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Characterization of the specific interaction between archaeal FHA domain-containing protein and the promoter of a flagellar-like gene-cluster and its regulation by phosphorylation

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ABSTRACT

The mechanism and target genes of regulation by Forkhead (FHA) domain-containing transcription factors have not yet been documented in Archaea. In this study, using a bacterial one-hybrid technique, we successfully screened and identified for the first time a target gene regulated by ST0829, an FHA domain-containing potential transcriptional factor in the hyperthermophilic archaeon *Sulfolobus tokodaii*. We show that ST0829 could specifically bind to the promoter region of ST2519p, the archaeal flagellar protein-encoding operon (including FlaG, FlaF, FlaH, FlaI, and FlaJ) by using both *in vitro* electrophoretic mobility shift assay and surface plasmon resonance experiments, and *in vivo* chromatin immunoprecipitation assays. Furthermore, phosphorylation of the FHA domain-containing protein was found to negatively regulate its specific DNA-binding activity. The interaction between ST0829 and ST2519p could be inhibited by wild-type Ser/Thr protein kinase ST1565, but was not significantly affected by its mutant variant ST1565-K166A that lacks kinase activity. These findings not only increase our knowledge about the function of an archaeal FHA domain-containing regulator but also offer important insights for further understanding the signaling mechanism of environmental adaptation in archaea.

1. Introduction

Transcriptional regulation is essential to all three domains of life: *Bacteria*, *Eukarya*, and *Archaea*. The crenarchaeon *Sulfolobus tokodaii,* like any other extremophile, has an innate ability to respond to environmental stress while living under extreme conditions [1]. However, the role and mechanism of transcriptional regulation in promoting its environmental adaptation are unclear.

Protein phosphorylation plays an important role in regulating many cellular processes in both bacteria and eukaryotes [2–6] and several studies have shown its critical importance in Archaea [7–9]. Forkhead-Associated [FHA] domain has been described as a region of homology within a subset of Forkhead transcription factor family members, and represents a phosphoprotein recognition unit with preference for phospho-threonine peptides [10–12]. A functional FHA domain spans approximately 100–150 amino acids and has been found in many regulatory proteins, kinases, phosphatases, and transcription factors [13–18]. ST0829, the archaeal FHA domain-containing protein, has recently been characterized as a substrate of a typical eukaryote-like Ser/Thr protein kinase, ST1565, in the archaeon *S. tokodaii* [9]. However, the transcriptional targets of ST0829 have not yet been characterized.

In order to fill this gap in our understanding of FHA domain-containing proteins in Archaea, we conducted a bacterial one-hybrid screen using ST0829 as a bait in the archaeon *S. tokodaii*. We successfully identified a target gene regulated by ST0829 and confirmed that ST0829 could bind to the promoter of the target gene, an archaeal flagellar protein-encoding operon (archaeal flagellar protein FlaG, FlaF, FlaH, FlaI, and FlaJ), both *in vitro* and *in vivo*. Furthermore, we found that phosphorylation of ST0829 reduced its specific DNA-binding activity. These results offer essential information for further understanding the mechanism of archaeal transcriptional regulation and the function of FHA domain-containing transcription factors.

2. Materials and methods

2.1. Strains, enzymes, plasmids, and reagents

Escherichia coli BL21 (DE3) cells and pET28a were purchased from Novagen and were used to express archaeal proteins. DNA polymerase, deoxynucleoside triphosphates (dNTPs), restriction enzymes, T4 ligase, modification enzymes for molecular cloning and all antibiotics were obtained from NEB and TaKaRa Biotech.

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The pBT and pTRG vectors, *Escherichia coli* XR host strains, and the reagents for the one-hybrid assay were purchased from Stratagene. PCR primers were synthesized by Invitrogen (Supplemental Table S2).

2.2. Construction of S. tokodaii promoter library

Primer pairs for a total of 1825 promoter sequences (upstream DNA of each gene or operon) of *S. tokodaii* were designed. Each promoter DNA fragment was amplified by PCR and cloned into the pBXcm vector as described previously [19]. A promoter library for *S. tokodaii* was thus constructed.

2.3. Cloning and purification of archaeal proteins

Archaeal genes were amplified using specific primers and were cloned into a pTRG or pET28a vector (Supplemental Tables S1 and S2). *E. coli* BL21 (DE3) cells transformed with the recombinant plasmid were grown at 37 °C. Protein purification was carried out as described previously [9].

2.4. Bacterial one-hybrid analysis

Bacterial one-hybrid analysis was performed according to previously published procedures [19]. On a selective screening medium plate, the recombinant plasmid pTRG-ST0829 was used to screen the library for target *S. tokodaii* promoters. The plates were incubated at 30 °C for 3–4 d together with the self-activation control. Positive growth co-transformants were selected on a screening medium plate containing 20 mM 3-AT, 16 μg/mL streptomycin, 15 μg/mL tetracycline, 34 μg/mL chloramphenicol, and 50 μg/mL kanamycin. The plates were incubated at 30 °C for 3–4 days. CK^+ refers to co-transformant containing pRomoter-Rv2031p and pTRG-Rv3133c as a positive control. CK^- refers to co-transformant containing pRomoter-Rv2031p and pTRG-Rv3133c-deltaC as a negative control [19].

2.5. Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) analysis was performed according to previously published procedures [19]. Briefly, biotin-labeled promoter DNA was immobilized on SA chips (BIAcore). His-tagged archaeal proteins, to be used as the ligand, was diluted in HBS buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 50 μM EDTA, 5 mM ATP, 0.005% BIAcore surfactant P20 and injected at 100 μL/min for 1 min at 25 °C. GST protein was used as a negative control. Each analysis was performed in triplicate.

2.6. Electrophoretic mobility shift assay (EMSA)

The binding of proteins to their DNA targets was analyzed using an EMSA assay. ^{32}P-labeled DNA fragments were incubated with various amounts of phosphorylated and dephosphorylated ST0829 at 25 °C for 30 min. The reaction mixtures were incubated in a total volume of 15–20 μL with an EMSA buffer consisting of 50 mM Tris–HCl, pH7.5, 10 mM $MgCl_2$, 1 mM DTT, and 50 mM NaCl. After incubation, free DNA and protein–DNA complexes were resolved by 4% non-denaturing polyacrylamide gels containing 0.5× Tris–borate-EDTA (TBE) buffer. Gels were dried and exposed to a storage-phosphor screen at room temperature for 3 h, then subjected to autoradiography in a Typhoon Scanner (GE healthcare).

2.7. Protein kinase activity assay

In vitro protein phosphorylation assays were performed according to previously published procedures [9]. 400 nM ST0829 was incubated with 40 nM *S. tokodaii* protein kinase in a buffer [20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$ containing 300 μCi [γ-^{32}P] ATP for 60 min at 55 °C. The reaction was stopped with SDS sample buffer, and proteins were separated by 12% SDS–PAGE. Gels were dried and exposed to a storage-phosphor screen at room temperature for 3 h, then subjected to autoradiography in a Typhoon Scanner (GE healthcare).

2.8. Chromatin immunoprecipitation (ChIP) assay

Interaction of ST0829 with the promoter of the archaeal flagellar protein operon *in vivo* was analyzed using the ChIP assay as described previously [20]. Preimmune or immune sera raised against ST0829 was used in the assay. DNA recovered from immunoprecipitates was amplified with primers for the ST2519 promoter or a negative control, the ST2189 promoter.

3. Results

3.1. ST0829 binds with ST2519p, the promoter DNA of a flagellar protein-encoding operon

To uncover the as yet unknown target gene regulated by the archaeal FHA domain-containing regulator, ST0829, we first screened the *S. tokodaii* promoter library. Using a one-hybrid technique [19], we isolated the promoter of a flagellar protein-encoding operon ST2519 (ST2519p) that could interact with ST0829. As shown in Fig. 1A, the co-transformants containing pBX- ST2519p/pTRG-ST0829 grew well on the screening medium. In contrast, no growth was observed for their self-activated controls or for the co-transformant with pBX-ST2519p and pTRG-ST0015, an unrelated archaeal gene promoter cloned in the pTRG vector. In addition, positive controls (CK^+) also grew well on the medium, whereas negative controls (CK^-) were incapable of growth on the same screening medium (Fig. 1A). An additional negative control, co-transformants with pBX-Rv1688p (an unrelated mycobacterial gene) and pTRG-ST0829, did not grow on the screening medium. These results suggested that the selective system we used worked well and ST0829 could interact with ST2519p, the promoter DNA of a flagellar protein-encoding operon.

3.2. ST0829 could specifically bind to the promoter of ST2519 both in vitro and vivo

An EMSA assay further confirmed the binding of ST0829 with ST2519p. As shown in Fig. 1B, ST0829 was observed to strongly bind with ST2519p DNA because an obvious protein/DNA complex appeared on the gel as the concentration of St0829 was increased stepwise from 1 to 4 μM (Fig. 1B, lanes 3–6). In contrast, no complex was observed for an unrelated DNA, the promoter of ST0015, under similar experimental conditions (Fig. 1B, lanes 1–2). These results strongly suggested to us that ST0829 could bind with ST2519p, the promoter DNA of a flagellar protein-encoding operon.

To further examine the specificity of the binding of ST0829 with ST2519p, SPR assays were conducted. Biotin-labeled ST2519p DNA was first immobilized on the surface of SA chips (BIAcore). As shown in Fig. 2A, when different amounts of the ST0829 protein (90 nM, 180 nM, 270 nM, 360 nM, and 450 nM) were passed over the chip, a clear DNA-binding activity of ST0829 was observed. No significant response was observed for GST protein alone, which

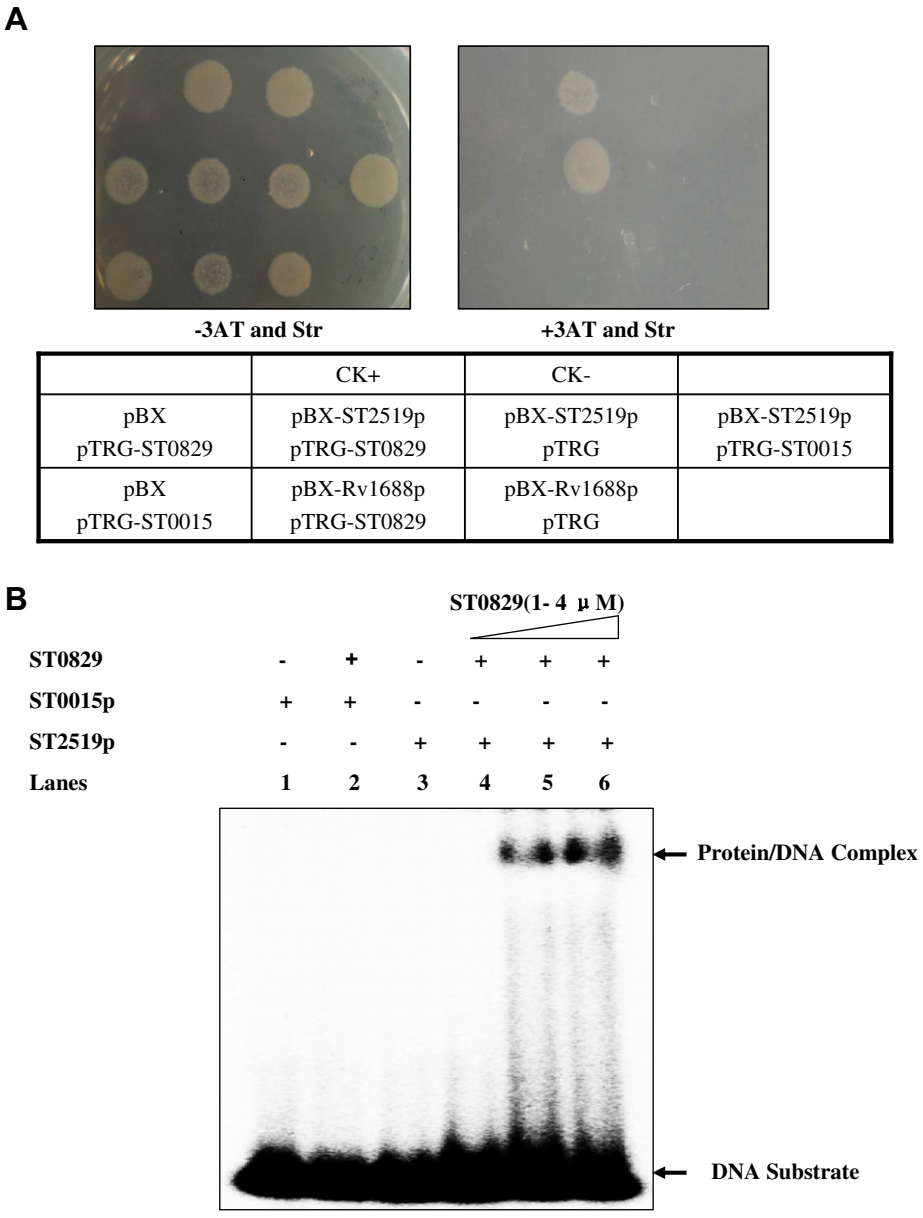

Fig. 1. Bacterial one-hybrid and EMSA assays for the interaction between ST0829 and the promoter of ST2519. (A) Bacterial one-hybrid (Stratagene) assay for the interaction between ST0829 and the promoter of ST2519. Experiments were performed as described in Section 2. (B) EMSA assay for the interaction between ST0829 and the promoter of ST2519. ^{32}P-labeled ST2519p or ST0015p (a non-specific DNA) DNA substrates were co-incubated with various amounts of the ST0826 protein (1, 2, 4 μM).

was used as a negative control, even with a high protein concentration (450 nM). In a further competition experiment, the response for the binding of biotin-labeled ST2519p with ST0829 was observed to sharply decrease when unlabeled ST2519p DNA (200 nM) was added into the reaction. In contrast, no significant effect was found if an unrelated ST0015p DNA was added (Fig. 2B). This indicated that ST0829 could specifically bind to the promoter of ST2519 *in vitro*.

The physiological significance of the binding of ST0829 with ST251p *in vitro* as revealed by EMSA and SPR assays was investigated further with chromatin immunoprecipitation (ChIP) experiments. As shown in Fig. 2C, our *in vitro* findings were confirmed by the observation that the ST2519 promoter could be amplified from immunoprecipitates pulled down using the ST0829 antibody (Fig. 2C). In contrast, no PCR products could be amplified from the same immunoprecipitates using primers for ST2189p, an unrelated DNA (Fig. 2C, right panel). These results indicate that ST0829 can interact specifically with the promoter of ST2519 *in vivo* in the archaeon *S. tokodaii*.

3.3. The binding of ST0829 with the ST2519 promoter is negatively regulated by phosphorylation

In a previous study, we reported that ST0829 is a phosphorylation substrate of ST1565, a Ser/Thr protein kinase in the archaeon *S. tokodaii* and that the ST1565-K166A mutant exhibits a partial loss of its phosphorylation function [9]. Here, we examined the effect of the kinase activity of ST1565 and its mutant variant on the activity of ST0829. As shown in Fig. 3A, compared with wild-type ST1565 (1.2 nmol ^{32}P/min mg) (Fig. 3A, lane 2), ST1565-K166A showed a significantly reduced phosphorylation of ST0829 (about 0.6 nmol ^{32}P/min mg) (Fig. 3A, lane 3). This is consistent with our previously published result (11). Importantly, GST protein

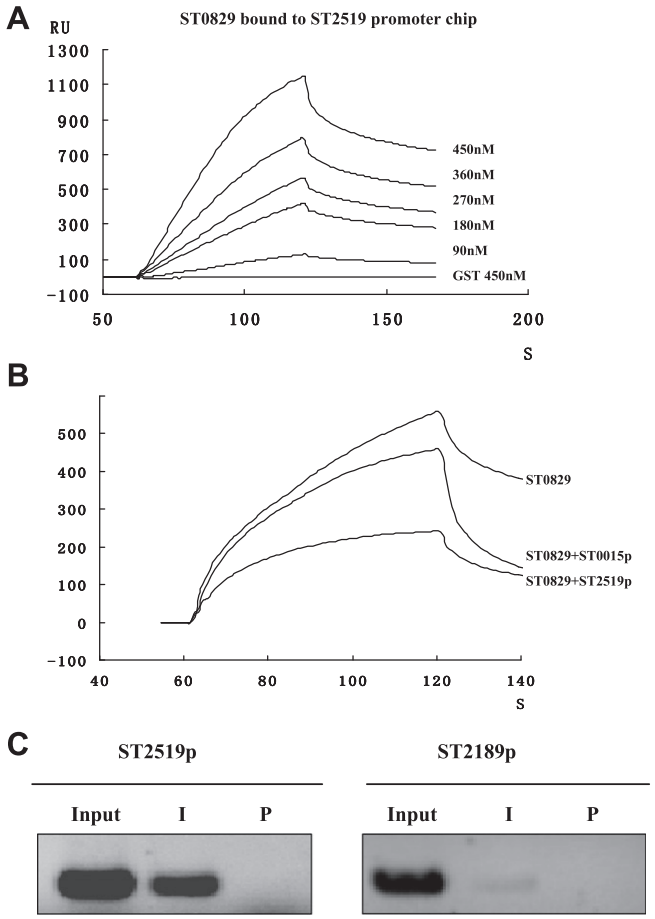

Fig. 2. SPR and ChIP assays for the interaction between ST0829 and the promoter of ST2519. (A) The SPR assay. The ST2519p DNA was biotin-labeled and immobilized on the SA chip. Various amounts of proteins (90, 180, 270, 360, and 450 nM) were then passed over the chip. GST protein was used as a negative control. (B) Competition assay for the interaction between ST0829 and the promoter of ST2519. Unlabeled ST2519p or non-specific ST0015 DNA were tested for their ability to compete with the binding of the ST0829 protein with biotin-labeled promoter ST2519p on the chip. (C) ChIP assays for the interaction between ST0829 and the promoter of ST2519 *in vivo*. Preimmune or immune sera raised against the ST0829 protein was used. DNA recovered from immunoprecipitates was amplified with primers specific for the ST2519 promoter or a negative control ST2189 promoter. I: immune and P: preimmune.

used as a negative control did not result in phosphorylation of ST0829 under similar conditions (Fig. 3A, lane 1).

We then examined the effect of the phosphorylation of ST0829 on its ability to bind with the target promoter through EMSA assays. In contrast to the negative control protein GST, wild-type ST1565 and ST1565-K166A (Fig. 3B, lanes 1–4), ST0829 alone (4 μM) was observed to bind with ST2519p DNA, as an obvious protein/DNA complex appeared on the gel (Fig. 3B, lane 5). Strikingly, no complex was observed when ST0829 (4 μM) pre-incubated with ST1565 (0.8 μM) was added to the reactions (Fig. 3B, lane 6). Interestingly, when ST0829 pre-incubated with ST1565-K166A (0.8 μM) was added, a fainter band representing a protein/DNA complex of the same size as that formed with ST0829 alone was observed (Fig. 3B, lane 7). These results suggested to us that phosphorylation of ST0829 reduces its DNA-binding activity.

An SPR assay further confirmed the effect of phosphorylation of ST0829 on DNA binding. As shown in Fig. 3C, different responses were observed when 400 nM ST0829 alone or its mixture with 40 nM ST1565 or GST were passed over the chip. DNA-binding activity of ST0829 decreased when wild-type ST1565, but not GST, was added together in the reaction, indicating that phosphorylation inhibited the DNA-binding activity of ST0829. No significant response was observed for the ST1565 protein alone.

In summary, our results indicate that phosphorylation of ST0829 negatively regulates its binding with the ST2519 promoter DNA.

4. Discussion

The mechanism and target genes of regulation by FHA domain-containing transcriptional factors have not yet been characterized clearly in *Archaea*. In this study, ST0829, a FHA domain-containing regulator, was found to interact specifically with the promoter of a flagellar protein-encoding operon in the archaeon *S. tokodaii* both *in vivo* and *in vitro*. Furthermore, phosphorylation of the FHA domain-containing protein was shown to negatively regulate its DNA-binding activity. To our knowledge, this is the first report on the transcriptional target and regulatory mechanism of an FHA domain-containing transcription factor in archaea.

Although much is known about the function of FHA proteins in eukaryotes and bacteria, little is known about them in archaea. In a previous study, we discovered that ST0829, an FHA domain-containing protein, is a substrate of a typical eukaryote-like Ser/Thr protein kinase ST1565 in the archaeon *S. tokodaii* [9]. ST0829 has also been found to contain an N-terminal Zn finger-RanBP domain, which is usually responsible for DNA-binding. Therefore, it has been suggested as a potential transcriptional regulator [9]. However, the transcriptional targets and regulatory mechanism of ST0829 have not been characterized. In the current study, we found that ST0829 can bind to the promoter of an operon encoding archaeal flagellar proteins (FlaG, FlaF, FlaH, FlaI, and FlaJ). Archaeal flagella are considered to be built in a similar manner as of bacterial type IV pili [21]. Bacterial type IV pili are responsible for essential biological processes such as cell–cell interaction, surface adhesion, auto-aggregation, conjugation, and twitching motility. However, archaeal flagella is known to be important for drive swimming motility [22]). Recent studies have revealed that all subunits of FlaC, FlaF, FlaG, FlaH, FlaI or FlaJ in the archaeon *Methanococcus maripaludis* are essential for flagella formation and function. Deletion mutants that lack any of the subunits are not motile [23,24]. In addition to swimming motility, certain archaeal flagella could also function in facilitating surface attachment. For example, deletion of genes that encode flagellin subunits in the crenarcheon *S. solfataricus* prevent it from attaching to solid surfaces [25]. Therefore, by virtue of its regulation of flagellar proteins, ST0829 protein of the archaeon *S. tokodaii* could be involved in regulating its ability to thrive in extreme environments such as high temperature, saturating salt concentration, or extremely acidic pH.

Regulation of protein function by phosphorylation can be critical for extreme acidothermophilic archaeon such as *S. tokodaii* to exhibit appropriate adaptive responses to environmental cues. In the present study, we found that the specific DNA-binding activity of ST0829 decreased upon phosphorylation by the Ser/Thr protein kinase ST1565. Based on our current findings and previous observations [9], ST1565 may convey environmental signals to the transcriptional apparatus through phosphorylation of ST0829, which further regulates formation of the archaeal flagella and may promote its environmental adaptation. Therefore, these data support a model in which a Ser/Thr protein kinase regulates the DNA-binding activity of an FHA-domain-containing regulator and consequently the expression of a flagellar protein-encoding operon, which may together underlie a response to stressful environmental conditions in the archaeon *S. tokodaii.*

In summary, we have successfully characterized the first target gene of an archaeal FHA domain-containing regulator and found that phosphorylation by a Ser/Thr kinase negatively regulates its

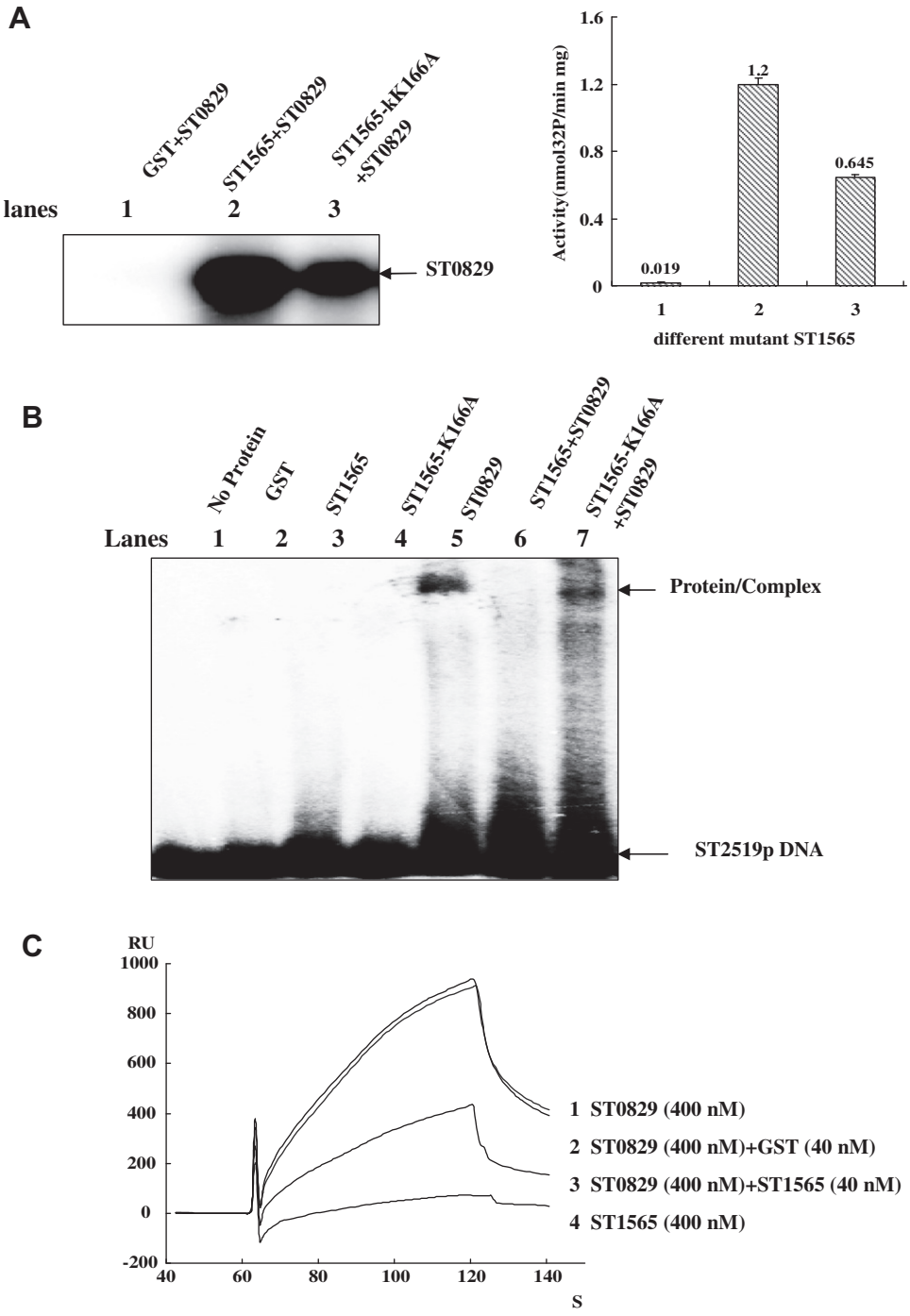

Fig. 3. Effect of phosphorylation on the DNA-binding activity of ST0829. (A) Kinase activity assays for wild-type and mutant ST1565. GST protein was used as a negative control for the phosphorylation reaction. (B) EMSA assays for the effect of ST0829 phosphorylation on its DNA-binding activity. ST1565 or ST1565-K166A (0.8 μM) was pre-incubated with ST0829 (4 μM) and the mixture was added to the reactions. GST protein was used as a negative control. The phosphorylation experiments and EMSA assays were performed as described under Section 2. (C) The effect of phosphorylation by ST1565 on the DNA-binding activity of ST0829 was examined using surface plasmon resonance on a BIAcore 3000. All experiments were performed as described under Section 2. Overlay plots depicting the interactions were then produced.

DNA-binding activity. These data not only increase our understanding of the function and regulation of archaeal FHA-domain-containing proteins but also offer important insights into possible signaling mechanisms of environmental adaptation in archaea.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.011.

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