2007

Contribution of invariant residues to the function of Rgg family transcription regulators

Jennifer Loughman
Washington University School of Medicine in St. Louis

Michael G. Caparon
Washington University School of Medicine in St. Louis

Follow this and additional works at: http://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation
http://digitalcommons.wustl.edu/open_access_pubs/2488

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact engeszer@wustl.edu.
Contribution of Invariant Residues to the Function of Rgg Family Transcription Regulators

Jennifer A. Loughman and Michael G. Caparon

Published Ahead of Print 10 November 2006.

Updated information and services can be found at:
http://jb.asm.org/content/189/2/650

**SUPPLEMENTAL MATERIAL**

Supplemental material

**REFERENCES**

This article cites 39 articles, 24 of which can be accessed free at: http://jb.asm.org/content/189/2/650#ref-list-1

**CONTENT ALERTS**

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
The Rgg family of transcription regulators is widely distributed among gram-positive bacteria, yet how these proteins control transcription is poorly understood. Using Streptococcus pyogenes RopB as a model, we demonstrated that residues invariant among Rgg-like regulators are critical for function and obtained evidence for a mechanism involving protein complex formation.

The Rgg-like regulators constitute a conserved family of proteins that modulate transcription in gram-positive bacteria. This group is widely distributed, and the members occur in both pathogenic and commensal species and include Rgg of Streptococcus gordonii, which is required for extracellular glucosyltransferase expression (29, 30); GadR of Lactococcus lactis, which is required for glutamate-dependent acid tolerance (27); MutR, which is required for expression of the mutacin lantibiotic, MutA, of Streptococcus mutans (23); and the plasmid-encoded LasX protein of Lactobacillus sakei, which regulates the synthesis of and immunity to the lantibiotic lactocin S (25, 28). Additional uncharacterized Rgg-like proteins are encoded by the genomes of Streptococcus pneumoniae (31), Streptococcus agalactiae (13), Streptococcus oralis (10), Streptococcus sanguis (34), Streptococcus equi (http://www.sanger.ac.uk), and Listeria monocytogenes (12), and some genomes, like those of S. pyogenes (9), S. gordonii (15, 33), S. pneumoniae (31), and S. mutans (1), contain multiple rgg-like genes.

How the members of this extensive family function to regulate gene expression is not well understood. Rgg-like proteins have a helix-turn-helix motif in the amino terminus of the polypeptide, which is a conserved DNA-binding domain found in other families of transcription regulators (17). Only recently has it been established that any Rgg-like proteins bind specifically to DNA to regulate transcription. For example, association with nucleic acid has been demonstrated for Rgg of S. gordonii (35), RopB (21), and LasX (24), but there is only a weak consensus binding site (24). The absence of a conserved regulatory motif in the promoter regions of genes regulated by Rgg-like proteins and the functional diversity of the regulated gene products suggest that Rgg-like proteins interact with additional regulatory networks to alter gene expression. Experimental data supporting this hypothesis were obtained in an analysis of the speB regulatory program in S. pyogenes, where RopB is necessary but not sufficient for activation of transcription (21) and may influence gene expression via its ability to influence the expression of other regulators (5). The integration of Rgg pathways with other regulatory pathways could also be established through protein-protein interactions. For example, RopB has been shown to associate with a negative regulator, LacD.1, which may be a mechanism for maintaining temporally controlled expression programs (16a).

Although the members of the Rgg family have been adapted to individual regulatory programs, it is likely that these proteins have a common structure and mechanism of action. Thus, an understanding of how any individual member functions may provide crucial insight into the general way that members of this family function. In S. pyogenes, RopB (also referred to as Rgg) influences the production of proteins during the transition to the stationary phase (3, 6), including the production of the secreted cysteine protease, SpeB (4, 18). Protease expression is dependent on RopB transcriptional activation (4, 18), and its activity is readily detectable, providing a convenient assay for modeling Rgg family function. The present genetic and biochemical studies were undertaken to determine if amino acids that are universally conserved throughout the Rgg family contribute to the ability of RopB to regulate speB expression in S. pyogenes. The results confirmed the importance of these invariant residues and provided insight for understanding the functional domains of RopB and related transcription factors.

Conserved Rgg residues are essential for RopB function. A recent influx of genomic information has revealed that the Rgg-like proteins constitute a large conserved family of regulators unique to gram-positive bacteria. To obtain insight into the common structure and mechanism of action that have been adopted by this group of proteins, the primary amino acid sequences of all predicted Rgg-like proteins for which protein sequence data were available were aligned by the Clustal W method (32). Proteins were considered candidates for this analysis if they were significantly homologous (E-value, <0.001) to the prototype protein from S. gordonii (accession number AAA26968) as determined by a BLAST analysis (8) of the GenBank database of microbial genomes (blastp; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). If sequence data were available for multiple isolates of a species, only one isolate was selected for analysis in order to avoid redundancy.
This search returned 57 open reading frames, and the alignment revealed numerous highly conserved and very similar amino acids, including three amino acids that were invariable (Fig. 1A; see the supplemental material).

We reasoned that because these residues were the most conserved, they might make an important contribution to the structure and/or function of Rgg-like regulators. To test this hypothesis, each of the invariant residues in RopB (G4, R11, and W142) (Fig. 2) was targeted for site-directed mutagenesis, and the ability of the mutant protein to function as an activator of \textit{speB} transcription was assessed using protease indicator plates and a quantitative assay for activity in culture supernatants, as described previously (16, 18, 21). An expression vector encoding wild-type RopB with a C-terminal hemagglutinin (HA) epitope tag was used as the template for inverse PCR with the primers described in Table 1 to construct mutant derivatives with alanine substitutions (RopBG4A, RopB R11A, and Rop W142A) or a more conservative mutation at W142 (RopBW142F). The resulting plasmids were used to transform wild-type \textit{S. pyogenes} strain HSC5 (14) or a RopB \textit{H11002} mutant derivative (MNN100 [21]) to generate the strains described in Table 2.

As noted above, RopB is required for \textit{speB} expression (4, 18); therefore, a strain with a deletion in \textit{ropB} produced only background levels of proteolytic activity compared to the wild-type strain (Fig. 1, compare the RopB \textit{H11002} strain with no plasmid to the wild-type strain with no plasmid). When wild-type \textit{ropB} was introduced into the RopB \textit{H11002} strain on a plasmid, the protease activity was restored to wild-type levels (Fig. 1B and C, compare the RopB \textit{H11002} strain with the wild-type allele to the wild-type strain with no plasmid). The behavior of a RopB \textit{H11002} strain expressing RopBG4A was similar to that of the wild-type complemented strain (Fig. 1B, compare the RopB \textit{H11002} strain with the G4A allele to the wild-type complemented strain).
and was indistinguishable from that of the wild-type strain (Fig. 1C, compare the RopB\textsubscript{H11002} strain with the G4A allele to the wild-type strain with no plasmid). In contrast, strains expressing RopB alleles modified at the arginine (R11A) and tryptophan (W142A or W142F) residues were completely nonfunctional when they were examined on protease indicator plates (Fig. 1B, compare the RopB\textsubscript{H11002} strain with the R11A, W142A, or W142F allele to the RopB\textsubscript{H11002} strain with the wild-type allele) and quantitatively (Fig. 1C) ($P < 0.001$).

When the relative transcript abundance of \textit{ropB} and \textit{speB} was determined by real-time reverse transcription-PCR performed as described elsewhere (2) using the primers listed in Table 1, we found that \textit{ropB} transcription from a plasmid was increased relative to expression from the chromosome. However, the magnitudes of \textit{ropB} overexpression were similar in all of the complemented RopB\textsubscript{H11002} strains (Fig. 2), and there was a correlation between protease activity and \textit{speB} transcript abundance. In the strains that did not exhibit protease activity (e.g.,

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J135</td>
<td>BL21(DE3)</td>
<td>pJL58</td>
<td>Expression of GST-RopB</td>
<td>This study</td>
</tr>
<tr>
<td>J143</td>
<td>BL21(DE3)</td>
<td>PGEX</td>
<td>Expression of GST</td>
<td>Amersham 27-4587-01</td>
</tr>
</tbody>
</table>

S. pyogenes strains |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC5</td>
<td>Wild-type</td>
<td>None</td>
<td>Wild type</td>
<td>14</td>
</tr>
<tr>
<td>J136</td>
<td>Wild type</td>
<td>pLZ12</td>
<td>HSC5 (vector)</td>
<td>This study</td>
</tr>
<tr>
<td>J187</td>
<td>Wild type</td>
<td>pJL77</td>
<td>HSC5(pRopB-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J189</td>
<td>Wild type</td>
<td>pJL79</td>
<td>HSC5(pRopB G4A-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J191</td>
<td>Wild type</td>
<td>pJL92</td>
<td>HSC5(pRopB R11A-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J193</td>
<td>Wild type</td>
<td>pJL83</td>
<td>HSC5(pRopB W142A-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J196</td>
<td>Wild type</td>
<td>pJL105</td>
<td>In-frame deletion of \textit{ropB}</td>
<td>This study</td>
</tr>
<tr>
<td>MNN100</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>none</td>
<td>MNN100 (vector)</td>
<td>21; this study</td>
</tr>
<tr>
<td>J410</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>pLZ12</td>
<td>MNN100(pRopB-HA; Km\textsuperscript{3})</td>
<td>This study</td>
</tr>
<tr>
<td>J139</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>pJL60</td>
<td>MNN100(pRopB-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J207</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>pJL77</td>
<td>MNN100(pRopB-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J209</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>pJL79</td>
<td>MNN100(pRopB G4A-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J211</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>pJL92</td>
<td>MNN100(pRopB R11A-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J213</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>pJL83</td>
<td>MNN100(pRopB W142A-HA)</td>
<td>This study</td>
</tr>
<tr>
<td>J218</td>
<td>HSC5 \textit{ropB}\textsubscript{H9004}\textsubscript{6-274}</td>
<td>pJL105</td>
<td>MNN100(pRopB W142F-HA)</td>
<td>This study</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Gene designations are based on annotation of the SF370 genome of \textit{S. pyogenes}.
the RopB− strains with the R11A, W142A, and W142F alleles) the level of the speB transcript was reduced nearly 100,000-fold compared to the level in the wild type (Fig. 2). Thus, these data indicate that the mutations rendered RopB incompetent as a transcriptional activator. Although RopB-G4A was competent for promoting the production of protease activity, the allele was not as efficient at promoting speB transcription as the wild-type allele, as shown by a twofold decrease observed when the complemented RopB− strain with the G4A allele was compared to the wild type (Fig. 2) (P < 0.01) or the fivefold decrease observed when the RopB− strain complemented with RopB-G4A was compared with the RopB− strain complemented with RopB-WT (Fig. 2) (P < 0.01). The relative expression and stability of the wild-type and mutant epitope-tagged proteins in the RopB− strain were assessed by Western blotting of whole-cell extracts (26) prepared from S. pyogenes whole-cell lysates. The strains used were the strains described in the legend to Fig. 1; two independent isolates of the strains expressing RopB-W142A were analyzed. Equivalent amounts of total protein were loaded in the lanes. WT, wild-type.

RopB expression plasmids were used to transform the wild-type strain, and SpeB activity was measured on protease indicator plates (Fig. 1B) and quantitatively (Fig. 1C). As expected, overexpression of functional RopB had no significant effect on protease expression (Fig. 1B and C, compare the wild-type strain with no plasmid to the wild-type strain with the G4A and wild-type RopB alleles). In contrast, the presence of stable, nonfunctional RopB (R11A, W142F) was dominant negative, resulting in significantly reduced SpeB activity (Fig. 1C) (P < 0.02). The stability of the dominant-negative proteins was confirmed by Western blot analysis (Fig. 3B). Taken together, these data suggest that RopB monomers may oligomerize or interact with another protein(s) in order to promote activation of speB transcription.

Evidence for RopB dimerization in vitro. The dominant-negative phenotype indicated that there was a potential for RopB-RopB interaction. This was not surprising, as it is typical for the functional unit of DNA-binding transcriptional regulators to be a dimer (17). It has been shown previously that RopB specifically interacts with DNA in the speB promoter that contains inverted repeats (21). In addition, analysis of conserved domains in the RopB primary amino acid sequence revealed a putative DNA-binding helix-turn-helix motif at the N terminus (20). Plasmid pJLS8 encoding a glutathione S-transferase (GST) fusion to the N terminus of RopB was constructed and expressed in E. coli using the pGEX-2TK expression vector (Amersham) and the primers described in Table 1. For expression and purification of the recombinant fusion protein and GST alone we followed the recommendations of the manufacturer (Amersham).

The fusion protein was used as bait for an interaction with proteins in a whole-cell extract (26) prepared from S. pyogenes expressing epitope-tagged RopB (RopB-HA). Glutathione-Sepharose beads were incubated with the GST-RopB fusion protein or GST and washed twice with phosphate-buffered saline prior to addition of streptococcal extracts. The extracts were incubated with the GST-fusion protein-coated beads and washed three times with phosphate-buffered saline, and bound proteins were eluted with reduced glutathione (10 mM) and subjected to immunoblotting with polyclonal anti-HA antiserum (Sigma). This analysis revealed that RopB-W142A was probably unstable; however, substitution of another aromatic amino acid at position 142 and alanine substitutions at other positions were tolerated (Fig. 3A), indicating that the tryptophan contributes to both the stability of RopB and its ability to regulate gene expression. Introduction of alanine at position 142 did create a rare codon (7), raising the possibility that the reduced level of detectable RopB-W142A in streptococcal extracts was the result of translational inefficiency rather than protein instability. This interpretation is less likely, however, since expression of RopB-R11A, which used the same rare codon, was efficient. Furthermore, a Western blot analysis of E. coli extracts was consistent with the S. pyogenes data (not shown), indicating that failure to detect RopB-W142A was due to a general problem rather than codon bias. Taken together, these data demonstrate that the conserved arginine (R11), tryptophan (W142), and, to a lesser extent, glycine (G4) are required for fully active RopB and are thus likely to be important for other Rgg-like regulators.

Expression of nonfunctional RopB mutants inhibits native RopB function. To obtain insight into the mechanism of RopB regulation, we examined whether the various mutants were dominant negative when they were expressed in trans. The

FIG. 3. Conserved tryptophan contributes to RopB protein stability and regulation: Western blot analyses of ectopically expressed RopB in S. pyogenes whole-cell lysates. The strains used were the strains described in the legend to Fig. 1; two independent isolates of the strains expressing RopB-W142A were analyzed. Equivalent amounts of total protein were loaded in the lanes. WT, wild-type.

Evidence for RopB dimerization in vitro. The dominant-negative phenotype indicated that there was a potential for RopB-RopB interaction. This was not surprising, as it is typical for the functional unit of DNA-binding transcriptional regulators to be a dimer (17). It has been shown previously that RopB specifically interacts with DNA in the speB promoter that contains inverted repeats (21). In addition, analysis of conserved domains in the RopB primary amino acid sequence revealed a putative DNA-binding helix-turn-helix motif at the N terminus (20). Plasmid pJLS8 encoding a glutathione S-transferase (GST) fusion to the N terminus of RopB was constructed and expressed in E. coli using the pGEX-2TK expression vector (Amersham) and the primers described in Table 1. For expression and purification of the recombinant fusion protein and GST alone we followed the recommendations of the manufacturer (Amersham).

The fusion protein was used as bait for an interaction with proteins in a whole-cell extract (26) prepared from S. pyogenes expressing epitope-tagged RopB (RopB-HA). Glutathione-Sepharose beads were incubated with the GST-RopB fusion protein or GST and washed twice with phosphate-buffered saline prior to addition of streptococcal extracts. The extracts were incubated with the GST-fusion protein-coated beads and washed three times with phosphate-buffered saline, and bound proteins were eluted with reduced glutathione (10 mM) and subjected to immunoblotting with polyclonal anti-HA antiserum (Sigma). This analysis revealed that RopB-W142A was probably unstable; however, substitution of another aromatic amino acid at position 142 and alanine substitutions at other positions were tolerated (Fig. 3A), indicating that the tryptophan contributes to both the stability of RopB and its ability to regulate gene expression. Introduction of alanine at position 142 did create a rare codon (7), raising the possibility that the reduced level of detectable RopB-W142A in streptococcal extracts was the result of translational inefficiency rather than protein instability. This interpretation is less likely, however, since expression of RopB-R11A, which used the same rare codon, was efficient. Furthermore, a Western blot analysis of E. coli extracts was consistent with the S. pyogenes data (not shown), indicating that failure to detect RopB-W142A was due to a general problem rather than codon bias. Taken together, these data demonstrate that the conserved arginine (R11), tryptophan (W142), and, to a lesser extent, glycine (G4) are required for fully active RopB and are thus likely to be important for other Rgg-like regulators.

Expression of nonfunctional RopB mutants inhibits native RopB function. To obtain insight into the mechanism of RopB regulation, we examined whether the various mutants were dominant negative when they were expressed in trans. The
FIG. 4. Evidence of in vitro RopB dimerization. (A) RopB-HA from streptococcal whole-cell lysates was specifically bound by a GST fusion to RopB (GST-RopB). Extracts from S. pyogenes strain JL139 [RopB (pRopB-HA)] were incubated with GST or GST-RopB, and specifically bound proteins were resolved by SDS-PAGE and immunoblotted with HA-specific antibody (oHA). The flowthrough fraction (FT) contained proteins that did not bind the immobilized GST bait, and the elution fraction (E) contained the GST bait and bound proteins. (B) A whole-cell lysate of S. pyogenes strain JL139 was prepared and heated for 10 min at 95°C or not treated prior to resolution by SDS-PAGE and immunoblotting with HA-specific antibody. The positions of molecular weight standards are indicated on the left in panel A, and the positions of bands corresponding to the expected sizes of a RopB-HA monomer and dimer (2×RopB-HA) are indicated between the panels.

Implications for the Rgg protein family. In the present report, we show that common Rgg-like sequence motifs are essential for RopB function, which may indicate a common mechanism used by members of the Rgg-like family to process regulatory signals. The mechanism of Rgg family regulation has yet to be fully elucidated, but the data indicate that it likely involves interaction with both DNA and other proteins. For example, two of the invariable residues, G4 and R11, are located within the predicted DNA-binding domain. While the role of glycine has not been well studied, structural analysis of other XRE-type helix-turn-helix domains has revealed that arginine in this position contributes to the stability of the DNA-protein complex through formation of a fold-stabilizing salt bridge with a conserved glutamic acid residue (22) or side chain contacts with phosphate groups in target DNA (11, 36–38). Invariant tryptophan residues are often involved in protein-protein interactions (19), and W142 may localize to the interface of a critical RopB-protein complex. Substitution of a nonhydrophobic residue can interfere with complex formation, resulting in an unstable protein. In this context, the decreased stability of RopB_W142A is not surprising. Also consistent with this, we obtained evidence that RopB forms protein complexes in vivo through generation of dominant-negative mutant alleles and in vitro through pull-down assays with purified protein. These studies provide a foundation for investigation of the molecular mechanism of speB regulation and general insights into the specificity and functional domains of the family of Rgg-like proteins.

We thank M. Neely for providing strain MNN100 (21). We also thank Travis Jewett and L. David Sibley for providing reagents and technical advice. This work was supported by Public Health Service grant AI463303 to Michael G. Caparon.

REFERENCES


