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A eukaryotic-type serine/threonine protein kinase StkP of *Streptococcus pneumoniae* acts as a dimer *in vivo*

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Abstract

Streptococcus pneumoniae carries a single Ser/Thr protein kinase gene *stkP* in its genome. Biochemical studies performed with recombinant StkP have revealed that this protein is a functional membrane-linked eukaryotic-type Ser/Thr protein kinase. Here, we demonstrate that the deletion of its extracellular domain negatively affects the stability of a core kinase domain. In contrast, the membrane anchored kinase domain and the full-length form of StkP were stable and capable of autophosphorylation. Furthermore, evidence is presented that StkP forms dimers through its transmembrane and extracellular domains.
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Signal transduction pathways in both prokaryotes and eukaryotes utilize protein phosphorylation as a key regulatory mechanism. Formerly, signaling pathways mediated by Ser/Thr- and Tyr-specific protein kinases were thought to be unique to eukaryotes, whereas two-component systems consisting of histidine kinases and response regulators were considered to be widespread in prokaryotes. Recent studies have proven that eukaryotic-type Ser/Thr protein kinases (Hanks-type) [1] are abundantly present in prokaryotic genomes [2–5].

In our previous study [6], we characterized the biochemical properties of a single eukaryotic-type Ser/Thr protein kinase, StkP, found in *Streptococcus pneumoniae* [6]. The N-terminal part of StkP is composed of a conserved kinase domain joined through a hydrophobic linker to a predicted extracellular domain containing four repeats of the PASTA signature sequence (Penicillin-binding protein and Ser/Thr protein kinase associated domain) [7]. Aside from experimental evidence demonstrating catalytic activity of the kinase domain, the mode of action of StkP is unknown since it cannot be derived from the primary structure. Here, by using genetic and biochemical approaches we demonstrate that both the transmembrane and extracellular domains promote dimerization of StkP.

Materials and methods

Bacterial strains, plasmids, phages, and growth conditions. Bacterial strains, plasmids, and phages used in this study are listed in Table S1. *Escherichia coli* XL1-Blue (Stratagene) and *E. coli* JM 109 (Promega) were used to propagate plasmids in cloning experiments and were grown in Luria–Bertani medium. *E. coli* 61F [8] was used as a host strain for fusion constructs in dimerization assays and was grown in NZ medium [9]. *S. pneumoniae* was grown in casein–tryptone medium (CAT) [10]. When necessary, media were supplemented with antibiotics at the following final concentrations: *E. coli* hosts: ampicillin, 100 mg L^{-1}, chloramphenicol 10 mg L^{-1}, kanamycin 50 mg L^{-1}, tetracycline 12.5 mg L^{-1}; *S. pneumoniae* strains: chloramphenicol 10 mg L^{-1}. λ cI and λ vir phages used in

Abbreviations: cat, chloramphenicol acetyltransferase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid disodium salt; PSB, protein storage buffer; BCA, bicinchoninic acid; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminiscence; IgG, immunoglobulin G; TMD, transmembrane domain; pfu, plaque forming units.

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dimerization assays were grown and titrated on *E. coli* 61F by standard methods [11].

DNA manipulations and plasmid constructions. Chromosomal DNA of the *S. pneumoniae* wild-type strain Cp1015 [12] was used as a template for PCR amplifications. Primers used in this study are listed in Table S2. To construct epitope labeled forms of StkP the reverse primers that were used contained the sequence motif encoding 10 histidine residues and a stop triplet as a 5′ extension. To prepare a tagged kinase domain, a fragment containing the 3′ end of the *stkP* kinase domain was amplified by using the primers KD-f and KD-r (positions 105–270 in StkP). The fragment containing the *stkP* downstream sequence was amplified with the primers X-f and X1-r. To construct a membrane-anchored kinase domain of StkP, the primers KD-f and TM-r (positions 105–372 in StkP) were used yielding an 850 bp product. The downstream region was amplified as described above. To create an epitope labeled full-length form of StkP, the C-terminus of the gene (positions 271–659 in StkP) was amplified using the primers STK-f and STK-r yielding a 1200 bp product. The downstream fragment of a similar length was amplified with the primers X-f and X2-r. The plasmid pEVP3 [13] was used as a source of the *cat* cassette, which was amplified with the primers CAT1 and CAT2. The pBluescript II SK+ vector was used for cloning and sequencing experiments.

To determine the dimerization capability of StkP, different portions of the protein were fused to the cI repressor headpiece by cloning PCR products. The primers StkP-f and Kd-r were used for the amplification of the core kinase domain (positions 1–270 in StkP), the primers StkP-f and Kdtm-r for the amplification of the membrane-anchored kinase domain (positions 1–372 in StkP) and the primers C-term-f and StkP-r for the C-terminal domain (positions 367–659 in StkP). PCR products were digested with *Afl*III and cloned into the unique *Afl*III site of the plasmid pJAH01 [8].

The sequences of all clones used in this study were confirmed to ensure accuracy.

Allelic exchange. Preparation of the *stkP* mutant strains was achieved by transformation of the *S. pneumoniae* wild-type strain Cp1015 with vectorless DNA fragments containing oligohistidine tagged portions of the *stkP* gene, the *cat* cassette and the downstream sequence of the *stkP* gene (5′-*stkP*-*cat*-flanking region-3′), similarly to the method described in [14]. Integrity of the mutants was verified by PCR using appropriate primers (Table S2).

Cell fractionation. *Streptococcus pneumoniae* mutant strains were grown to $OD_{400} = 1$ in CAT medium and harvested by centrifugation. The cell pellet was resuspended in 0.5 ml of PSB [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol] supplemented with protease inhibitor cocktail (Sigma), desintegrated by sonication and centrifuged. The clarified cell lysate was ultracentrifuged at 100,000*g* for 1 h at 4 °C. The pellet (membrane fraction) was resuspended in PSB. The protein concentration in individual fractions was determined with the BCA Protein Assay Kit (Pierce).

In vitro kinase assay. The standard *in vitro* kinase reaction mixture contained 30 μg of proteins (subcellular fraction) in an assay buffer (50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol). The reaction was initiated by the addition of 1 μCi [γ-^{32}P]ATP (167 TBq/mmol; MP Biomedicals) at a final concentration of 10 μM and was terminated after 10 min of incubation at room temperature by the addition of 5× SDS sample buffer and heating at 100 °C for 3 min. After separation by SDS–PAGE, phosphorylated proteins were scanned with the BAS 5000 PhosphorImager (Fuji) and evaluated using the AIDA 2.11 program.

Western blots and immunodetection. Protein samples were mixed with 5× SDS sample buffer, heated to 100 °C for 3 min and separated by SDS–PAGE. Proteins from the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane and analyzed by immunodetection. The monoclonal anti-polyhistidine antibody produced in mouse clone HIS-1 (Sigma) and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham) were used at a dilution of 1:2000 and 1:1000, respectively. Detected proteins were visualized by ECL (Super Signal Chemiluminescent Substrate, Pierce).

Dimerization assay. Bacteriophage λ cI immunity assays were performed as described by Leeds and Beckwith [8].

Blue-native electrophoresis. Electrophoresis of proteins under native conditions was performed as described by Schagger [15].

Results

In vitro kinase assay in subcellular fractions of S. pneumoniae strains

Immunodetections and *in vitro* kinase assays performed with the cellular fractions of the wild-type and *stkP* deletion mutant strains localized StkP to the membrane fraction [6]. To assess the role of distinct protein domains we created the corresponding mutants (Fig. 1) expressing the epitope labeled full-length and truncated forms of StkP by allelic exchange and the catalytic activity of these proteins was tested. As expected, in the strain expressing the full-length StkP, a phosphorylated protein of the molecular mass corresponding to that of recombinant StkP was detected in either the crude extract or the membrane fraction (Fig. 2A, lanes 2 and 4). Analysis of cellular fractions of the mutant expressing the core kinase domain did not reveal the presence of any phosphorylated protein of corresponding molecular mass (Fig. 2A, lanes 6–8). Further, the mutant expressing a kinase domain anchored to the membrane through its TMD was tested for the activity. The results clearly showed that association of StkP with the membrane brought about restoration of its autophosphorylation activity (Fig. 2A, lanes 9–11).

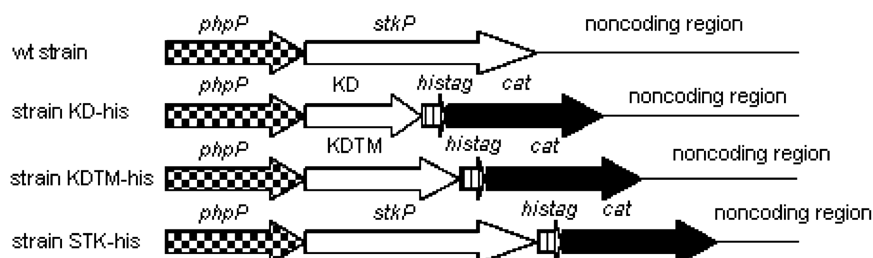

Fig. 1. Schematic representation of *stkP* locus of *S. pneumoniae* wild-type and mutant strains achieved by allelic exchange. Abbreviations: *phpP*, protein phosphatase gene; *stkP*, protein kinase gene; KD, *stkP* kinase domain; KDTM, *stkP* kinase domain with transmembrane sequence; *histag*, oligohistidine tag; *cat*, chloramphenicol resistance cassette.

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