Molecular Interaction between Proteins Involved in EvgAS Signal Transduction of Escherichia coli

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EvgA and EvgS constitute one two-component signal transduction system in Escherichia coli. Although probable signaling domains of these proteins have been estimated, the molecular mechanism of their interaction remains to be elucidated. Here, we investigated protein to protein interactions between EvgA and EvgS and also between the EvgAS system and other related signaling pathways by means of surface plasmon resonance. EvgA and EvgS interacted directly and inhibition of phosphorylation of their functional domains abolished formation of the EvgAS complex. No interaction was observed either between EvgA and Bordetella BvgS or BvgA and EvgS. OmpR, a response regulator for the osmoregulatory gene expression of E. coli, had similar but not identical behavior towards EvgS to that of EvgA. These results indicate that interaction between the signaling proteins is closely related to phosphorylation of the functional domain of the proteins.

Key words: EvgAS; OmpR; two-component system; signal transduction; surface plasmon resonance

A major mechanism of signal transduction that is widespread in bacteria is the two-component system that responds to a wide variety of environmental stimuli.1) This system consists of a membrane receptor (sensor) and a cytoplasmic DNA-binding protein (response regulator), and also seems to exist in eukaryotes.2,3) Environmental signals detected by the sensor protein are passed to the response regulator via phosphorylation, which activates the latter protein, resulting in an alteration of gene expression. The phosphotransfer between the two proteins requires two distinctive domains, namely a transmitter and a receiver. The transmitter domain found in sensor proteins has an autokinase activity that attaches a phosphate group from ATP to a His residue. The resulting phosphoHis serves as a high-energy intermediate for subsequent transfer of the phosphate group to an Asp residue in the receiver domain of response regulators. Response regulators sometimes receive input signals from a sensor that is part of other two-component systems. Such cross-talk may be an important physiological function of bacteria, allowing them to identify and respond to complex environmental information.4,5)

Using the cross-talk phenomenon, we isolated from Escherichia coli, a novel two-component system comprised of the response regulator EvgA and the sensor protein EvgS.6) EvgA and EvgS are highly homologous to BvgA and BvgS of Bordetella spp.,7–9) respectively. The Bvg signaling system has been extensively studied.10) The BvgS protein has a more complex structure than most sensors: BvgS contains additional sequences including the receiver domain found in reponse regulators and a C-terminal domain.11) Mutational analysis has shown that the BvgS transfer, receiver, and C-terminus are all required for the signal transduction and virulence control of Bordetella pertussis.11–13) In addition to these three modules, the periplasmic and linker portions of BvgS are also essential for its function.13–16)

BvgA is a typical response regulator with Asp-54, a putative phospho-accepting site in the receiver, and BvgS has three phosphorylation sites: His-729 of the transmitter, Asp-1023 of the receiver, and His-1172 of the C-terminus.10) The Evg signaling factors also contain these conserved amino acid residues: Asp-52 in the EvgA receiver, and His-721, Asp-1009, and His-1137 in the EvgS cytoplasmic region.10) An in vivo study showed that amino acid conversion of Asp-52 to Ala or His-721 to Arg suppresses Evg signaling activity.17) Therefore, these amino acid residues are supposed to control the EvgAS phosphorylation pathway. The aim of this study was to characterize the intermolecular interaction between proteins related to the Evg signaling circuitry.

Materials and Methods

Preparation of His-tagged proteins. Table is a list of bacterial strains and plasmids used to overexpress the gene that encodes a wild-type protein or its mutant. EvgAm is a mutant protein of EvgA, in which Asp52 of EvgA has been changed to Ala. EvgSm1 and EvgSm2 are mutant derivatives of EvgS, in which the amino acid sequences were altered at His721 and His1137 to Arg, respectively. To prepare EvgAm, BvgA, and OmpR, the entire open reading frame was subcloned from the respective template plasmids into pQE30 (Qiagen) and overexpressed in E. coli SG13009 (pREP4) as described previously for EvgA.17) For EvgS, EvgSm1, EvgSm2, and BvgS, a DNA fragment encoding the cytoplasmic portion in the respective template plasmids was engineered by the polymerase chain reaction to bear a BamHI and a HindIII site at the 5′- and 3′-ends, respec-

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Abbreviations: BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Molecular Interaction between *E. coli* Evg Signaling Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size*</th>
<th>Amino acid substitution</th>
<th>Template plasmid</th>
<th>Expression vector</th>
<th>Host strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>EvgA(7)</td>
<td>Full length</td>
<td>None</td>
<td>pSK001(6)</td>
<td>pQE30</td>
<td>SG13009(pREP4)</td>
</tr>
<tr>
<td>EvgAm</td>
<td>Full length</td>
<td>Asp52→Ala</td>
<td>pKM12(7)</td>
<td>pQE30</td>
<td>SG13009(pREP4)</td>
</tr>
<tr>
<td>BvgA</td>
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<td>None</td>
<td>pDM20(9)</td>
<td>pQE30</td>
<td>SG13009(pREP4)</td>
</tr>
<tr>
<td>OmpR</td>
<td>Full length</td>
<td>None</td>
<td>pAT22E(8)</td>
<td>pQE30</td>
<td>SG13009(pREP4)</td>
</tr>
<tr>
<td>EvgS</td>
<td>a.a. 556–1197</td>
<td>None</td>
<td>pSK001(6)</td>
<td>pET-21a</td>
<td>BL21(DE3)</td>
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<td>EvgSm1</td>
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<td>a.a. 566–1238</td>
<td>None</td>
<td>pDM(9)</td>
<td>pET-21a</td>
<td>BL21(DE3)</td>
</tr>
</tbody>
</table>

* a.a.: amino acids

tively, and then subcloned in-frame into the BamHI/ HindIII site of pET-21a (Novagen). DNA sequencing confirmed that all the DNA fragments were inserted in-frame and, concerning the plasmid encoding EngAm, EvgS, EvgSm1, or EvgSm2, every mutation was introduced into the proper site. The resultant plasmid was used to transform *E. coli* BL21 (DE3) and the subcloned fragment was overexpressed as described previously for EvgA under conditions in favor of induction of signaling, i.e., at 37°C in the absence of signaling-modulation agents such as nicotinic acid and magnesium sulfate.\(^\text{17}\)

All the proteins produced in this manner, carried a His hexamer at the end of the peptide chain and were purified on an Ni-nitrilotriacetic acid agarose column.\(^\text{17}\)

**Experiments of surface plasmon resonance.** Surface plasmon resonance allows real-time analysis of specific protein to protein interactions, in which binding of an analyte to a ligand immobilized on a sensor chip is measured as a change in refractive index and is directly proportional to the amount of the bound analyte.\(^\text{21–23}\)

Ligands were immobilized and the binding to analytes was monitored automatically by a BIAcore system (Pharmacia) according to the manufacturer's instructions. Purified ligand proteins were covalently coupled to a BIA sensor chip at an amount of about 10,000 resonance units. One resonance unit corresponds to 1 pg of bound protein per mm² of the sensor chip. Analytes were dissolved in HBS buffer (10 mm HEPES-NaOH, pH 7.4, 150 mm NaCl, 3.4 mm EDTA, and 0.05% Surfactant P20). The concentration and amount of injected analytes were 10 ng/μl and 40 μl, respectively. Protein concentration was measured by the method of Bradford.\(^\text{24}\)

The binding reaction that proceeded at 37°C was monitored on a sensorgram where a resonance unit was plotted as a function of the reaction time. Chips were regenerated after each binding experiment, which was done by injecting 15 μl of 2 M NaCl followed by extensive washing with HBS buffer.

**Results and Discussion**

**Purification of signaling proteins**

The proteins listed in Table were produced in an *E. coli* overexpression system. They were tagged with six consecutive His residues and purified by affinity chromatography to electrophoretic homogeneity (Fig. 1). The eluate containing the proteins was dialyzed against HBS buffer and used for analysis by surface plasmon resonance.

**Association of EvgA with EvgS**

Our previous phosphotransfer study using purified EvgA and a cell membrane fraction suggested that EvgA and EvgS react in the same manner as a typical responsive regulator and sensor kinase.\(^\text{17}\) We tested whether or not this EvgAS interaction was direct by surface plasmon resonance, using purified forms of both Evg (Fig. 2a). Since these proteins were prepared under signaling-inducing conditions,\(^\text{17}\) each preparation should be a mixture of phosphorylated and unphosphorylated forms. After initial equilibration of the sensor surface with HBS buffer, a sample was injected (time 0). At 100 s, the sample reached the sensor surface, resulting in an instantaneous increase in the response signal due to a difference in the refractive index between the sample and the running buffer. The high signal remained during sample flow. At 330 s, the sample pulse was replaced with HBS buffer, and dissociation of the bound sample protein
was followed. At this phase, association between the sample protein and the immobilized ligand was evident. When EvgS was passed over an EvgA surface, considerable response could still be detected after the HBS wash. In a negative control to define the background level where bovine serum albumin (BSA) was used as the ligand, the response curve presented a rapid drop to near the baseline on the wash. These sensorgrams demonstrate a direct and specific interaction between EvgA and EvgS, and correlated with our previous result of in vivo phosphotransfer from EvgS-containing cell membrane to EvgA. 17) We presume that the EvgAS interaction observed here reflects phosphotransfer from a phosphorylated subpopulation of EvgS to an unphosphorylated subpopulation of EvgA. We also injected EvgA onto the EvgA surface. Although phosphorylated EvgA should be present both in the ligand and the analyte, no signal for EvgA to EvgA interaction was detected (data not shown), indicating that, unlike other response regulators, EvgA does not take multimeric forms even after it has been phosphorylated.

Effects of phosphorylation on EvgAS interplay
As mentioned above, point mutation in the putative
phosphorylation sites of EvgA and EvgS abolished the \textit{in vivo} signaling cascade.\textsuperscript{17} Therefore, phosphorylation of these amino acids is likely to be essential for interaction between EvgA and EvgS. To further understand the interaction, point mutations in the receiver, transmitter, and C-terminus were tested for binding ability by surface plasmon resonance. When EvgAm was used instead of EvgA as the ligand for EvgS, the resonance signal declined to near the basal level (Fig. 2a). This finding suggests that EvgAm is defective in association with phosphorylated EvgS and, therefore, phospho-acceptability of Asp52 on the EvgA receiver is indispensable for EvgAS association as well as the subsequent acceptance of a phosphoryl group from EvgS. Next, EvgSm1 and EvgSm2 were injected separately onto the immobilized EvgA to estimate the function of the His residue on the transmitter and C-terminal domains of EvgS (Fig. 2b). Both EvgSm1 and EvgSm2 similarly lowered the affinity to EvgA compared with wild-type EvgS, implying that these EvgS mutants are partially deactivated in binding to unphosphorylated EvgA due to loss of one of the phospho-donor sites. Boucher \textit{et al.} examined DNA-binding activity of BvgA treated with alkaline phosphatase and demonstrated that BvgA was effectively dephosphorylated by the treatment.\textsuperscript{27} We used the method of enzymatic dephosphorylation to further investigate the relation between phosphorylation of EvgS and its ability for binding to EvgA. To treat EvgS with phosphatase, wild-type EvgS (100 ng) was incubated with 2 units of calf intestine alkaline phosphatase for 30 min at 37°C before injection. The reaction profile of the phosphatase-treated EvgS and the immobilized EvgA is given in Fig. 2b. This EvgS had even less binding activity than EvgSm1 and EvgSm2. We suppose that extensive dephosphorylation of EvgS by the phosphatase treatment abolished its ability to bind to EvgA. Thus, these data indicate that interaction between EvgA and EvgS is closely related to phosphorylation of the functional sites on these proteins. The reason for the difference in affinity for EvgA between the EvgS mutants and the phosphatase-treated EvgS is not clear. The two His residues of EvgS may contribute equally and cumulatively to the physical contact with EvgA. Alternatively, other probable phospho-donor residue(s) of EvgS, \textit{e.g.}, Asp-1009, may be actually involved in EvgAS interaction. The use of a double/triple mutant EvgS eliminating these residues should further clarify their role.

\textbf{Interaction between Evg and Bvg systems}

BvgA accepts a phosphate group from wild-type BvgS but not from a mutant BvgS lacking a phosphorylated amino acid on its receiver region.\textsuperscript{19} This finding was supported by our results from an experiment on surface plasmon resonance in which immobilized BvgA bound to BvgS but not to BvgS that was treated with alkaline phosphatase as EvgS was (Fig. 2c), implying that unphosphorylated BvgA is able to associate with phosphorylated BvgS to receive a phosphoryl group but does not respond to dephosphorylated BvgS. As EvgAS and BvgAS are structurally and functionally very similar, the notion of cross-talk between the two systems is plausible. However, no binding was evident either between EvgA and BvgS (Fig. 2b) or between BvgA and EvgS (Fig. 2c). Beier \textit{et al.} reported that substitution of the C-terminus of BvgS by that of EvgS abolished the signaling activity of BvgS.\textsuperscript{12,13} These data that contradict the BvgA/EvgS association may explain the molecular basis of their finding. Although there is no direct evidence to rule out cross-talk between Evg and Bvg signaling systems, our observations together with the results of Beier \textit{et al.} strongly suggest that EvgAS and BvgAS are not functionally interchangeable despite their high sequence similarity. The final conclusion awaits construction of a system for simultaneous overexpression of EvgA/BvgS or BvgA/EvgS.

\textbf{Involvement of OmpR in Evg signaling cascade}

OmpR is a transcriptional regulator that is required for the osmolarity-sensitive expression of genes for OmpC and OmpF, the major outer membrane proteins of \textit{E. coli}.\textsuperscript{20} EvgA and EvgS were originally isolated as factors that promote the expression of non-cognate \textit{ompC},\textsuperscript{6} but the relationship between the Evg system and OmpR remains unknown. We investigated this relationship by surface plasmon resonance in which EvgA, EvgS and OmpR were introduced to an OmpR sensor chip (Fig. 2d). OmpR assumes oligomer structures,\textsuperscript{29} which can be seen from the OmpR-OmpR interaction profile in this sensorgram. EvgS had an even higher response to OmpR than OmpR, and EvgA was not active on OmpR. Thus, we propose that the environmental signals sensed by EvgS are directly transferred to OmpR through cross-talk, ending in the stimulation of \textit{ompC} expression. Phosphotransfer to the non-cognate OmpR has been identified in the \textit{E. coli} Arc signaling system.\textsuperscript{30,31} We also examined the ability of EvgSm1, EvgSm2, and phosphatase-treated EvgS to bind to OmpR (Fig. 2e). The affinity of EvgSm1 and EvgSm2 for OmpR was lower than that of wild-type EvgS, but unlike the pattern of response to EvgA (Fig. 2b), the extent of the activity loss was comparable to that of phosphatase-treated EvgS, implying a difference in how EvgA and OmpR receive signals from EvgS. Another difference between EvgA and OmpR was interaction with BvgS: OmpR has a weak but significant binding activity to BvgS in comparison to that to phosphatase-treated BvgS (Fig. 2e), but no detectable signal of response between EvgA and BvgS was obtained (Fig. 2b). This finding is compatible with an earlier report on the cross-talk found between the Bvg and OmpR systems,\textsuperscript{12} again showing an intimate relationship of phosphotransfer between signaling proteins with their physical interaction. The approach using surface plasmon resonance technology in combination with phosphorylation/phosphotransfer experiments should further understanding of how signaling complexes transmit intracellular messages.

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References

23) J. Davies, Nanobiology, 3, 5-16 (1994).