −Trp	+Trp	Inhibition ratio (−Trp/+Trp)
No TRAP	768 ± 31	734 ± 24	1.0
WT TRAP	80 ± 5	0.4 ± 0.1	200
T30V TRAP	50 ± 4	46 ± 5	1.1

[a]*B.subtilis* strain, BYC1 *(ΔmtrB trpE′-′lacZ)* transformed with pVP5, or pVP5 containing either the WT or T30V *mtrB* gene. Cells were grown in the absence or presence of 50 μg/ml tryptophan.
[b]β-Galactosidase activity is given in Miller Units.

Trypsin cleavage of WT and T30V TRAP

Relatively little is known about the mechanism by which tryptophan binding activates TRAP to bind RNA. The binding sites for tryptophan and RNA are distinct and the closest atom of a bound tryptophan is ~7.5 Å from the bound RNA (4). Several studies suggest that tryptophan binding induces a conformational change in the protein that alters the RNA binding region (15,27). In particular, NMR studies indicate that tryptophan binding reduces the conformational flexibility of the protein in the vicinity of the bound tryptophan and that these changes in dynamics are propagated to the RNA binding surface of TRAP (15). These authors also showed that tryptophan-bound TRAP is resistant to cleavage by trypsin whereas apo-TRAP (in the absence of tryptophan) is cleaved after Arg31, which is in close proximity to the tryptophan binding pocket (15).

Recent studies have shown that T30V TRAP and tryptophan-activated WT TRAP recognize similar features of the (G/U)AG RNA repeats, which suggests that these two proteins may function similarly (17). To test the possibility that the T30V substitution alters the mutant protein so that it resembles tryptophan activated WT TRAP, we examined the susceptibility of T30V TRAP to trypsin cleavage. As seen previously with *Bacillus stearothermophillus* TRAP (15), WT *B.subtilis* apo-TRAP was much more susceptible to trypsin cleavage than the TRAP–tryptophan complex (Figure 1). The migration of the fragments obtained by trypsin cleavage of both *B.subtilis* and *B.stearothermophillus* TRAP was similar on SDS gels (data not shown), suggesting that the site of cleavage is the same (after residue 31) in both proteins.

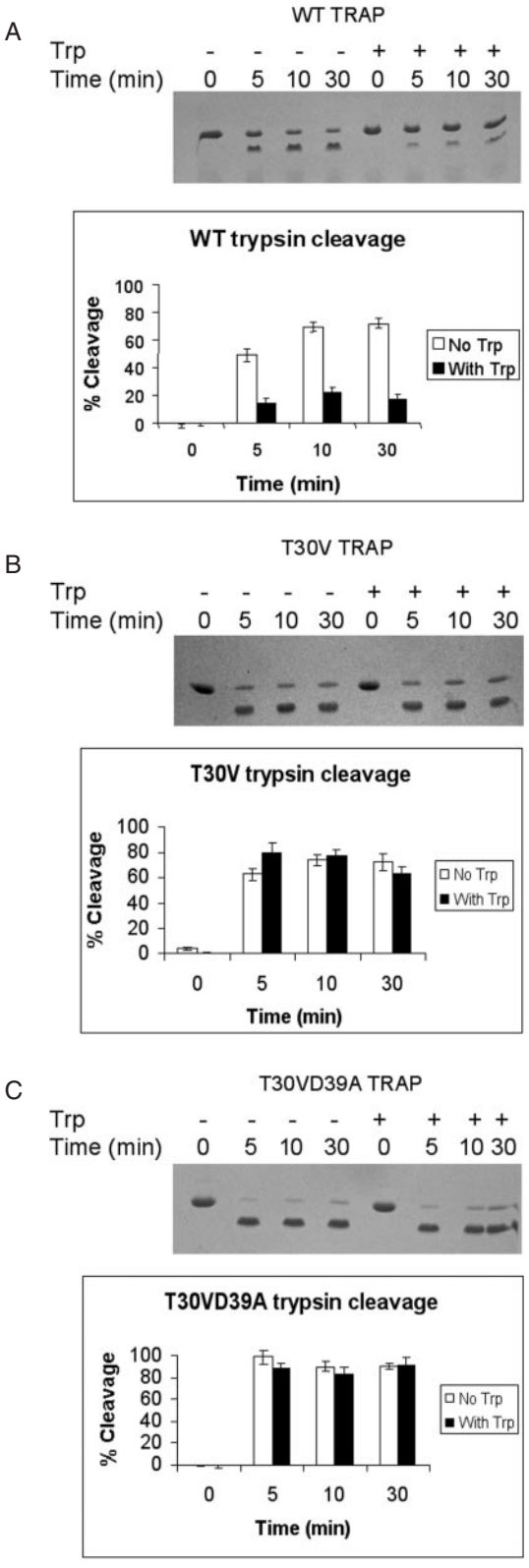

Figure 1. Trypsin cleavage of WT and mutant TRAP proteins. TRAP proteins were digested with 5% trypsin either in the absence (lanes 1–4) or presence (lanes 5–8) of tryptophan and samples were removed at various times. Cleavage was monitored on 16% SDS–polyacrylamide gels that were stained with GelCode Blue (Pierce). Bands were quantitated using ImageQuant (Molecular Diagnostic) and the results were plotted as percentage cleavage versus incubation time with trypsin.

Table 2. RNA and tryptophan binding affinities of WT and TRAP mutants with substitutions at position 30

TRAP	$(GAGUU)_{11}$ RNA binding K_d (nM)[a]		Tryptophan binding $S_{0.5}$ (μM)[b]
	+Tryptophan	−Tryptophan	
Wild Type	1.0 ± 0.5	NB[c]	1.5 ± 0.2
T30A	NB	NB	NB
T30V	144 ± 22	115 ± 16	NB
T30I	110 ± 10	177 ± 20	NB
T30S	1.0 ± 0.5	NB	2.0 ± 0.3
T30C[d]	8.0 ± 0.1	NB	3.0 ± 0.1
T30L	158 ± 18	NB	1.4 ± 0.3
T30P	180 ± 30	NB	1.1 ± 0.3
T30D	230 ± 6	NB	3.3 ± 0.3
T30E	335 ± 5	NB	4.5 ± 0.2
T30N	70 ± 7	518 ± 70	4.5 ± 1.0
T30Q	200 ± 30	620 ± 50	3.4 ± 0.3

[a]K_d values were determined in the absence or presence of 1 mM tryptophan. The data represent averages of the results of at least two experiments each performed in duplicate.
[b]$S_{0.5}$ is the concentration of free tryptophan at which the concentration of bound tryptophan reaches 50% of the saturation level.
[c]NB, no binding detected.
[d]Assays performed in the presence of DTT.

If the T30V substitution activates TRAP to bind RNA by a similar mechanism as tryptophan binding, then we might expect the mutant protein to be resistant to trypsin cleavage. However, contrary to this prediction, the efficiency of trypsin cleavage of T30V TRAP was comparable to that of apo-TRAP (Figure 1). T30V TRAP was equally sensitive to trypsin cleavage in the absence or presence of tryptophan, which is consistent with earlier findings demonstrating that this protein does not bind tryptophan (16,17). Hence the decrease in accessibility of residue 31 to trypsin cleavage seen with WT TRAP is not essential for RNA binding to TRAP, at least in the case of T30V TRAP.

Other substitutions at position 30

Previous studies of TRAP with Ala and Val substitutions at position 30 suggested that this residue plays a key role in tryptophan-induced activation of TRAP (16). We further examined the role of this residue in activation of TRAP by introducing several other substitutions that altered the size and/or the hydrogen bonding properties of the side chain of residue 30. In each case we tested the tryptophan- and RNA-binding activities of the resulting mutant protein (Table 2). The mutant proteins that we examined can be divided into four classes based on their RNA binding properties: (i) those that do not bind RNA in either the absence or presence of tryptophan; (ii) those similar to T30V, which bind RNA constitutively and are not affected by the presence of tryptophan; (iii) those similar to WT TRAP, which bind RNA in a tryptophan-dependent manner and (iv) those that bind RNA in the absence of tryptophan but their affinity for RNA is increased in the presence of tryptophan, which we call partially constitutive. T30A TRAP is the only member of the first class of mutant proteins that do not bind RNA irrespective of the absence or presence of tryptophan. Other than T30V, only T30I TRAP displayed strictly constitutive RNA binding. T30I bound RNA with similar affinity as T30V TRAP and also showed no detectable tryptophan binding (Table 2).

Five of the mutant proteins displayed tryptophan-dependent RNA binding with affinities ranging from $K_d \approx 1$ nM (similar to WT TRAP) to over 300 nM (Table 2). Of these, T30S and T30C showed the highest affinity for RNA. Like Thr in WT TRAP, both Ser and Cys contain uncharged polar side-chains that can accept a hydrogen bond from the amino group of tryptophan, suggesting that these are important properties for residue 30 in tryptophan-mediated activation of TRAP. Several other proteins in this group showed 160- to 330-fold lower affinity for RNA than WT TRAP. These include T30D ($K_d = 230$ nM) and T30E ($K_d = 335$), which have side chains that can hydrogen bond with the NH group of bound tryptophan but also introduce a negative charge into the binding pocket. Two other members of this group, T30P and T30L, do not have a polar side chain, and thus cannot accept a hydrogen bond from the amino group of tryptophan. Proline's cyclic side chain introduces conformational constraints on the protein backbone. Introduction of proline at residue 30 may alter the structure of the tryptophan binding site in a manner that allows tryptophan to bind to T30P TRAP in the absence of this hydrogen bond, and activate the protein to bind RNA. The most unusual member of this group is T30L TRAP. Both leucine and valine have similar aliphatic side chains with leucine's being just one methyl group larger than valine's, yet the T30V and T30L TRAP proteins display rather different properties (Table 2). One difference between these amino acids is that valine is β-branched, whereas leucine is γ-branched.

Because all the mutant proteins in this class bound tryptophan with affinities no more than 3-fold lower than WT TRAP (Table 2), the reduced affinity for RNA seen in some cases cannot be due to partial saturation with tryptophan under the conditions of the RNA binding assay (1 mM L-tryptophan) but must be due to partial activation the proteins.

In the last group, which we designate partially constitutive, T30N or T30Q TRAP bound RNA in the absence of tryptophan ($K_d = 500$–600 nM) but their affinity for RNA was enhanced 3- to 7-fold in the presence of tryptophan (1 mM). Both proteins bound tryptophan with affinity similar to WT TRAP ($S_{0.5}$ 3–5 μM). Hence the reduced affinity for RNA that these proteins display in the presence of tryptophan is not due to partial saturation of their tryptophan binding sites. Both of these mutant TRAP proteins contain residues at position 30 that are larger than Thr, and that have polar uncharged side chains with amino groups.

Substitution of RNA binding residues in T30V TRAP

Previous studies demonstrated that, in general, T30V TRAP recognizes similar features of the (G/U)AG repeats in RNA as does tryptophan-activated WT TRAP (17). However, from these studies it was not clear whether the same amino acid residues on both proteins are involved in binding RNA. For WT TRAP these residues are principally Glu36, Lys37, Lys56 and Arg58 (10–12,28). As seen previously (12), replacing any of these residues with an Ala residue reduced the affinity of WT TRAP for RNA from 26-fold to over 400-fold while having only minor effects on tryptophan binding (Table 3). To determine if these residues are important for RNA binding in T30V TRAP, we examined the effects of combining Ala substitutions at each of these positions with the T30V substitution. Replacing Glu36, Lys56 or Arg58, with Ala reduced the affinity of T30V TRAP for RNA (Table 3). With T30VK56A TRAP the effect was 2.5-fold while the T30VR58A and T30VE36A doubly-altered TRAPs showed no detectable RNA binding. Hence these three residues play significant roles in binding RNA with both the WT and T30V proteins.

Table 3. Substitutions of RNA binding residues in WT and T30V TRAP

TRAP	$(GAGUU)_{11}$ RNA binding K_d (nM)[a]		Tryptophan binding $S_{0.5}$ (μM)[b]
	+Tryptophan	−Tryptophan	
Wild-type	1.0 ± 0.5	NB[c]	1.5 ± 0.2
K37A	130 ± 20	NB	6.8 ± 0.4
K56A	35 ± 4	NB	5.8 ± 2.0
R58A	26 ± 3	NB	2.0 ± 0.5
E36A	407 ± 42	NB	8.5 ± 1.1
D39A	0.25 ± 0.05	NB	2.7 ± 0.2
T30V	144 ± 22	115 ± 16	NB
T30VK37A	101 ± 25	92 ± 4	3.4 ± 1.1
T30VK56A	360 ± 24	NB	2.1 ± 0.2
T30VR58A	NB	NB	2.2 ± 0.4
T30VE36A	NB	NB	2.9 ± 0.3
T30VD39A	6 ± 1	7 ± 1	2.8 ± 0.1
T30VD39V	126 ± 6	127 ± 5	5.3 ± 0.5

[a]K_d values were determined in the absence or presence of 1 mM tryptophan. The data represent averages of results from at least two experiments, each performed in duplicate.
[b]$S_{0.5}$ is the concentration of free tryptophan at which the concentration of bound tryptophan reaches 50% of the saturation level.
[c]NB, no binding detected.

Substituting Lys37 with an Ala had different effects on RNA binding to WT TRAP as compared to T30V TRAP (Table 3). Several structures of TRAP–RNA complexes show that the main-chain amino and carboxyl groups of Lys37 hydrogen bond with the base of the A residue of each GAG or UAG repeat (10,11). In addition, the aliphatic portion of side-chain of Lys37 makes hydrophobic interactions with the first base of the repeat. Consistent with these structural data, a series of substitutions at residue 37 showed that decreasing the length of the side chain resulted in corresponding decreases in affinity of the resulting mutant proteins for RNA (10,12). In contrast, substituting Lys37 with Ala in T30V TRAP had no significant affect on the affinity of T30VK37A TRAP for $(GAGUU)_{11}$ RNA as compared to T30V TRAP (Table 3). Similar results were obtained when comparing T30V and T30VK37A TRAP binding to an RNA with U in the first position of the repeats: $(UAGUU)_{11}$ RNA ($K_d = 136$ and 115 nm, respectively). These observations indicate that the aliphatic side chain of Lys37 does not play the same role in T30V TRAP binding to RNA that it does in WT TRAP. This difference may, at least in part, explain the lower affinity of T30V TRAP for RNA as compared to tryptophan-activated WT TRAP.

Substitutions at residue 39 of T30V TRAP

The results above, comparing the role of Lys37 in WT and T30V TRAP, suggest that these two proteins interact differently with the first residue of the (G/U)AG repeats. The

crystal structures of several TRAP–RNA complexes indicate that the side chain of Asp39 forms a hydrogen bond with the first G or U of the triplet repeats of the bound RNA (10,11). However we previously observed that replacing Asp39 with Ala does not reduce the affinity of TRAP for RNA (12) (also see Table 3). To further examine the interaction of T30V TRAP with the first G or U of the repeats, we determined the effects of substituting Asp39 with an Ala residue. The T30VD39A double mutant protein retained tryptophan-independent RNA binding and showed a 24-fold increased affinity for $(GAGUU)_{11}$ RNA as compared to T30V TRAP (Table 3). Similar results were obtained for binding to $(UAGUU)_{11}$ RNA (data not shown). Hence, while substituting Asp39 with Ala has only a limited (4-fold) effect on tryptophan-activated WT TRAP, this substitution significantly (24-fold) increases the affinity of T30V TRAP for RNA.

The Ala side chain is neutral and smaller than the negatively charged side chain of Asp. To investigate the importance of the size of the side chain at position 39 on the RNA binding properties of T30V TRAP, we substituted Val at this position, which is also neutral but is larger than Ala. T30VD39V TRAP showed $\approx$ 20-fold lower affinity for $(GAGUU)_{11}$ RNA than T30VD39A TRAP, resulting in similar affinity for this RNA as T30V TRAP; binding remained tryptophan independent (Table 3). Thus a side chain that is larger than Ala at position 39 interferes with the binding of T30V TRAP to RNA.

Trypsin cleavage of T30VD39A TRAP

Prior studies demonstrated a correlation between tryptophan activation of TRAP and protection from trypsin cleavage after residue 31 (15). There are several possible explanations for these observations. One possibility is that the bound tryptophan directly hinders trypsin's ability to cleave TRAP after residue 31. Alternatively, tryptophan binding may bring about a conformational change in TRAP that reduces the susceptibility to trypsin; this change may, or may not be, responsible for activating the protein to bind RNA. We have shown that T30V TRAP binds RNA but is susceptible to cleavage by trypsin (Figure 1). However, since this mutant protein does not bind tryptophan it is not possible to test the first possibility using this protein. Moreover, since T30V binds RNA with $\approx$100-fold lower affinity than tryptophan-activated WT TRAP, it is also possible that the conformational change which protects residue 31 may only be associated with the higher affinity RNA binding seen with WT TRAP. The T30VD39A double mutant protein provides an opportunity to test both possibilities. This protein binds RNA constitutively with high affinity ($K_d \approx 6$ nM) and also binds tryptophan with similar affinity as WT TRAP (Table 3). Hence if the bound tryptophan directly protects residue 31 from trypsin then T30VD39A should be less sensitive to cleavage in the presence of tryptophan than in its absence. On the other hand, if protection of residue 31 reflects a conformational change required for high affinity binding to RNA then this protein might be resistant to trypsin cleavage in the absence or presence of tryptophan. In contrast to both predictions, we found that T30VD39A TRAP was cleaved efficiently by trypsin both in the presence and absence of tryptophan (Figure 1). This observation indicates that the presence of tryptophan in the binding pocket is not sufficient to protect residue 31 from trypsin. Moreover, any conformational changes that protect residue 31 are not essential for high affinity RNA binding (at least in the case of T30VD39A TRAP). Hence the significance of the protection of residue 31 by tryptophan binding to WT TRAP remains unclear.

Effects of substitutions of RNA binding residues on tryptophan binding to T30V TRAP

Altering any of the residues in the RNA binding region of T30V TRAP affected the tryptophan binding properties of the resulting double mutant proteins. T30V TRAP does not bind tryptophan (16,27), whereas all of these double mutant proteins bound tryptophan with similar affinity as WT TRAP (Table 3). We addressed whether tryptophan binding to these mutant proteins affects their RNA binding properties. For T30VK37A, tryptophan binding had no appreciable effect on the affinity of the protein for RNA ($K_d \approx 100$ nM in the absence or presence of tryptophan). For T30VE36A and T30VR58A TRAP, RNA binding was undetectable in the absence or presence of tryptophan. However, since both Glu36 and Arg58 are crucial residues for binding RNA (replacing either of these resides with Ala severely affects the interaction with WT TRAP to RNA (12) (Table 3), the lack of RNA binding to T30VE36A and T30VR58A TRAP may not be indicative of whether tryptophan binding has any effect on the RNA binding region of these proteins.

For T30VK56A TRAP, the affinity for RNA increased from being undetectable in the absence of tryptophan to a K_d of 360 nM in the its presence (Table 3). This protein therefore provided an opportunity to further investigate why changes in the RNA binding region affect tryptophan binding at a site $\approx$ 7.5 Å away (there are over 10 Å separating the closest atoms of Lys56 and the bound tryptophan). Replacing Lys56 with Ala both shortens the side chain and eliminates its charge. We made several additional substitutions at position 56 to examine the importance of both of these properties on tryptophan binding and activation of double mutant proteins that also contain the T30V change (Table 4).

Table 4. Effect of substitutions at position 56 in T30V TRAP on tryptophan and RNA binding properties

TRAP	$(GAGUU)_{11}$ RNA binding K_d (nM)[a]		Tryptophan binding $S_{0.5}$ (μM)[b]
	+tryptophan	−Tryptophan	
Wild-type	1.0 ± 0.5	NB[c]	1.5 ± 0.2
K56A	35 ± 4	NB	5.8 ± 2.0
T30V	144 ± 22	115 ± 16	NB
T30VK56A	360 ± 24	NB	2.0 ± 0.3
T30VK56V	98 ± 9	NB	1.3 ± 0.6
T30VK56L	93 ± 7	NB	4.6 ± 0.4
T30VK56M	NB	NB	NB
T30VK56E	NB	NB	NB

[a]K_d values were determined in the absence or presence of 1 mM tryptophan. The data represent averages of results from at least two experiments each performed in duplicate.
[b]$S_{0.5}$ is the concentration of free tryptophan at which the concentration of bound tryptophan reaches 50% of the saturation level.
[c]NB, no binding detected.

Changing residue 56 to Val or Leu, both of which have small hydrophobic side chains, resulted in double mutant proteins with similar tryptophan- and RNA-binding properties as the T30VK56A mutant protein. However, increasing the size of the side chain at position 56 by substituting with Met eliminated tryptophan and RNA binding to T30VK56M. Substituting Lys56 with Glu, which is negatively charged and larger than Ala or Val, also eliminated tryptophan and RNA binding (T30VK56E). Therefore the presence of a small uncharged side chain at position 56 appears to be important in allowing tryptophan to bind to and activate the double mutant proteins that also contain the T30V substitution.

DISCUSSION

Tryptophan-activation of TRAP to bind its target RNAs is central to regulation of expression of the *trp* genes in *B.subtilis*. However, the precise mechanism of tryptophan-activation is not known, in part due to lack of a high resolution structure of apo-TRAP (i.e. TRAP in the absence of bound tryptophan). In this study, we provide insight into tryptophan-activation of TRAP by studying a mutant protein (T30V) that binds RNA in the absence of bound tryptophan. T30V TRAP recognizes similar RNA features as does WT TRAP (17). Our results show that three of the four principal RNA binding residues, Glu36, Lys56 and Arg58, are important for interacting with RNA in both WT and T30V TRAP (Table 3). Lys37 is important for RNA binding to WT TRAP but not for binding to T30V TRAP.

The side chain of Thr30 hydrogen bonds with the amino group of the bound tryptophan (4). Our results with mutant proteins bearing substitutions at position 30 indicate that as long as the hydrogen bonding potential of this side chain is maintained, TRAP can bind tryptophan and become activated to bind RNA. Some mutant proteins bind RNA with lower affinity than WT TRAP even though they are fully saturated with tryptophan (Table 2: T30D, T30E, T30N and T30Q). The tryptophan analog indole-3-propionic acid lacks the amino group of tryptophan and thus is unable to hydrogen bond with Thr30. This analog does not bind to either WT or T30V further demonstrating the importance of this interaction (17). Together these observations provide further support for the importance of residue 30 in both tryptophan binding and activation of RNA binding as well as show that these processes can be distinct from each other.

To date, mutants with substitutions at Thr30 are the only examples of altered TRAP proteins that bind RNA constitutively in the absence of bound tryptophan. These include T30V and T30I, which suggests that constitutive binding is the result of the presence of a intermediate sized hydrophobic side chain at position 30 that cannot hydrogen bond with tryptophan (16). However, T30L TRAP, with Leu at position 30, does not bind RNA constitutively but instead shows tryptophan-dependent RNA binding. Hence, a hydrophobic residue at position 30 alone is not sufficient to activate TRAP to bind RNA (and the ability of the side chain at residue 30 to hydrogen bond is not essential for tryptophan binding or activation). While Val, Ile and Leu all have small aliphatic side chains, only the Val and Ile side chains are

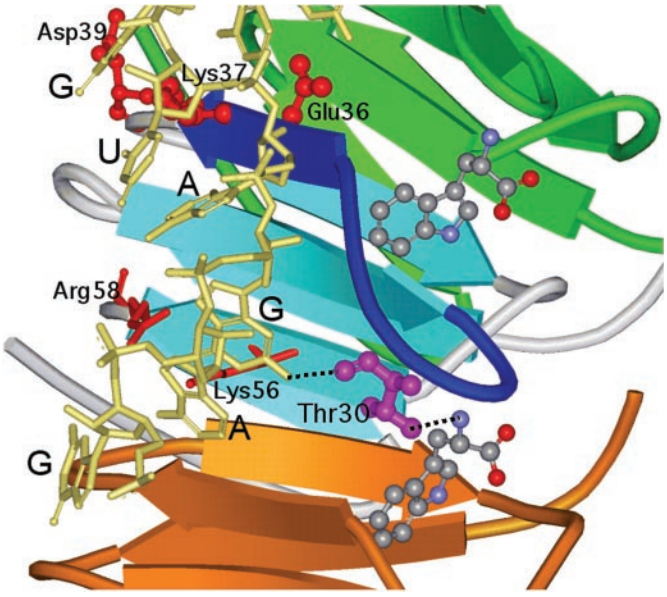

Figure 2. Ribbon diagram of TRAP complexed with RNA. One complete subunit (light blue) and parts of the two adjacent subunits (green and brown) in TRAP are shown. The loop formed from residues 25–33 and the adjacent β-strand (residues 34–38) proposed to be key in tryptophan-mediated activation of RNA binding are highlighted in dark blue. The β-strands are depicted as arrows and the bound tryptophans as ball and stick models. The side chains of several key amino acids are shown in either ball and stick models on the complete subunit. RNA binding residues are in red, Thr30 is in magenta. The RNA is shown as golden stick diagrams and the bases from one GAGAU repeat are labeled. The hydrogen bonds between Thr30 and the bound tryptophan as well as to the third G are shown as dashed lines.

β-branched, suggesting this property may be important to induce tryptophan-independent RNA binding. β-Branching of the side chain restricts the conformations that the peptide backbone can adopt due to steric considerations (29,30). Hence, the presence of the β-branched Val or Ile residues at position 30 may bring about changes in the protein backbone that activate these mutant proteins to bind RNA.

Perhaps one reason that Thr30 plays a key role in activation of TRAP is that it is the only residue in the protein that interacts with both the bound tryptophan and bound RNA. The side chain hydroxyl of Thr30 hydrogen bonds with the amino group of the bound tryptophan. In the complex with $(GAGAU)_{11}$, the main chain carbonyl oxygen of Thr30 hydrogen bonds with the NH2 of the third G of each repeat (Figure 2), and makes a hydrogen bond to the 2′ sugar hydroxyl of the preceding A via a bridging water molecule (11). Therefore, changes at residue 30 have the potential to directly affect both the tryptophan and RNA binding properties of TRAP.

NMR studies have demonstrated that tryptophan binding reduces the flexibility of TRAP (15). These authors also showed that tryptophan binding to WT TRAP results in changes in the vicinity of residue 31 that reduce accessibility of this residue to trypsin. However, it was not clear whether these changes affecting residue 31 are directly tied to activation of TRAP to bind RNA. Given that we have shown that Thr30 plays a key role in activation of TRAP, it was reasonable to propose that these changes in the protein in the vicinity of residue 31 might be associated with activation.

However our results with several mutant proteins do not support this hypothesis. Both T30V and T30VD39A TRAP, which bind RNA constitutively, are susceptible to cleavage by trypsin. Hence the changes that protect residue 31 are not essential for RNA binding, at least not with these mutant proteins. Moreover, comparing the properties of WT, T30V, T30VD39A TRAP shows that there is no correlation between the affinities of these proteins for RNA and their sensitivity to trypsin cleavage. In addition, T30VD39A TRAP binds tryptophan (with no effect on the affinity for RNA) yet this protein is cleaved by trypsin equally well in the absence or presence of tryptophan. Together these observations show that the changes in the vicinity of residue 31 which protect TRAP from trypsin cleavage are not required for TRAP to bind RNA. It is also possible that the changes at residue 31 reflect an alteration in the protein required for tryptophan-activation of WT TRAP, and the mutant proteins are activated to bind RNA by a different means. However our data showing that T30V TRAP recognizes similar features of the RNA and uses most of the same residues in the protein to bind to it, suggests that the mutant proteins are activated by at least a similar mechanism as tryptophan binding to WT TRAP.

Our results suggest that although WT and T30V TRAP recognize similar features of RNA (17), these two proteins interact differently with the first base of each (G/U)AG repeat. Several crystal structures of TRAP complexed with RNA show that both Lys37 and Asp39 interact with the first G or U of the repeats (10,11) (Figure 2). We found that substitutions at Lys37 and Asp39 have different effects on WT and T30V TRAP. Consistent with the structural data, substitution of Lys37 with Ala in WT TRAP is deleterious to binding RNA (Table 3). In contrast, this change has no significant effect on the RNA binding affinity of the T30V protein. Conversely, changing Asp39 to Ala has no effect on WT TRAP binding to RNA yet this change increases affinity for RNA with T30V TRAP. The results with Asp39 are particularly interesting because several crystal structures indicate that this residue forms a hydrogen bond with the first G or U in the RNA repeats, yet we can find no evidence that this interaction contributes to the stability of the complex of WT TRAP with RNA [Table 3; (12)]. In T30V TRAP it seems that the side chain of residue 39 interferes with RNA binding when it is larger than the methyl group of Ala. These differences between T30V and WT TRAP were observed regardless of whether there was a G or a U in the first position of the RNA repeats. Together these observations suggest that the orientations of Lys37 and Asp39 are different between complexes of T30V and tryptophan-activated WT TRAP with RNA. These differences may, at least in part explain the lower affinity of T30V TRAP than WT TRAP for RNA.

Altering residues in the RNA binding region of T30V TRAP to Ala had the unexpected effect of restoring tryptophan binding activity to this protein, which otherwise does not bind tryptophan (Table 3). Further investigation of this finding by making changes at Lys56 showed that residues with smaller side chains at position 56 allow the mutant proteins to bind tryptophan, whereas those with larger side chains do not (Table 4). There are two possible explanations for these observations. First, the side chain of residue 56 could directly interfere with tryptophan accessing its binding site. Alternatively, changes at residue 56 could affect the conformation of the protein and thereby influence its tryptophan binding properties indirectly. The structure of TRAP complexed with RNA shows that Lys56 is over 10 Å from the bound tryptophan (4). This would seem to argue against the simple steric hindrance model. However, the location of Lys56 in apo-TRAP is not known. Several observations suggest there must be a significant difference in the arrangement of the tryptophan binding site in apo-TRAP as compared to the complex with tryptophan, which could also be the case in T30V TRAP. For example, in the WT TRAP–tryptophan complex the bound tryptophan is almost completely buried in the binding pocket (4). Hence for tryptophan to enter the binding site, it must be in a more open conformation in apo-TRAP. Consistent with this proposal, NMR studies show that the tryptophan binding region of the protein is disordered in the absence of tryptophan and then becomes ordered when tryptophan binds (15). Thus with T30V TRAP it is possible that a large side chain at position 56 might directly create steric hindrance to tryptophan binding in its binding pocket.

In addition to TRAP-mediated regulation of the *trp* genes in response to changes in intracellular free tryptophan, *B.subtilis* also regulates expression of these genes in response to accumulation of uncharged $tRNA^{Trp}$ (31–33). Accumulation of uncharged $tRNA^{Trp}$ leads to expression of the anti-TRAP (AT) protein, which antagonizes TRAP activity, thus increasing expression of the *trp* genes (33,34). AT does not bind to apo-TRAP but specifically binds to tryptophan-activated TRAP and prevents it from binding to RNA (34,35). Studies of AT binding to mutant TRAP proteins suggest that AT recognizes similar features of TRAP that are essential for binding RNA, including Lys37, Lys56 and Arg58 (35). Preliminary studies indicate that AT does not bind to T30V TRAP *in vitro* (Y. Chen, D. Paul and P. Gollnick unpublished data). These observations suggest that while T30V TRAP functions similarly as tryptophan-activated WT TRAP, the RNA binding surfaces of the two proteins are not identical. Our studies suggest that Lys37 may be positioned differently between the two proteins, which may explain why T30V TRAP is not recognized by AT.

One of the most extensively studied proteins that shows ligand induced activation of nucleic acid binding is the Trp repressor of *E.coli* (36). Upon binding two molecules of L-tryptophan, the Trp repressor dimer binds to its operator DNA and blocks RNA polymerase from binding to the promoter. Each monomer is composed of six α helices, two of which form a helix–turn–helix (HTH) motif that contains the amino acid residues that interact directly with the DNA (37–39). Tryptophan binding induces the HTH substructures from each subunit to move apart so that they can fit into the two successive major grooves of the DNA, which is essential for operator specific recognition (40). In addition, tryptophan binding also stabilizes the HTH substructures, which are more flexible in the absence of tryptophan (37,41). Therefore, in part, tryptophan binding activates the Trp repressor to bind operator DNA by a similar mechanism involving changes in protein flexibility that has been proposed for TRAP activation (15).

Our results suggest that changes in the RNA binding and tryptophan binding regions of TRAP influence one another.

These changes may reflect the pathway that normally conveys changes when tryptophan binds to activate RNA binding. One possible means for this transfer of information between the two binding regions would be via the residues that most directly connect the two binding sites. According to this model, tryptophan binding would introduce changes in residues in its binding site—particularly Thr30—and these changes would be transmitted to the RNA binding site via residues most directly adjacent along the polypeptide backbone to one or more residues that contact the RNA. Two key residues that contact the RNA, Glu36 and Lys37, are directly linked to Thr30 by a portion of the loop made up of residues 25–33 and by the β-strand composed of residues 34 to 38 (Indicated in dark blue on Figure 2). The hypothesis that this means of communication is important in the activation mechanism is supported by results from previous mutagenesis studies (12). These studies showed that Ala substitutions in several of these connecting residues, including Lys31 and His33, interfere with TRAP-mediated regulation of the *trp* operon *in vivo* even though neither residue directly interacts with either tryptophan or RNA. Determining the tryptophan and RNA binding properties of these mutant proteins may provide additional insight into the role of these residues in the activation mechanism of TRAP.

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