The *Dictyostelium* dual-specificity kinase splA is essential for spore differentiation

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SUMMARY

We have studied the structure and function of the *Dictyostelium* kinase splA. A truncated form of the splA protein exhibited primarily tyrosine kinase activity in vitro; however, it also autophosphorylated on serine and threonine residues. The kinase domain of splA exhibits approximately 38% identity to the CTR1 kinase of Arabidopsis, which is a member of the Raf family. Outside its kinase domain, splA shares homology with the byr2 kinase of S. pombe. By aligning the sequences of splA, byr2 and STE11, a homologue of byr2 in S. cerevisiae, we have identified a conserved motif that is also found in members of the Eph family of growth factor receptor tyrosine kinases. SplA is expressed throughout development with a peak during the mound stage of morphogenesis. Strains in which the splA gene had been disrupted completed fruiting body formation; however, spore cells spontaneously lysed before completing their differentiation. Northern analysis revealed the expression of the prespore marker cotB and the prestalk markers ecmA and ecmB in the mutant strain during development. The spore differentiation marker splA was detected in the mutant spores both by northern and immunoblotting, but these cells failed to assemble spore coats. Immunoblot analysis of the developmental pattern of tyrosine phosphorylation revealed a protein that was phosphorylated in mutants but was not phosphorylated in the wild-type cells. SplA is a novel dual specificity kinase that regulates the differentiation of spore cells.

Key words: sporulation, phosphorylation, differentiation, *Dictyostelium*, protein tyrosine kinases (PTK)

INTRODUCTION

Protein tyrosine kinases (PTKs) are key elements of signal transduction pathways that regulate cell proliferation and differentiation during development in multicellular eukaryotes. We have begun studying PTKs in *Dictyostelium discoideum*, an organism that provides unique experimental opportunities.

The developmental cycle of *Dictyostelium* is simple and short in duration, but it exhibits features of development that are common to all multicellular eukaryotes, such as cell migration, signaling between cells and the differentiation of specific cell types. When a population of vegetative amoebae is starved of nutrients, the cells proceed through a coordinated, developmental pathway lasting approximately 26 hours and leading to the formation of fruiting bodies (for review see Loomis, 1975). Each fruiting body consists of approximately 10^9 cells, of which 70%-80% are spores in a spherical mass that rests atop a stalk generated by the remaining cells. Spores remain dormant until germination is induced and each spore gives rise to a new amoeba to continue the cycle.

As with higher eukaryotes, tyrosine phosphorylation has been shown to play a role in regulating the development of *Dictyostelium*. Western blots and immunoprecipitations with anti-phosphotyrosine antibodies reveal changes in the overall pattern of tyrosine phosphorylation over the course of development (Schweiger et al., 1990; Howard et al., 1992). A developmentally regulated MAP kinase in *Dictyostelium* called erkB is tyrosine phosphorylated in vivo (Segall et al., 1995). Several non-receptor type tyrosine phosphatases have been identified in *Dictyostelium* that cause fruiting body malformations when overexpressed (Howard et al., 1992; Ramalingam et al., 1993; Howard et al., 1994).

Work in our laboratory has led to the identification of two PTK genes in *Dictyostelium*, which were found to increase in expression during development (Tan and Spudich, 1990). These genes were named *DPYK1* and *DPYK2*, but we propose the name *splA* ('spore lysis A') to replace *DPYK1* because of the phenotype of the loss-of-function mutants described in this report. The *DPYK1* partial cDNA encodes an enzyme that phosphorylates proteins on tyrosine when expressed in *E. coli*. However, splA (DPYK1) has been referred to as putative dual specificity kinase because sequences in its kinase catalytic domain, thought to be important in determining hydroxyamino acid substrate specificity, deviate from the consensus of PTKs (Lindberg et al., 1992).

We have performed this study of splA with the aim of identifying its role in *Dictyostelium* development. We have disrupted the gene and characterized defects in the maturation of spore cells. We determined the complete splA amino acid sequence and characterized its enzymatic specificity, thus con-
Genomic cloning of the splA gene

Genomic Southern mapping indicated the presence of an XbaI site 1.1 kb upstream from the 5′ end of the cDNA fragment that we had previously cloned (Tan and Spudich, 1990). We constructed a plasmid library by digesting Dictyostelium genomic DNA with PstI and XbaI. The 1.0-1.6 kb fragments were ligated into the vector pJDC9 (Chen and Morrison, 1988). Positive clones that hybridized with the DPKY1 cDNA were sequenced and found to be identical at their 3′ end with the cDNA. This genomic fragment of the splA gene was named splA.1.

We obtained complete genomic clones of the splA gene using the technique of integration/excision cloning (Hildebrandt and Nellen, 1991). In detail, the Thy1 gene, a Dictyostelium selectable marker (Dynes and Firtel, 1989) as a BamHI fragment was ligated into the BamHI site of the plasmid pDPKY1 (Tan and Spudich, 1990) upstream of the cDNA. The resulting plasmid was linearized with BglII and transformed into the thymidine auxotrophic Dictyostelium strain JH10 (Madsen and Firtel, 1990; Hadwiger and Firtel, 1992) by electroporation (Howard et al., 1988). Colonies that arose under selection in HL5 medium were pooled and grown in suspension culture. Genomic DNA from these transformants was cut with SaII followed by extraction with phenol/chloroform. SaII cut at one site in the plasmid, between the Thy1 marker and the vector, and also cut a genomic site 3.3 kb 5′ from the site of the integrated plasmid. The liberated plasmid and contiguous chromosomal DNA was ligated and transformed into E. coli. Plasmid DNA was restriction mapped and Southern blotted using the XbaI to BglII fragment of splA.1 as a probe. Clones were recovered that had an identical restriction map corresponding with the splA genomic Southern map. This 3.3 kb genomic fragment of the splA gene up to the SaII site, was called splA.2. We then used a 900 bp fragment from splA.2 in a second round of integration/excision cloning to recover an additional 3.8 kb of genomic clone upstream to the SpeI site. These clones were found to match the genomic Southern map of the splA gene and they hybridized to a probe from upstream of the 900 bp fragment from splA.2. To clone genomic DNA 3′ from the region from which the DPKY1 cDNA was derived, we excised the plasmid integrated into the strain HS9117 (described below) using HindIII.

Sequencing and sequence analysis

The splA genomic clones were sequenced as double or single stranded DNA using Sequenase 2.0 (USB). After sequencing one strand manually, the other strand was sequenced by primer walking using automated fluorescent techniques. All automated sequencing was performed by the Protein and Nucleic Acid facility of the Beckman Center for Molecular and Genetic Medicine.

The program Gene Works (IntelliGenetics Inc.) was used to manipulate the sequence. Searching of databases was performed at the National Center for Biotechnology Information using the BLAST algorithm (Altschul et al., 1990). The splA sequence was analyzed for motifs present in the PROSITE database (Bairoch, 1992) using the Quest program as part of the IntelliGenetics Suite. We used the program PEPTIDESTRUCTURE as part of the GCG software package to analyze the hydrophobicity and surface probability of the splA sequence using the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) and a window of 19.

cDNA cloning of a portion of the splA message

The sequence of the genomic clone of splA indicated two introns, which we confirmed by cloning a segment of cDNA using RT-PCR. RNA was purified from wild-type (JH10) Dictyostelium that had been starved on non-nutrient agar plates for six hours. This total RNA was reverse transcribed using a primer matching the sequence 3′ from the second intron (5′-GCCGATCCACGTCTCGGTGAAAACCT) and PCR was performed adding a primer from 5′ of the first intron (5′-GCGGATCCAAAACACCAACCACTATCCCAA). This reaction resulted in a single band of 422 bp, the correct size for the fragment after editing. This cDNA fragment was subcloned into pGEM7, making the plasmid pSPLA-introns and sequenced.

splA expression pattern

The temporal pattern of splA expression during the developmental cycle of Dictyostelium was assayed using semiquantitative, competitive RT-PCR (Riedy et al., 1995). Axenically grown wild-type (Ax2) cells were developed on non-nutrient agar and total RNA was prepared for each time point. A homologous RNA (called the standard template) was synthesized in vitro and added to the RNA sample from each developmental time point as an internal control. The standard template was generated by digesting the cDNA clone pSPLA-introns with NdeI, removing approximately 200 nt using Bal31, and religating the plasmid. Standard template RNA was synthesized from this resulting plasmid using SP6 RNA polymerase.

For determining the relative amount of splA message at each developmental time point, RT-PCR was performed using the same primers as described above and 0.2 μg of 32P-alpha dATP was added to each reaction, and the amplification was limited to 20 cycles. Each reaction contained 0.75 μg of total RNA from developed Dictyostelium and a constant amount of the standard template. The amplified products were electrophoretically separated and the radioactive bands were quantitated on a phosphorimager. The relative amount of splA message from each developmental time point was calculated as the ratio of the counts in the target band to the counts in the standard band in that reaction.

Gene targeting constructs

Two plasmids were constructed for targeting the splA gene. The first plasmid was constructed by ligating the 0.9 kb BglII/PstI fragment from the genomic clone splA.1 into the vector pTZ-18R (Pharmacia). The Thy1 selectable marker was inserted into the SplH site of this plasmid making pSPLA-int as is shown in Fig. 5A. 10 μg of this plasmid was linearized with EcoRV and electroporated into the cell line JH10. Cloned, independent transformants were screened for homologous integration by genomic Southern analysis. The second plasmid for gene targeting was built by first subcloning the 3.3 kb splA.2 genomic fragment into pGEM7zf(-) making pGEM-splA.2. Then the 1.3 kb BglII fragment, including half of the kinase domain was replaced with the Thy1 gene making pSPLA-del (Fig. 5A). This plasmid was digested with EcoRV and with EcoRI prior to electroporation into JH10 cells, and transformants were characterized by Southern blotting.

Spore viability assay

Cells were grown in suspension and washed in LPS buffer (20 mM KCl, 0.24 mM MgCl2, 40 mM phosphate buffer pH 6.4) with 60 U/ml penicillin, 60 μg/ml streptomycin sulfate (PS) and developed on filters. For some experiments, fruiting bodies were developed by the addition of vegetative cells to a lawn of Klebsiella aerogenes. At the end of development, spores were collected in LPS buffer and passed through an 18 gauge needle to disperse aggregates. Samples of spores were counted on a hemocytometer and adjusted to the same concentration with LPS. An aliquot of each of the cell suspensions was incubated at 40°C for 20 minutes to induce spore germination and kill non-spore cells (Cotter and Raper, 1966). This heat-treated aliquot was serially diluted and plated on bacterial lawns. The viability of the splA mutant spores was compared to that of wild-type spores by determining the number of plaque forming units present in each cell suspension.
Microscopic examination of spores
Wild-type and splA strains were developed both on filters and by spotting a few cells on lawns of K. aerogenes. Spores from several sorocarps were assayed for viability by staining with 20 μg/ml of propidium iodide (Molecular Probes) in LPS, or stained for spore coats with 1 μg/ml calcofluor (Sigma) in LPS. Photographs were taken on a Zeiss Axiohot with a 40x Plan Neofluar objective.

Bacterial expression and phosphorylation assays
The splA kinase domain and C-terminal tail was expressed in E. coli with the addition of the myc epitope tag (Kolodziej and Young, 1991) on the C-terminus (Fig. 2C). The myc tag was fused to the splA sequence in the plasmid pDPYK1 using a PCR strategy with the primers, 5'-CGCGAATTCTCGATATCCGGCGCATTTGGTGGTGG and 5'-GCGGAATTTCTTATAAATCTTCTTCTGAAAATTATT- TTTGTTCGATATCAGATACACCATGATCAAAG. This PCR product was cloned into the expression vector pET11d (Novagen) and transformed into the E. coli strain BL21(DE3) (pLysS).

For anti-SpiA immunoblots, protein was loaded on the gel for each sample. Western blots were stained with the primary anti-phosphotyrosine antibody PY20 (Zymed) at a concentration of 0.5 μg/ml and an HRP-conjugated goat anti-rabbit antibody (BioRad) at a dilution of 1:3000. For anti-SpiA immunoblots, rabbit antiserum (Richardson et al., 1991) was diluted to 1:5000. An HRP-conjugated goat anti-rabbit antibody (BioRad) was used at a dilution of 1:5000. All blots were developed using the ECL Western kit (USB) and exposed on Kodak Scientific Autoradiography film.

Northern analysis
SplA- or AX4 wild-type cells were developed on filters and collected at the indicated time points. Total RNA was prepared using Trizol reagent (GibcoBRL) and analyzed on northern blots as previously described (Fosnaugh et al., 1991).

Selection of phenotype reversion
Spores with wild-type viability were selected out of populations of splA mutants using the following protocol. Amoebae from the splA mutant strains HS9117 and HS13103 were each spotted onto three lawns of K. aerogenes and incubated until the plate was covered with fruiting bodies (approximately 5 days). The spores were collected and spread on a second lawn, and these plates were incubated at 37°C for 1 hour. After this heat treatment, the naires were incubated at 21°C to grow fruiting bodies and this cycle was carried out for a total of three rounds of selection. At the end of the selection, one clone was isolated from each of the plates. The six independent clones of survivors were assayed for growth in medium with and without supplemented thymidine, and the morphology of spores derived from each of these strains was evaluated by phase-contrast microscopy. Genomic DNA was prepared from these survivors and the splA locus was analyzed by Southern blotting.

RESULTS
Cloning of the splA gene
Using the technique of integration/excision cloning (Hildebrandt and Nellen, 1991), we recovered four overlapping fragments spanning approximately 8.5 kb of the chromosome containing the splA gene. These genomic clones were restriction mapped and found to correspond to the map of the splA locus derived from genomic Southern blotting (see Fig. 5B). We sequenced these genomic fragments and identified a long open reading frame interrupted near the 5′ end by two introns. We cloned a cDNA fragment by RT-PCR using primers indicated by dashed lines in Fig. 1 and confirmed the exon splice junctions.

Primary structure of splA
Fig. 1 presents the complete nucleotide and amino acid sequence of the splA gene, which has been assigned the GenBank accession number U32174. The open reading frame predicts a protein of 1584 amino acids with the kinase catalytic domain (aa 1296-1559, boxed in Fig. 1) near the C terminus. The predicted relative molecular mass of the protein is approximately 174x103. Analysis of the amino acid sequence did not reveal potential membrane spanning segments. Computer program analysis identified Y855 (circled in Fig. 1) as a possible site for tyrosine phosphorylation, matching the PROSITE database (Bairoch, 1992) consensus pattern (R or K)xxx(D or E)xYxxxF where x is any amino acid and J and O represent charged and polar amino acids respectively, although we confirmed experimentally that other tyrosines of the protein can serve as autophasphorylation sites as described below. Many PTKs have an autophasphorylation site within the activation loop of the kinase domain between subdomains VII and VIII (Hanks et al., 1988). At an equivalent site in splA there is a serine (S1460, double boxed in Fig. 1) that otherwise matches this consensus pattern.

In comparison to other kinase sequences, splA is most homologous to CTR1 from Arabidopsis (Kieber et al., 1993), with 37.5% identity over the 264 amino acids of splA’s kinase catalytic domain. We identified a 41 amino acid domain of splA that is 41% identical to the S. pombe kinase byr2 (see Fig. 2A). This domain in byr2 (indicated by a triangle in Fig. 2C) is located eight residues from the amino terminus and approximately 350 residues from the kinase domain. Its position in splA (Fig. 2C) is also approximately 350 residues aminoterminal from its kinase domain, byr2 has a functional (Styrkarndottir et al., 1992; Neiman et al., 1993) and structural homolog in S. cerevisiae called STE11 (Rhodes et al., 1990). When we aligned the homologous domains of splA and byr2 with the sequence of STE11, we identified a motif that is shared among these three kinases (Fig. 2B). We then searched databases with the sequence of this domain from splA and identified this same motif in the carboxy-terminal tail (Fig. 2C) of several receptor type PTKs of the Eph family from chicken, rodents and human.

Characterization of kinase activity
We performed experiments to determine whether the splA kinase would phosphorylate serine and threonine residues in addition to tyrosine. For these experiments, we expressed in E.
Fig. 1. Nucleotide and amino acid sequence of the splA gene. The nucleotides are numbered on the left and the amino acids on the right. The splA gene contains two introns near its 5’ end the positions of which were confirmed by RT-PCR. Primers for this PCR are indicated by dashed lines. A tyrosine that matches a tyrosine kinase substrate recognition consensus is circled. The domain found to have homology with the byr2 kinase is indicated by bold lines. The kinase catalytic domain is boxed, within which there is a position indicated by an additional box that is an autophosphorylation site in many tyrosine kinases. The GenBank accession number for the splA sequence is U32174.

coli a 39×10^3 M_r, truncated form of the kinase, appended with a C-terminal epitope tag (see Fig. 2C) and purified this protein by immunoprecipitation. This protein was found to autophosphorylate in vitro in the presence of [32P]-gamma ATP. Quantitative phosphoamino acid analysis revealed that approximately 77% of the radioactive phosphate coupled to the protein during the kinase reaction was on tyrosine residues while 18% and 5% was on serine and threonine residues respectively (Fig. 3). Histone 2B or Ras was added to the kinase reaction to serve as substrates. The histone protein was phosphorylated by the splA kinase and phosphoamino acid analysis indicated that greater than 95% of this phosphorylation was on tyrosine residues (Fig. 3). The Ras protein was not phosphorylated to a detectable level in these assays.

**Pattern of expression**

We examined the timing of expression of the splA gene during multicellular development in *Dictyostelium* using semiquantitative RT-PCR. A target segment of the splA gene was amplified from RNA purified from wild-type cultures at time points throughout development. The relative amount of splA

Fig. 2. (A) An alignment of sequences shared between splA and the byr2 kinase of *S. pombe*. (B) The VWLIYF motif is conserved among divergent groups of kinases. For the shaded residues, J indicates charged amino acids, O indicates polar and B indicates hydrophobic. The amino acids were classified according to Taylor (1986). Each listed member of the Eph family represents a separate branch of the family’s phylogenetic tree (Tuzi and Gullick, 1994). GenBank accession numbers: byr2, M74293; STE11, X35341; eph, M18391; eck, M59371; elk, X59290; hek, M83941; erk, D31661; eek, X13411; and cek7-10, Z19058-61. (C) These diagrams of the domain structure of splA, the truncated and myc tagged form splA-myc, byr2 and Eph are drawn to scale. The open box in each domain structure of splA, the truncated and myc tagged form splA-myc, byr2 and Eph are drawn to scale. The open box in each domain represents the kinase catalytic domain and the triangle indicates the position of the domain containing the VWLIYF motif. In splA, the shaded box indicates an asparagin-rich region and the solid box is the myc epitope tag. PM indicates the position of the transmembrane domain of Eph with the carboxy-terminus oriented in the cytoplasm.

Fig. 3. A truncated 39×10^3 M_r form of the splA kinase with a C-terminal myc epitope tag was expressed in E. coli and purified by immunoprecipitation. In vitro kinase reactions were performed with the kinase immunoprecipitate alone or with the addition of histone 2B as a substrate. Phosphoamino acid analysis was performed on the products and the incorporated radioactivity was quantitated. The amount of phosphorylation of splA itself (autophosphorylation) or histone 2B on tyrosine (Y) serine (S) or threonine (T) is presented as a percentage relative to the total amount of phosphorylation on these amino acids.

Fig. 4. The relative level of expression of the splA message (the target) was determined at time points throughout development by RT-PCR. A homologous control RNA (the standard) was added to each reaction and the level of expression was determined by the ratio of the target to the standard after amplification (A_t/A_s). The peak between 8 and 12 hours corresponds to the mound stage of morphological development. Fruiting body formation was complete by 26 hours.
message at each time point of development was determined from the ratio of the amplified target to a homologous control RNA amplified in the same reaction. Fig. 4 shows a graph of the pattern of expression of the splA gene as determined by this approach. The peak level of expression appeared between 8 and 12 hours of development, which corresponds to the mound stage of morphological development, although the message was detectable throughout development and in vegetative cells.

**Disruption of the splA gene**

The splA gene was targeted by homologous recombination using two different strategies that both resulted in the same phenotype. The first strategy deleted from the genome 1.3 kb of the splA gene including half of the kinase domain coding region. An axenic Dictyostelium cell line was transformed with the plasmid pSPLA-del (Fig. 5A) and transformants were screened for the deletion by genomic Southern blotting. We identified multiple strains resulting from independent homologous recombination events. Fig. 5B shows the expected changes in the map of the splA locus and Fig. 5C shows a genomic Southern blot confirming the deletion. In a second strategy, the entire plasmid pSPLA-int (Fig. 5A) was inserted into the splA locus in a homologous integration event. After integration, genomic Southern blotting confirmed that this plasmid, carrying the Thy1 selectable marker, was flanked by identical segments of the splA gene. Since the disruption of the splA gene caused a loss of spore viability (described below), we then selected for the homologous ‘loop

![Disruption of the splA gene by homologous recombination](image)

**Fig. 5.** Disruption of the splA gene by homologous recombination. (A) Two plasmids were constructed to disrupt the splA gene. The plasmid pSPLA-del was designed to delete 1.3 kb of the splA gene. The plasmid pSPLA-int was designed to disrupt the splA gene by integrating in a reversible manner. (B) A map of the splA locus was derived from genomic Southern analyses. The splA locus has been mapped to chromosome 4 near the locus gpaA (Kuspa and Loomis, 1996). After transformation with the plasmid pSPLA-del, the map of the splA locus was altered as indicated. The plasmid pSPLA-int integrated into the splA gene resulting in the genomic rearrangements indicated. (C) The genomic Southern was probed for the splA gene with the fragment splA.2, shown on the wild-type diagram of B. The pattern of the wild-type splA locus digested with either EcoRV and PstI (lane 1) or NdeI (lane 3) is shown. Lane 2 shows the EcoRV and PstI digest of one of the strains (HS13103) in which the transformation with pSPLA-del resulted in the deletion of a portion of the splA gene and the removal of an EcoRV site. Lane 4 shows an NdeI digest of one of the strains (HS9117) in which a single copy of pSPLA-int, which carries an NdeI site, integrated into the splA gene. Lane 5 shows a strain that was derived from HS9117 but was selected for wild-type spore viability. This revertant strain lost the integrated plasmid and the splA gene returned to its wild-type configuration. Restriction enzyme site abbreviations: B, BglII; (B), BamHI/BglII fusion; E, SpeI; H, HindIII; I, EcoRI; N, NdeI; P, PstI; S, SalI; Sp, SphI; V, EcoRV; X, XbaI.
out’ of the integrated plasmid by selecting for viable spores (described in Materials and Methods). The mutant strain HS9117 was subjected to this selection procedure and three independent strains were generated that all displayed a wild-type phenotype. These three revertant strains grew as vegetative cultures only when supplemented with thymidine, indicating the loss of the Thy1 marker. We analyzed these revertant strains by genomic Southern blotting, which confirmed that the plasmid had looped out in the revertants, returning the splA locus to its wild-type arrangement (Fig. 5C). HS13103, which was disrupted at the splA locus by the irreversible deletion event, was also subjected to this procedure of selecting for phenotype reversion. HS13103 cells survived the selection procedure much less readily and those that did survive grew in liquid culture in the absence of supplemented thymidine. Southern analysis of one such survivor showed the deletion in the splA gene like that of the HS13103 parent strain (data not shown).

Phenotype of the splA− strains
Strains in which the splA gene had been disrupted by either method (splA− strains) were found to divide at a normal rate when grown vegetatively (data not shown). Under appropriate conditions, splA− strains formed pseudoplasmodia that appeared normal both in their morphology and phototaxis behavior when compared to wild-type strains (data not shown). However, the splA− strains formed fruiting bodies with shorter stalks and sori (spore heads) that were larger and more translucent than those of the wild-type strain (Fig. 6).

Spore viability
We collected wild-type spores and spores from the splA− mutants from fruiting bodies on bacterial lawns and examined them by phase-contrast microscopy. As shown in Fig. 7A, the wild-type spores were characteristically oblong and surrounded by a phase bright region. The splA− spores, however,
as shown in Fig. 7C, were spherical or irregular in shape and appeared less phase bright. To test the viability of these spores, we stained them with the fluorescent vital dye propidium iodide, which only stains lysed cells. Fig. 7B shows that only a few of the wild-type spores stained with this vital dye, while almost all of the splA− spores stained (Fig. 7D). In a functional assay (see ‘Spore Viability Assay’ in Materials and Methods), we found that the viability of spores generated by splA− strains developed on filter pads or on bacterial lawns was less than 0.5% of that of wild-type spores.

The splA− phenotype was found to be cell autonomous. Mixed cultures of 90% wild-type cells and 10% splA− cells were proportionally no more effective at generating viable splA− spores than were cultures of 100% splA− cells (data not shown). Furthermore, the viability of wild-type spores was impaired when developed in a fruiting bodies containing a high percentage of splA− cells.

Expression of developmental markers
Developmental northern blots of the irreversible splA− strain or a wild-type strain were probed for cell-type-specific markers. As shown in Fig. 8A, the splA− strain expressed the prespore marker cotB (Fosnaugh et al., 1991) and the spore differentiation marker spia (Richardson and Loomis, 1992). This strain also expressed the prestalk genes ecmA and ecmB. The peak of expression of cotB, spia and ecmB was found to be delayed by two to four hours in the splA− strain as compared to the wild type (Fig. 8A), although no consistent difference in developmental timing was detected when monitoring the morphology of this strain during fruiting body formation (data not shown). Spia, which is normally expressed in the last few hours of spore development (Richardson et al., 1991, 1994; Richardson and Loomis, 1992), was detected in the splA− strain by immunoblotting (Fig. 8B).

We then assayed for the formation of spore coats in wild-type and splA− spores by staining with the fluorescent dye calcofluor. Calcofluor binds to the cellulose that is normally present in spore coats. Fig. 9 shows a phase-contrast micrograph of splA− spores (C) and the corresponding calcofluor fluorescence image (D). As compared to the calcofluor staining of wild-type spores (Fig. 9B), the splA− spores stained only weakly for cellulose indicating incomplete spore coat formation.

Tyrosine phosphorylation
To further characterize the phenotype caused by the loss of expression of the splA PTK, we examined the overall pattern of tyrosine phosphorylation during Dictyostelium development by anti-phospho-tyrosine immunoblots. Fig. 10 shows the pattern of tyrosine phosphorylation of proteins in wild-type and splA mutant cells late in development. The most prominent difference is a polypeptide of approximately 55×103 Mw that was phosphorylated beginning at 28 hours of development in the splA− strain that is not detectable in the wild-type strain. This band was also prominent in blots performed on splA− spore cells collected after 43 hours of development.

**DISCUSSION**

We have studied the structure and function of the splA kinase and find it to be a dual-specific enzyme that is required for spore differentiation. Comparison of the sequence of the splA kinase domain to other known kinases revealed the greatest homology to CTR1 of Arabidopsis (Kieber et al., 1993). CTR1 is a protein serine/threonine kinase (PSK) and a member of the Raf family. We have found that splA exhibits PSK activity as well as PTK activity in vitro, although the PSK activity may be reserved only for autophosphorylation. By comparing the sequence of the kinase domain of splA with that of the insulin receptor, a PTK for which the crystal structure has been solved (Hubbard et al., 1994), we can better appreciate how this kinase may acquire its dual specificity. It has been proposed (Lindberg et al., 1992; Hubbard et al., 1994) that substrate specificity is determined in part by the conformation of the P+1 loop, which interacts with the residues surrounding the substrate’s phosphorylation site. A proline at a specific position of this loop is conserved among nearly all PTKs and, in most PSKs, there is a serine or threonine in an equivalent position. The P+1 loop of splA (aa 1467-1474) has a cysteine (C1468) at this site and a proline two residues away. This loop in splA may have an unusual conformation that contributes to the dual specificity of the enzyme. Adjacent to the P+1 loop is the activation loop which, in the insulin receptor and in many other PTKs, contains an autophosphorylation site that is involved in regulating the activity of the kinase. SplA has a serine (S1460) at this site that may be a site of autophosphorylation in our kinase assays.

Fig. 8. The splA− strains expressed prestalk and prespore markers as well as the spore coat protein spia. (A) RNA samples were collected at various times following initiation of development from wild-type AX4 and mutant splA− cells. Northern blots were probed for cotB, spia, ecmA and ecmB mRNA expression as described in Materials and Methods. (B) Whole cell lysates of wild-type JH10 (WT) and splA− HS9117 (splA−) spores were immunoblotted with an antibody to spia. The same amount of total protein was loaded in each lane. Mutant spores expressed this antigen and another band that we interpreted as a proteolytic breakdown product.
We identified a domain of splA with homology to the byr2 kinase of \textit{S. pombe} (Wang et al., 1991). Strains of \textit{S. pombe} with the \textit{byr2} gene deleted are defective in conjugation and sporulation (Wang et al., 1991). Sporulation in \textit{Dictyostelium} is very different from that in yeasts and does not involve conjugation or meiotic cell division. Therefore it is especially intriguing that kinases that share homology are required for regulating sporulation in these two evolutionarily distant organisms.

The VWLIYF motif is found within this domain of homology between splA and byr2. This motif is also found in the byr2 homolog STE11 (Styrkardottir et al., 1992; Neiman et al., 1993) and in members of the largest family of growth factor receptors, the Eph family (Tuzi and Gullick, 1994). An analysis of the amino acid sequence of splA using the algorithm of Emini (Emini et al., 1985) shows a high probability that the domain containing the VWLIYF motif is on the surface of the molecule and could be accessible for binding to other proteins, despite the hydrophobicity of these conserved residues. It is possible that the VWLIYF motif is involved in the binding of these kinases to other proteins, perhaps substrates and may contribute to overall substrate specificity.

A gene targeting approach was aimed at determining the function of the splA kinase and the pathway in which it acts. Our analysis of the splA\textsuperscript{−} strains revealed the expression of prestalk- and prespore-specific markers, as well as the expression of a marker for spore differentiation. The peak of expression of several of these markers was delayed by 2-4 hours as a result of the disruption of the splA gene. This delay suggests that the splA kinase may normally be active during the early stages of cell differentiation, but its activity was not required for the development of prespore or prestalk cells from undifferentiated vegetative cells. Despite the delay in expression of these markers, the splA\textsuperscript{−} strains developed fruiting bodies that exhibited only slight morphological abnormalities. The larger spore heads and shorter stalks found in these mutants were likely a consequence of spore lysis and a decrease in structural integrity of the fruiting body. The requirement of the splA gene for spore cell maturation was apparent late in development. Despite expression of the spore coat genes \textit{cotB} and \textit{spiA}, splA\textsuperscript{−} spores failed to assemble spore coats and lysed. These data suggest a role for the splA kinase in a late stage of spore differentiation, the maturation phase. Late in normal development,
spore coat proteins are secreted from the prespore vesicles and assemble the extracellular spore coats (Hohl and Hamamoto, 1969). SplA may regulate this exocytosis.

Alternatively, splA may function early in development while prespore and prestalk cells are generated. The peak of splA expression was found to be between 8 and 12 hours of development and a delay in cotB expression was detected from 16 to 20 hours in the mutant. Mutant prespore cells and possibly the prestalk cells may have been defective in ways that did not become apparent until very late in development, during spore maturation. For example, during development, Dictyostelium cells arrest in their cell cycle and there is little or no replication of nuclear DNA (Shaullsky and Loomis, 1995). If spore cells were to continue in their cell cycle while completing their morphological differentiation, this could lead to the type of cellular catastrophe that we have seen in splA− strains.

By developing mixtures of wild-type and splA− cells at different proportions, we have made several interesting findings. The presence of wild-type cells did not promote the normal sporulation of mutant cells. This indicates that there is no extracellular factor that can be supplied by the wild-type cells to correct for the defect in the mutants, despite the fact that some secreted spore coat components become incorporated into the capsules of neighboring spores (West and Erdos, 1992). Mixing experiments also demonstrated that the splA− sporulation defect was dominant over wild-type spores, most likely due to proteases and other hydrolyzing enzymes that were liberated by the lysis of the mutant spores.

By analyzing the tyrosine phosphorylation of proteins in wild-type and splA mutant strains as they completed development, we identified a band that increased in its level of tyrosine phosphorylation as a result of the loss of the splA kinase. It is possible that the splA kinase is normally involved in either inhibiting the kinase that phosphorylates this substrate, or the splA kinase may activate the phosphatase that dephosphorylates this substrate.

It is likely that splA is one step in a signal transduction pathway that leads to the differentiation of spores. Spore differentiation can be a good model system for studying fundamental aspects of eukaryotic cell regulation because it involves programmed changes in overall metabolism, morphological changes and arrest of the cell cycle. The disruption of the splA gene generated a conditionally lethal phenotype since spores are the means by which Dictyostelium survive starvation. It may be possible to complement this phenotype through the overexpression of genes whose products lie downstream of splA in the same signal transduction pathway.

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REFERENCES


The splA kinase of Dictyostelium 3305


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