HepK, a protein-histidine kinase from the cyanobacterium *Anabaena* sp. strain PCC 7120, binds sequence-specifically to DNA

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Abstract

Two-component phosphorelay systems are minimally consisted of a sensory protein-histidine kinase (HK) and a response regulator (RR). HK autophosphorylates its conserved histidine residue in response to stimulus from an environment, this phosphate group then is transferred to a conserved aspartic acid residue of an RR, which is generally a transcription factor. HepK is a member of the family of sensory protein-histidine kinases in two-component phosphorelay systems (TCPS). We previously showed that HepK is an autokinase, and that DevR is its cognate RR, together comprising a mini two-component phosphorelay system that mediates developmental regulation of biosynthesis of a heterocyst envelope polysaccharide in the cyanobacterium *Anabaena* sp. PCC 7120. Unlike a typical TCPS, both HepK and DevR lack known DNA-binding domains. However, mutations in *hepK*, *hepC* and *hepA* all block the synthesis of heterocyst envelope polysaccharide. A *hepK* mutation of *Anabaena* blocks the induction of *hepA* expression. We hypothesized that HepK may regulate transcription of *hepA* or *hepC* by binding to DNA. To test this hypothesis we have performed a gel-shift analysis and have shown that although lacking a known DNA-binding motif, a truncated, soluble version of HepK binds sequence-specifically to a fragment of DNA found upstream from *hepC*, a gene that is located immediately upstream from *hepA* and also required for the synthesis of heterocyst envelope polysaccharide. The conserved phosphorylation histidine residue of HepK kinase is not required for this DNA-binding activity. Therefore, regulation of the synthesis of heterocyst envelope polysaccharide by HepK may be, at least in part, independent of two-component phosphorylation. The membrane-anchored HepK kinase with specific DNA-binding activity may serve as a membrane-tethered transcription factor, which may require an activation of regulated intramembrane proteolysis. We have found no other example of a protein histidine kinase without a known DNA binding motif that binds DNA sequence-specifically. Our finding may enable development of small DNA molecule as highly specific anti-microbial drugs because protein histidine kinases are broadly conserved in microbial pathogens but absent in humans.

Keywords: Cyanobacterium, heterocyst, *hepA*, *hepC*, histidine kinase binds DNA, DNA drug

Introduction

Two-component phosphorelay regulatory systems are widespread in prokaryotes, and are found also in fungi, amoebae and plants [1,2]. Typically, a sensory protein-histidine kinase phosphorylates its own conserved histidine residue in response to an environmental stimulus. Histidine kinases are not commonly known to function by binding to DNA, although they have been shown to regulate the transcription of target genes indirectly by activation of a specific response regulator. Response regulators generally contain an N-terminal regulatory domain with a conserved aspartate residue, to which the phosphoryl group of the kinase is transferred, and a variable C-terminal effector domain [3]. The response-regulator protein usually regulates the expression of certain genes to effect an adaptive response [4]. Most known
response regulators are transcription factors whose effector domains bind DNA. However, some response regulators have no known effector domain [1] and the effector domain of some others are enzymatically active [5, 6].

Three proteins with similarity to members of two-component regulatory systems are known to influence heterocyst development in filamentous cyanobacteria. HepK was identified as a sensory protein-histidine kinase [7] and DevR and PatA resemble response regulators. All three lack known DNA-binding domains [7, 9, 10]. Mutations in hepK, hepC and hepA all block the synthesis of heterocyst envelope polysaccharide, rendering the heterocysts incapable of N₂ fixation under aerobic conditions [10, 11]. A hepK mutation of Anabaena sp. strain PCC 7120 blocks the induction of hepA, whereas inactivation of hepC, located directly upstream from hepA, leads to constitutive expression of hepA [10].

The catalytic core of histidine kinases, including of HepK, contain highly conserved regions (the H, N, D/F, G1 and G2 boxes) that are distinct from the previously characterized Ser/Thr/Tyr kinase domain [12]. That catalytic core does not appear to be obligatorily dependent upon catalytic phosphorylation of a His rather than a Ser, Thr, or Tyr, because histidine kinase homologs are known in which the phosphorylated His is substituted by Tyr [13] or Ser [14]. Unanticipated similarities of the catalytic core to DNA gyrase B and to DNA-mismatch-repair enzyme MutL [12, 15, 16] provide hints that a histidine kinase might be able to interact directly with DNA. We previously demonstrated that HepK is a sensory protein-histidine kinase in HepK-DevR two-component regulatory system [7]. Because HepK may regulate the transcription of hepA or hepC, we tested whether HepK can bind DNA upstream from these genes. We report here that a water-soluble version of HepK that lacks the two putative transmembrane regions of that protein is a histidine kinase that binds sequence-specifically to DNA upstream from hepC. Therefore, the regulation of the synthesis of heterocyst envelope polysaccharide by HepK may be, at least in part, independent of two-component phosphorylation. Except for a kinase that resembles response regulators. All three lack known DNA-binding domains [7].

Materials and methods

Production and purification of truncated HepK

Unable to produce catalytically active, intact HepK (GenBank accession no. U68034) by expression in Escherichia coli, we produced a truncated, water-soluble version of HepK that lacks two presumptive transmembrane regions, as follows. A 3’-terminal portion of hepK encoding residues 267-575 of HepK was amplified by PCR with primers 5’-GGAATTCCATATGGC-GACTAGTGGAGGATCCT-3’ and 5’-CGGGATCTCACTTTGCTC-TGAAAGTG-3’ (introduced Ndel and BamHI sites are underlined). The PCR product was cloned between the Ndel and BamHI sites of plasmid pET-14b (Novagen, Inc., Madison, WI), which provided an N-terminal hexa-histidine tag. The resulting plasmid was denoted pRL2406, and the tagged, truncated product was denoted HepK. DNA sequencing confirmed that the PCR product was error-free. To overproduce HepK, Escherichia coli strain BL21 (DE3) transformed with pRL2406 was grown in 500 ml LB medium supplemented with 100 µg ml⁻¹ ampicillin at 37°C to an OD₆₀₀ of 0.5-0.6, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added, and incubation was continued for 5 h. HepK was purified to homogeneity as follows: E. coli suspended in 50 mM Na phosphate, 100 mM NaCl, pH 7.0, was broken with a French press (American Instrument Co. Div. Travenol Laboratories, Inc., Silver Spring, MD). The supernatant solution from centrifugation for 20 min at 20,000 x g at 4°C was applied to a cobalt-based resin column (Clontech Laboratories, Inc., Palo Alto, CA), which was then washed thrice with 50 mM Na phosphate, 300 mM NaCl, 5 mM imidazole, pH 7.0. HepK eluted with 50 mM Na phosphate, 300 mM imidazole, pH 5.5, was loaded onto a Sephadex G-100 gel-filtration column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) from which it was eluted at 4°C with 50 mM Na phosphate, 100 mM NaCl, pH 7.0, at 20 ml h⁻¹. Hexa-histidine-tagged DevR (H₆-DevR) and hexa-histidine-tagged mutant HepK (H₆-‘HepK-H348A) were prepared as described previously [7].

Gel mobility shift assay in vitro for DNA-protein complexes

Mobility shift assays [19] were performed with a “DIG Gel Shift Kit” (Roche Molecular Biochemicals, Indianapolis, IN) according to [20]. The DNA probes used in these experiments are shown in Figure 1. The DNA probes were prepared by PCR with the following primers: A1, 5’-GGATATAAGACTCTGTAGAAATG-3’ and 5’-GAATGGATTGAATACAATCCTC-3’; B2, 5’-CCGAATTC-TACAGAGCTTTGTTCAG-3’ and 5’-GATAATGTTCAACAC-TCAATTTTAAAC-3’; X1, 5’-ATCGATTAAAACTACAATTTCGCCC-3’ and 5’-TACTGTTCATTTGCAACATATTTTC-3’; X2, 5’-CTATGACTATT-TAAAAAGG-3’ and 5’-TACTTTGTTTCTCGACAAATTTT-3’; X3, 5’-CCGTTTTTAAAAATCG- TACCTCC-3’; and X3, 5’-TCCCCAATATTTAGATTTT-3’ and 5’-GGATAAGAGCTCTGATAGATTGTAG-3’. The DNA fragments were 3’-end-labeled by using DIG-11-ddUTP and terminal transferase. Labeled DNA probes (0.4-0.8 ng) were incubated with 0.3 µg of purified HepK or HepK-H₆-‘HepK-H348A (protein) in binding buffer (25 mM HEPES, pH 7.9, 5 mM MgCl₂, 25 mM NaCl, 0.5 mM DTT, 5% glycerol, 5 µg BSA) containing 0.5 µg of poly-(di-dC/di-dC) in a final volume of 20 µl. For gel shift competition, HepK or HepK-H₆-‘HepK-H348A was bound with at least 75-fold excess (75- to 270-fold molar ratios based on the molecular weight of the competitor DNAs) of unlabeled competitor DNA for 15 min at room temperature before addition of the probe. Incubation with the probe lasted for 20 min. The mixture was then loaded on an 8% polyacrylamide gel (30:1 acrylamide-bisacrylamide) in Tris-glycine buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5) [19] that had been pre-run for 1 h at 4°C and 140 V. Electrophoresis was performed at 4°C in a Tris-glycine buffer, for 2 to 5 h at a constant voltage of 140 V (ca. 30 mA). The gel was then electroblotted onto a Hybond-N+ Nylon membrane.
(Amersham Pharmacia) at 200 mA in the same Tris-glycine buffer at 4°C for 1 h, the membrane baked at 80°C for 2 h, and chemiluminescence detected according to the manufacturer’s instructions.

Results
Recombinant H$_6$-‘HepK, overexpressed in E. coli and purified to homogeneity (Figure 2A, lane 3), binds fragment X1 (Figure 3A, lane 2), and also X13 (Figure 3B, lane 2), a subfragment of X1 (Figure 1), but not fragments X2, B2, A1 and X12 (Figure 3A, lanes 3-10 and Figure 1). Competition experiments with unlabeled A1 showed that a 75-fold excess of A1 (by mass; 270-fold molar excess) does not significantly affect the formation of a complex between X1 and H$_6$-‘HepK (Figure 3C, lanes 3 and 5), whereas a 75-fold excess of X1 reduces complex-formation by approximately 90% (Figure 3C, lanes 4 and 3). Phosphorylation of H$_6$-‘HepK is labile to acid and stable to alkali, supporting the idea that its site of autophosphorylation is a histidine residue [7,22]; the H348A substitution abolishes autophosphorylation [7]; and (unphosphorylated) H$_6$-‘HepK-H348A binds X1 specifically (Figure 4, lane 2). Competition experiments with unlabeled A1 (Figure 4, lane 4) and unlabeled X1 (Figure 4, lane 3) showed similar results to that of wild-type H$_6$-‘HepK (Figure 3C, lanes 5 and 4). These results provide additional information that the specific binding does not require phosphorylation. The control experiment, the purified H$_6$-DevR protein (Figure 2B, lane 3) produced no mobility shift of X1 (Figure 3C, lane 2), showing that the site-specific binding by H$_6$-‘HepK is not due to the hexa-histidine tag.

Discussion
Several bacterial proteins with high homology to histidine kinases are apparently not kinases [1]. For example, Azotobacter vinelandii NifL contains five conserved blocks characteristic
of histidine sensor kinases, including the conserved His residue [23], but purified NifL has not been observed to autophosphorylate [21,24] nor is that residue required for in vivo function of NifL [25]. However, HepK evidently is an autokinase: purified H$_5^+$HepK autophosphorylates highly efficiently in vitro [7]. Some transcriptional activators, e.g., GAL4 [26], NtrC [27,28] and Spo0A [29] bind DNA independent of their state of phosphorylation, but activate transcription efficiently only when phosphorylated. Purified, intact HepK, complete with its two presumptive transmembrane domains [10], had no detectable autokinase activity in vitro [7]. It is possible that the two presumptive transmembrane domains of intact HepK in vivo are responsible for sensing a signal specific to heterocyst development, and subsequently trigger autokinase activity of HepK in vivo. Although the soluble, truncated, recombinant form of HepK binds DNA independent of phosphorylation, the biological consequences of that binding may depend on whether HepK is phosphorylated. ToxR is a transmembrane transcriptional activator with a cytoplasmic DNA-binding domain [30]. Because truncated HepK binds site-specifically to DNA but has no known DNA-binding motif, it may be a prototype for a new class of protein-histidine kinase that, like ToxR, serves also as a membrane-tethered transcription factor, which may also require an intramembrane proteolytic activation [31-33] in response to a signal specific to heterocyst development.

Genomic sequence data have identified no presumptive protein histidine kinase genes in animal genomes including human genome [34,35]. Since some two-component phos-

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**Figure 3.** Mobility shift assays with H$_5^+$HepK (in even-numbered lanes) probed with labeled DNA fragments (in parentheses). A: Lanes 1 and 2 (fragment X1), 3 and 4 (fragment X2), 5 and 6 (fragment B2), 7 and 8 (fragment A1), and 9 and 10 (fragment X12). B: Lane 1, X13 probe only; Lane 2, X13 probe+H$_5^+$HepK. C: Labeled DNA fragment X1 as probe, with the following additions. Lane 1, no added protein; lane 2, H$_5^-$DevR; lane 3, H$_5^-$HepK; lane 4, H$_5^-$HepK +75-fold excess of unlabeled X1; lane 5, H$_5^-$HepK +270-fold molar excess of unlabeled fragment A1.

**Figure 4.** Mobility shift assays with or without mutant protein H$_5^+$HepK-H348A and labeled DNA fragment X1 as probe. Lane 1, no added protein; lane 2, as in lane 1 plus H$_5^+$HepK-H348A; lane 3, as in lane 2 plus 75-fold excess of unlabeled X1; lane 4, as in lane 2 plus 270-fold molar excess of unlabeled DNA fragment A1.
phorelay proteins are essential for the viability, virulence, and drug resistance of microbial pathogens including human fungal and bacterial pathogens [36-39], novel anti-microbial drugs targeted to protein histidine kinase in two-component phorelay systems may prove high specificity and minimal toxicity [40,41]. Several series of inhibitors to bacterial histidine kinase have been reported in the literature [42-44], however, most appear to suffer from high hydrophobicity, poor selectivity, and excessive protein binding and/or limited bioavailability [45]. The strong hydrophobicity of these molecules makes formulation and drug delivery impossible. Unlike these compounds, small DNA molecules bound specifically by a protein-histidine kinase HepK are able to bypass the drawbacks of conventional inhibitors. These sequence-specific small DNA molecules have several unique advantages in developing novel anti-microbial drugs, such as high solubility, high specificity, minimal toxicity, efficient synthesis, easy of formulation and delivery. Therefore, our finding provides an exciting opportunity for developing small DNA molecules as novel anti-microbial drugs.

Conclusion
We have shown that although lacking a known DNA-binding motif, a truncated, soluble version of sensory protein-histidine kinase HepK binds sequence-specifically to a fragment of DNA found upstream from hepC, a gene that is required for the synthesis of heterocyst envelope polysaccharide of cyanobacteria. The conserved phosphorylation histidine residue of HepK kinase is not required for this DNA-binding activity. Therefore, regulation of the synthesis of heterocyst envelope polysaccharide by HepK may be, at least in part, independent of two-component phosphorylation. For our best knowledge this is the first example of a protein histidine kinase without a known DNA binding motif that binds DNA sequence-specifically. Our finding may enable development of small DNA molecule as highly specific anti-microbial drugs because protein histidine kinases are broadly conserved in microbial pathogens but absent in humans.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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