A New Class of Signal Transducer in His-Asp Phosphorelay Systems*

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Nitrate transport activity of the LtnT permease of the cyanobacterium Synechococcus elongatus is activated when LtnA, a response regulator without an effector domain, is phosphorylated by LtnB, a hybrid histidine kinase. We identified a protein (LtnC) that is required for activation of LtnT. LtnC consists of an N-terminal histidine-containing phosphoacceptor (HisKA) domain, a receiver domain, and a unique C-terminal domain found in some cyanobacterial signals. Because LtnC lacks an ATP-binding kinase domain of a histidine kinase, it is incapable of autophosphorylation, but LtnC is phosphorylated by LtnA. The histidine residue in the HisKA domain but not the aspartate residue in the receiver domain is essential for phosphorylation of LtnC and activation of LtnT. LtnC phosphorylation leads to oligomerization of the protein. Fusion of the C-terminal domain of LtnC to glutathione S-transferase, which forms oligomers, also activates LtnT, suggesting that oligomerization of the LtnC C-terminal domain causes LtnT activation. These results indicate that the C-terminal domain of LtnC acts as an effector domain that directs the output of the signal from the phosphorelay system. The two-step (His-Asp-His) phosphorelay system, composed of the LtnB, LtnA, and LtnC proteins, is distinct from the known phosphorelay systems, namely, the typical two-component system (His-Asp-His) and the multistep phosphorelay system (His-Asp-His-Asp), because the HisKA domain of LtnC is the terminal phosphoacceptor that determines the signal output. LtnC is a new class of signal transducer in His-Asp phosphorelay systems that contains a HisKA domain and an effector domain.

To adapt to fluctuating environments, living organisms possess various mechanisms for monitoring environmental signals and regulating cellular activities to achieve optimal growth under new conditions. One of the main regulatory mechanisms for signal transduction in bacteria, yeast, and plants is the two-component system consisting of a sensor histidine kinase and a response regulator (1–3). Sensor histidine kinases consist of an N-terminal domain that detects various stimuli, a histidine-containing phosphoacceptor domain (histidine kinase A domain or HisKA, NCBI accession number cd00082), and an ATP-binding kinase domain (histidine kinase ATPase or HATPase, NCBI accession number cd00075). The conserved histidine residue of the HisKA domain is autophosphorylated by the HATPase domain. Most of the response regulators consist of an N-terminal receiver domain (REC, NCBI accession number cd00156) and an effector domain. The conserved aspartate residue of the REC domain receives the phosphoryl group from the histidine residue of the HisKA domain of the sensor histidine kinase, and this phosphorylation regulates the activity of the C-terminal effector domain. Most response regulators have a DNA-binding effector domain and function as transcription factors, although some lack effector domains, and a few have effector domains with enzymatic activity. To date, two types of His-Asp phosphorelay system, namely, the typical or classic two-component system described above and the multistep phosphorelay system, have been demonstrated. The typical two-component system mediates a single phosphoryl transfer reaction (His-Asp) from the HisKA domain of the sensor histidine kinase to the REC domain of the response regulator (e.g. Ref. 4; see also Fig. 5A). In contrast, the multistep phosphorelay system additionally requires both an intermediate REC domain and a histidine-containing phosphotransfer (HPT) domain. This system catalyzes three phosphoryl transfer reactions (His-Asp) from the HisKA domain of the sensor histidine kinase, the intermediate REC domain, the HPT domain, and the REC domain of the terminal response regulator in this order (e.g. Ref. 5; see also Fig. 5B). In both systems, the REC domain of a response regulator is the terminal acceptor of the phosphoryl group, and the phosphorylated response regulators act as molecular switches to elicit cellular events by regulating the activity of their C-terminal effector domains.

The cyanobacterium Synechococcus elongatus has an ATP-binding cassette-type (ABC-type) nitrate transport system (NRT) encoded by the four genes, nrtA, -B, -C, and -D (6–8). The NA3 mutant, which is constructed by deleting the nrtA, -B, -C, and -D genes, lacks NRT activity and is unable to grow in low concentrations of nitrate (<5 mM) (9). Genetic character-

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* This work was supported by a Grant-in-aid for Scientific Research in Priority Areas (13206027) and in part by the 21st Century COE Program and a Grant-in-aid for Scientific Research in Priority Areas (18056008) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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37868 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 49 • DECEMBER 8, 2006

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 49, pp. 37868 –37876, DECEMBER 8, 2006

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Two-step (His-Asp-His) Phosphorelay System

Cyanobacterial strains and plasmids used

| Strains or plasmid | Relevant characteristics | Reference or source |
|--------------------|--------------------------|---------------------|
| pSE1               | Km<sup>+</sup>, *Synechococcus* shuttle expression vector | 9                    |
| pLtnA-B-(408–735)  | pSE1 derivative encoding LtnA and LtnB lacking N-terminal region and both REC domains | This study |
| pLtnC              | pSE1 derivative encoding LtnC | This study |
| pLtnC(H16E)        | pSE1 derivative encoding LtnC(H16E) | This study |
| pLtnC(D223N)       | pSE1 derivative encoding LtnC(D223N) | This study |
| pLtnCc             | pSE1 derivative encoding LtnC lacking HisKA and REC domains | This study |
| pLtnD              | pSE1 derivative encoding LtnD | This study |
| pLtnDc             | pSE1 derivative encoding LtnD lacking a CAP_ED domain | This study |
| pSE1GST            | pSE1 derivative encoding glutathione S-transferase | This study |
| pGSTLtnCc          | pSE1GST derivative encoding the C-terminus region of LtnC | This study |
| pGSTLtnCc          | pSE1GST derivative encoding the C-terminus region of LtnD | This study |
| pQE30, pQE31       | Amp<sup>+</sup>, *E. coli* His-tagged expression vector | Qiagen |
| pQELtnA            | pQE30 derivative encoding LtnA | 10 |
| pQELtnB-(408–977)  | pQE31 derivative encoding LtnB lacking the N-terminal region | 10 |
| pQELtnB-(408–735)  | pQE31 derivative encoding LtnB lacking the N-terminal region and both REC domains | 10 |
| pQELtnC            | pQE30 derivative encoding LtnC | This study |
| pQELtnC(H16E)      | pQE30 derivative encoding LtnC(H16E) | This study |
| pQELtnC(D223N)     | pQE30 derivative encoding LtnC(D223N) | This study |

**ExPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—A derivative of *S. elongatus* that was cured of the resident small plasmid pUH24 (R2-Scp<sup>1</sup>, hereafter designated as the wild-type strain) and the mutant strains derived from it were grown phototautrophically at 30 °C under continuous illumination provided by fluorescent lamps (70 microeinstein m<sup>-2</sup> s<sup>-1</sup>). The basal medium used was a nitrogen-free medium obtained by modification of the BG11 medium (12) as described previously (13). Ammonium-containing medium was prepared by adding 3.75 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to the basal medium. Nitrate-containing medium was prepared by adding KNO<sub>3</sub> at indicated concentrations to the basal medium. Solid medium was prepared by adding 1.5% Bacto Agar (Difco) to the liquid medium. All media were buffered with 20 mM HEPES-KOH (pH 8.2). When appropriate, kanamycin (25 μg/ml) or chloramphenicol (10 μg/ml) or both was added to the media. The *Synechococcus* strains and plasmids used in this study are listed in Table 1.

**Construction of the Insertional Mutants**—Site-directed insertional mutants were constructed as described previously by Williams and Szalay (14). DNA fragments that contained complete or partial sequences of the target open reading frames (ORFs) of *Synechococcus* were amplified by PCR and cloned into pGEM-T Vector (Promega Corp.). A chloramphenicol-resistant (Cm<sup>+</sup>) marker (15) was subsequently inserted at a suitable restriction site in each of the ORFs in the same orientation as the ORFs. The resulting plasmids were used to transform *Synechococcus* to chloramphenicol resistance through homologous recombination. The transformants were allowed to grow on solid medium supplemented with chloramphenicol. After
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serial streak purifications to promote segregation of alleles and to isolate homozygous mutants, genomic DNA from selected clones was analyzed by PCR to confirm the presence and position of the Cmr marker.

Expression of Plasmid-encoded Proteins in Synechococcus—A shuttle expression vector (pSE1) (9) was used to express cloned genes in Synechococcus. The coding regions of ltnC and ltnD were amplified from the Synechococcus chromosomal DNA by PCR. The 5′ primers carried mismatches with the genomic sequence, which created a BspHI recognition site at the translation start site without changing the encoded amino acid sequence, and the 3′ primers carried a BglII or a BamHI recognition site immediately downstream of the termination codon. Two ltnC derivatives were generated by overlap extension PCR (16): one had CAT-to-GAG base substitutions at nucleotides 46–48 that change the histidine residue at position 16 to a Glu residue (H16E), and the other had a G-to-A base substitution at nucleotide 667 that changes the Asp residue at position 223 to an Asn residue (D223N). To express the C-terminal portion of the LtnC and LtnD proteins, the relevant regions of ltnC and ltnD were amplified by PCR using 5′ primers carrying mismatches with the genomic sequence to create a NcoI recognition site, which provides the initiation codon, at nucleotide position 898 of ltnC and 532 of ltnD. The PCR-amplified ltnC, ltnD, and their derivatives were digested with a combination of BspHI and BglII, BspHI and BamHI, Ncol and BglII, or Ncol and BamHI and cloned between the NcoI and BamHI sites of pSE1.

To express the translational fusions of glutathione S-transferase (GST) and the C-terminal region of LtnC and LtnD, a shuttle expression vector (pSE1GST) was constructed from the genomic sequence to create a BglII recognition site at the translation start site without changing the encoded amino acid sequence, and the 3′ primer carried a BglII or a BamHI recognition site immediately downstream of the termination codon. Two ltnC derivatives were generated by overlap extension PCR (16): one had CAT-to-GAG base substitutions at nucleotides 46–48 that change the histidine residue at position 16 to a Glu residue (H16E), and the other had a G-to-A base substitution at nucleotide 667 that changes the Asp residue at position 223 to an Asn residue (D223N). To express the C-terminal portion of the LtnC and LtnD proteins, the relevant regions of ltnC and ltnD were amplified by PCR using 5′ primers carrying mismatches with the genomic sequence to create an NcoI recognition site, which provides the initiation codon, at nucleotide position 898 of ltnC and 532 of ltnD. The PCR-amplified ltnC, ltnD, and their derivatives were digested with a combination of BspHI and BglII, BspHI and BamHI, Ncol and BglII, or Ncol and BamHI and cloned between the Ncol and BamHI sites of pSE1.

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Identification of the ltnC Gene Required for Activation of LtnT—In a previous study (10), we identified three genes (ltnA, ltnB, and ltnT) required for nitrate transport by the NA3R mutant, a pseudorevertant of the NA3 mutant of S. elongatus lacking the ABC-type NRT. Downstream of the ltnA and -B genes involved in activation of the permease encoded by ltnT are two ORFs syc2279_d and syc2280_d, which are oriented in the same direction as ltnAB and which overlap with the upstream genes by 8 and 77 bases, respectively (Fig. 1). syc2279_d encodes a protein of 411 amino acids, which is similar to hybrid histidine kinase and carries a HisKA domain in the N-terminal region and a REC domain in the central region,
although it lacks an HATPase domain. syc2280_d encodes a protein of 271 amino acids and has an effector domain of the cAMP receptor protein family (CAP_ED domain, NCBI accession number cd00038) in the N-terminal region. The C-terminal regions of Syc2279_d and Syc2280_d are 47% identical, but they do not belong to known domains proposed in the conserved domain database of NCBI, and their function is unknown. To characterize the syc2279_d and syc2280_d genes, we inactivated them by inserting a CmR cassette in the NA3R pseudorevertant (Fig. 1) and compared the growth of the resulting mutants with that of the wild-type strain, the NRT-deficient mutant (NA3), the NA3R pseudorevertant, and the previously constructed NA3R derivatives (Fig. 2, strains a–g). The wild-type strain grew equally well on ammonium- or nitrate-containing medium (a). The NA3 mutant grew as well as the wild-type strain on ammonium-containing medium but hardly grew on a medium containing nitrate and showed yellow color because of reduced pigmentation (b), as described previously (9). NA3R cells grew as well as the wild-type strain on plates containing nitrate (c), but interruption of ltnA or ltnB in NA3R abolished the ability to grow on nitrate (d and e), as described previously (10). Interruption of syc2279_d but not syc2280_d in NA3R abolished the ability to grow on nitrate (f and g), indicating that syc2279_d but not syc2280_d was required for the LtnT-dependent nitrate transport activity of NA3R. syc2279_d was named ltnC.

Structure-Function Relationship of LtnC—To study the structure-function relationship of LtnC in activating LtnT, we used the pSE1 shuttle expression vector (9) to introduce the ltnC gene and its derivatives into the NA3R derivative carrying the LtnC pseudorevertant (Fig. 1) and examined the growth of the resulting transformants (Fig. 2). Expression of the LtnC protein abolishing the ability to grow on nitrate (Fig. 3, lane 1), as reported previously (10). When incubated for 20 min at 30 °C with 0.05 mM [γ-32P]ATP, LtnB-(408–977) and LtnB-(408–735) were radiolabeled (Fig. 3B, lanes 2 and 8), as reported previously (10), but LtnC was not (Fig. 3B, lane 1), indicating that LtnC is not capable of autophosphorylation. The radiolabel in the LtnB derivatives did not decrease when LtnC was incubated with LtnB-(408–977) or LtnB-(408–735) (Fig. 3B, lanes 3 and 9), indicating that the phosphoryl group was not transferred to LtnC from either the HisKA domain or the REC domains of the C-terminal region carrying the two REC domains, were expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 1–3), as reported previously (10). LtnC, LtnC(H16E), and LtnC(D223N) were also expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 4–6). When incubated for 20 min at 30 °C with 0.05 mM [γ-32P]ATP, LtnB-(408–977) and LtnB-(408–735) were radiolabeled (Fig. 3B, lanes 2 and 8), as reported previously (10), but LtnC was not (Fig. 3B, lane 1), indicating that LtnC is not capable of autophosphorylation. The radiolabel in the LtnB derivatives did not decrease when LtnC was incubated with LtnB-(408–977) or LtnB-(408–735) (Fig. 3B, lanes 3 and 9), indicating that the phosphoryl group was not transferred to LtnC from either the HisKA domain or the REC domains of the C-terminal region carrying the two REC domains, were expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 1–3), as reported previously (10). LtnC, LtnC(H16E), and LtnC(D223N) were also expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 4–6). When incubated for 20 min at 30 °C with 0.05 mM [γ-32P]ATP, LtnB-(408–977) and LtnB-(408–735) were radiolabeled (Fig. 3B, lanes 2 and 8), as reported previously (10), but LtnC was not (Fig. 3B, lane 1), indicating that LtnC is not capable of autophosphorylation. The radiolabel in the LtnB derivatives did not decrease when LtnC was incubated with LtnB-(408–977) or LtnB-(408–735) (Fig. 3B, lanes 3 and 9), indicating that the phosphoryl group was not transferred to LtnC from either the HisKA domain or the REC domains of the C-terminal region carrying the two REC domains, were expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 1–3), as reported previously (10). LtnC, LtnC(H16E), and LtnC(D223N) were also expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 4–6). When incubated for 20 min at 30 °C with 0.05 mM [γ-32P]ATP, LtnB-(408–977) and LtnB-(408–735) were radiolabeled (Fig. 3B, lanes 2 and 8), as reported previously (10), but LtnC was not (Fig. 3B, lane 1), indicating that LtnC is not capable of autophosphorylation. The radiolabel in the LtnB derivatives did not decrease when LtnC was incubated with LtnB-(408–977) or LtnB-(408–735) (Fig. 3B, lanes 3 and 9), indicating that the phosphoryl group was not transferred to LtnC from either the HisKA domain or the REC domains of the C-terminal region carrying the two REC domains, were expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 1–3), as reported previously (10). LtnC, LtnC(H16E), and LtnC(D223N) were also expressed as His-tagged proteins in E. coli and purified to near homogeneity (Fig. 3A, lanes 4–6). When incubated for 20 min at 30 °C with 0.05 mM [γ-32P]ATP, LtnB-(408–977) and LtnB-(408–735) were radiolabeled (Fig. 3B, lanes 2 and 8), as reported previously (10), but LtnC was not (Fig. 3B, lane 1), indicating that LtnC is not capable of autophosphorylation. The radiolabel in the LtnB derivatives did not decrease when LtnC was incubated with LtnB-(408–977) or LtnB-(408–735) (Fig. 3B, lanes 3 and 9), indicating that the phosphoryl group was not transferred to LtnC from either the HisKA domain or the REC domains of
LtnB. When LtnA was incubated with the LtnB derivatives, the radiolabel in the LtnB derivatives decreased due to the transfer of the phosphoryl group to the LtnA protein (Fig. 3), indicating that the fused protein forms hexamer and higher order oligomers. These results show that phosphorylation of LtnC results in the formation of high order oligomers of the C-terminal region of LtnC. However, gel-filtration chromatography analysis (Fig. 4) showed that the His-tagged LtnC protein, which has a calculated molecular mass of 47,844 Da, was eluted in a peak corresponding to a calibrated molecular mass of 100 kDa (Fig. 4A, open square), indicating that unphosphorylated LtnC is a dimeric form. The GST protein, which has a calculated molecular mass of 27,795 Da, was eluted in a peak corresponding to a molecular mass of 50 kDa (Fig. 4A, open circle), confirming that GST is in a dimeric form, as reported previously (20). The fusion of the C-terminal region of LtnC and GST, having a calculated molecular mass of 39,279 Da, was eluted in a broad peak corresponding to a molecular mass range of >230 kDa (Fig. 4A, closed circle), indicating that the fused protein forms hexamer and higher order oligomers. These results indicate that dimerization of LtnC is not sufficient for activation of LtnT and that higher order oligomerization of the protein is required. To examine whether phosphorylation of LtnC causes high order oligomerization of the protein, His-tagged LtnC protein was coexpressed with untagged LtnA and LtnB-(408–735) proteins in E. coli cells, purified to homogeneity, and subjected to gel-filtration chromatography. The purified His-tagged LtnC was eluted in two peaks (Fig. 4A, closed square), one of which corresponded to a molecular mass range of >400 kDa but was not observed when LtnC alone was expressed in E. coli (Fig. 4A, open square). These results show that phosphorylation of LtnC results in the formation of octamer or higher order oligomers. The results also suggest that LtnT permease is activated by formation of high order oligomers of the C-terminal region of LtnC in the cell.

Identification of the ltnD Gene—Although Syc2280_d is dispensable for manifestation of the LtnT activity in NA3R (discussed above), its C-terminal region is 47% identical to the C-terminal region of LtnC, suggesting that the protein may activate LtnT under certain circumstances. Expression of the full-length Syc2280_d protein or the C-terminal region of Syc2280_d did not confer on NA3 the ability to grow on nitrate (Fig. 2, q and r). However, expression of the fusion of the C-terminal region of Syc2280_d to GST supported cell growth on plates containing 5 mM nitrate (Fig. 2, s). These results suggest that the C-terminal region of Syc2280_d, which is similar to the corresponding region of LtnC, can activate the LtnT permease upon formation of high order oligomers. Thus, we named syc2280_d ltnD, although it is currently unknown when and how the protein forms oligomers in vivo.

DISCUSSION

By characterizing the NA3R mutant of S. elongatus, in which a nitrate transport system distinct from the ABC-type NRT is...
activated, we previously identified two regulatory genes (*ltnA* and *ltnB*) required for activation of the permease encoded by the *ltnT* gene (10). *ltnB* encodes a hybrid histidine kinase and *ltnA* encodes a response regulator with no effector domain. Phosphotransfer from *ltnB* to *ltnA* is essential for activation of the *ltnT* permease (10). However, the next step of this signal transduction pathway was unknown. In the present study, we identified the third component of this phosphorelay system, which is encoded by the gene (*ltnC*) located downstream of the *ltnA* and *ltnB* genes (Fig. 1). *ltnC* contains a HisKA domain and a REC domain, but because it lacks an HATPase domain, the protein is incapable of autophosphorylation (Fig. 3, lane 1). *ltnC* is nevertheless capable of accepting the phosphoryl group from *ltnA* (Fig. 3, lanes 5 and 11), showing that it comprises a phosphorelay system with *ltnB* and *ltnA*. Analyses including site-specific amino acid substitution in *ltnC* showed that the His16 residue in the HisKA domain is essential for phosphorylation of *ltnC* in *E. coli* cells (11). Glutathione *S*-transferase (GST) (500 μg, ○), and the fusion of GST and the C-terminal region of *ltnC* (1000 μg, ●) were fractionated on a prepacked Superdex 200-pg column. The molecular mass markers used were ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA, 67 kDa), chymotrypsinogen A (25 kDa), and cytochrome c (12 kDa). B, elution profile of the column. A standard curve was drawn using the peak fraction numbers for molecular mass markers (+). The positions of the eluted proteins are shown by the same symbols used in A.

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FIGURE 5. Schematic diagrams depicting the modular organization representative of the His-Asp phosphorelay systems. Variable stimulus detection domains are shown by black bars, histidine kinase A (HisKA) domains by hexagons, histidine kinase ATPase (HATPase) domains by gray boxes, receiver (REC) domains by gray circles, histidine-containing phosphotransfer (HPt) domains by triangles, DNA-binding effector domains by white boxes, and high order oligomerization-dependent regulatory (HODR) domains by white diamonds. The histidine residues in the HisKA and HPt domains are denoted by H, and the aspartate residues in the REC domains are denoted by D. Arrows indicate the presumed flow of phosphoryl (P) groups. A, the *E. coli* osmoregulatory system uses a single phosphoryl transfer event between the sensor histidine kinase (EnvZ) and its cognate response regulator (OmpR) (4). B, the *Bacillus subtilis* sporulation control system uses three phosphoryl transfer events. Spo0F, which contains an intermediate REC domain, receives a phosphoryl group from a sensor histidine kinase (KinA) and subsequently transfers the phosphoryl group to Spo0B, which contains an HPt domain. The phosphoryl group is then transferred to the terminal response regulator (Spo0A) (5). C, the *Synechococcus elongatus* regulatory system of transport activity of the LtnT permease uses two phosphoryl transfer events. LtnA, which contains an intermediate REC domain, receives a phosphoryl group from a sensor histidine kinase (LtnB) and transfers it to the HisKA domain of LtnC. Further details are given in the text.

FIGURE 4. Gel-filtration chromatography of purified proteins. A, purified samples of His-tagged *ltnC* (500 μg, □), His-tagged *ltnC* (500 μg) coexpressed with untagged *ltnA* and untagged *ltnB*-408-735 proteins in the *E. coli* cells (■), glutathione *S*-transferase (GST) (500 μg, ○), and the fusion of GST and the C-terminal region of *ltnC* (1000 μg, ●) were fractionated on a prepacked Superdex 200-pg column. The molecular mass markers used were ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA, 67 kDa), chymotrypsinogen A (25 kDa), and cytochrome c (12 kDa). B, elution profile of the column. A standard curve was drawn using the peak fraction numbers for molecular mass markers (+). The positions of the eluted proteins are shown by the same symbols used in A.
system (Fig. 5B), response regulators, whose aspartate residues of the REC domains are terminal phosphoacceptors, act as molecular switches to certain cellular events. Thus, the LtnB, LtnA, and LtnC proteins comprise a newly described two-step (His-Asp-His) phosphorelay system that terminates with a “HisKA-containing regulator” carrying an effector domain (Fig. 5C).

The NCBI conserved domain search program (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) shows 203 proteins that have a HisKA domain but lack an HATPase domain in addition to LtnC. Among these, 37 lack the conserved histidine residue of the HisKA domains and hence would not receive a phosphoryl group. Eight of the others consist only of the HisKA domain and may function as intermediate phosphotransfer molecules like HPt proteins. The remaining 158 proteins, found in the genomes of 4 archaea, 101 bacteria, and 2 eukaryotes, may include HisKA-containing regulators (supplementary Fig. S1). However, only two of these proteins have known effector domains. One, from Neisseria gonorrhoeae FA 1090, has a predicted permease domain (COG0730), a sigma-54 interaction domain; HTH-8, [factor for inversion stimulation] domain; REC, receiver domain; CyaA, adenylate cyclase catalytic domain; GAF, cGMP- and photopigment-binding domain; HAMP, HAMP linker domain; PAS, heme- and flavin-binding domain; PAC, C-terminal to a subset of PAS domains; COG3452, predicted periplasmic ligand-binding sensor domain. a, a,, amino acid.

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| Domain structure | Sequence number | Example Strains | Accession No. |
|------------------|----------------|-----------------|---------------|
| COG0730-HisKA-Sigma54-HTH_8 | 1 | Neisseria gonorrhoeae FA 1090 | AAC82507 |
| HisKA-REC-CyaA | 1 | Bradyrhizobium japonicum USDA 110 | NP_788928 |
| GAF-HisKA | 4 | Haloarcula marismortui ATCC 43049 | AAV45781 |
| HAMP-HisKA | 26 | Vibrio alginolyticus 12G01 | ZP_01258497 |
| PAS-HisKA | 17 | Salinibacter ruber DSM 13855 | YP_446492 |
| PAS-PAC-HisKA | 3 | Chlorobium phaeobacteroides BS1 | ZP_0053059 |
| COG3452-HisKA | 1 | Geobacter metallireducens GS-15 | ABB34979 |
| REC-HisKA | 2 | Wolinella succinogenes | CAE10412 |
| REC-PAS-HisKA | 2 | Methanococcales burtonii DSM 6242 | ZP_00633487 |
| HisKA-REC | 4 | Erythrobacter sp. NAP1 | ZP_01039115 |
| HisKA | 97 | Sphingopyxis alaskensis RB2256 | YP_616334 |

FIGURE 6. Domain organization of putative HisKA-containing regulators. COG0730, predicted permease domain; HisKA, histidine-containing phosphoacceptor domain; Sigma-54, sigma-54 interaction domain; HTH-8, [factor for inversion stimulation] domain; REC, receiver domain; CyaA, adenylate cyclase catalytic domain; GAF, cGMP- and photopigment-binding domain; HAMP, HAMP linker domain; PAS, heme- and flavin-binding domain; PAC, C-terminal to a subset of PAS domains; COG3452, predicted periplasmic ligand-binding sensor domain. a, a,, amino acid.

The C-terminal portion of LtnC activates LtnT when expressed as a fusion protein with GST in the S. elongatus NA3 mutant (Fig. 2, p). Because expression of GST does not activate LtnT in NA3 (Fig. 2, m), we conclude that the C-terminal portion of LtnC acts as the effector domain that activates LtnT permease. However, expressing the domain alone in NA3 failed to activate LtnT (Fig. 2, o). Because the effector domain formed insoluble material when expressed in E. coli,3 we infer that the fusion to GST contributes to stabilizing the LtnC effector domain in NA3 cells. Whereas GST by itself forms a dimer in solution (Ref. 20; see also Fig. 4A), the fusion of the LtnC effector domain to GST forms hexamer or higher order oligomers (Fig. 4A), indicating that the LtnC effector domain can promote the formation of the high order oligomers. However, LtnC is in a dimeric structure when expressed in E. coli, and the oligomers are formed when coexpressed with LtnB and LtnA in E. coli cells (Fig. 4A). These observations and the known ability of the

3 S.-i. Maeda, unpublished results.
HisKA domain to mediate dimer formation (25) suggest that LtnC dimer is formed by the interaction between the HisKA domains of two unphosphorylated LtnC molecules and, in these conditions, that the ability of the effector domain to promote oligomerization is inhibited. Phosphorylation of the HisKA domain presumably causes conformational change in LtnC to allow its C-terminal domain to promote oligomer formation. Because phosphorylation of the HisKA domain of LtnC is essential for activation of LtnT in vivo (Fig. 2, i and j), we conclude that phosphorylation-induced oligomerization of the effector domain somehow triggers activation of LtnT. We therefore define the effector domain of LtnC as a “high order oligomerization-dependent regulatory (HODR)” domain.

The gene located downstream of ltnC, which we named ltnD, encodes a protein whose C-terminal portion can activate LtnT when expressed as a fusion to GST (Fig. 2, s). Because the C-terminal portion of this protein and the LtnC effector domain are 47% identical in amino acid sequence (Fig. 7A), it seems reasonable to suppose that the C-terminal domain of LtnD is the second member of HODR domains, although oligomer formation remains to be verified experimentally. Because the N-terminal portion of the LtnD protein comprises a CAP_ED domain, this domain seems to accept an as-yet unidentified signal to promote oligomerization of the protein to activate the LtnT permease. It is currently unknown whether the oligomerized HODR domains interact with LtnT or whether one or more additional signaling proteins are involved in activation of LtnT.

Further study is required to clarify the molecular mechanism of LtnT activation.

Proteins containing HODR-like sequences are found only in proteins from cyanobacteria Thermosynechococcus elongatus BP-1 (Tll2374), Crocosphaera watsonii WH 8501 (Cwat_3664), Nostoc sp. PCC 7120 (All2034), Anabaena variabilis ATCC 29413, and Nostoc punctiforme PCC 73102 (Npun02004888, Npun02006412, and Npun02006607) with the exception of Methylococcus capsulatus str. Bath (MCA0452) (Fig. 7A). In all cases, the putative HODR domains are located in the C-terminal region of the respective protein (Fig. 7B). All of these proteins except for Cwat_3664 contain putative signal-accepting domain(s), such as HAMP, PAS, Cache (pfam02743) (26), and COG4192 (Fig. 7B). Thus, these proteins are likely to be involved in signal-transduction mechanisms in which their reception of a specific signal initiates the formation of high order oligomers to regulate certain cellular processes.

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