

Identification of a Substrate for Pkn2, a Protein Ser/Thr Kinase from *Myxococcus xanthus* by a Novel Method for Substrate Identification

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Abstract

Eukaryotic cells contain a large number of protein Ser/Thr kinases, which play important roles in signal transduction required for cell proliferation, differentiation, and stress response and adaptation. It is also known that some prokaryotes contain a family of protein Ser/Thr kinases. A major challenge in the characterization of these kinases is how to identify their specific substrates. Here we developed such a method using a protein Ser/Thr kinase, Pkn2 from *Myxococcus xanthus*, a Gram-negative soil bacterium. When Pkn2 is inducibly expressed in *E. coli*, cells are unable to form colonies on agar plates. This lethal effect of Pkn2 was eliminated in an inactive Pkn2 mutant in which the highly conserved Lys residue was changed to Asn, indicating that phosphorylation of a cellular protein(s) in *E. coli* resulted in growth arrest. Several clones from an *E. coli* genomic library were found to suppress the lethal effect when co-expressed with *pkn2*. Four out of seven multi-copy suppressors were identified to encode HU, (3 for HU α and 1 for HU β) a histone-like DNA binding protein. Purified HU α was found to be specifically phosphorylated by Pkn2 at Thr-59, and the phosphorylated HU α became unable to bind to DNA, suggesting that the phosphorylation of endogenous HU proteins by Pkn2 contributed at least in part to the lethal effect in *E. coli*. The present method termed the STEK method (Suppressors of Toxic Effects of Kinases) may be widely used for the substrate identification not only for prokaryotic protein Ser/Thr kinases but also for eukaryotic kinases.

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Introduction

Human cells are assumed to contain more than 1000 protein Ser/Thr and Tyr kinases (Hunter and Plowman, 1997). From the genomic sequence yeast contains 113 protein Ser/Thr kinases, but no protein Tyr kinase (Hunter and Plowman, 1997). These kinases play important roles in various signal transduction pathways involved in cell growth, regulation of the cell cycle, differentiation, cell-cell interaction, tissue-specific gene expression, and stress response and adaptation.

Interestingly, some prokaryotes having unique life cycles contain a family of eukaryotic-like protein Ser/Thr kinases such as *Myxococcus xanthus* (Munoz-Dorado *et al.*, 1991; Zhang *et al.*, 1992 and 1996; Udo *et al.*, 1995; and Hanlon *et al.*, 1997), *Streptomyces coelicolor* (Matsumoto *et al.*, 1994 and Ueda *et al.*, 1996), *Anabaena PCC7120* (Zhang, 1993 and Zhang *et al.*, 1998) and *Mycobacterium tuberculosis* (Cole *et al.*, 1998). These kinases are considered to play important roles in their life cycle and not to be replaceable with protein histidine kinases, which are the most prevalent kinase in the prokaryotes.

One of the major tasks in the studies on these protein Ser/Thr kinases is to identify their cellular targets in the signaling pathways as their specific substrates. In the present paper, we developed a novel genetic method to identify unknown substrates of Ser/Thr kinases. This method is based on the cytotoxic effect resulting from inducible expression of a protein Ser/Thr kinase in *E. coli*, which can be suppressed by overproducing its substrates, and thus termed the STEK method for Suppressors for Toxic Effects of Kinases. Here, using this method, we identified HU α and HU β , histone-like proteins as substrates for Pkn2, one of 13 protein Ser/Thr kinases found in *M. xanthus*. Pkn2 consists of 830 amino acid residues of which the amino-terminal 297-residue region shows high similarity with eukaryotic Ser/Thr kinases (Udo *et al.*, 1995). Pkn2 is autophosphorylated at serine and threonine residues. When Pkn2 is expressed in *E. coli*, it phosphorylates TEM- β -lactamase to block the secretion of β -lactamase across the cytoplasmic membrane (Udo *et al.*, 1995). We propose that the STEK method may be widely applied for other protein Ser/Thr and Tyr kinases using not only *E. coli*, but also other host cells for identification of their specific substrates. Recently this method has been used to identify a substrate for mouse minibrain kinase from a brain cDNA library (Dr. E. Kandel, personal communication).

Results

Cytotoxicity of Pkn2 Expression in *E. coli*

When the *E. coli* strain SB221 harboring pNIIIcM/pkn2 was plated on glucose-M9 agar plates in the presence or

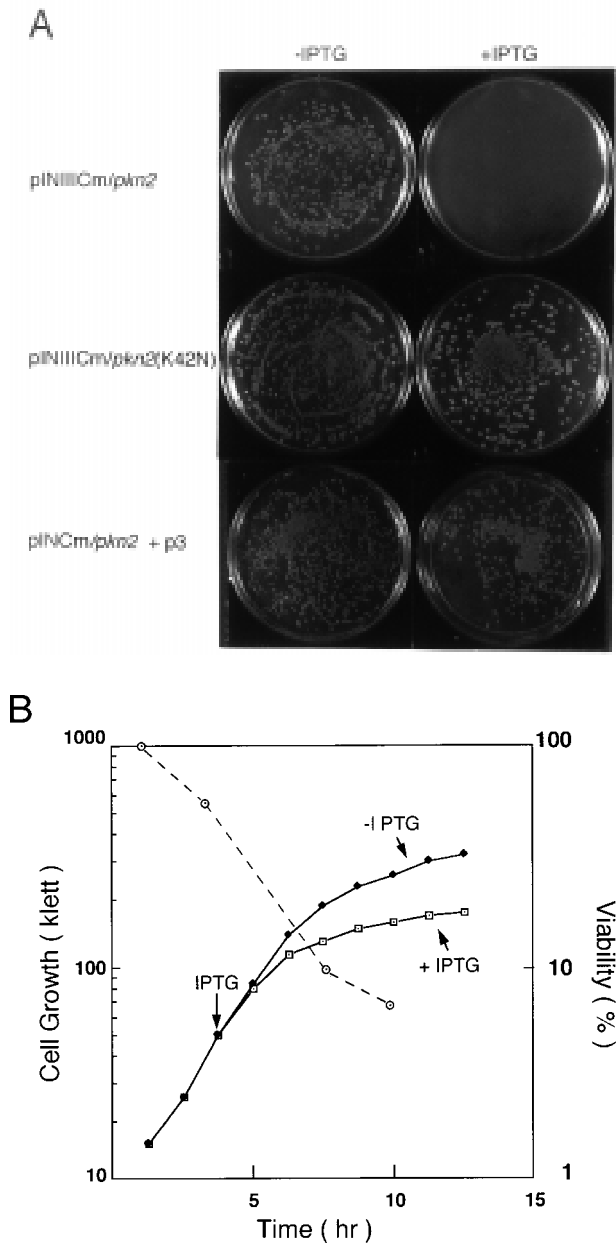


Figure 1. A. Cell growth inhibition by Pkn2 expression and suppression by plasmid p3. *E. coli* SB221 harboring pNIIICm/pkn2, pNIIICm/pkn2 (K42N), and pNIIICm/pkn2 and p3 cells were plated on glucose-M9 plates with or without 1 mM IPTG. The plates were incubated at 37°C for 36 hr. B. Growth curve and viability of SB221 harboring pNIIICm/pkn2 in glucose-M9 medium: At the Klett unit of 50, IPTG was added at the final concentration of 1 mM as indicated by an arrow. Viability of SB221 harboring pNIIICm/pkn2 after *pkn2* induction indicated by a dotted line was scored by plating cells at different time points on glucose-M9 plates. Open and filled squares indicate cell growth with and without IPTG addition. Open circles indicate cell viability.

the absence of 1 mM IPTG, cells were able to form colonies without IPTG, but not in the presence of IPTG as shown in Figure 1A, suggesting that the expression of Pkn2 is toxic in *E. coli*. pNIIICm is an expression vector, which consists of the *E. coli lpp* promoter and *lac* promoter and operator regions with the chloramphenicol acetyltransferase gene

(Ghrayeb *et al.*, 1984). The cloned gene is thus under the control of these promoters, and its expression is induced by the addition of IPTG. To examine whether the lethal effect of Pkn2 was due to the protein kinase activity of Pkn2, pNIIICm/pkn2(K42N), which expresses an inactive Pkn2 (Udo, *et al.*, 1995), was constructed. In this mutated Pkn2 (Pkn2K24N), the highly conserved Lys residue in the kinase catalytic subdomain II of Pkn2 was changed to an Asn residue, which inactivates the kinase activity of Pkn2. As shown in Figure 1A, *E. coli* SB221 harboring pNIIICm/pkn2(K42N) was able to form colonies on a plate containing 1 mM IPTG to a similar extent to a plate without IPTG. The same result was obtained when another *E. coli* strain, JM83 (Vieira and Messing, 1982) was used as a host cell (not shown). These results indicate that a cellular protein(s) was phosphorylated by Pkn2, resulting in a cytotoxic effect.

The inhibition of cell growth was also examined in a liquid culture as shown in Figure 1B. *E. coli* SB221 (pNIIICm/pkn2) was grown in glucose-M9 medium and *pkn2* was induced by adding 1 mM IPTG at a mid-exponential phase as indicated with an arrow. In comparison with the growth curve of SB221 (pNIIICm/pkn2) cells in the absence of IPTG, the growth rate of SB221 (pNIIICm/pkn2) gradually declined during the first 5 hr incubation after the addition of IPTG. When a Klett unit reached 270, the cell growth almost completely stopped. The viability of SB221 (pNIIICm/pkn2) cells steadily dropped during induction in the presence of 1 mM IPTG (Figure 1B).

Screening of Suppressor Genes in *E. coli*

If the cytotoxic effect of Pkn2 is due to the phosphorylation of a specific cellular protein(s) as described above, the cytotoxicity may be suppressed by overproducing the specific protein in *E. coli* expressing Pkn2, which may act as a Pkn2 substrate, an inhibitor for Pkn2 or a phosphatase for proteins phosphorylated by Pkn2. Therefore, we next attempted to search by screening an *E. coli* genomic library for multi-copy suppressor genes for the Pkn2 toxicity, whose products may serve as Pkn2 substrates. The *E. coli* genomic library was constructed by cloning of *Sau3A*I partial digests (3~4kb) of the *E. coli* chromosomal DNA into a unique *Bam*HI site of pBRKm which is a pBR322 derivative carrying a *Km^r* gene from Tn5 (Beck, *et al.*, 1982) at the *Dra*I site in order to prevent the formation of colonies from drug-sensitive cells after prolonged incubation when the *Amp^r* marker is used. *E. coli* SB221 cells harboring the genomic library were then electroporated with pNIIICm/pkn2. Colonies formed on glucose-M9 agar plates containing 1 mM IPTG with Kanamycin (30 µg/ml) and chloramphenicol (30 µg/ml) were isolated and plasmids were prepared. To confirm the suppression activity, each candidate plasmid was reexamined for the second screening. After the second screening, 7 out of 30 candidates were found to still retain the suppressor function. Seven plasmids designated p 3, 4, 7, 9, 10, 27, and 30 were digested with restriction enzymes to classify the type of the insert DNA fragments. Three out of seven candidate plasmids (p 3, 4, and 9) contained common DNA fragments (see Figure 3). The protein expression patterns of strains SB221 harboring seven candidate plasmids were also examined by SDS-PAGE. As shown in Figure 2, the strains SB221 harboring p3, 4, and 9 were found to produce a 9-kDa protein at a similar level (lanes 3, 4, and 6 in Figure

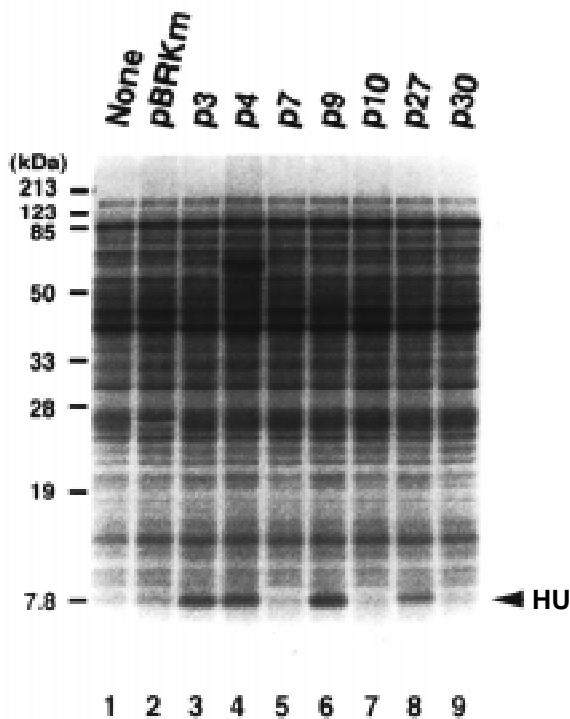


Figure 2. Protein expression patterns of SB221 harboring multicopy suppressor plasmids (p3, 4, 7, 9, 10, 27, and 30). Strains SB221 and SB221 (pBRKm) were used as controls. *E. coli* strains were grown in LB medium for 16 hr and protein samples were applied to 15% SDS-PAGE.

2). SB221 (p27) was also able to express the 9-kDa protein at a lower level (lane 8, Figure 2). Pkn2 expression levels in SB221 strains harboring both pINIICm/pkn2 and pBRKm, p3, 4, or 9 were examined by Western blot analysis with anti-Pkn2 antiserum, and found that the levels of Pkn2

expression were almost identical in all strains at least for 6 hr after IPTG (not shown), indicating that the expression of the suppressor gene did not affect the Pkn2 expression.

Identification of the Suppressor Genes

In order to map the suppressor gene on the *E. coli* chromosome, p3 DNA was used as a probe to screen the Kohara library (Kohara *et al.*, 1987). Two phages, No. 532 (9B9) and No. 533 (7B7) that were found positive, are located around the 3,550-kb region on the *E. coli* chromosomal DNA (Blattner *et al.*, 1997). Sequencing of the suppressor DNA revealed that p3 and p9 contain a DNA fragment from 65000 to 68714 bp (3,715 bp in length) and p4 contained a DNA fragment from 65000 to 68115 bp (3,116 bp in length), respectively, of U00006 in GeneBank. Thus, plasmids contained common ORFs of *hupA*, *o231*, and *f188* as shown in Figure 3.

The restriction sites *Bst*XI, *Nru*I, *Kpn*I, and *Hinc*II, were used to create deletion mutations of p3 as shown in Figure 3. The resulting plasmids and pINIICm/pkn2 were co-transformed to SB221 cells and strains were subjected to test for suppression of the Pkn2 toxicity as summarized in Figure 3. The plasmids, which lack *hupA*, *p3ΔNru*I, and *p3ΔKpn*I-*Hinc*II, failed to suppress the Pkn2 toxicity while a plasmid *p3ΔBst*XI, which contains *hupA*, retained the suppressor function. To further confirm whether the *hupA* gene product is the suppressor, a frame-shift mutation within the *hupA* gene was created by removing 4 bp at the *Kpn*I site. A resulting plasmid, *p3ΔKpn*I lost the suppression activity, clearly indicating that the *hupA* product is responsible for the suppression of the Pkn2 toxicity.

As described previously, SB221 strains harboring p3, 4, and 9 produce a 9-kDa protein (see Figure 2) whose molecular weight is very close to that of the *hupA* gene product, HU α (9.5kDa). Note that the gene products of ORF *o231* and *f188* have calculated molecular weights of 26 and 20 kDa, respectively.

As shown in Figure 2, p27 was also found to produce

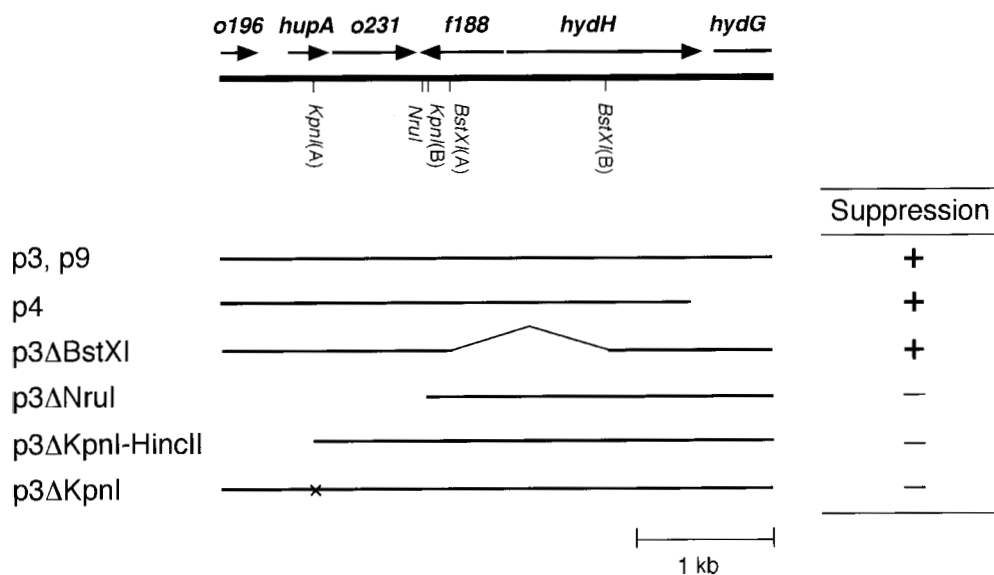


Figure 3. Physical map of the suppressor gene and suppression assay of deletion and frame-shift mutants. The multi-copy suppressor plasmids, p3, 4, and 9, were found to contain three common ORFs of *hupA*, *o231*, and *f188*. Deletion and frame-shift mutants were constructed using restriction sites of *Bst*XI, *Kpn*I, and *Nru*I, and suppression activities of resulting plasmids, *p3ΔBst*XI, *p3ΔNru*I, *p3ΔKpn*I-*Hinc*II, and *p3ΔKpn*I were shown as (-) and (+).

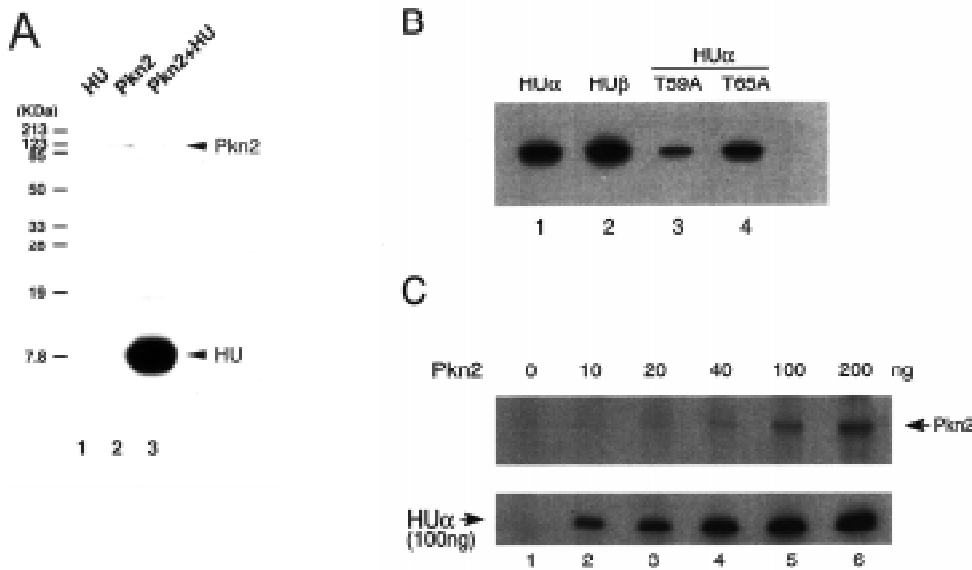


Figure 4. Phosphorylation of HU α and HU β by Pkn2. A. Phosphorylation of HU by Pkn2. HU $\alpha\beta$ (0.5 μ g) was incubated in the presence or the absence of Pkn2 (5 ng) with 1mMATP containing 5 μ Ci [δ - 32 P]ATP for 30 min at 30°C. B. Phosphorylation of HU α , HU β , HU α (T59A) and HU α (T65A) by Pkn2: Reaction was carried out as described above. C. Phosphorylation of HU α by various amounts of Pkn2: HU α (100 ng) was incubated with Pkn2 (10 to 200 ng) for 1 hr at 30°C as described above.

a 9-kDa protein and suppress Pkn2 toxicity at the same level as p3. It is known that *E. coli* carry two HU genes, *hupA* for HU α and *hupB* for HU β (Kano *et al.*, 1985 and 1987). PCR was performed using two oligonucleotides corresponding to the 5'-end and 3'-end sequences of the *hupB* as primers, and the p27 plasmid DNA as a template. The resulting DNA fragment was cloned into the *Nde*I and *Bam*HI sites of pET 11 (Studier *et al.*, 1990), its DNA sequence was determined, and found to be identical to the *hupB* gene. This plasmid (p27) was thus used for expression and purification of HU β . Two of the remaining three suppressors (p7 and p30) were found to contain a DNA fragment mapped at 57 min on the *E. coli* chromosome encoding YfgA and YfgB, hypothetical proteins. The DNA fragment carried by p10 has not yet been identified.

Phosphorylation of HU by Pkn2

The results described above suggest that HU α and HU β , histone-like protein in *E. coli*, are the targets of Pkn2 and that Pkn2 toxicity caused by phosphorylation of HU α and

HU β by Pkn2, is suppressed by the overproduction of HU α or HU β . To test this hypothesis, the phosphorylation of HU was examined using purified Pkn2 and HU $\alpha\beta$ heterodimers (gift from Dr. Goshima, Hiroshima University) with [δ - 32 P]ATP. As shown in Figure 4A, 0.5 μ g of HU $\alpha\beta$ was effectively phosphorylated by 5 ng of Pkn2 (lane 3) and no band was detected at the position of HU $\alpha\beta$ without Pkn2 (lane 2).

To further characterize phosphorylation of HU, HU α , and HU β were purified from *Escherichia coli* BL21(DE3) harboring pET11hupA and pET11hupB, as described in Experimental procedures. pET11hupA and pET11hupB contained the *hupA* and *hupB* gene, respectively, under the control of a T7 promoter (Studier *et al.*, 1990). As shown in Figure 4B, both HU α and HU β (100 ng) were phosphorylated by Pkn2 (40 ng) as in the case of HU $\alpha\beta$ (lane 1 and 2, respectively). The phosphorylation of HU α was dependent upon the concentration of Pkn2, as HU α phosphorylation linearly increased with increasing amounts of Pkn2 (Figure 4C). At the optimal condition, 19% of HU α was phosphorylated by Pkn2 (data not shown).

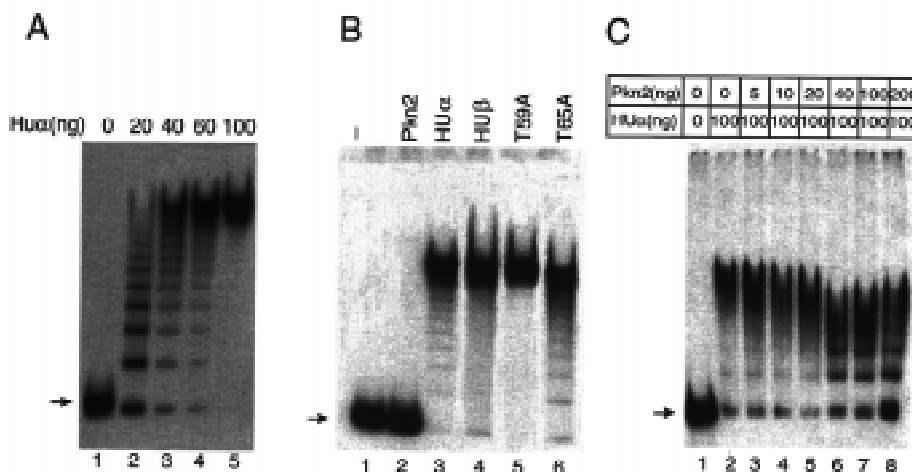


Figure 5. Gel retardation assays of 32 P-labeled 141-bp DNA fragment with various HU's. A. Different amounts of HU α were used for DNA binding as indicated on the top. B. 100 ng of HU α (lane 3), 100 ng of HU β (lane 4), 100 ng of HU α (T59A) (lane 5) and 100 ng of HU α (T65A) (lane 6) were used for DNA binding. In lane 2, 40 ng of Pkn2 was used. In lane 1, only DNA was added. C. Effects of phosphorylation of HU α by Pkn2 on DNA-binding: HU α (100 ng) was used for each reaction without (lane 2) and with Pkn2 (lanes 3 to 8).

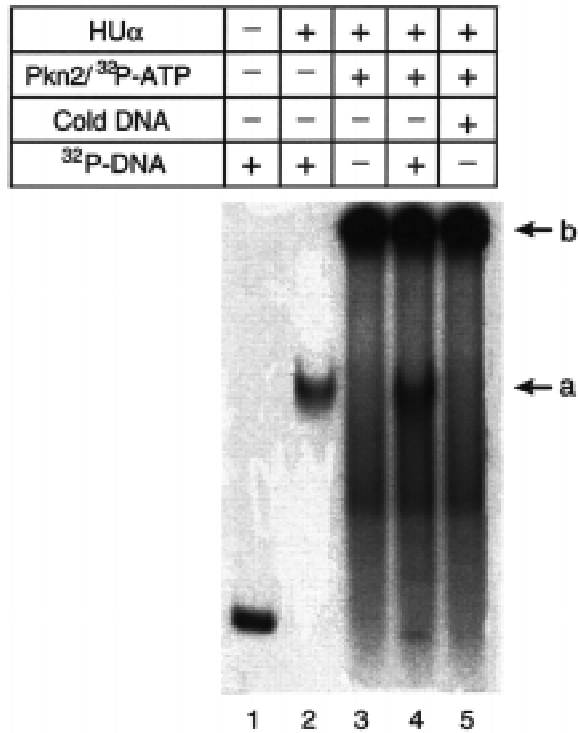


Figure 6. Effect of phosphorylation of HU α by Pkn2 on DNA mobility shift. A. DNA mobility shift assay using the 141-bp DNA and 32 P-phosphorylated HU α . 0.1 ng of 32 P-labeled DNA (lane 1), with or without 100 ng unphosphorylated HU α (lane 2) were used for the gel shift assay as described in Experimental Procedures. In lane 3, HU α was phosphorylated by 1 mM ATP containing 5 μ Ci [γ - 32 P]ATP and 40 ng Pkn2 was also added. Under the condition used, approximately 8% of HU α was phosphorylated. A HU α mixture (lane 3) was used for the DNA mobility shift assay with 32 P-labeled DNA (lane 4) or unlabeled DNA (lane 5). The HU α and DNA complex migrated to position a and phosphorylated HU α was retained in a well, as indicated with an arrow with letter b.

Phosphorylation Site of HU α

Pkn2 is known to autophosphorylate Ser and Thr residues and to phosphorylate threonine residues of TEM- β -lactamase *in vivo* (Udo *et al.*, 1995). Since HU α contains 4 Ser and 7 Thr residues, we next examined which residue(s) is phosphorylated by Pkn2. For this purpose, HU α was phosphorylated with [γ - 32 P]ATP and Pkn2 and then treated with Lys-C (Roche), which cleaved only after a lysine residue. When HU α containing 11 Lys and 3 Arg residues is digested with Lys-C, all 3 Arg residues are resided in a single peptide from Val52 to Lys67. This peptide can be easily identified by reverse phase column chromatography using a C18 column, since the peak corresponding to the peptide disappears by tryptic digestion cleaving at Arg residues. We found that a trypsin-sensitive peptide was phosphorylated by Pkn2 (not shown), and thus concluded that the phosphorylation site(s) resides between V52 and Lys67. Since this peptide contains two Thr residues, Thr59 and Thr65, but no Ser residues, phosphorylation of HU α by Pkn2 occurs at either Thr59 or Thr65 or both.

To elucidate which Thr residue was phosphorylated by Pkn2, Thr59 and Thr65 were mutated to a Ala residue

by site-directed PCR mutagenesis, as described in Experimental procedures. Then phosphorylation of HU α T59A and HU α T65A was examined. As shown in Figure 4B, HU α T65A was still phosphorylated as well as the wild-type HU α (lane 4), but HU α T59A was not (lane 3). Note that a faint band detected in lane 3 is considered to be due to phosphorylation of wild-type HU α , which was co-purified with HU α T59A from *E. coli* BL21(DE3) harboring pET11hupA(T59A), since *E. coli* BL21(DE3) carries the wild-type *hupA* gene.

Effect of Phosphorylation of HU α for DNA Binding Activity

Since HU is known to bind to single-stranded and double-stranded DNA, DNA binding activity of phosphorylated HU was examined. A double-stranded DNA of 141 bp in length was prepared from pUC9 digested with *Bam*HI and *Pvu*II and filled in with [α - 32 P]dGTP and [α - 32 P]dATP using the Klenow fragment of DNA polymerase I. When different amounts of HU α were added to 0.1 ng of DNA in a binding buffer as previously described (Tanaka *et al.*, 1993), ladders of radioactive bands were observed as shown in Figure 5A. At 20 ng HU α , eight to nine new bands appeared and as more HU α was added, the bands shifted to more slowly migrating positions. The addition of 100 ng HU α resulted in a single band at the most slowly migrating band. Since eleven bands can be detected in the ladder, eleven HU α dimers are considered to be able to bind to the 141-bp DNA. The DNA-binding abilities of HU β , HU α T59A, and HU α T65A were also examined. HU β and HU α T59A could bind to the 141 bp DNA as well as HU α , while HU α T65A showed slightly less ability to bind to DNA (Figure 5B).

To examine the effect of phosphorylation of HU α on DNA binding, 100 ng HU α was first phosphorylated with different amounts of Pkn2 and then added to the binding buffer containing the 32 P-labeled 141-bp DNA. As shown in Figure 5C, inhibition of DNA binding was observed as the amounts of Pkn2 increased. Since Pkn2 itself could not bind to DNA (Figure 5B, lane 2), the result indicates that Pkn2 inhibits DNA binding activity of HU α by phosphorylation.

To further investigate DNA binding ability of phosphorylated HU α , HU α was first phosphorylated by Pkn2 with [γ - 32 P]ATP and applied to 5% polyacrylamide gel in Tris-borate buffer. As a result of phosphorylation, 32 P-labeled HU α was retained at the well of the gel (Figure 6, lane 3; shown by an arrow **b**). Under this condition, 8% of HU was phosphorylated. Next, using the 32 P-labeled HU α , the binding assay with 32 P-labeled 141-bp DNA was carried out. As shown in lane 4, Figure 6, a new band indicated by arrow **a** appeared, which migrated at the position identical to the HU α -DNA complex (lane 2). However, if the same binding assay was performed with non-labeled 141-bp DNA, no band was detected at position **a** (lane 5), indicating that band **a** did not contain 32 P-labeled HU α . On the basis of these results we concluded that only non-phosphorylated HU α can bind DNA, and that the DNA binding activity of HU α is inhibited upon its phosphorylation by Pkn2.

Discussion

In the present paper, we established a novel method to identify the substrate(s) for protein Ser/Thr kinases by

isolating suppressor genes to reduce the toxic effect of kinases in *E. coli*. This method termed the STEK method (Suppressors for Toxic Effects of Kinases) may be applicable to Ser/Thr and probably Tyr kinases from bacteria to animal cells as far as they are toxic when expressed in *E. coli* or other host cells.

The lethal effect of *M. xanthus* *pkn2* in *E. coli* was found to be suppressed by either *hupA* or *hupB* genes of *E. coli*. The *hupA* and *hupB* genes of *E. coli* are known to encode the histone-like protein HU α and HU β , respectively and the HU proteins are highly conserved in bacteria (Kano *et al.*, 1986). HU α and HU β form a heterodimer *in vivo* and affect gene expression (Lewis, *et al.*, 1999). Strains mutated in both *hupA* and *hupB* genes show reduced viability, perturbed cell division (Wada *et al.*, 1988 and Huisman *et al.*, 1984) and more sensitive to UV (Li and Wartens, 1998). Purified HU $\alpha\beta$ heterodimers, HU α and HU β , were phosphorylated by Pkn2 (Figure 4A and 4B). Peptide-mapping digested with LysC and trypsin, and mutational analysis revealed that Thr-59, which is located in the tip of flexible arms of the three-dimensional structure determined by X-ray crystallography, was phosphorylated. These regions are known to associate with DNA (Drlica and Rouviere-Yanit, 1987). Therefore, the phosphorylation of Thr-59 is likely to affect the binding of HU to DNA. Although Pkn2 can phosphorylate no more than 19% of HU *in vitro* (the reason for this is unknown at present), HU binding to DNA was inhibited as Pkn2 was continuously added in the reaction mixture (Figure 5C), indicating that the phosphorylated HU is unable to bind to DNA.

At present, it is not unknown how the phosphorylation of HU causes the cytotoxic effect. Since it has been shown that a $\Delta hupA \Delta hupB$ strain is still viable at 37°C (Wada *et al.*, 1988), the cytotoxic effect may not be simply explained by dissociation of HU from DNA. Phosphorylated HU itself may cause a toxic effect to cells by affecting the DNA binding properties of other factors such as H-NS and IHF. Alternatively Pkn2 may have other targets whose phosphorylation by Pkn2 results in a lethal effect to *E. coli*. Indeed, there are two other suppressor genes yet to be identified, which may be the primary target for the *E. coli* lethality caused by Pkn2.

Pkn2 is a receptor type of Ser/Thr kinase which plays an important role in regulation of *M. xanthus* cell cycle presumably by transducing external signals to negatively regulate the transition from vegetative growth to developmental growth (Udo *et al.*, 1996). Since HU proteins are highly conserved in bacteria, and function in the chromosomal structure and gene expression, it is an intriguing question whether HU is also the substrate for Pkn2 in *M. xanthus*. Currently, we are attempting to isolate the suppressor gene(s) from *M. xanthus* library using the same method described in this paper. We are also attempting to isolate *M. xanthus* HU protein(s) to examine whether it can be phosphorylated by Pkn2. It should be noted that the phosphorylation site of *E. coli* HU α is R-A-E-R-T, which is the first sequence identified as the specific phosphorylation sequence for bacterial protein Ser/Thr kinases. The corresponding sequence of *E. coli* HU β is R-A-A-R-T.

The STEK method described here may be used with other host cells such as *Bacillus subtilis* and yeast. This method was successfully applied to a mouse minibrain kinase to identify substrates from a brain cDNA library (E.

Kandel, personal communication), implying a wide application of the STEK method to a large number of protein Ser/Thr kinases from bacteria to kinases, whose substrates have not yet been identified. In addition, the STEK method could be applied for screening for specific inhibitors of a given Ser/Thr kinase as these inhibitors would be expected to function as suppressors for the cytotoxic effect of the kinase.

Experimental Procedures

Materials

Restriction enzymes and Klenow enzyme were purchased from New England Biolabs. T4 DNA ligase was obtained from BRL. Alkaline phosphatase conjugated goat anti-rabbit IgG, 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) and nitro blue tetrazolium (NBT) were purchased from Bio-Rad. [α -³²P]dGTP, [α -³²P]dATP and [γ -³²P]ATP were purchased from Amersham.

Bacterial Strains and Growth Conditions

E. coli SB 221 (Nakamura *et al.*, 1982) and JM83 (Vieira and Messing, 1982) were used for cloning and suppression assay. Cells were grown at 37°C in LB and glucose-M9 media (Miller, 1972) supplemented with kanamycin (30 μ g/ml) and chloramphenicol (30 μ g/ml), when necessary.

Construction of *E. coli* Genomic Library

The chromosomal DNA was isolated from *Escherichia coli* SB221 (Nakamura, *et al.*, 1982) by the method described previously (Yee and Inouye, 1981). The chromosomal DNA was digested partially with *Sau*3AI and 3–4 kb fragments were extracted from a 0.7% agarose gel. These fragments were cloned into the *Bam*HI site in pBRKm in which the 615-bp *Dra*I fragment in the β -lactamase gene of pBR322 was replaced with the 1.3-kb *Hinc*II fragment consisting of the kanamycin-resistant gene from Tn5 (Beck *et al.*, 1982). After transformation into *E. coli* SB221, colonies were harvested and used as an *E. coli* genomic library of which more than 90% of plasmids were carrying *E. coli* DNA.

Screening of the *E. coli* Genomic Library

E. coli SB221 harboring the *E. coli* genomic library consisting of approximately 5x10⁴ independent transformants was grown in LB medium containing kanamycin until the Klett unit reached 100 and electroporated with pINIICm/pkn2 using a Bio-Rad Gene Pulser (2.5 kV, 25 μ F and 200 Ω). After incubation in LB medium for 1 hr, 100 μ l of cells together with 20 μ l of 1 M isopropyl thio- β -D-galactopyranoside (IPTG) were plated on glucose-M9 agar plates containing chloramphenicol (30 μ g/ml) and kanamycin (30 μ g/ml). Plates were incubated for 36 hr at 37°C, and 30 colonies were picked and plasmids were prepared. Since these plasmid DNA samples contain both pINIICm/pkn2 and the candidate plasmids, the plasmids isolated were transformed to *E. coli* SB221 using LB agar plates containing only kanamycin. After overnight incubation, the plasmids containing suppressor genes were isolated from the colonies. Cells expressing candidate suppressor genes were reexamined for their resistance to pINIICm/pkn2 in the presence of IPTG.

pINIICm/pkn2 was constructed by placing the *pkn2* gene under the control of the *E. coli* *lpp* promoter and *lac* promoter and operator using a pINIICm vector (Ghrayeb *et al.*, 1984).

Western Blot Analysis of Pkn2

E. coli SB221 harboring pINIICm/pkn2 and the suppressor plasmids were cultured in glucose-M9 medium containing both kanamycin and chloramphenicol. The *pkn2* gene was induced with 1 mM IPTG at Klett unit of 100 and incubated for 0, 2, 4, and 6 hr. Cells were harvested and washed with 50 mM Tris-HCl (pH 8.0). The cell pellet was suspended in sonication buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 2 mM 2-mercaptoethanol] and sonicated for 2 min at 4°C. The supernatant after centrifugation at 5,000 x g for 1 min at 4°C was used as the cellular extract. The protein amount was determined by Bradford method (Bradford, 1976) and 20 μ g of protein samples were applied to 15% SDS-PAGE. The protein gel was blotted onto PVDF membrane. After the membrane was blocked with 5% skim milk for 4 hr, anti-Pkn2 antiserum was added and secondary antibodies were then applied. Pkn2 bands were developed with alkaline phosphatase substrates, NBT and BCIP.

Construction of HU α (T59A) and HU α (T65A)

HU α (T59A) was constructed by two-step PCR using pUC/HU α as template and the following oligonucleotides as primers: For the first step, T7 primer; TTG CAG CAC AT C C C C C T T with GCG GCC AG C AG C T C A G, and CTG AG C G T G C T G G C C G C with T7 3'-end primer; TGCTTCGGCTC

GTATGT. The PCR products were gel-purified and used as template for the second PCR. HU α (T65A) was constructed by the same procedure described above using oligonucleotides TTTACCGCCTGCGGGT and ACCCGCAGCCGGTAAA as primers. After confirmed nucleotide sequences, HU α (T59A) and HU α (T65A) were placed under the control of a T7 promoter.

Purification of HU α , HU α (T59A), HU α (T65A), and HU β

In order to purify HU protein, expression plasmids of HU α , HU α (T59A), HU α (T65A) and HU β were constructed by placing these genes under the control of T7 promoter (Studier *et al.*, 1990). The production of HU α and its derivatives and HU β were induced by the addition of IPTG at the final concentration of 1 mM in glucose-M9 medium. Purification of HU was performed by the method described by Tanaka *et al.* (1993) with some modification including, hydroxyapatite (HA) column chromatography to remove DNA fragments.

In vitro Phosphorylation of HU

Purified HU was phosphorylated with purified 40 ng Pkn2 (Udo *et al.*, 1997) and 1 mM ATP containing 5 μ Ci [γ -³²P]ATP (3000Ci/m mol) in the kinase buffer (0.1 M Tris-HCl [pH 7.5]/5 mM MnCl₂/1 mM dithiothreitol (DTT)). The reaction mixture was incubated for 30 min at 30°C and terminated by the addition of SDS-sample buffer (80 mM TrisHCl [pH 6.8]/ 2% SDS/ 10% glycerol/ 100 mM β -ME). Proteins were resolved by SDS-PAGE and autoradiographed.

Gel Retardation Assay

The DNA fragment for binding assay was prepared by digestion of pUC9 (Vieira and Messing, 1982) with *Bam*HI and *Pvu*II and the 141-bp fragment was purified by 5% acrylamide gel electrophoresis. The 141-kb fragment was labeled with [α -³²P]dGTP and [α -³²P]dATP using the Klenow fragment of DNA polymerase I. Binding reaction was performed using 0.1 ng DNA probe by the method described by Tanaka *et al.* (1993).

The reaction mixtures were separated by 5% polyacrylamide gel electrophoresis in TBE buffer (45 mM Tris-HCl/ 45 mM borate/ 2 mM EDTA pH 8.3). The gels were dried and autoradiographed.

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