The Human Antimicrobial Peptide LL-37 Binds Directly to CsrS, a Sensor Histidine Kinase of Group A Streptococcus, to Activate Expression of Virulence Factors*

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Background: Group A Streptococcus (GAS) responds to the human antimicrobial peptide LL-37 by up-regulating virulence factors controlled by the CsrRS system.

Results: We provide genetic and biochemical evidence for direct binding between LL-37 and the CsrS receptor.

Conclusion: LL-37 signaling through CsrS is mediated by a direct binding interaction.

Significance: These findings advance our understanding of streptococcal virulence regulation in response to a host signal.

Group A Streptococcus (GAS), the etiologic agent of streptococcal pharyngitis, is also capable of causing invasive infections including streptococcal toxic shock and necrotizing fasciitis that are associated with high mortality rates (1). Invasive infections are thought to be preceded by local oropharyngeal infection or colonization (1). However, the mechanisms by which a local infection is established and progresses to an invasive infection are not completely understood.

GAS harbors multiple regulatory systems that enable the organism to respond to environmental signals. Among these are the two-component systems, typically characterized by a sensor histidine kinase, which transduces an extracellular signal by phosphotransfer to a second intracellular component, the response regulator. Phosphorylation of the response regulator modulates its binding to target promoters where regulator binding acts to repress or activate gene transcription (2). GAS has up to 13 two-component systems, among which the CsrRS (also known as CovRS) system is the best studied (3–7). The CsrRS system has recently been hypothesized to play a role in oropharyngeal colonization and persistence by GAS (8–10). Up-regulation of virulence factors under regulatory control of this system results in increased resistance to opsonophagocytosis and intracellular survival in epithelial cells, neutrophils, and macrophages, which may facilitate oropharyngeal colonization (8, 11, 12). The CsrRS system also plays a role in the transition of a local infection to a more invasive process. Bacteria recovered from sterile sites both in a mouse model of infection and in patients with invasive GAS disease frequently have inactivating mutations of this two-component system (6, 7, 13–15). For most genes that encode CsrS-regulated virulence factors, inactivation of CsrR or CsrS results in constitutive up-regulation of gene expression (4, 15).

LL-37 is a 37-residue amphipathic antimicrobial peptide (AMP) that is produced by leukocytes and epithelial cells of the skin, respiratory tract, and gastrointestinal tract (16, 17). A member of the cathelicidin class of AMPs, LL-37 has antimicrobial activity against many bacteria, as well as immunomodulatory effects (16–18). The structure of the peptide is well described as a helix, and its antimicrobial properties have been localized to the C-terminal domain consisting of amino acids 17–32 (FK-16) (19–22). The smallest fragment that maintains any antimicrobial properties for Escherichia coli is 12 residues in length and corresponds to amino acids 18–29 (KR-12) of the LL-37 sequence (22, 23).

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† The abbreviations used are: GAS, group A Streptococcus; AMP, antimicrobial peptide; qRT-PCR, quantitative RT-PCR; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBP, maltose-binding protein; cfu, colony-forming units.
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Gryllos et al. (24) first described the signaling relationship between LL-37 and the CsrS sensor. They found that exposure of GAS to subinhibitory concentrations of LL-37 resulted in increased expression of multiple virulence factors including the has operon, responsible for capsular polysaccharide biosynthesis, and that CsrS was essential for this effect. The CsrRS system regulates 10–15% of the GAS genome (7); the effects of LL-37 signaling confer an “invasive phenotype” to the pathogen, reflected in a marked increase in resistance to osmophasocytic killing by human blood leukocytes (11). Multiple additional genes, including sda1, slo, ska, and spyCEP/scpC, which encode virulence factors streptodornase D/DNase, streptolysin O, streptokinase, and the IL-8 protease SpyCEP/ScpC, respectively, respond to LL-37 through the CsrRS system (1, 11, 24). Testing of multiple AMPs for CsrS-mediated signaling revealed that only LL-37 and to a lesser degree the rhesus macaque cathelicidin RL-37 (68% identity with LL-37) had the observed effect (24). Human alpha and beta defensins and cathelicidins from mouse (mCRAMP) and sheep (SMAP 29) were inactive in signaling through CsrS. Tran-Winkler et al. (11) subsequently demonstrated that replacement of three acidic amino acids in the extracellular domain of CsrS with uncharged residues abrogated the signaling effects of LL-37. These results suggested the targeted acidic residues might be part of an LL-37 binding site on CsrS.

In this study, we investigated the mechanism by which LL-37 interacts with CsrS and results in up-regulation of virulence factors. We considered two hypotheses. The first was that CsrS transduces a stress response, as has been demonstrated for other two-component receptors, because of sufficient disruption of the bacterial membrane to cause a conformational change that results in signal transduction by modulating the autokinase and/or phosphatase activity of CsrS (25). The second hypothesis we entertained was that LL-37 binds directly and specifically to CsrS to induce a conformational change and downstream signaling in a classic ligand/receptor interaction. We favored the latter hypothesis because CsrS signaling seemed to be specific to LL-37 and was not observed for other cationic AMPs (24). In this study, we identified a 10-residue fragment of LL-37 (RI-10) that has similar activity to full-length LL-37 with respect to its ability to signal through CsrS. This short peptide lacks all antimicrobial activity against GAS and exhibits specific amino acid and chirality requirements for its signaling effect. We also demonstrate a physical interaction of LL-37 with CsrS in pulldown experiments. These data provide strong evidence that LL-37 signaling through CsrS reflects a specific binding interaction between LL-37 and the extracellular domain of CsrS.

EXPERIMENTAL PROCEDURES

Bacterial Strains—GAS strain 854 (24) was used for all experiments involving pathogenic GAS. This is an M1T1 strain originally isolated from a retroperitoneal abscess. Bacterial growth, unless otherwise indicated, was in Todd Hewitt broth supplemented with 0.5% yeast extract (THY; BD Biosciences) at 37 °C under static conditions or on THY agar supplemented with 5% defibrinated sheep blood (Northeast Laboratory Services, Portland, ME).

LL-37, SMAP 29, mCRAMP, and LL-37 Fragments—LL-37, SMAP 29, mCRAMP, SMAP 29, and peptides corresponding to portions of the LL-37 molecule, including RI-10 with specific amino acid substitutions and D-RI-10, were synthesized by Anaspec (Frement, CA), and purity was confirmed by HPLC and mass spectrometry. Additional SMAP 29, a kind gift from Robert I. Lehrer (UCLA, Los Angeles, CA), was synthesized as previously described, and purity was confirmed by HPLC and mass spectrometry (11, 26). All peptides were dissolved in deionized water at 1 mg/ml and stored at −20°C until use.

Virulence Factor Expression Analysis by qRT-PCR—qRT-PCR analyses were performed essentially as previously described (3, 8). Briefly, GAS 854 was grown in THY broth. When indicated, the growth medium was supplemented with LL-37 or a peptide fragment at a concentration of 300 nM. Cells were grown to midexponential phase (A600 nm 0.3–0.4), and 4.5 ml of culture were harvested for total RNA isolation using standard and previously described protocols (Qiagen RNeasy™) (4). The quality and concentration of RNA were determined spectrophotometrically using a Nanodrop ND-1000 instrument. Five ng of total RNA were used for qRT-PCR using the SYBR Green RT-PCR system (Qiagen) in an ABI Prism 7300 real time PCR instrument (Applied Biosystems/Invtrogen). Previously described primers were utilized for quantification of hasB, recA, sda1, slo, ska, and spyCEP/scpC transcripts (4, 11). The change in gene expression during growth in the presence of peptide compared with that in unsupplemented THY was calculated by the ΔΔCt method and normalized to the housekeeping gene recA. Experiments were performed three or four times in triplicate. The data shown represent mean values ± standard deviation. Statistical significance of differences in gene expression was evaluated by unpaired two-tailed Student’s t test of log10 normalized values. The data are depicted as fold increase in gene expression on a log2 scale as compared with GAS grown in the absence of added peptide.

Measurement of GAS Hyaluronic Acid Capsule—Capsular polysaccharide was measured as previously described (27, 28). Briefly, GAS 854 was grown in 10 ml of THY or the same medium supplemented with 300 nM LL-37, RI-10, or scrambled LL-37. Bacterial cells were harvested at midexponential phase (A600 nm 0.3–0.4) by centrifugation and resuspended in 500 μl of 10 mxi Tris, pH 7.5. An equal volume of chloroform was added, and the mixture was vortexed vigorously. The quantity of hyaluronic acid in the aqueous phase was determined by adding 1-ethyl-2-[3-(1-ethylthiopheno[1,2-d][thiazol-2-ylidene]-2-methylpropenyl]naphtho[1,2-d][thiazolium bromide (Stains-All; Sigma-Aldrich) to 100 μl, measuring absorbance at 640 nm, and comparing against a standard curve. The values were corrected for background absorbance determined from a capsular derivative of GAS 854 (8). The experiment was repeated three times. The results are expressed as fg/cfu.

Live/Dead Staining, Fluorescence Microscopy, and Microplate Live/Dead Quantification—GAS 854 was grown to midexponential phase (A600 nm 0.3–0.4) in THY broth. One-ml aliquots of broth culture were collected by centrifugation, washed in PBS, and resuspended in 0.5× PBS. AMP was added to the desired concentration, and suspensions were incubated with occasional mixing for 1 h at room temperature. Cells were collected by centrifu-
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PCR amplification from GAS 854 chromosomal DNA and cloning into pORI23 (33) with a C-terminal His6 (pORI23CsrSHis). Primers used for this amplification were CsrSHisF (5'-AAGCG-GATCCCGGCACTGGGATCGTTATCCTGGTAGG-3') and CsrSHisR (5'-AAGCGCTGCCAGTGTGATTCTGGA-TATGCTTTCCTGATACACTCCTTATGAGCTGGGCG- AAAGG-3'). The selected CsrS coding region was then amplified by PCR from plasmid pORI23CsrSHis with primers pColdECDF (5'-AAGCGACTATAGAGTATCACAAGATTCTTTACTTTT-ttcttattgaag-3') and CsrSedN183R (5'-AAGCGCTGACGCTTAATGCCTAAATCATGAGACATTGAC-3'). The product was cloned into restriction sites SpeI and PstI of the PColdMBP multiple cloning site to create plasmid pCold MBP-ECDF. For expression of MBP, pCold MBP-ECDF was digested with XbaI and SpeI and religated to remove the CsrS gene and to introduce a translation termination codon at the multiple cloning site (pColdMBPstop). Finally, for construction of an expression vector harboring the CsrS ECD with a triple amino acid substitution as previously described (11), the full-length CsrS was introduced into the pGEM-T vector system (Promega, Madison, WI) after amplification from pORI23-CsrSHis with Easy-A Hi-Fi Polymerase (Agilent Technologies, Santa Clara, CA) using primers CsrSHisF and CsrSHisR. Three acidic residues clustered in the extracellular domain (Asp148, Glu151, and Asp152) were changed to polar uncharged residues (Asn148, Gln151, and Asn152) by QuikChange mutagenesis (Agilent Technologies, Santa Clara, CA) using previously described primers css418-F (muNHIQN) and css480-R (muNHIQN) (11) to create pGemCsrSHis3×mut. The CsrS extracellular domain was then amplified from this plasmid, using primers pColdECDF and CsrSedN183R, and cloned into pColdMBP, as described for the wild-type extracellular domain, to form pColdMBP-ECDF3×mut. Fidelity of all cloned sequences was confirmed by DNA sequencing (Genewiz, South Plainfield, NJ).

Protein Expression and Purification—To purify MBP-ECDF, pCold MBP-ECDF was expressed from E. coli BL21 DE3-T1 (Sigma). Cells were grown in an Applikon (Foster City, CA) 5-liter Bioreactor to A600 nm 5–7, at which point expression was induced by decreasing the temperature to 15 °C and adding isopropyl-β-D-Thiogalactopyranoside to a final concentration of 0.8 mM. Cells were allowed to express for 2–3 h, harvested, and frozen (−80 °C) for later use. For purification, bacterial pellets were thawed and resuspended in buffer A (50 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 8.0) with 0.1% Tween 20 and EDTA-free protease inhibitors (EMD Millipore, Darmstadt, Germany). Resuspended bacteria were exposed to lysozyme at 1 mg/ml for 30 min and DNase I at 0.05 mg/ml for an additional 30 min on ice. Bacterial cells were then lysed with a Sonic Dismembrator (Thermo Fisher Scientific, Waltham, MA) using a half-inch probe at amplitudes of 40–50% for a total time of 30 min, including cooling for 1 min between 30-s bursts, all on ice. Cleared lysate was filtered and loaded onto a 5-ml nickel-nitrilotriacetic acid-agarose (Invitrogen) column equilibrated with buffer A on an AKTA Purifier FPLC system (GE Healthcare). The column was washed with 20 column volumes of buffer A at 1 ml/min. Protein was then eluted with a gradient of 20–250 mM imidazole over 20 column volumes. The eluted protein was then loaded onto 5 ml of amylose resin (New Eng-

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Our laboratory has previously demonstrated that GAS 854, when exposed to 100–300 nM LL-37, responds through the CsrRS two-component system to up-regulate expression of multiple virulence factors including the has operon responsible for capsular polysaccharide synthesis (24). Using chloramphenicol acetyltransferase to report activity of the has operon promoter, Gryllos et al. (24) also showed that the mouse (mCRAMP) and sheep (SMAP 29) cathelicidins had little or no CsrRS signaling activity in GAS strain DLS003. In the current study, we used the M1T1 strain 854, an invasive clinical isolate that produces larger quantities of hyaluronic acid capsule in response to LL-37 as compared with GAS DLS003. Using qRT-PCR, we found that exposure of GAS 854 to 300 nM mCRAMP resulted in increased expression of the CsrRS-regulated genes hasB and sda1 (Fig. 1). The increase in gene expression, however, was only 50% of that seen in response to human LL-37. Exposure to the sheep SMAP-29 had no effect on transcription of either hasB or sda1 in GAS 854. However, assays of MIC and MBC showed that mCRAMP and SMAP 29 had similar or more potent antimicrobial activity against GAS as LL-37 (Table 1). These results indicate that the toxicity of the peptides, particularly SMAP-29, does not directly correlate with signaling through CsrS.

A 10-Amino Acid Fragment of LL-37, RI-10, Signals through CsrS—The fact that AMPs with similar antimicrobial activity against GAS had vastly different CsrRS signaling activity suggested that these two properties are not necessarily linked. Accordingly, we sought to identify the core region of LL-37 responsible for CsrS signaling and to determine whether there was a correlation between CsrS signaling capacity and antimicrobial activity for GAS. We tested a series of 13 synthetic peptides designed to correspond to fragments of LL-37 (Table 2). The peptide sequences spanned the region of LL-37 shown pre-
viously to be most active in damaging bacterial cell membranes (16, 19). The peptides were tested for CsrS signaling activity by exposing GAS to 300 nM peptide and then quantifying CsrS signaling by qRT-PCR analysis of expression of hasB. As expected, full-length LL-37 resulted in a 30-fold increase in hasB transcription, whereas a 37-amino acid peptide containing the same amino acids in random order (scrambled LL-37) had no activity. We identified several shorter peptides that retained CsrS signaling activity, as reflected in up-regulation of hasB transcription (Fig. 2A). The smallest fragment of LL-37 that retained signaling activity was 10 residues in length (RI-10). Signaling effects of RI-10 were confirmed for additional genes known to be regulated by CsrRS (sda1, spyCEP/scpC, slo, and ska; Fig. 2B) (4, 11, 27). We also tested the quantity of hyaluronic acid expressed by GAS 854 in the presence of LL-37 and RI-10 and found increases that were consistent with the qRT-PCR results (Fig. 2C). RI-10 had no effect on transcription of hasB or sda1 in a GAS 854 strain with a deletion of CsrS (not shown). Signaling was not solely dependent on peptide length. KR-10 and IV-10, both also 10 residues in length, had no significant CsrS signaling activity (Fig. 2A). These peptides differ from RI-10 by a shift of one residue toward the N terminus (KR-10) or C terminus (IV-10) of the LL-37 parent sequence (Table 2). Together, these results identify the LL-37 domain represented by RI-10 as critical for LL-37 signaling. Of note, we observed that the entire RI-10 sequence is present in each of the LL-37 fragments that had CsrS signaling activity (Table 2).

| Peptide Name | Peptide Sequence |
|--------------|------------------|
| LL-37        | LGDFRKSEKI GKEFKRIVQRIKDFLRNLVRPRES |
| RK-19        | RSKSEKI GKEFKRIVQRIK |
| FK-16        | FKRIVQRIKDFLRNLVR  |
| FK-13        | FKRIVQRIKDFLRNLVR  |
| FK-9         | FKRIVQRIKDFLRNLVR  |
| KR-12        | KRIVQRIKDFLRNLVR  |
| KR-11        | KRIVQRIKDFLRNLVR  |
| KR-10        | KRIVQRIKDFLRNLVR  |
| RI-11        | RIIVQRIKDFLRNLVR  |
| RI-10        | RIIVQRIKDFLRNLVR  |
| IV-10        | IVQRIKDFLRNLVR    |
| IV-9         | IVQRIKDFLRNLVR    |
| QR-11        | QRQRIKDFLRNLVR    |
| KD-8         | KDFLRNLVR         |

**TABLE 2**

Amino acid sequences of synthetic peptides corresponding to fragments of mature, fulllength LL-37

Fragments are named by the first two amino acids followed by the total number of residues in the peptide. All peptides found to signal through CsrRS include the 10-amino acid sequence represented by RI-10 (highlighted in blue).

**FIGURE 2. Stimulation of CsrRS-regulated gene expression by LL-37 fragments.** A, fold increase in transcript abundance for hasB in GAS grown in the presence of 300 nM peptide (Table 2) compared with that for GAS grown in the absence of peptide. *, p < 0.05 for comparison with the value for scrambled LL-37. B, fold increase in transcript abundance for selected CsrRS-regulated genes in GAS grown in the presence of 300 nM LL-37 or RI-10 compared with that for GAS grown in the absence of peptide. C, cell-associated hyaluronic acid produced by GAS 854 grown in the absence or presence of 300 nM LL-37, RI-10, or scrambled LL-37. *, p < 0.05 for comparison to the value for GAS 854 grown in the absence of peptide.
Neither LL-37 nor Any of Its Fragments Cause Significant GAS Membrane Damage at a Subinhibitory Concentration of 300 nM—We next sought to investigate whether LL-37 and its fragments signaled through CsrS by damaging the GAS cell membrane. We tested the effect of select peptides to induce membrane damage at a concentration of 300 nM, the concentration at which LL-37 and some of its fragments were shown to signal through CsrS, described above. GAS membrane injury was assessed by live/dead staining with SYTO 9 (green, penetrates intact cell membranes) and propidium iodide (red, stains cells with membrane damage). At this concentration of peptide, bacteria treated with LL-37 and its fragments signaled through CsrS by damaging the GAS cell membrane. We tested the effect of select peptides to induce GAS membrane injury serves to dissociate CsrS signaling from membrane damage.

**MICs and MBCs of LL-37 and Its Fragments**—We used two additional methods to assess the GAS antimicrobial activity of LL-37 and its fragments: 1) determination of the MIC of each peptide, defined as the concentration of peptide that did not allow growth of GAS 854 in overnight culture, and 2) determination of the MBC, defined as the minimal peptide concentration that achieved 99.9% killing of GAS 854 in 0.5× PBS (Table 3). Consistent with previous reports for other GAS strains (30, 35), we found that LL-37 had antimicrobial activity for GAS strain 854, with an MIC of 50 μM and an MBC of 20–30 μM. The slightly lower concentration of peptide required for killing compared with that for inhibition of growth reflects the different conditions of the two assays: the killing assay was performed under hypotonic conditions that enhance antimicrobial activity. FK-16, which corresponds to the region considered to encompass the core of LL-37 antimicrobial activity (19, 20), demonstrated more potent antimicrobial activity than full-length LL-37, with lower MIC and MBC of 1 and 0.75–1 μM, respectively. KR-12 and RI-11, both of which are competent for CsrS signaling, had less potent antimicrobial activity than LL-37. RI-10, which is also signaling-competent, did not exhibit detectable antimicrobial activity against GAS at the concentrations and under the conditions we tested. In contrast, RK-19, which lacks CsrS signaling activity, had inhibitory activity in the MIC assay at a concentration of 200 μM. These data indicate that GAS antimicrobial activity by LL-37 fragments is neither sufficient nor necessary for signaling through CsrS and demonstrate a dissociation between the signaling and antimicrobial properties of the peptides.

**TABLE 3**

| Peptide     | MIC (μM) | MBC (μM) |
|-------------|----------|----------|
| LL-37       | 50       | 20–30    |
| RK-19       | >200     | >200     |
| FK-16       | 1        | 0.75–1.0 |
| FK-13       | 25       | 30       |
| FK-9        | >200     | >200     |
| KR-12       | 100      | 200      |
| KR-11       | >200     | >200     |
| KR-10       | >200     | >200     |
| RI-11       | 200      | >200     |
| RI-10       | >200     | >200     |
| IV-10       | >200     | >200     |
| IV-9        | >200     | >200     |
| QR-11       | >200     | >200     |
| KD-8        | >200     | >200     |

**FIGURE 3. GAS membrane damage by antimicrobial peptides. A, fluorescence microscopy of GAS after exposure to antimicrobial peptides in 0.5× PBS for 1 h followed by staining with SYTO 9 (green, penetrates intact cell membranes) and propidium iodide (red, stains cells with membrane damage). B, quantification of cell viability by fluorescence measurement in a microplate fluorometer and comparison with a standard curve of different ratios of live/dead bacteria.**
Amino acid specificity of RI-10 binding to the extracellular domain of CsrS. The data represent fold increase in transcript abundance for four CsrS-regulated genes in GAS grown in the presence of 300 nM peptide (RI-10 amino acid substitutions; Table 4) compared with that for GAS grown in the absence of peptide. * denotes three of four and ** denotes four of four virulence factors for which expression in the presence of peptide is significantly different ($p < 0.05$) from that for GAS grown in the presence of RI-10.

CsrS-regulated genes as described above (Fig. 4). We found that a conservative change from arginine to lysine at the first amino acid position did not change the signaling activity of RI-10 as reflected in transcription of *hasB*, *sda1*, *slo*, or *spyCEP/scpC*. However, a change to alanine at the same position, removing the positive charge, decreased signaling (statistically significant for three of four genes tested). Changing the C-terminal leucine to either alanine or phenylalanine, removing the large hydrophobic side chain or increasing its bulk, respectively, both resulted in complete abrogation of signaling by these peptides. Signaling by RI-10, therefore, has specific amino acid requirements and is not simply dependent on nonspecific properties such as hydrophobicity. Of interest, the mouse cathelicidin mCRAMP has phenylalanine rather than leucine at this position. Because mCRAMP exhibits CsrS signaling activity, albeit less than LL-37, it appears that the substitution of phenylalanine for leucine does not completely abrogate signaling in the context of the full-length cathelicidin. We also tested an all-D enantiomer of RI-10 (D-RI-10), *i.e.* a mirror image of RI-10 that is predicted to have a similar amphipathic nature and charge distribution. We hypothesized that if the binding and signaling capacity of RI-10 were due to nonspecific properties such as charge or amphipathicity, then an all-D enantiomer should behave similarly. However, if RI-10 signaling depends on specific binding to CsrS, then the corresponding D peptide is likely to have reduced activity in signaling through CsrS. We found that D-RI-10 had no signaling activity (Fig. 4). These data support specific binding of RI-10 (and, by inference, LL-37) to CsrS and a critical role for particular amino acid residues within this signaling core region of LL-37.

### Table 4

| Peptide | Peptide sequence |
|---------|-----------------|
| RI-10   | R I V Q R I K D F L |
| R1A     | A I V Q R I K D F L |
| R1K     | R I V Q R I K D F L |
| L10A    | R I V Q R I K D F A |
| L10F    | R I V Q R I K D F F |
| D8N     | R I V Q R I K N F L |
| D-RI10  | i v q r k d f i   |

* All D-amino acids.

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triple amino acid substitution (MBP-ECD 3X mutant). LL-37 bound to the mutant construct to a greater extent than to MBP alone, but far less than to the wild-type MBP-ECD construct (Fig. 5A). Thus, the impaired responsiveness to LL-37 signaling of a GAS strain expressing a CsrS ECD harboring amino acid substitutions D148N, E151Q, and D152N correlates with decreased affinity of LL-37 binding. To address the possibility of a nonspecific charge interaction between LL-37 and the CsrS ECD, we tested whether MBP-ECD could pull down the LL-37 fragment RK-19 (Fig. 5B) or the sheep cathelicidin SMAP 29 (Fig. 5C), both cationic peptides and neither able to signal an increase in transcription of virulence factors through CsrS. Neither control peptide was detected in the eluate, indicating that they were not able to bind to MBP-ECD and that the effect seen with LL-37 is unlikely to be a nonspecific electrostatic interaction. Taken together, these data provide evidence for a direct binding interaction between LL-37 and the extracellular domain of CsrS.

DISCUSSION

GAS exposure to subinhibitory concentrations of LL-37 stimulates the coordinated up-regulation of expression of multiple virulence determinants controlled by the CsrRS two-component system (11, 24). A critical consequence of this regulatory program is a marked and multifaceted increase in GAS resistance to effective killing by host immune effectors, notably phagocytic leukocytes (8, 11). LL-37 is produced by neutrophils where it is found as a component of granules. Production by epithelial cells is induced by injury, inflammation, and exposure to microbial products. LL-37 has been detected in mucosal secretions in human airways and has been found in saliva of healthy individuals at concentrations ranging from 30 to 700 nM (36, 37). LL-37 is not produced by skin keratinocytes under resting conditions but can reach concentrations as high as 300 µM in inflamed skin, e.g. in psoriasis (38). At concentrations of 100–300 nM, LL-37 signals through CsrS to increase expression of GAS genes that control synthesis of the hyaluronidase capsule, an important antiphagocytic factor; of SpyCEP, an IL-8 protease that inactivates the chemotactic cytokine, IL-8, to reduce neutrophil influx to the site of infection; of SLO and its co-toxin NADase that damage phagocytes and reduce their bacterial killing activity; and of DNase that degrades neutrophil extracellular traps, permitting bacterial escape (11, 24). Together, these and other changes in gene expression triggered by LL-37 confer a virulence phenotype that is strongly associated with invasive infection in animal models and that mimics the behavior of naturally occurring CsrS mutants in human infection (6, 7, 11, 13–15). LL-37-induced changes in virulence factor expression, like inactivating mutations in CsrS or CsrR, result in a striking increase in GAS resistance to osonophagocytic killing in human blood in vitro (11). The virulence phenotype induced by LL-37 has been suggested to mediate the transition from asymptomatic colonization to invasive GAS infection (11). Therefore, defining the mechanism through which LL-37 signaling occurs is of central importance to development of new strategies for preventing or treating invasive GAS disease.

The goal of the present investigation was to study the nature of the interaction of LL-37 with GAS to distinguish more clearly whether CsrRS signaling is a consequence of LL-37-induced membrane damage or rather a direct effect of specific binding of LL-37 to CsrS. Our results strongly support the latter hypothesis. We initially made the observation that nonspecific membrane damage did not lead to signaling through CsrS by testing the sheep cathelicidin SMAP 29. We then were able to extend this observation to LL-37 by testing the CsrS signaling and antibacterial activities of a series of LL-37 fragments. We found that these two activities could be dissociated in RI-10, a 10-amino acid internal fragment of LL-37 that exhibited CsrRS signaling activity that was only slightly reduced compared with full-length LL-37 but had no detectable antimicrobial activity against GAS as assessed by assays of bacterial killing, growth inhibition, and cell membrane injury. Similarly, the closely related peptides RI-11, KR-11, and KR-12 had reduced antimicrobial activity compared with LL-37 but similar CsrS signaling activity. This dissociation of CsrRS signaling from antimicrobial activity provides compelling evidence that LL-37 signaling is not simply a consequence of membrane injury but rather reflects a specific interaction with CsrS, an interaction that appears to depend on a 10-amino acid core sequence of LL-37 represented by the RI-10 peptide. Indeed, all signaling-competent peptides in the qRT-PCR signaling assays harbored all 10 of these residues (Table 2). Additional evidence for binding specificity is presented from amino acid substitutions of RI-10. Both conservative and nonconservative changes (RI-10 L10A, L10F, and R1A) resulted in decreased signaling ability, suggesting that binding and/or signaling was affected by specific amino acids in the peptide. An all-D enantiomer (D-RI-10) also was unable to signal through CsrS, suggesting that the interaction between the two is not simply because of the amphipathic nature of the peptide, further supporting the specific nature of LL-37 binding to CsrS. These data all support the hypothesis of a direct ligand/receptor interaction between LL-37 and CsrS.

If CsrRS signaling by LL-37 reflects a specific interaction rather than a response to membrane damage, the interaction is expected to occur with the ECD of the cell surface-associated histidine kinase, CsrS. Previous work showed that CsrS has a surface-exposed domain that can be labeled by biotinylation of surface proteins on intact GAS cells (11). We found that a fusion protein representing the predicted CsrS ECD fused to MBP could pull down LL-37 from solution, whereas a control construct of MBP could not. We also tested an analogous construct in which a cluster of three acidic amino acids in the ECD were substituted with their uncharged analogs, a change shown previously to abrogate LL-37 signaling through CsrS (11). The triple-mutant construct showed detectable binding to LL-37, but binding was much reduced compared with the native ECD. These results indicate that LL-37 binds to the CsrS ECD and that binding is in part dependent on a cluster of three acidic residues shown previously to be required for LL-37-induced signaling through CsrS.

The GAS proteases SpeB and Sic have been shown to cleave LL-37, reducing its antimicrobial activity (39–41). In addition, LL-37 may be degraded by the proteolytic action of human
plasmin associated with the GAS cell surface upon exposure of the bacteria to human blood plasma (35). It is therefore likely that GAS is exposed to fragments of LL-37 during the process of establishing infections. However, the antimicrobial and signaling effects of LL-37 fragments for GAS had not previously been investigated. Signaling-competent LL-37 fragments generated by proteolysis could induce an invasive phenotype during infection, even as the antimicrobial activity of LL-37 is inactivated by GAS and/or host proteases.

The observation of direct binding of LL-37 to the CsrS ECD, together with the finding that CsrS signaling can be dissociated from GAS antimicrobial activity, provides new and direct support for specific binding to CsrS as the mechanism for LL-37-mediated signaling through CsrRS. The striking specificity of CsrRS signaling by LL-37 is consistent with a role of this two-component system in adaptation of GAS to survival in the human host in the face of attack by innate immune effectors. The ability of GAS to detect and respond to LL-37 as an indicator of the host innate immune response enables rapid deployment of virulence factors that enhance bacterial resistance to host control of GAS infection.

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