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Rapid Communication

A Plant Receptor-Like Gene, the *S*-Locus Receptor Kinase of *Brassica oleracea* L., Encodes a Functional Serine/Threonine Kinase[1]

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To investigate the catalytic properties of the *Brassica oleracea* S-locus receptor kinase (SRK), we have expressed the domain that is homologous to protein kinases as a fusion protein in *Escherichia coli*. Following in vivo labeling of cultures with ^{32}P-labeled inorganic phosphate, we observed phosphorylation of the fusion protein on serine and threonine, but not on tyrosine. In contrast, labeling was not observed when lysine-524, a residue conserved among all protein kinases, was mutated to arginine, thus confirming that SRK phosphorylation was the result of intrinsic serine/threonine kinase activity.

The *Brassica oleracea* L. *SRK* gene belongs to a family of genes recently cloned in plants that encode receptor-like proteins. In common with its relatives, which include the *Zea mays* protein kinase 1 (Walker and Zhang, 1990) and *Arabidopsis thaliana* receptor kinase 1 genes (Tobias et al., 1992), the *SRK* sequence predicts a transmembrane protein having an extracellular domain homologous to the product of *SLG* and a cytoplasmic domain homologous to protein Ser/Thr kinases (Stein et al., 1991). Owing to this structure, it has been proposed that proteins in this family function as signal-transducing receptors, able to phosphorylate intracellular substrates in response to extracellular stimuli. To understand the function of *SRK*, we are investigating the catalytic properties of its protein product.

The expression of protein kinase genes in *Escherichia coli* as a means to study catalytic activity is a commonly used strategy that does not require protein purification from native tissues (Wang et al., 1982; Tan and Spudich, 1990; Ben-David et al., 1991; Crews et al., 1991; Muñoz-Dorado et al., 1991; Stern et al., 1991; Wu et al., 1991; Lin et al., 1992). In addition, because most protein kinases are able to autophosphorylate, the prior knowledge or use of exogenous substrates is often not essential. We demonstrate here that the product of SRK_6, expressed in bacteria, is a functional protein kinase, able to autophosphorylate on Ser and Thr residues. As has been shown for other protein kinases, mutation of the Lys residue at the putative ATP-binding site abolishes the catalytic activity of SRK.

MATERIALS AND METHODS

SRK Fusion Protein Constructs

Because the SRK_6 kinase-like domain was originally obtained on separate overlapping cDNA clones, pJS30 and pS6–27 (Stein et al., 1991), it was necessary to reconstitute the coding region by ligating the 1.1-kb *Xho*I fragment of pJS30 into the *Xho*I site of pS6–27, and selecting for a clone, pJS31, with the correct orientation. Four oligonucleotides, synthesized at the Cornell Biotechnology Analytical and Synthetic Facility, were used in the preparation of constructs: primer A (5′-TATGAACGGGATCCTACTATC-3′), primer B (5′-CCCTCTTTCACATATGATATTGAATC-3′), primer C (5′-CTTTGATAGCCTTCTTACTGCA-3′), and primer D (5′-TGCAGTAAGAAGGCTATCAA-3′). To obtain a subfragment suitable for bacterial expression, the pJS31 template was amplified by PCR with the upstream primer A and the downstream primer B, using the GeneAmp kit (Perkin-Elmer Cetus). The resulting 1.1-kb fragment was first cloned into the pCR1000 vector (Invitrogen), where its sequence on one strand was confirmed by dideoxy-sequencing with Sequenase (United States Biochemical Corp.). It was then subcloned into the *Bam*HI/*Eco*RI site of the bacterial expression vector pGEX-3X (Pharmacia). The resulting construct, pJS37, fuses the GST coding region to that of SRK_6, starting 27 codons downstream of the putative transmembrane domain and ending with the cDNA-encoded stop codon.

A second plasmid, pJS37K524R, was constructed in which the SRK_6 codon for Lys^{524} was mutagenized to Arg. Site-directed mutagenesis was accomplished using the overlap extension PCR method (Ho et al., 1989). Briefly, two PCR reactions were performed using pJS37 as a template. In the first, primer A and the mutagenic primer C were used; in the second, the mutagenic primer D and primer B were used.

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Abbreviations: GST, *Schistosoma japonicum* glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; *SLG*, *Brassica oleracea S*-locus glycoprotein gene; SRK, *B. oleracea S*-locus receptor kinase; *SRK*, gene encoding SRK; SRK_6, *SRK* allele derived from the *B. oleracea* S_6 haplotype; SRK_6, the protein product of SRK_6.

Because primers C and D are complementary, the products of these two reactions could be combined and amplified in a third PCR reaction with primers A and B to produce a single mutagenized product of 1.1 kb. This fragment was cloned, sequenced, and inserted into pGEX-3X as described above. Aside from the Lys-to-Arg codon change, pJS37 and pJS37K524R are identical.

Expression and Purification of SRK Fusion Proteins

Overnight cultures of *E. coli* strain JM109, transformed with the above constructs, were diluted 1:10 in LB broth plus ampicillin and grown for 1 h at 37°C. After induction with 0.1 mM IPTG for 3 h at 30°C, cells were lysed by sonication in Dulbecco's PBS (pH 7.4) containing 1% (v/v) Triton X-100. Fusion proteins were affinity purified using GSH-agarose beads (Sigma) essentially as described (Smith and Johnson, 1988). Purified proteins were resolved by electrophoresis on SDS/10% polyacrylamide gels and detected by Coomassie blue staining or by immunoblotting.

Labeling Cells with ^{32}P and Phosphoamino Acid Analysis

Cultures were grown and induced as described above. Pulse-labeling of cells was carried out with 100 μCi of ^{32}Pi (NEN) per 10 mL of induced culture for 15 min at 37°C, according to a previously described method (Wang et al., 1982). After SDS-PAGE of affinity-purified proteins, the gel was stained with Coomassie blue, dried, and exposed to Kodak XAR 5 film at −70°C with an intensifying screen.

To determine which amino acids on SRK_6 were phosphorylated, cells containing pJS37 were labeled with ^{32}Pi as described above and sonicated in a buffer containing 50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 10 mM NaF, 10 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 5 μg of aprotinin per mL, and 0.5 μg of leupeptin per mL. The ^{32}P-labeled SRK_6 fusion protein was purified with GSH-agarose beads, subjected to SDS-PAGE, and blotted onto Immobilon-P (Millipore). The radiolabeled fusion protein was identified by autoradiography and then hydrolyzed in 6 N HCl at 110°C for 1 h, according to Kamps and Sefton (1989). The hydrolysate was dissolved in a solution containing nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine, and was spotted onto a 0.1-mm cellulose plate (Merck). One-dimensional thin-layer electrophoresis was carried out at 1500 V in glacial acetic acid:pyridine:H_2O (50:5:945, v/v/v), as described by Hunter and Sefton (1980). The positions of the phosphoamino acid standards were determined by ninhydrin staining. The plate was then subjected to autoradiography.

Immunodetection of the SRK Fusion Proteins

A synthetic peptide (Cys-Nle-Val-Arg-Arg-Ser-Pro-Tyr-Glu-Leu-Asp-Pro-Ser-Ser-Ser) conjugated to ovalbumin was prepared at the Cornell Biotechnology Analytical and Synthetic Facility. The peptide sequence corresponds to the predicted residues 790 to 803 within the C-terminal region of SRK_6. Norleucine was included in the peptide as a marker to facilitate quantitation. A total of 500 μg of the peptide conjugate was injected into a single rabbit to obtain antiserum 1–254. For immunoblotting, proteins were subjected to SDS-PAGE and transferred to Immobilon-P. The membrane was blocked with 1% (w/v) BSA in 10 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.5% (v/v) Tween-20, and then reacted with a 1:2500 dilution of 1–254 antiserum for 1 h at room temperature. Subsequent reaction with goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Promega) and detection with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) were carried out according to their respective manufacturers' instructions.

RESULTS

To characterize the gene product of SRK_6, we subcloned the protein kinase-homologous domain of the SRK_6 cDNA into the bacterial expression vector pGEX-3X. The resulting construct, pJS37, was expected to produce a GST-SRK_6 fusion protein of 68 kD. In addition, we constructed pJS37K524R, a plasmid identical to pJS37 except that it carried a conservative mutation that substituted Arg for the Lys^{524} codon of SRK_6. Corresponding to the ATP-binding site, this Lys is essential in protein kinases (Hanks et al., 1988) and is a common target for site-directed mutagenesis as a means to obtain kinase-defective proteins (Snyder et al., 1985; Kamps and Sefton, 1986; Weinmaster et al., 1986; Chou et al., 1987; Ebina et al., 1987; Denhez et al., 1988).

When induced with IPTG, cultures bearing pJS37 and pJS37K524R each produced an approximately 64-kD protein that was not observed in noninduced cultures or in cultures containing the pGEX-3X vector without insert (data not shown). Only a small fraction of this protein was produced in soluble form, but this fraction could be substantially purified by affinity to GSH-agarose beads (Fig. 1A). In addition to the 64-kD band, a series of lower molecular mass bands, possibly the products of proteolytic cleavage, and on occasion a larger band (75 kD) were copurified. Immunoblotting with the anti-SRK_6 antibody 1–254 demonstrated that the 64-kD band and several of lower molecular mass bands are in fact the products of the SRK_6 gene (Fig. 1B). The Lys to Arg mutation had little effect on protein stability, because the wild-type and mutant proteins accumulated to equivalent levels.

Several attempts were made to demonstrate protein kinase activity in vitro using the purified GST-SRK_6 fusion protein, but without success. As an alternative approach, we investigated SRK_6 phosphorylation in vivo. Cultures bearing pJS37, pJS37K524R, and the control vector pGEX-3X were grown under inducing conditions and pulse-labeled with ^{32}Pi (see "Materials and Methods"). Examination of whole cell extracts by SDS-PAGE and autoradiography revealed that cultures bearing pGEX-3X and pJS37K524R possessed identical profiles of phosphorylated bands, whereas the pJS37 culture produced a single additional phosphorylated band that comigrated precisely with the GST-SRK_6 fusion protein (data not shown). To demonstrate unambiguously that the recombinant SRK_6 protein was phosphorylated, the proteins were affinity purified. Coomassie blue staining showed that approximately equal quantities of the wild-type and mutant fusion proteins were isolated (Fig. 2A). Yet, exposure to

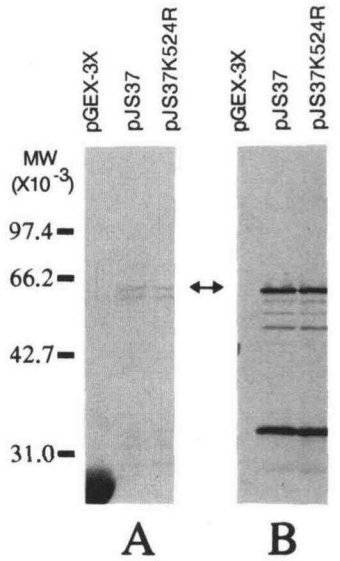

Figure 1. Bacterial expression and purification of wild-type and mutant SRK_6 fusion proteins. Wild-type GST-SRK_6 and Lys^{524}-to-Arg mutant GST-SRK_6 fusion proteins were expressed in JM109 cells transformed with plasmids pJS37 and pJS37K524R, respectively. Cells bearing the indicated plasmids were induced with IPTG. Proteins were purified by affinity to GSH-agarose and analyzed by SDS-PAGE and Coomassie blue staining (A) and immunoblotting with rabbit anti-SRK_6 antibodies, 1–254 (B).

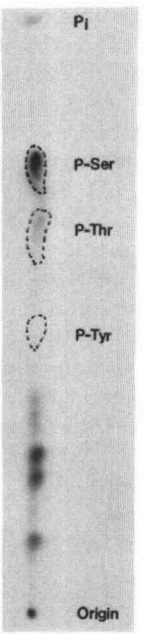

Figure 3. Phosphoamino acid analysis of ^{32}P-labeled SRK_6 fusion protein. Cells transformed with pJS37 were pulse-labeled with ^{32}Pi, and the purified fusion protein was subjected to SDS-PAGE and autoradiography. The phosphoamino acid content of the labeled band was analyzed as described. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) standards are circled.

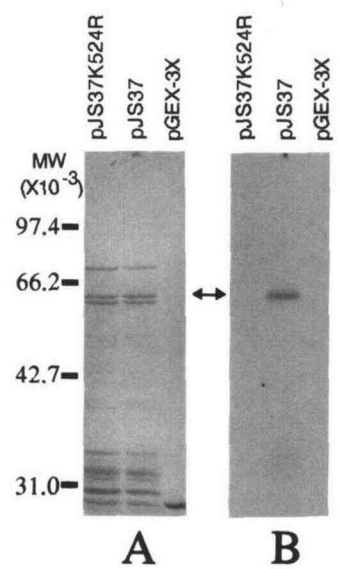

Figure 2. Phosphorylation of recombinant SRK_6. Cultures bearing the indicated plasmids were pulse-labeled with ^{32}Pi. After purification with GSH-agarose, proteins were analyzed by SDS-PAGE and Coomassie blue staining (A), followed by autoradiography (B).

x-ray film revealed that only the wild-type SRK_6 protein was detectably phosphorylated (Fig. 2B). The inability of either the mutant fusion protein or the control GST protein to become labeled supports the view that an active catalytic domain in SRK_6 was required for the observed phosphorylation of the fusion protein, and that this occurred as a result of autophosphorylation.

Next, we determined which amino acids on SRK_6 were phosphorylated. The affinity-purified ^{32}P-labeled GST-SRK_6 fusion protein was hydrolyzed and subjected to phosphoamino acid analysis as described in "Materials and Methods." As shown in Figure 3, the ^{32}P label comigrated predominantly with the phosphoserine and to a lesser extent with phosphothreonine standards, but not with phosphotyrosine.

DISCUSSION

We have provided compelling evidence that the product of *SRK_6* is a functional Ser/Thr kinase and that it is capable of autophosphorylation. Although we were unable to find conditions under which the bacterially expressed protein was active in vitro, our experiments showed that wild-type SRK_6, but not a catalytically defective mutant protein, was phosphorylated on Ser and Thr residues in vivo. These findings are an important step forward in our understanding of a potentially new class of receptors in plants. The existence of receptor Ser/Thr kinases is not unprecedented. In animals, members of the TGF-β family of receptors recently have been shown to be functional Ser/Thr kinases (Lin et al., 1992).

These receptors mediate numerous and diverse processes of cell growth and differentiation (Massagué, 1992).

The biological function of *SRK* is not yet established. However, several lines of evidence suggest that *SRK* functions in the perception of self and nonself between pollen and stigma. First, *SRK* maps genetically and physically to the *S*-locus (Chen and Nasrallah, 1990; Stein et al., 1991; Boyes and Nasrallah, 1992), the locus that controls mating self-incompatibility. Second, several alleles of *SRK* have been cloned from different *S*-locus haplotypes, and these display a high degree of protein sequence polymorphism, as would be expected for molecules that function in recognition determination (Stein et al., 1991; and unpublished data). Finally, *SRK* is expressed exclusively within pistil and anther tissues (Stein et al., 1991). The role of SRK in pollen/stigma recognition will be clarified by an understanding of how native SRK catalytic activity is regulated.

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NOTE ADDED IN PROOF

While this article was in review, the product of the *SRK-910* gene, an *SRK* allele cloned from *Brassica napus*, was shown to possess protein Ser/Thr kinase activity in vitro (Goring and Rothstein, 1992).

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LITERATURE CITED

Ben-David Y, Letwin K, Tannock L, Bernstein A, Pawson T (1991) A mammalian protein kinase with potential for serine/threonine and tyrosine phosphorylation is related to cell cycle regulators. EMBO J **10:** 317–325

Boyes DC, Nasrallah JB (1993) Physical linkage of the SLG and SRK genes at the self-incompatibility locus of *Brassica oleracea*. Mol Gen Genet (in press)

Chen C-H, Nasrallah JB (1990) A new class of S sequences defined by a pollen recessive self-incompatibility allele of *Brassica oleracea*. Mol Gen Genet **222:** 241–248

Chou CK, Dull TJ, Russell DS, Gherzi R, Lebwohl D, Ullrich A, Rosen OM (1987) Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. J Biol Chem **262:** 1842–1847

Crews CM, Alessandrini AA, Erikson RL (1991) Mouse *Erk-1* gene product is a serine/threonine protein kinase that has the potential to phosphorylate tyrosine. Proc Natl Acad Sci USA **88:** 8845–8849

Denhez F, Heimann B, d'Auriol L, Graf T, Coquillaud M, Coll J, Galibert F, Moelling K, Stehelin D, Ghysdael J (1988) Replacement of lys 622 in the ATP binding domain of $p100^{gag\text{-}mil}$ abolishes the in vitro autophosphorylation of the protein and the biological properties of the *v-mil* oncogene of MH2 virus. EMBO J **7:** 541–546

Ebina Y, Araki E, Taira M, Shimada F, Mori M, Craik CS, Siddle K, Pierce SB, Roth RA, Rutter WJ (1987) Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. Proc Natl Acad Sci USA **84:** 704–708

Goring DR, Rothstein SJ (1992) The S-locus receptor kinase gene in a self-incompatible *Brassica napus* line encodes a functional serine/threonine kinase. Plant Cell **4:** 1273–1281

Hanks SK, Quinn M, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:** 42–52

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:** 51–59

Hunter T, Sefton BM (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci USA **77:** 1311–1315

Kamps MP, Sefton BM (1986) Neither arginine nor histidine can carry out the function of lysine-295 in the ATP-binding site of $p60^{src}$. Mol Cell Biol **6:** 751–757

Kamps MP, Sefton BM (1989) Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal Biochem **176:** 22–27

Lin HY, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish H (1992) Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell **68:** 775–785

Massagué J (1992) Receptors for the TGF-β family. Cell **69:** 1067–1070

Muñoz-Dorado J, Inouye S, Inouye M (1991) A gene encoding a protein serine/threonine kinase is required for normal development of M. xanthus, a gram-negative bacterium. Cell **67:** 995–1006

Smith DB, Johnson KS (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene **67:** 31–40

Snyder MA, Bishop JM, McGrath JP, Levinson AD (1985) A mutation at the ATP-binding site of $pp60^{v\text{-}src}$ abolishes kinase activity, transformation, and tumorigenicity. Mol Cell Biol **5:** 1772–1779

Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB (1991) Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc Natl Acad Sci USA **88:** 8816–8820

Stern DG, Zheng P, Beidler DR, Zerillo C (1991) Spk1, a new kinase from *Saccharomyces cerevisiae*, phosphorylates proteins on serine, threonine, and tyrosine. Mol Cell Biol **11:** 987–1001

Tan JL, Spudich JA (1990) Developmentally regulated protein-tyrosine kinase genes in *Dictyostelium discoideum*. Mol Cell Biol **10:** 3578–3583

Tobias CM, Howlett B, Nasrallah JM (1992) An *Arabidopsis thaliana* gene with sequence similarity to the *S*-locus receptor kinase of *Brassica oleracea*. Plant Physiol **99:** 284–290

Walker JC, Zhang R (1990) Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica*. Nature **345:** 743–746

Wang JYJ, Queen C, Baltimore D (1982) Expression of an Abelson Murine Leukemia Virus-encoded protein in *Escherichia coli* causes extensive phosphorylation of tyrosine residues. J Biol Chem **257:** 13181–13184

Weinmaster G, Zoller MJ, Pawson T (1986) A lysine in the ATP-binding site of $p130^{gag\text{-}fps}$ is essential for protein tyrosine kinase activity. EMBO J **5:** 69–76

Wu J, Rossomando AJ, Her J-H, Vecchio RD, Weber MJ, Sturgill TW (1991) Autophosphorylation in vitro of recombinant 42-kilodalton mitogen-activated protein kinase on tyrosine. Proc Natl Acad Sci USA **88:** 9508–9512