Identification of a Region of Bacillus subtilis Ffh, a Homologue of Mammalian SRP54 Protein, That Is Essential for Binding to Small Cytoplasmic RNA*

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Kei Kurita, Kiyofumi Honda, Satoru Suzuki, Hiromu Takamatsu, Kouji Nakamura, and Kunio Yamanishi

From the Institute of Biological Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki 305, Japan

Bacillus subtilis Ffh and scRNA are homologues of mammalian SRP54 and SRP RNA, respectively, which are components of the eukaryotic signal recognition particle (SRP). Ffh (446 amino acids) interacts with scRNA to form a stable complex in vivo. Here, we identified an RNA-binding domain of Ffh. The results obtained using a series of deletion mutants show that amino acid positions 364 to 432 in the C-terminal region of Ffh correlates with its ability to bind RNA. The amino acid sequence of this region is well conserved among members of the SRP54 protein family. This sequence contains two hydrophobic regions (h2, 364 to 391, and h3, 416 to 435), separated by the positively charged amino acid motif, 398RRKRIAKGSG407. Among the basic amino acid residues in this region, Arg-401 was essential for binding to scRNA, but Arg-399 and Lys-400 were not. The corexistence of Arg-398 and Lys-404 was necessary for the same affinity as wild type Ffh. The two glycine residues of the 405GSG407 were also essential. MH23 peptide (91 amino acids) encompassing from 356 to 446, consisting of h2-RRKRIAKGSG-h3, bound scRNA with the same affinity as wild type Ffh, whereas a 24-amino acid synthetic peptide 392DIINASRRKRIAKGSGTSVQE-VNR415 did not. The region containing two hydrophobic segments separated by the positively charged motif is the minimal requirement of Ffh for RNA binding.

In mammals, the signal recognition particle (SRP) plays an important role in targeting secretory proteins to the membrane of the endoplasmic reticulum. Mammalian SRP is a ribonucleoprotein particle composed of one RNA molecule (7S RNA here referred to SRP RNA) and six polypeptides of 9, 14, 19, 54, 68, and 72 kDa (1, 2). SRP interacts with a signal sequence of a nascent polypeptide as it emerges from the ribosome, then the elongation of the nascent chain is inhibited (3, 4). The complex consisting of SRP, nascent polypeptide chain, and ribosome is targeted and binds to the heterodimeric SRP receptor in the endoplasmic reticulum membrane (3–5).

The SRP54 subunit of SRP has a central function in recognizing and binding signal sequences (6–8). This protein comprises the structurally distinct, N-terminal G- and C-terminal M-domains (9, 10). The G-domain contains three GTP-binding motifs, and it may play an essential role in mediating the interaction between SRP and the SRP receptor (11). The M-domain is characterized by a high content of predominantly positively charged residues and an abundance of methionine residues. Moreover, there are three putative amphipathic helices in the M-domain (9). The M-domain is a binding site for the signal sequence and SRP RNA (12–15). The three-dimensional structure of SRP54 protein has been determined by scanning transmission electron microscopy (STEM) (16). The scanning transmission electron microscopy image consisting of one larger and one smaller domain (probably representing the G- and M-domain, respectively) joined by slender linker, was in agreement with genetic and biochemical data.

A protein homologous to SRP54 has been identified in Eukaryotes and Eubacteria (3, 17–24). The features of the two structural domains (G- and M-domains) of SRP54 are highly conserved in these proteins. Furthermore, these proteins form an SRP-like complex with RNA molecules that are structurally related to mammalian SRP RNA (25–28). The binding of Ffh to 4.5 S RNA, which are homologues of SRP54 and SRP RNA in Escherichia coli, respectively, is essential for the signal recognition function (29). Larsen and Zweib (30) proposed that SRP RNA consists of 8 helices (numbered 1 to 8). The nucleotide sequence in the region corresponding to the helix 8 is highly conserved among SRP RNA homologues, and this loop region mediates the binding to SRP54 and its homologues (17, 31, 32). The SRP RNA homologue of Bacillus subtilis scRNA (small cytoplasmic RNA) lacks helices 6 and 7, whereas those of E. coli 4.5 S RNA lack helices 1, 2, 3, 4, 6, and 7 as well as part of 5 (30). Furthermore, mammalian SRP54 can bind to E. coli 4.5 S RNA in vitro (33). These findings indicated that the RNA-binding form of SRP and related complexes contain common features. The RNA-binding domain of the homologue of SRP54 in Mycoplasma mycoides is located in the M-domain (34), and it has been proposed that the hydrophilic faces of the predicted amphipathic α-helices in the M-domain contact SRP RNA (11, 14, 15). However, the RNA-binding region of Ffh has not yet been analyzed in detail.

B. subtilis can produce large amounts of extracellular enzymes. We demonstrated that it contains SRP-like particles and that they are closely involved in enzyme secretion (26). B. subtilis Ffh protein has features very similar to those of SRP54 (23). The Ffh also has G-domain and M-domain. A phylogenetic comparison of SRP54 protein has revealed three highly hydrophobic regions (hydrophobic regions 1, 2, and 3 are referred to as h1, h2, and h3) in the M-domain (23). The h1, h2, and h3 regions correspond to amino acid positions 307 to 331, 364 to 391, and 416 to 435, respectively, in B. subtilis Ffh. To deter-
mine the essential region of Ffh protein for the binding to sRNA of a homologue of SRP RNA, we constructed several mutant Ffh proteins of B. subtilis in which deletions and amino acid replacements were introduced. We then determined their RNA-binding activity using the filter assay (34). On the basis of the RNA-binding activity of wild-type and mutants of Ffh, we defined a minimal RNA-binding domain encompassing h2 to h3 in the M-domain. Moreover, in the 398RRKRI- amino acid replacements were introduced. We then determined mutant Ffh proteins of (35, 36). The nucleotide sequence of ACCGTC at position 1951 to 1953(TAKARASHUZOCo., Ltd., Tokyo) based on the method of Kunkel HI restriction fragment of plasmid pTUE920 was introduced into plasmid expressing -TTCAG-9. (5'-CTCATGCTGCTGCTGGC-3'), K371M (5'-GCAATCTGCTGCTGGC-3'), and QQQR-K404Q derivatives, RRKR-K404Q, RQQR-K404Q, and QQQR-K404Q, was constructed using the same oligonucleotide (5'-CCGGTCTCTTG- GAACTC-3'), PSS (5'-GGATGTCGGCTTGTATCTGCTGCTGCTGGC-3'), and GPS (5'-GGATGTCGGCTTGTCTCGTGTATCTGCTGCTGCTGGC-3'). Using the oligonucleotides and the single-stranded DNA described above as the template, the respective replacements and a short deletion were introduced, then the BstXI-BamHI fragment was ligated into the BstXI-BamHI site of pQE60BS. The plasmid expressing the three K404Q derivatives, RRKR-K404Q, RQQR-K404Q, and QQQR-K404Q, was constructed using the same oligonucleotide (5'-CCGGTCTCTTG-GAACTC-3'), and respective templates of single-stranded DNA. The single-stranded DNA templates for RRKR-K404Q, RQQR-K404Q, and QQQR-K404Q derivatives were prepared from M13mp19 which contained HindIII and BamHI fragments of the plasmid expressing Ffh-WT (pTUE920), RRKR, and QQQR, respectively. These plasmids were introduced by the BstXI-BamHI site ligated into BstXI-BamHI site of pQE60BS. Plasmid Construction to Prepare MH23 Polypeptide—The plasmid expressing the Ffh-MH23 was constructed as follows. A DNA fragment containing the synthetic oligodeoxynucleotide N3 (5'-GCCATGGGTGACGTGAATATCGAG-3') was inserted into the BstXI-BamHI site (pQE815) and the latter of which contained a novel Ncol site. This fragment was digested with BgII and NcoI and inserted into the BgII and NcoI site of pQE60BS. Synthesis of Peptide—A synthetic 24-mer peptide corresponding to the region between B. subtilis Ffh amino acid positions 392 to 415 (DINASRRKRIAGKGGTSQVSKRQQR, N1, K404Q) was synthesized on a 2000 peptide synthesizer (Lewisville, TX) by solid phase Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry (37). Upon completion of synthesis, the resin is treated with 95% trifluoroacetic acid, containing 1% triisopropylsilane as a scavenger. The peptide is purified using the BIOCAD perfusion chromatography system from PerSeptive Biosystems (Framingham, MA). The synthetic peptide is lyophilized and redissolved in 50% aqueous, also from PerSeptive Biosystems. A gradient from 20% solvent A to 100% solvent B is used. Solvent A is 20% aqueous acetonitrile containing 0.1% trifluoroacetic acid. Solvent B is 100% acetonitrile containing 0.1% trifluoroacetic acid. The purity of the peptide was more than 95%.

Expression and Purification of Ffh and Its Derivatives with the Hexahistidine Tag—Each recombinant protein was expressed in E. coli M15 harboring pRE4 (Qiagen) grown for 5 h at 37 °C in L-broth (150 ml) supplemented with 2 m IPTG. Cells were harvested and suspended in 10 ml of sonication buffer (20 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 150 mM NaCl, 10% glycerol, 0.05% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride). After freezing and thawing the suspension, a half-volume of glass beads was added, vortex-mixed for 10 min at 30 g, and centrifuged. This vigorously vortex-extracted for 10 min at 4 °C. The supernatant was separated from the centrifuged glass beads that went to the bottom and centrifuged at 10,000 × g for 15 min at 4 °C. The precipitate was dissolved in 10 ml of buffer A (0.1 mM NaH2PO4, 0.01 mM Tris-HCl (pH 8.0), 8 M urea) and centrifuged again at 12,000 × g for 15 min at 4 °C. The supernatant was dialyzed overnight against buffer A containing 100 mM NaPO4, 0.01 mM Tris-HCl (pH 8.0), 8 M urea, and 50 mM NaCl.)

Gel Retardation Assay—To prepare radiolabeled sRNA in vitro, a 120-bp DNA fragment of the B. subtilis rrnB operon was inserted into the EcoRI-BamHI sites of plasmid pSP64 (Pharmacia Biotech Inc.). The resulting plasmid, in which the gene encoding scRNA404 RNA (nucleotide positions 116 to 219 in the mature form of scRNA404) was positioned downstream of SP6 promoter in the correct direction, was designated pTUE953. To transcribe scRNA404 RNA in vitro, the plasmid pTUE953 linearized by BamHI digestion was incubated with 35 units of SP6 RNA polymerase in the transcription buffer (40 m Tris-HCl (pH 7.5), 2 mM MgCl2, 10 mM NaCl, 10 mM dithiothreitol, 2 mM spermidine) containing 0.5 mM each of dGTP, dATP, dUTP, 100 μM CTP, and 50 μCi of [γ-32P]CTP.
**FIG. 1. The binding of wild-type Ffh with scRNA.** A, gel retardation assay. 

$^{32}$P-labeled sc104 RNA was incubated with (lane 2) or without (lane 1) 0.5 pmol of Ffh for 30 min at 30°C. The specificity of the interaction between Ffh and scRNA was estimated in the presence of a 250-fold excess of unlabeled wild-type scRNA (sc271, lane 3), sc104 RNA (lane 4), and tRNA$^{\text{met}}$ (lane 5), as competitors in the presence of Ffh. Slowly migrating bands are indicated by an arrowhead. B, assay of the complex between wild-type Ffh and sc104 RNA on nitrocellulose filters. Various concentrations of wild-type Ffh were incubated with 0.15 nm labeled sc104 RNA, and the retention of the Ffh-sc104 RNA complex on nitrocellulose filters was monitored. RNA bound was defined as follows: percent RNA retained = retained labeled RNA on filter (cpm) / 100 added labeled RNA (cpm) at 37°C for 1 h.

To evaluate the binding activity, the proteins (5 pmol each) were incubated with labeled sc104 RNA (2 $\times$ 10$^3$ cpm) in a buffer (15 mM HEPES (pH 7.9), 50 mM KC1, 6 mM MgCl$_2$, 50 mM NaCl, 20% glycerol, 0.3% VRC, 1 unit of poly(d1-dC) for 1 min at room temperature. The mixtures were then diluted with 0.5 ml of wash buffer (20 mM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, 100 mM NaCl, 10% glycerol). The resulting solutions were immediately passed through nitrocellulose filters (ADVANCEC, 0.45 µm or 0.20 µm pore size; TOYO ROSHI KAISHA Ltd., Tokyo) and autoradiographed. The density of the shifted bands was quantified using a Bio Image Analyzer (Millipore/MilliGen).

**Nitrocellulose Filter Assay—The** $^{32}$P-labeled sc104 RNA (0.15 nm, 1.3 $\times$ 10$^3$ cpm/mmol) was incubated with various concentrations of Ffh and its derivatives (1 nm to 10 µM) in 10 µl of a buffer (10 mM Tris-HCl (pH 7.4), 10 µM MgCl$_2$, 50 mM NaCl, 0.3% VRC, 1 unit of poly(d1-dC)) for 1 min at room temperature. The mixtures were then diluted with 0.5 ml of wash buffer (20 mM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, 100 mM NaCl, 10% glycerol). The resulting solutions were immediately passed through nitrocellulose filters (ADVANCEC, 0.45 µm or 0.20 µm pore size; TOYO ROSHI KAISHA Ltd., Tokyo) and washed with 1.5 ml of wash buffer. The levels of radioactivity remaining on the nitrocellulose filters were determined by a liquid scintillation counter (Beckman, LS5000TA).

**RESULTS**

**Binding of Wild-type Ffh with sc104 RNA in Vitro—To prepare the purified Ffh protein, we constructed the E. coli plasmid pTUE920 that encoded Ffh with a hexahistidine tag at its C terminus. We then examined the effect of adding the hexahistidine tag to Ffh in vivo. The ffh conditional mutant, B. subtilis NA208F, in which the expression of ffh is controlled by the Pspsac-1 promoter (23), cannot grow in the absence of 1 mM IPTG. However, the growth in the absence of IPTG was complemented by the introduction of pTUBE911, in which the B. subtilis ffh gene was placed under the control of the protein A promoter, into the cells as well as by that of pTUBE917, in which a gene for ffh with a hexahistidine tag at its C terminus was placed under the control of the protein A promoter. Therefore, we constructed pTUE920 and prepared B. subtilis Ffh with a hexahistidine tag from an E. coli transformant as the wild-type Ffh of B. subtilis. The preparation was designated as Ffh-WT. The purity of the sample was over 85% according to laser densitometry of the Coomassie Brilliant Blue stained gel after SDS-polyacrylamide gel electrophoresis.**

As shown in Fig. 1A, a distinct band migrated much more slowly than the free sc104 RNA in a nondenaturing gel when $^{32}$P-labeled sc104 RNA, a derivative of scRNA, was incubated with Ffh-WT (lane 2). This band disappeared upon adding a 250-fold excess of non-radio-labeled scRNA (sc271) or sc104 RNA, but not 250-fold excess of tRNA as a competitor (lanes 3–5). Although a slowly migrating band was also detected using scRNA (sc271) as a probe in the presence of Ffh, it was closer to the band of the free probe than those of sc104 RNA (data not shown). Wild-type scRNA consisting of 271 nucleotides (sc271) was too large to be used as a probe in the gel retardation and filter binding assays. The Zuker minimal energy program (38) predicted that sc104 RNA could almost form a hairpin structure similar to that of wild-type of scRNA. Therefore, to monitor the formation of the complex between B. subtilis Ffh and scRNA in vitro, we used sc104 RNA corresponding to positions 116 to 219 of scRNA, which lacked helix 1–4, and most of helix 5, but remained helix 8. The size of sc104 RNA is similar to that of E. coli 4.5 S RNA, which is a counterpart of scRNA. Furthermore, a scRNA of smaller size is functional in vivo, since the sc104 RNA complements the growth defect caused by depleting scRNA from B. subtilis (39). In this study, sc104 RNA was used for the gel retardation and filter assays instead of wild-type scRNA (sc271). The dose dependence of the binding of wild-type Ffh and sc104 RNA was also examined by a filter binding assay. Up to 10 µM Ffh, the amount of sc104 RNA bound to Ffh increased as Ffh added (Fig. 1B). The binding curve of M. mycoides Ffh protein is also sigmoidal (34). The half-maximal binding (M$_{1/2}$) was approximately 0.15 µM.

**scRNA Binding by the Large Deletion Mutants of Ffh—To search the RNA-binding region of Ffh protein, purified mutant proteins were used in the RNA-binding assay. Ffh-3G lacks most of the G-domain (Fig. 2A), and it bound to sc104 RNA in the gel retardation (Fig. 2B) and filter assays (Fig. 2C). In contrast, Ffh-3M lacking the M-domain had no affinity for sc104 RNA in either assay (Fig. 2, B and C). Therefore, like mammalian SRP54, the RNA-binding domain of B. subtilis Ffh is also located in the M-domain. Since the binding activity data determined by the gel retardation and filter assays were consistent, we employed the filter assay to further studies as the quantitative analysis of the interaction between derivatives of Ffh and scRNA.**

Using a series of large deletion mutant proteins of Ffh (Fig. 3A), we searched the region that binds to scRNA in Ffh (Fig. 3B). Neither Ffh-3H3, Ffh-3H$^3$ (deleted amino acid positions 392 to 446 and 413 to 446, respectively), nor Ffh-3H2 (deleted amino acid positions from 338 to 397) bound to sc104 RNA. In contrast, Ffh-3H1 (deleted amino acid positions from 311 to 362) and Ffh-3C (deleted 14 amino acids at the C-terminal region) bound to sc104 RNA with the same affinity as that of wild-type Ffh. These results show that the region of Ffh spanning positions 364 to 432 and corresponding to the region from h2 to h3, is necessary for scRNA binding. Furthermore, the results using the other Ffh derivatives, Ffh-3H23 (deleted amino acids from position 364 to 446 including the h2 and h3 regions)
regions) and Ffh-Δh12 (deleted amino acids from position 311 to 415 including the h1 and h2 regions), were consistent with this conclusion, because these two Ffh derivatives could not bind sc104 RNA (Fig. 3B). When these mutant Ffh proteins were introduced into B. subtilis NA208F, only Ffh-ΔC completely restored the growth defect of NA208F in the absence of IPTG, whereas the others only partially did so (data not shown). All mutant Ffh protein except for Ffh-ΔG mutant was shown to hydrolyze GTP the same as Ffh-WT (data not shown).

scRNA-binding Region of Bacillus subtilis Ffh

A schematic structure of deletion derivatives of Ffh. The numbers below the name of each mutant protein indicate the deleted amino acid positions (Fig. 2). The numbers above indicate the approximate amino acid position of Ffh. The numbers on each diagram indicate the amino acid positions of the termini of deletion. The numbers below the name of each mutant protein indicate the amino acid length for Ffh-WT and the deleted amino acid positions for Ffh-ΔG and Ffh-ΔM. Gel retardation assays were performed by incubating 32P-labeled sc104 RNA with each Ffh protein (0.5 pmol) for 30 min at 30 °C. The Ffh proteins added to the reaction mixture were: lane 1, no Ffh proteins; lane 2, wild-type Ffh; lane 3, Ffh-ΔM; lane 4, Ffh-ΔG; arrowheads indicate the slowly migrating bands. C, filter assay of Ffh-ΔG and ΔM. Ffh-WT (●), Ffh-ΔG (○), and Ffh-ΔM (△) were monitored by means of the filter assay as described under “Experimental Procedures.”

RNA-binding activities of large deletion derivatives of Ffh. A, schematic structures of deletion derivatives of Ffh. The numbers below the name of each mutant protein indicate the deleted amino acid positions. B, gel retardation assay of Ffh-ΔG and ΔM. Gel retardation assays were performed by incubating 32P-labeled sc104 RNA with each Ffh protein (0.5 pmol) for 30 min at 30 °C. The Ffh proteins added to the reaction mixture were: lane 1, no Ffh proteins; lane 2, wild-type Ffh; lane 3, Ffh-ΔM; lane 4, Ffh-ΔG; arrowheads indicate the slowly migrating bands. C, filter assay of Ffh-ΔG and ΔM. Ffh-WT (●), Ffh-ΔG (○), and Ffh-ΔM (△) were monitored by means of the filter assay as described in the legend to Fig. 2.
The shorter region between h2 and h3 of the Ffh M-domain is very important for the binding to sc104 RNA, and that the positively charged amino acids in h2 (amino acids position 346 to 391) and h3 (amino acids position 416 to 435) are less important than the shorter region. The short peptide sequence between h2 and h3 constitutes one of the most highly conserved regions in the M-domains of SRP54 proteins. Furthermore, the two arginine residues among sequences RRKR (italic), and GSG were especially highly conserved in the short region between the h2 and h3 regions among SRP54 homologues (Fig. 4).

RNA-binding Activity in Mutant Ffh Proteins at the RRKR Sequence—The cluster of four positively charged amino acids located between the hydrophobic region h2 and h3 has also been found in the SRP54 homologues of M. mycoides and in chloroplasts of Arabidopsis thaliana (22) (Fig. 4). Furthermore, a similar structure, RXKR, has been identified in the h2 and h3 regions of Eukarya, Canis familiaris (10), Mus musculus (9), Lycopersicon esculentum (18), A. thaliana (21), Schizosaccharomyces pombe (19), and Saccharomyces cerevisiae (19, 20), as shown in Fig. 4. Since the QQQQ derivative of B. subtilis Ffh completely lacked RNA-binding activity, we prepared three novel derivatives (RQQR, RQQQ, and QQQR). As shown in Fig. 6, the RQQR and QQQR derivatives had similar RNA-binding activities, whereas that of RQQQ was dramatically reduced. Therefore, the arginine residue at position 401 (R401) of 398RRKR401 in B. subtilis Ffh was essential for the RNA-binding activity.

RNA-binding Activity of Mutant Ffh Proteins at the GSG Sequence—The GSG sequence is highly conserved among SRP54 and its homologues. To determine whether or not the secondary structure of this region is closely involved in RNA-binding activity, ASA (two glycines were replaced with alanine) and PSP (two glycines were replaced with proline) derivatives were prepared. The RNA-binding activity of both ASA and PSP derivatives was reduced dramatically (Fig. 7). We then prepared the GSP and PSG derivatives to determine which glycine residue of the GSG sequence is more important for RNA binding. Neither PSG nor GSP derivatives bound to sc104 RNA (Fig. 7). These results indicated that the GSG sequence is crucial for the RNA-binding activity of Ffh.

Lys-404 Replacements of Ffh Protein Affected the RNA-binding Activity—A positively charged amino acid between RRKR and GSG sequences, such as Lys-404 of B. subtilis Ffh protein, was conserved among most SRP54 and its homologues, except...
for E. coli Ffh. To confirm that Lys-404 of B. subtilis Ffh is necessary for RNAbinding, we constructed three novel replacement mutants in which Lys-404 was replaced with Gln (K404Q), RRKR-K404Q, RQQR-K404Q, and QQQR-K404Q and monitored the RNA-binding activity. All of the K404Q replacements significantly reduced the RNA-binding activity. The half-maximal bindings for Ffh-WT, RRKR-K404Q, RQQR-K404Q, and QQQR-K404Q were 0.15, 0.5, 0.7, and 2.0 mM, respectively (Fig. 8). Since replacing Arg-398 with Gln with K404Q replacement reduced the binding activity even more (comparing that of RQQR-K404Q and QQQR-K404Q), both Arg-398 and Lys-404 are necessary for binding to scRNA.

RNA-binding Activity of MH23 Peptide and a Synthetic Peptide (a 24-Amino Acid Peptide)—Based upon the findings that the region from position 364 to 432 of Ffh is necessary for RNAbinding, we determined whether the peptide of this region was sufficient for the binding to scRNA. We constructed the MH23 peptide corresponding to amino acid positions 356 to 446, which included the peptide region from h2 to h3 (Fig. 9A) and assayed the RNA-binding activity using nitrocellulose filters. The MH23 peptide bound to sc104 RNA with the same affinity as Ffh-WT (Fig. 9B). Furthermore, to determine whether or not the region between h2 and h3 is sufficient for the binding to scRNA, we synthesized a peptide consisting of 24 amino acid residues (amino acid positions 356 to 415) corresponding to the stretch between these regions (Fig. 9A). This synthetic peptide did not bind sc104 RNA according to the nitrocellulose filter assay (Fig. 9B). Additionally, under the conditions shown in Fig. 9B, a 1000-fold excess of the synthetic peptide did not compete with the RNA-binding activities of either Ffh-WT or MH23 in the nitrocellulose filter assay (data not shown).

In total, these results showed that the 24 amino acids contained a region that was essential, but not sufficient, for bind-

**DISCUSSION**

The results of this study indicated that in B. subtilis Ffh-scRNA complex the peptide from amino acid position 364 to 432 of B. subtilis Ffh, including two conserved hydrophobic regions (h2 and h3), is necessary and sufficient for scRNA binding. Point mutations within the region between h2 and h3 dramatically affected the RNA-binding activity. Substitutions of Arg-401 and substitution of two glycine residues in the GSG sequence abolished the binding, suggesting that these amino acid residues bind directly to RNA. The RQQR derivative of B. subtilis Ffh showed the same binding activity as Ffh-WT (Fig. 5). The RXXR sequence in this region is found in SRP54 of C. familiaris in which the amino acid sequence of the corresponding region is RQRVARQSG (the conserved amino acids are underlined). Moreover, mammalian SRP54 can bind bacterial SRP RNA (33). These data indicated that two conserved positively charged residues in this sequence are necessary for RNA binding. On the other hand, effect of the introduction of amino acid substitutions into the h2 and h3 regions is less than that positively charged residues. Computer-assisted secondary structure analysis of this region suggested that both hydrophobic regions (h2 and h3) fold into an amphipathic α-helix, with methionine and other hydrophobic residues clustered on one face (9). The positively charged amino acid residues clustered in the predicted boundaries between two helices. Therefore, it
is more plausible that h2 and h3 cannot bind directly to RNA, but that a secondary or tertiary structure formed by h2 and h3 plays a pivotal role in RNA binding. This model is supported by our data showing that a chemically synthesized oligopeptide (corresponding to amino acid positions 392 to 415) could not bind to RNA by itself (Fig. 9) and did not interfere with RNA binding of Ffh-WT (data not shown). Furthermore, Ffh-WT binds to RNA by itself (Fig. 9) and did not interfere with RNA binding. Consequently, the RNA-binding region of Ffh for further understanding the structure-function relationship of double-stranded RNA motifs.

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