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RshA mimetic peptides inhibiting the transcription driven by a *Mycobacterium tuberculosis* sigma factor SigH

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Abstract

SigH, an alternative σ factor in *Mycobacterium tuberculosis*, is a central regulator in responses to the oxidative and heat stress. This SigH activity is specifically controlled by an anti-σ factor RshA during expression of stress-related genes. Thus, the specific interaction ($k_{on} = 1.15 \times 10^5$ (M^{-1} s^{-1}), $k_{off} = 1.7 \times 10^{-3}$ (s^{-1}), $K_D = 15$ nM, determined in this study) between SigH and RshA is crucial for the survival and pathogenesis of *M. tuberculosis*. Using phage-display peptide library, we defined three specific regions on RshA responsible for SigH binding. Three RshA mimetic peptides (DAHADHD, AEVWTLL, and CTPETRE) specifically inhibited the transcription initiated by SigH in vitro. One of them (DAHADHD) diminished the extent of binding of RshA to SigH in a dose-dependent manner. The binding affinity (K_D) of this peptide to SigH was about 1.2 μM. These findings might provide some insights into the development of new peptide-based drugs for TB.
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Keywords: SigH–RshA interaction; Phage-display; Surface plasmon resonance; *Mycobacterium tuberculosis*; Anti-σ factor

Tuberculosis arises from the infection of *Mycobacterium tuberculosis*, a common human pathogen in the world. *M. tuberculosis* is an obligate aerobic pathogen, which has a predilection for the oxygen-rich lung tissue. In general, this pathogen enters the body via the respiratory route and spread from lung through lymphatic and blood to other parts of the body [1]. Therefore, *M. tuberculosis* must express some genes to overcome many stresses produced by host defense during infection [2].

RNA polymerase in bacteria is a multi-subunit entity consisting of a core enzyme ($\alpha_2\beta\beta'$) and a σ factor to form the holoenzyme. Bacterial σ factor makes a direct contact with conserved promoter elements and initiates transcription together with other subunits of RNA polymerase. In addition to its role in promoter recognition, the σ factor is a target for a variety of transcriptional regulators, including both DNA-bound activators and anti-σ factors [3]. Like other bacterial species, *M. tuberculosis* has multiple σ factors and anti-σ factors encoded in its genome [4–6].

Among these sigma factors, one principal σ factor is responsible for the transcription of the housekeeping genes, and alternative σ factors regulate gene expression in response to the specific extracellular environmental signals: misfolded proteins in the periplasm, light, changes in osmolarity or barometric pressure, and toxic molecules in the external environment [7,8]. Because alternative σ factors play crucial roles in regulating how cells communicate with the extracellular environment and adapt to outside stress, these proteins are considered as potential targets to control the virulence of pathogens [9].

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Mycobacterium tuberculosis SigH belongs to the ECF (extracytoplasmic function) σ family and acts as a central regulator in responses to the oxidative and heat stress. Upon induction of these stresses, cells have large increase of expression of sigH gene as well as genes encoding additional regulators and effectors in order to respond to these stress [10,11]. Although σ factor genes are frequently induced under a given condition, their enzyme activities are also regulated by a family of proteins, called anti-σ factors. The anti-σ factor binds a specific σ factor, keeping it in an inactive form. Upon the presence of a specific stimulus, the anti-σ factor releases the σ factor, which becomes active. RshA, a polypeptide of 101 amino acids encoded by a gene in the *sigH* operon, is an anti-σ factor for SigH in *M. tuberculosis*, and it specifically inhibits SigH-dependent transcription [2,12].

Here, we isolate small RshA peptides capable of binding to SigH using phage display peptide libraries and analyze their roles in the transcription in vitro and interaction between SigH and RshA using surface plasmon resonance (SPR).

Materials and methods

Materials. Two Peptide Library Kits Ph.D.-C7C and 12 were purchased from New England Biolab. Sodium dodecyl sulfate (SDS), acrylamide, *N*,*N*′-methylene-bisacrylamide, ammonium persulfate, dithiothreitol (DTT), urea, Tris base, and glycine were purchased from Bio-Rad or USB. Imidazole, PMSF, and Heparin were obtained from σ. Isopropyl-β-D-thiogalactoside (IPTG) and NTP set were from Fermentas. X-gal and tetracycline were from Dong IN Biotech (Korea). Sequenase Version 2.0 DNA Sequencing Kit was from USB. Ni–NTA–agarose beads were purchased from Qiagen. SUPERase-IN was from Ambion, and *Escherichia coli* RNA polymerase core enzyme was obtained from Epicentre Technologies. Four small synthetic RshA peptides (peptide 1, DAHADHD; peptide 2, AEVWTLL; peptide 3, CTPETRE; control peptide, ALIGTKC) were obtained from Peptron (Korea). Peptide 1 and control peptide were dissolved in H_2O, and peptides 2 and 3 were dissolved in DMSO.

Expression and purification of SigH or RshA. The *M. tuberculosis sigH* or *rshA* gene expression constructs were transformed into *E. coli* BL21 (DE3) and purified as described previously [12]. In brief, cells were grown in Luria broth containing ampicillin (100 μg/ml) with shaking at 37 °C until an OD_{600} reaches 0.6 and then induced with 1 mM IPTG to overproduce proteins at room temperature for 3 h. Cells were collected by centrifugation at 6000 rpm at 4 °C for 15 min. The cell pellet was suspended in the binding buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, and 10 °C glycerol) with 2 mM imidazole, 2 mM β-mercaptoethanol, and 1 mM DTT. Cells were lysed by sonication on ice six times, and soluble proteins were separated by centrifugation at 25,000 rpm at 4 °C for 30 min. The supernatant was loaded on 1 ml Ni-column, washed with binding buffer containing 30 mM imidazole, and eluted with binding buffer with 200 mM imidazole. After pooling fractions, the protein solution was dialyzed in the buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM DTT, and 0.005% P-20 surfactant) at 4 °C for 4 h. Aliquots of protein solution were stored at −70 °C after being frozen in liquid nitrogen.

Kinetic analysis of SigH–RshA interaction on the biosensor chip. The real-time binding analysis of SigH–RshA was carried out in BIAcore 2000 as described previously [13]. The purified SigH protein (30 μl, 100 ng/ml) was immobilized on the BIAcore biosensor chip (CM5) by a NHS (*N*-hydroxysuccinimide)/EDC (*N*-ethyl-*N*′(dimethylamino propyl) carbodiimide) coupling reaction. From this immobilization process, about 180 RU (resonance unit) of SigH was coupled to the chip surface. Several different concentrations (5 μl of 100, 200, 500 nM, and 1 μM) of RshA protein were independently injected over the chip coupled with SigH at 5 μl/ml flow rate in the binding buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM DTT, and 0.005% P-20 surfactant). As a control, RshA was injected on the blank chip without SigH. In the peptide competition experiment, the protein solution containing RshA (500 nM) and peptide (0, 60, or 120 μM) was injected to the chip immobilized with SigH (about 300 RU) at the same condition. The chip was regenerated by injection of 5 μl of 10 mM glycine–HCl, pH 2.5. The kinetic parameters were analyzed by BIAevaluation software (version 3.0).

Selection of phage clones binding to SigH. Peptide selection using phage display peptide libraries (closed 7-mer or linear 12-mer) was carried out by the manual provided by the company (New England Biolab). Target protein, SigH (100 μg/ml) in 0.1 M $NaHCO_3$, pH 8.6, was coated on the plate and incubated overnight. The plate was further incubated for 1 h at 4 °C in blocking buffer (5 mg/ml BSA in 0.1 M $NaHCO_3$, pH 8.6) and washed six times with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 °C, and Tween 20). Phage display peptide library was diluted to 4×10^{10} in 1 ml TBST and added to the plate, and the plate was incubated for 10–60 min at room temperature and washed 10 times with TBST. Bound phages were eluted with 1 ml elution buffer (1 mg/ml BSA in 0.2 M glycine–HCl, pH 2.2), and phage solution was neutralized by 150 μl of 1 M Tris–HCl, pH 9.1. Eluted phages were amplified in *E. coli* ER2738 strains and their titers were determined after being purified by PEG/NaCl precipitation (1/6 volume of 20% polyethylene glycol 8000/2.5 M NaCl). Phage particles were enriched by panning four times as described in the company manual.

Preparation of phage DNA and sequencing. Plaques from the fourth round panning were inoculated in the overnight 100-fold diluted ER2738 culture and incubated at 37 °C with shaking for 5 h. Phages were purified by PEG/NaCl precipitation and suspended in thoroughly in 100 μl iodide buffer. Phage DNA was precipitated in ethanol and then suspended in 30 μl TE buffer. The sequencing of all phage DNA was carried out by the manual method using DNA sequenase (version 2.1, USB) and a M13 common primer (CCCTCATAGTTAGCGTAACG). Phage DNA labeled with [α-^{35}S]dATP was separated on a 7 M urea 8% denaturing polyacrylamide gel at a 70-W constant power and visualized by autoradiography.

Enzyme-linked immunosorbent assay SigH protein (200 μl of 100 μg/ml in 0.1 M $NaHCO_3$, pH 8.6) was coated in Enzyme-linked immunosorbent assay (ELISA) plate by incubating at 4 °C overnight in a humidified box. And then each well was filled with blocking buffer (50 mg/ml BSA in 0.1 M $NaHCO_3$, pH 8.6) and incubated for 2 h at 4 °C. The plate was washed six times with 1× TBST. Fourfold serial dilutions of purified phages were prepared in 200 μl TBST per well ; starting with 2×10^5 virions in the first well of a row and ending with 10^{12} virions in the 12th well. Each diluted phage was applied to the SigH-coated plate and incubated for 2 h with agitation at room temperature. The plate was washed six times with TBST. HRP (horseradish peroxidase)-conjugated anti-M13 antibody (Pharmacia, diluted 1: 5000 in 200 μl blocking buffer) was added to each well and incubated at room temperature for 1 h with agitation. The plate was washed six times with TBST. Two hundred microliters of HRP substrate solution (A mix of 21 ml ABST stock (22 mg ABST (2,2′-azio-bis(3-ethylbenzthiazoline-6-sulfonic acid)) in 100 ml of 50 mM sodium citrate, pH 4.0) and 36 μl of 30% H_2O_2) was added to each plate and incubated at room temperature for 60 min. Absorbance at 415 nm was measured using an ELISA microplate reader.

In vitro transcription assay. In vitro transcription analysis was preformed as described previously [12] except the pre-incubation of SigH with RshA or/and peptides (1.8 μM) before reconstitution with *E. coli* RNA polymerase core enzyme. Purified RshA protein (1.0 μg) was mixed with SigH (0.5 μg) in transcription buffer (50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 25 mM KCl, 0.05 mM EDTA, 250 μg/ml BSA, and 0.5 U/μl RNase inhibitor (SUPERase-In) for 30 min at 37 °C). Following reconstitution with 0.2 U *E. coli* RNA polymerase core enzyme for 20 min at 37 °C in 30 μl final transcription buffer, 0.09 pmol DNA template (PCR-amplified sigH promoter region) was incubated with the above reconstitution for 5 min at 37 °C. A mixture of [α-^{32}P]UTP (4 μCi) and three other nucleotides (final 0.15 mM each) in total reactions was incubated for 2 min

at 37 °C, then a mixture of heparin (200 μg heparin per ml) and unlabeled UTP was further incubated for 5 min at 37 °C. Reactions were stopped by addition of 21 μl of 95% formamide-dye solution and 50 mM EDTA, then samples were electrophoresed in a 7 M urea-6% denaturing polyacrylamide gel. Radio-labeled RNA transcripts were visualized by autoradiography.

Results

Binding kinetic analysis of Mycobacterium tuberculosis SigH and RshA on the real time biosensor chip

SigH, a key component of RNA polymerase holoenzyme in *M. tuberculosis*, specifically recognizes the promotor regions of stress-related genes upon induction of extracellular signals and initiates transcription [2]. This SigH activity during transcription is regulated by an anti-σ factor, RshA [12]. To determine the kinetic parameters of SigH/RshA interaction, we applied real-time SPR analysis. Both 6× histidine-tagged RshA and SigH proteins were overexpressed in *E. coli* BL21 (DE3) cells and purified using nickel affinity chromatography as described under Materials and methods.'' Both purified proteins were nearly homogeneous and fully active although RshA was relatively poorly expressed in this *E. coli* cell (data not shown).

SigH was first captured to the sensor chip (at response units of 180 RU), and SigH/RshA interaction was observed by injecting RshA over the chip at the several different concentrations (100 nM to 1 mM). The extent of binding of RshA to SigH gradually increased in a dose-dependent manner, and RshA binding response to SigH reached the plateau at about 85 resonance units (data not shown). Based on these binding responses, the apparent binding stoichiometry between RshA and SigH was calculated as about 1.02 (stoichiometry = [analyte response (RU)/immobilized ligand response (RU)] × [ligand MW/analyte MW] = [85 RU/180 RU] × [24.2/11.2 kDa]); that is, approximately one RshA binds to one molecule of SigH. As shown in Fig. 1 (a solid-line sensorgram), RshA associated with SigH at the fast rate ($k_{on} = 1.15 \times 10^5\ M^{-1}\ s^{-1}$) when RshA was injected over the SigH-immobilized chip, and it slowly dissociated from SigH ($k_{off} = 1.7 \times 10^{-3}\ s^{-1}$) upon buffer injection, but RshA was not bound to the blank control chip without SigH immobilization (a dot-line sensorgram), indicating that this RshA-SigH interaction is specific. From these results, we determined that the binding affinity (K_D) between SigH and RshA was about 15 nM. In conclusion, these data suggest that the transcriptional initiation activity of SigH is very specifically regulated by the direct interaction with RshA in *M. tuberculosis*.

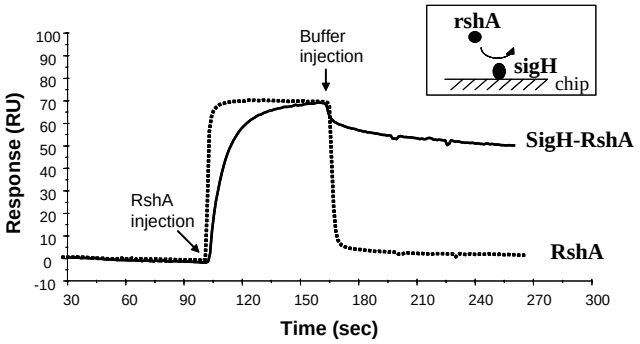

Fig. 1. SigH–RshA real time interaction on the biosensor chip. SigH proteins were immobilized on the CM5 chip (about 180 RU) as described in Materials and methods. The purified RshA (500 nM) was injected into the SigH chip at 5 μl/min flow rate. The real-time interaction between two proteins was represented by sensorgrams. The solid-line represents the SigH–RshA interaction, and the dot-line indicates the RshA binding to the blank chip (control). The experimental design for the SigH–RshA interaction is depicted in the box. Kinetic parameters were determined using BIAevalution software (version 3.0).

Selection of phage clones binding to SigH

In order to isolate small RshA mimetic peptides capable of binding to SigH, a powerful selection technology using peptide display phage libraries has been applied as described [14]. We used two different types of phage libraries obtained from a company: one type is 7-mer peptides closed by a disulfide linkage, and the other one is linear 12-mer peptides. Phage libraries were applied to the plate coated with SigH proteins, and bound phage clones were eluted for the next round of selection. Total four panning processes have been carried out to isolate the specific clones interacting with SigH. The final phage clones were subjected to DNA sequencing to determine their peptide sequences. From 10 independent attempts of this selection process, we isolated about 80 individual clones, and each of them was aligned to RshA amino acid sequences using an sequence alignment program. Peptide sequences of many isolated clones (about 70%) were mainly matched to three specific regions of RshA (Fig. 2A). The peptide 1 region (HADH) included two histidine residues. Also, one other histidine is placed downstream away from three amino acid residues (Fig. 2A), suggesting that these RshA histidines might play a role in the interaction with SigH. The peptide 2 region (EVWTL) contained several hydrophobic residues such as valine, tryptophan, and leucine. Among them, tryptophan was found in most phage clones. The peptide 3 region of RshA (TPETR) had a threonine and a proline as conserved residues. Based on these data, we synthesized three 7-mer RshA peptides spanning these peptide sequences (DAHADHD, AEVWTLL, and CTPETRE) and one control peptide (ALLGTKC). These peptides are underlined in the RshA amino acid sequence (Fig. 2A).

Some phage clones isolated from final panning have been tested their binding ability to SigH using ELISA. Correspondence of each phage clone to peptide is described in the figure legend. Most of phage clones were much more strongly bound to SigH (open bar in Fig. 2B) than to the BSA control (closed bar), although SigH binding of phage clones (#1-1, #1-2, and #1-3) in the peptide 1 region was slightly weaker than that of other clones (#2-1, #2-2, #2-3, #3-1, #3-2, and #3-3) aligned to two other peptide regions in this ELISA. Also, two out of three clones (#4-2, #4-3) matched

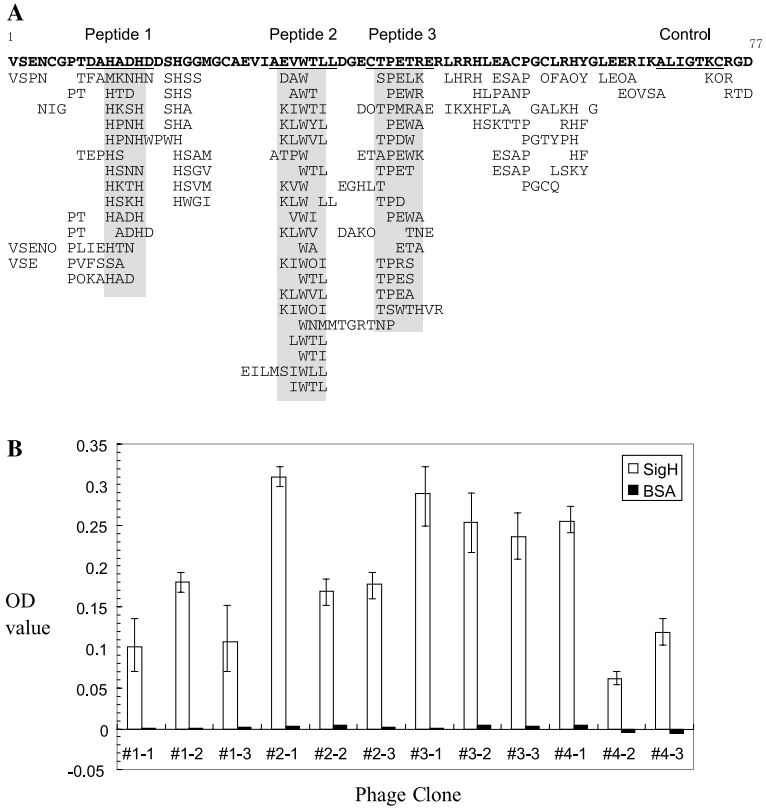

Fig. 2. (A) Alignment of selected phage clones to the RshA amino acid sequence. Each individual phage clone was sequenced and its peptide sequence was aligned to the RshA sequence (1–77 amino acids) using an alignment program (DNASTAR). Three major peptide regions (peptide 1, peptide 2, and peptide 3) of RshA were highlighted, and these synthetic peptides including a control peptide were underlined. (B) ELISA of selected phage clones. Three kinds of phage clones within peptide 1 region (#1-1 (HPNH), #1-2 (HADH), #1-3 (HKSH)), peptide 2 (#2-1 (KLWVL), #2-2 (KIWTI), #2-3 (LWTL)), peptide 3 (#3-1 (SPELK), #3-2 (ETAPEWK), #3-3 (TPEA)) or other regions #4-1 (GALKH), #4-2 (HSKTTP), #4-3 (HSAM) were used for the ELISA binding analysis. Amino acid sequences in parentheses are the correspondence of each phage clone to peptide. Phage clones were applied to the plates coated with either SigH or BSA (control) after a series of dilution. OD values presenting in this figure are the number determined at the same dilution factor among phage clones.

to the outside region of three peptides revealed their specific binding to SigH although they were weak. Furthermore, a phage clone (#4-1) not corresponding to three peptide regions was strongly interacted with SigH.

Synthetic RshA peptides inhibit in vitro transcription

To confirm whether these RshA mimetic peptides can inhibit the transcription reaction by blocking the interaction between SigH and RNA polymerase, we performed in vitro transcriptional analysis as described previously [12]. *E. coli* RNA polymerase core, purified SigH, and DNA amplified from *M. tuberculosis* genomic DNA as template have been incubated in the transcription reaction containing either RshA or RshA peptides. As expected, the native RshA protein severely inhibited the transcription (Fig. 3A, lanes 4 and 5) in this assay system. In addition, three RshA peptides isolated from phage selection similarly showed 80–90% of transcriptional inhibition in comparison of the reaction without anti-σ factor in this in vitro assay. However, the inhibition by a control peptide aligned to a non-specific region was not significant (Fig. 3A, lanes 12 and 13). Overall, all three RshA peptides had a similar inhibitory effect on the in vitro transcriptional activity driven by SigH (Fig. 3B). Interestingly, the reaction containing only RNA polymerase core (no σ factor) also showed some degrees of transcriptional activity, similar to the activities of reactions with RshA peptides (Fig. 3A, lane 1). This might be because of the contamination of a trace amount of σ factor in the RNA polymerase core purchased from a company.

Peptide inhibits the SigH–RshA interaction

To test whether these three RshA peptides are able to inhibit the direct interaction between SigH and RshA proteins, we added a specific peptide (peptide 1, dissolved in H_2O) to the SPR real-time binding reaction of SigH/RshA as mentioned in Fig. 1. In case of the reaction containing peptide 1, the maximum binding unit (RU) between SigH and RshA was gradually decreased with respect to increase of peptide concentration (Fig. 4, upper sensorgram), suggesting that this peptide competes with RshA to bind the same site of SigH. However, the

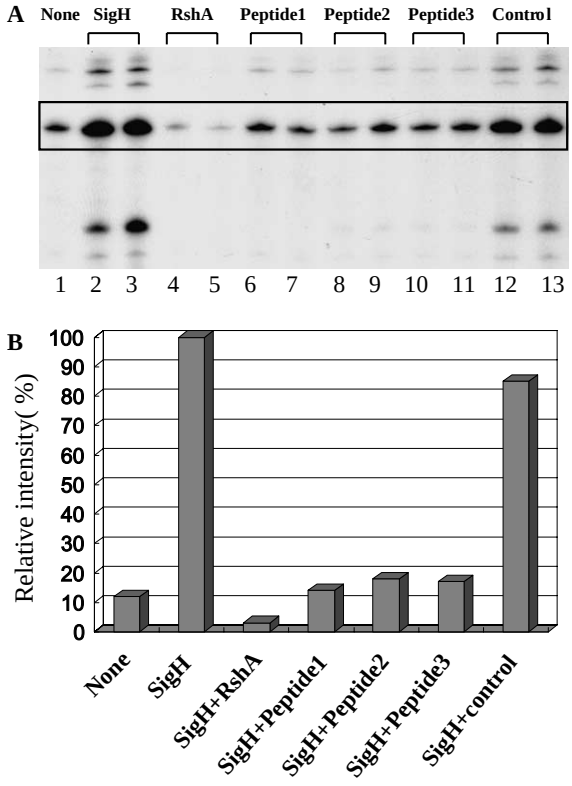

Fig. 3. Inhibition of in vitro transcription reactions by RshA peptides. (A) In vitro transcription reactions. In vitro transcription was performed as described in Materials and methods. After transcription reaction, RNA transcripts were analyzed in a 6% of 7 M urea polyacrylamide gel and visualized by autoradiography. The major RNA transcript of each duplicated reaction was boxed. None (first lane) indicates the reaction with RNA polymerase core only; SigH lanes represent the transcription reactions with RNA polymerase core and SigH; other lanes indicate the reactions with RNA polymerase, SigH or RshA peptides (1.8 μM). (B) Densitometric scan. Density of bands in the (A) was determined by a densitometric scan. The numbers in the figure are relative values to the reaction with SigH (100%). They are an average number of two reactions.

control RshA peptide (dissolved in H_2O) did not affect the binding capacity of RshA to SigH (Fig. 4, lower sensorgram). From these sensorgrams, we calculated the kinetic parameters of peptide binding to SigH using the competition fitting mode in which two analytes (RshA and peptide) compete to bind the same ligand molecule (SigH). The association rate (k_{a1}) and dissociation rate (k_{d1}) of RshA to SigH in the presence of peptide were not greatly changed rather than in the absence of peptide, although values were slightly decreased in a peptide concentration-dependent manner (Table 1). Based on the same fitting mode, the equilibrium constant (K_D) of peptide 1 binding to SigH was about 1.2 μM ($k_{a2,}$ association rate, 23 ($M^{-1}\,s^{-1}$); $k_{d2,}$ dissociation rate, 2.76×10^{-5} (s^{-1})). Two other RshA peptides have been tested in the same binding reaction, but we were not able to analyze the results (10–30 RU change, as shown in Fig. 4) that obtained from peptide addition because of the extremely high reflex index change (over 20,000 RU) by the DMSO solvent used for dissolving peptides.

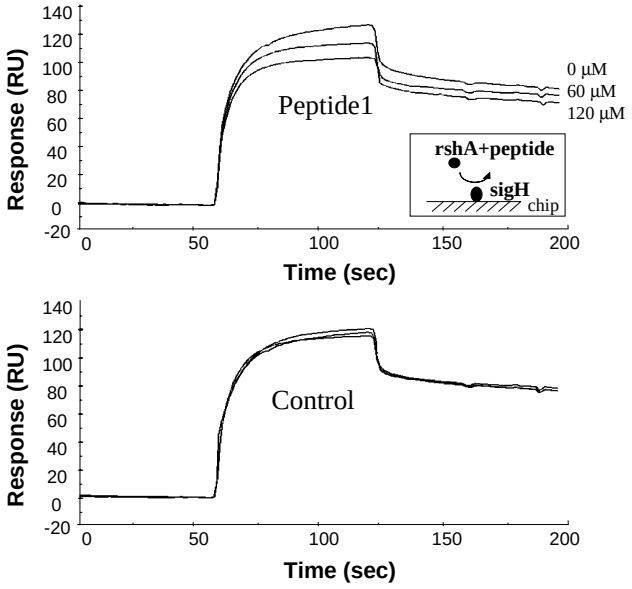

Fig. 4. Inhibitory effect by peptide 1 on the SigH–RshA interaction. The SPR binding analysis between SigH and RshA was carried out as described in Fig. 1, except addition of RshA peptides (peptide 1 or control). The experimental detail is depicted in the box. RshA (500 nM) without or with peptides (60 or 120 μM) was injected to the chip immobilized with SigH (about 300 RU). The upper sensorgram is the reaction with peptide 1, the bottom sensorgram is the reaction with control peptide (described in Fig. 2A).

Discussion

In this study, two purified proteins SigH and RshA were shown to strongly interact in a SPR analysis. The equilibrium dissociation constant (K_D) between two proteins was about 15 nM. As shown in Fig. 1, RshA bound SigH at the fast rate, and the dissociation rate between two proteins was relatively slow, suggesting that RshA specifically controls the SigH activity at the initiation of transcription. This strong complex formation between SigH and RshA prevented the RNA polymerase-SigH interaction and reduced the overall transcription activity as shown in Fig. 3 (lanes 4 and 5). Therefore, control of the SigH–RshA interaction might provide an important clue for regulating many genes involving in *M. tuberculosis* survival and proliferation.

Phage display is a powerful tool to identify ligand mimics [14], but sometimes isolation of low-affinity phage particles causes decrease of their specificity. To minimize the chance of these non-specific phage selections, we used two different types of phage display libraries: 12 mer-linear or 7 mer-closed peptide libraries and performed panning at least four times. From phage clone alignment created from 10 independent selections, three major peptides were selected at the N-terminal region of RshA. All these peptides did not share a common consensus sequence. Peptide 1 contains two histidines, and peptide 2 possesses a major tryptophan. A proline residue was found as a major amino acid in peptide 3. This suggests that RshA might interact with SigH through several contact sites. In fact, several other previous studies revealed that other bacterial anti-σ factors

Table 1
Kinetic parameters of peptide inhibition reactions in the real-time binding analysis

Peptide concentration	RshA association rate, k_{a1} (M^{-1} s^{-1})	RshA dissociation rate, k_{d1} (s^{-1})	Peptide association rate, k_{a2} (M^{-1} s^{-1})	Peptide dissociation rate, k_{d2} (s^{-1})	RshA equilibrium constant, K_{D1} (M)	Peptide equilibrium constant, K_{D2} (M)
0 nM	1.12×10^{5}	1.72×10^{-3}			1.53×10^{-8}	
60 μM	1.02×10^{5}	1.75×10^{-3}	15	2.10×10^{-5}	1.7×10^{-8}	1.4×10^{-6}
120 μM	1.00×10^{5}	1.99×10^{-3}	31	3.12×10^{-5}	1.99×10^{-8}	1.0×10^{-6}
Average*	1.01×10^{5}	1.87×10^{-3}	23	2.76×10^{-5}	1.84×10^{-8}	1.2×10^{-6}

*These kinetic parameters were determined from sensorgrams in Fig. 4 (upper) using BIAevaluation software (version 3.0).
The fitting model to determine kinetic parameters of RshA and peptide is based on the competing reaction in which two analytes (RshA and peptide) compete to bind the same ligand molecule (SigH) as the following equation.

$A_1 + A_2 + B \rightarrow A_1B + A_2B$

(A_1: RshA, A_2: peptide, B: SigH)

form a complex with σ factor through multiple contact sites. Two separate domains of an *Escherichia coli* ECF (extracytoplasmic factor) σ factor σ^E are involved in binding to the N-terminal cytoplasmic region of anti-σ factor, RseA [15]. Several hydrophobic residues (RseA-Trp 33 and Leu 20) and polar amino acids (RseA-Asp11 and Ser 7) directly participate to this interaction with σ^E. In *Bacillus stearothermophilus*, σ factor σ^F binds to spoIIAB, anti-σ factor, at three contact sites, and they form a complex at the 1:2 ratio [16,17]. Like σ^E–RseA, several crucial SpoIIAB residues including hydrophobic or polar amino acids have been determined at the binding interface with σ^F. In particular, Val 135 Glu mutation in SpoIIAB disrupted not only SpoIIAB activity but also σ^F binding [16]. The *Salmonella typhimurium* σ^{28} and FlgM associate together at multiple contact site and 1:1 ratio [18]. AsiA, T4 phage factor, inhibits the host transcription during infection by forming a complex with host σ^{70} at a 1:1 ratio [19]. *Streptomyces coelicolor* ECF σfactor σ^R, the homolog of *M. tuberculosis* SigH, also forms 1:1 complex with RsrA [20,21]. From real-time binding study in this report, we also suggest that one molecule of RshA directly interacts with one molecule of SigH although the details of structural features at the interface between SigH and RshA remain to be determined.

All three RshA mimetic peptides similarly inhibited the in vitro transcription reaction driven by a σfactor SigH and *E. coli* RNA polymerase core. Although the inhibition by peptides was not severe as much as by RshA, over 80% activity of the reaction with SigH was inhibited by addition of peptides. In addition, peptide 1 lowered the RshA binding to SigH in a dose-dependent manner. The binding affinity (K_D) between this peptide and SigH was about 1.2 μM (Table 1). Taken together, these data suggest that the small RshA peptides, selected by phage display, can regulate the activity of SigH, a transcription factor necessary for *M. tuberculosis* pathogenesis. Therefore, they might be good candidates for new TB drug development.

Acknowledgments

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