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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 *spiA* 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 10^5 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-

tributing to an understanding of the structure and function of a novel dual-specificity kinase.

MATERIALS AND METHODS

Genomic cloning of the *splA* gene

Genomic Southern mapping indicated the presence of an *Xba*I 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 *Pst*I and *Xba*I. The 1.0-1.6 kb fragments were ligated into the vector pJDC9 (Chen and Morrison, 1988). Positive clones that hybridized with the DPYK1 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 *Bam*HI fragment was ligated into the *Bam*HI site of the plasmid pDPYK1 (Tan and Spudich, 1990) upstream of the cDNA. The resulting plasmid was linearized with *Bgl*II and transformed into the thymidine auxotrophic *Dictyostelium* strain JH10 (Mann and Firtel, 1991; 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 *Sal*I followed by extraction with phenol/chloroform. *Sal*I cut at a single 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 *Xba*I to *Bgl*II 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 *Sal*I 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 *Spe*I 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 DPYK1 cDNA was derived, we excised the plasmid integrated into the strain HS9117 (described below) using *Hin*dIII.

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-Dolittle 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′-GCGGATCCAACGTCTCCGCTGCTAAAACT) and then PCR was performed adding a primer from 5′ of the first intron (5′-GCGAATTCAACAACACCAACCATACCAA). 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 *Nde*I, removing approximately 200 nt using *Bal*31, 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 μCi of ^{32}P-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 *Bgl*II/*Pst*I fragment from the genomic clone *splA.1* into the vector pTZ-18R (Pharmacia). The Thy1 selectable marker was inserted into the *Sph*I site of this plasmid making pSPLA-int as is shown in Fig. 5A. 10 μg of this plasmid was linearized with *Eco*RV 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 *Bgl*II fragment, including half of the kinase domain was replaced with the *Thy1* gene making pSPLA-del (Fig. 5A). This plasmid was digested with *Eco*RV and with *Eco*RI 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 $MgCl_2$, 40 mM phosphate buffer pH 6.4) with 60 U/ml penicillin, 60 μg/ml streptomycin sulfate (P/S) 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 Axiophot with a 40× 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′-CGCGAATTCGATATCCGCCCATTTGGTGGTTGG and 5′-GCGGAATTCTTATAAATCTTCTTCTGAAATTAATT-TTTGTTCGATATCAGCATAAACACCAGTATCAATAG. This PCR product was cloned into the expression vector pET11d (Novagen) and transformed into the *E. coli* strain BL21(DE3) pLysS. The myc tagged kinase was immunoprecipitated from bacterial lysates using the antibody 9E10 (Evan et al., 1985). A kinase reaction was performed with the precipitate in the presence of ^{32}P-gamma-ATP with or without histone 2B (Sigma) or Ras (a gift from K. Maegley, Stanford University) as an exogenous substrate. The reactions were western blotted and processed for phosphoamino acid analysis using standard protocols. After two-dimensional thin-layer electrophoresis, the radioactivity of the phosphoamino acids was quantitated by phosphorimager scanning.

SpiA and anti-phosphotyrosine immunoblotting

Gel samples of wild-type and splA mutant strains were collected during development on non-nutrient agar. The same amount of total 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-mouse antibody (BioRad) diluted 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 plates 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 174×10^3. Analysis of the amino acid sequence did not reveal potential membrane spanning segments. Computer based sequence 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)xxY, although we confirmed experimentally that other tyrosines of the protein can serve as autophosphorylation sites as described below. Many PTKs have an autophosphorylation 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 (Styrkardottir 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. Fig. 2B shows an alignment of sequences from splA, the two yeast kinases and a member from each branch of the Eph family phylogenetic tree (Tuzi and Gullick, 1994) revealing the conserved VWLIYF motif. When we define the motif as VxxWLJxIxxxOYxxxF where x is any amino acid and J and O represent charged and polar amino acids respectively, database searches yielded only splA, byr2 and several of the Eph family members.

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.*

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   1  AATCTTTCTGGTTTAAATAGTTTTCATTAAAGCAATCAACAGATTCATTAACACCACCACCAAATTCACAACAATCAACTGTATCATCAAATTCAACACCAATTGCAGCCACACCAATCT
 121  CACCACTAACTGCACCAACATCACCACCCCCACCACCACCTCCACCAACAAATTTTAATAGTAAATTTAATAATAATAATAATAATATATAAATAATTCATCAAATAACAACACAACTG
 241  TACCACCATCACCACCACCAATAATTGTATTGCCAAAATCACCATCTTAAGATCACCAAGACCAGAAGTGCACAGTTCAGTGCACATTTTTAGTAAATAATCGAGTAATAAATACATCAT
 361  CGAGTAGTAATATTAGTACAAATAATACAGATTTAAATTTATCATCATCGTCTTCATCATCACCCCCATTAAATATTTCGACTGCTTCACCATCAAAATCTGAAGATTCCCCACCAACAG
 481  TATCCCCTTCATATAAGCAGCAGCAACAACAACAACAACAGCAACAACAACAACAACAATAAATTAATTCATCAAATTCTTCATATTCACCAAAACCAAGATCACCATCTGTATCAT
 601  CACCACCACCTTCATCAATTTCACCAAATTCATCTCCTATTGGTTCACCAAATATATCATTTCATCATCACCAACAACATCAAATCCCACCACCACCTCCTGTATTATCAAACACCAACA
 721  ACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAATAACAACAATAATAATAACAATAACAACAACAACAACCAACCATACCAACAAAAAAGAAG
 841  GAGATTCTAGTTGGTTTAATATGAGCTTTAAATTCTTTAAAAAGAAATTGGTCCCATCCAATGAATATAGATGGGATTTAAGAAAATCAAATTCACTTACATTAAATATTGAAGATAAAA
                       M  S  F  K  F  F  K  K  K  L  V  P  S  N  E  Y  R  W  D  L  R  K  S  N  S  L  T  L  N  I  E  D  K  S   34
 961  GTAGATGTTCTTACAGACTACCAACATCTGGTTCAAAAGGTGTAAGTATTTTTCTTTTTTTAAAATTTTATTTTCTTTTTTTATAAAAATAATATTAATTTTTATTTTATTTTAAAAAAA
        R  C  S  Y  R  L  P  T  S  G  S  K  G                                                                              47
1081  AGATTGCAAAATCAACACAACCATTTTCAAGTTCTTTTACTTATTTTGAATTATTCATTACAAATGGTAATGGAGAGTAAGTTTTTTTCTATTTTTTTTTTTTAAAATTTTAATAGATTT
        I  A  K  S  T  Q  P  F  S  S  S  F  T  Y  F  E  L  F  I  T  N  G  N  G  D                                        72
1201  ACTAATTTTTATTATTAAAAAAGTAAAATATGTTTTGGTTTAACAACAAATGATCATCCAATTGAAGTATATCCAGGTAATTATCAAGGATCATATGGATATAGTGGAGATGGTAAATGT
                             K  I  C  F  G  L  T  T  N  D  H  P  I  E  V  Y  P  G  N  Y  Q  G  S  Y  G  Y  S  G  D  G  K  C  104
1321  TATTTTGGAACCAATGAAGGTAGAGTTTATGGACCAAGTTTTAGCAGCGGAGACGTTGTAGGATGTGGTTATGATTCAAGTTCAAAAACATTATATTTTACAAAGAATGGTGTATATTTA
        Y  F  G  T  N  E  G  R  V  Y  G  P  S  F  S  S  G  D  V  V  G  C  G  Y  D  S  S  S  K  T  L  Y  F  T  K  N  G  V  Y  L  144
1441  GGAGTTGCAGCACAAAAAGTGAATCTAATTGGATTGTATCCAACCGTTGGTTTACAAAATCCCGGTGAATCAGTGGTAATCAATTTCTTTGGACCATTCTCTTATCGTGGTGCACCCGAA
        G  V  A  A  Q  K  V  N  L  I  G  L  Y  P  T  V  G  L  Q  N  P  G  E  S  V  V  I  N  F  F  G  P  F  S  Y  R  G  A  P  E  184
1561  AAACCAAGTAAACAAAGTACCATCAAAGATAGTGGTGGTAGTTCAATTATACCATCAGAGGATTTAATACCGAAGGAAGAGTTTGAAGTATGTCGTTGGAGCGAAAAGAAAAACTATCAT
        K  P  S  K  Q  S  T  I  K  D  S  G  G  S  S  I  I  P  S  E  D  L  I  P  K  E  E  F  E  V  C  R  W  S  E  K  K  N  Y  H  224
1681  GGTAAGCATGTAGTGGTTCGAAATAGAACAGCATTCTTACCTTTGGACTCACCAAAGGATACAATCGGTGGAGTTCGTGCCACTCAACCATTTGGAGAGGGATTTTGTTATTTCGAAGTA
        G  K  H  V  V  V  R  N  R  T  A  F  L  P  L  D  S  P  K  D  T  I  G  G  V  R  A  T  Q  P  F  G  E  G  F  C  Y  F  E  V  264
1801  ATCATCGATCAATTAGACAAAGGTCAACTTTCAATTGGTTTAGCAAATCTTGAATATCCAACATTTTATCATGTTGGATGGATGCCAAGATCCTATGGTTATCACAATGACGATGGTAGA
        I  I  D  Q  L  D  K  G  Q  L  S  I  G  L  A  N  L  E  Y  P  T  F  Y  H  V  G  W  M  P  R  S  Y  G  Y  H  N  D  D  G  R  304
1921  AAATTTAGATGGAGAGAAGAACCTGGCGTTAACGAAGGTGAGTCTTATGGTTCCTCCTATAAAAAAGGTGATATTATTGGTTGTGGTCTATCTTTTACGAGTAGAGAAATTTTTTTCACA
        K  F  R  W  R  E  E  P  G  V  N  E  G  E  S  Y  G  S  S  Y  K  K  G  D  I  I  G  C  G  L  S  F  T  S  R  E  I  F  F  T  344
2041  AAAAATGGTATGTACTTGGGTACTGCCTTTTCAAATGTATATGGTGTTTTCTATCCATCTGTAGCTTTTAATGAGCCTGGAATCTCTATAACAGGAGTTTTTGGACCTCCATTTAAATTC
        K  N  G  M  Y  L  G  T  A  F  S  N  V  Y  G  V  F  Y  P  S  V  A  F  N  E  P  G  I  S  I  T  G  V  F  G  P  P  F  K  F  384
2161  TCTCAAGTTACTTTAATGCTTAAAAATGTAAATTCAACTTCAATTTTAGTACCTAATGGTAATAATAATAATAATAGTAATAATAATAATAATAATAATAATAATAATATAATAGGTAAT
        S  Q  V  T  L  M  L  K  N  V  N  S  T  S  I  L  V  P  N  G  N  N  N  N  N  S  N  N  N  N  N  N  N  N  N  I  G  N  424
2281  GGTAAAATAACCACTACCACAACAACATCAACATCACCTTCATCAATAAATAATAATGAAGATATTTCATCAAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAAT
        G  K  I  T  T  T  T  T  S  T  S  P  S  S  I  N  N  N  E  D  I  S  S  N  N  N  N  N  N  N  N  N  N  N  N  N  N  N  N  464
2401  AATAATAATAATAATAATAATAATAATAATAATAATAGTAATAGTAGTAATACTAATAATAACAATATTAATAATACTACTAATAATAATAATAGTAATAATAATAATAATAATAAT
        N  N  N  N  N  N  N  N  N  N  N  N  N  N  S  N  S  S  N  T  N  N  N  N  I  N  N  T  T  N  N  N  N  S  N  S  N  N  N  N  504
2521  AATAATAATAATAGTAACAGCAATAGCAACAGCAATAATAATAATATTAATAACAACAATAATAATAATAATAATAACAATAATATATATTTAACAAAAAAACCATCAATAGGGTCGACA
        N  N  N  N  S  N  S  N  S  N  N  N  N  N  I  N  N  N  N  N  N  N  N  N  N  N  N  N  I  Y  L  T  K  K  P  S  I  G  S  T  544
2641  GATGAATCATCAACAGGTAGTTTAGGTGGTAATAATAGCAGCGGCAATAATAATAGTAGTAGTGGTAGTATAGGTAATAATAGTAGTATAATTAAACAAAGATCACCTCCTCACTCTATA
        D  E  S  S  T  G  S  L  G  G  N  N  S  S  G  N  N  N  S  S  S  G  S  I  G  N  N  S  S  I  I  K  Q  R  S  P  P  H  S  I  584
2761  AATGGACCATTAATGTTACCTCCATCTTCAACCAATAATAATAATATTTATTCTTCATATAATTCAACAACAGCAGGCTCATCGACAACTATCCTGCCAACATTAAATCATCCAATA
        N  G  P  L  M  L  P  P  S  S  T  N  N  N  N  N  I  Y  S  S  Y  N  S  T  T  A  G  S  S  T  T  I  L  P  T  L  N  H  P  I  624
2881  TTTGGTAACACCACTTCAAACAACAACTCATCATCAACTCTTTCAGTAGGTGGTAATAATAATCTATTAGGTAGACATTGTCAATCATTACCAATTACAGCTTCAACCAATCATACTTTA
        F  G  N  T  T  S  N  N  N  S  S  S  T  L  S  V  G  G  N  N  N  L  L  G  R  H  C  Q  S  L  P  I  T  A  S  T  N  H  T  L  664
3001  TCATCATCATTGGGTGTATCATTTTCATCACCATCAAGTTCACCAAAAACATCACCACGTAAAATTGTCAATAGTAGTGAAGATTTAGGTTTTGTTCAAACATTCCAAGATCAAGATGGT
        S  S  S  L  G  V  S  F  S  S  P  S  S  S  P  K  T  S  P  R  K  I  V  N  S  S  E  D  L  G  F  V  Q  T  F  Q  D  Q  D  G  704
3121  CAACCACCATCAGCTTGGAGACGTTGTGGTAAATCAATAAAGACCAAAGATGATATCACTCTCACCATCATAAAGAAAAAGACATCGGTTGCAATGGCCGATCGTCCATTCTCATCCAAT
        Q  P  P  S  A  W  R  R  C  G  K  S  I  K  T  K  D  D  I  T  L  T  I  I  K  K  T  S  V  A  M  A  D  R  P  F  S  S  N  744
3241  TCAAGCTCAACCATTTGTTACTTTGAAGTTTATTTAGAGGGTCATGATAAAAAAGGTTCAATCACTGTGGGTTTAAGTCATTCAACATATCCATTTATTAAACATATTGGACGTGAACCA
        S  S  S  T  I  C  Y  F  E  V  Y  L  E  G  H  D  K  K  G  S  I  T  V  G  L  S  H  S  T  Y  P  F  I  K  H  I  G  R  E  P  784
3361  AAATCCTACGGTTTCTCAAGTGAAGGAGAAAAGTATGGTGGAAGTGAAATTGGTGAACCTTATGGTCCTTTCTTTTTCTTTGATGGTGATTCAATCGCAAGTAGTTGTGTAATTGGTTGT
        K  S  Y  G  F  S  S  E  G  E  K  Y  G  G  S  E  I  G  E  P  Y  G  P  F  F  F  F  D  G  D  S  I  A  S  S  C  V  I  G  C  824
3481  GGTATCAATACCTCCACCAGAGACATATTCTTTACAAAGAATGGTCACTATTTAGGCGTTGCTTTCTCTAGAGTTACCTCTGATCCACTCTATCCTTCCATCTCATTCAGAGGTGTTGTG
        G  I  N  T  S  T  R  D  I  F  F  T  K  N  G  H  Y  L  G  V  A  F  S  R  V  T  S  D  P  L  (Y) P  S  I  S  F  R  G  V  V  864
3601  GGTGGACTTTGTGTTGCAACTTTCCCAGGTGGTCATTTCAGATTCAATATCGAAGATTTACCTGGTATTTCTCCATCAGTTTGGACTGAAGCCTTGGGACCAGATCGTCAAGGTTCAGGT
        G  G  L  C  V  A  T  F  P  G  G  H  F  R  F  N  I  E  D  L  P  G  I  S  P  S  V  W  T  E  A  L  G  P  D  R  Q  G  S  G  904
3721  TTCAAAAATTGGGGCACCAAACGACGTTGCAATTTGGTTGGAATCTTTCAACTATGGTCAATATAGAAAGAATTTCCGTGATAACAATATCTCTGGTCGTCATTTAGAAGGAATCACTCAT
        F  K  N  W  A  P  N  D  V  A  I  W  L  E  S  F  N  Y  G  Q  Y  R  K  N  F  R  D  N  N  I  S  G  R  H  L  E  G  I  T  H  944
3841  GCAATGTTAAAGAATGATTTAGGTATTGAACCCTATGGACATCGTGAGGATATTATCAATCGTCTCAATAGAATGATTCAAATTTGGAATGACAAGAGTCCAGATTCTTATCCAAAGATT
        A  M  L  K  N  D  L  G  I  E  P  Y  G  H  R  E  D  I  I  N  R  L  N  R  M  I  Q  I  W  N  D  K  S  P  D  S  Y  P  K  I  984
3961  GCTATCGATAGTAGTGATAAGATTAGATGGCCAGCCAGTGGCGGCAGTAGTGGTGGAATCAATATTAGCGGAGGAGTTGTCATTGGTAGTAGTAGTGGTAGTGATGATGGTATTACAGAG
        A  I  D  S  S  D  K  I  R  W  P  A  S  G  G  S  S  G  G  I  N  I  S  G  G  V  V  I  G  S  S  S  G  S  D  D  G  I  T  E  1024
4081  ATCTCAAGCTCATCAAAGAATATAAGACCCTACAAAAGTTATACACAAAAGGAAATTGAAGATAGAAATCGCAGAAGTACAATTAGTGGAGGTGAAAAGAAAAATAAATATTATATCGAT
        I  S  S  S  S  K  N  I  R  P  Y  K  S  Y  T  Q  K  E  I  E  D  R  N  R  R  S  T  I  S  G  G  E  K  K  N  K  Y  Y  I  D  1064
4201  AACCAAATGGATCCACATCAAATTGGTTCAATGGATTCCGATGGTTTACTTCCAGATTTTGGTCAAGGTCCACCCGATGAAAAGAATTCTTCAAAAACCTTATCCAATGAACAAATTAGA
        N  Q  M  D  P  H  Q  I  G  S  M  D  S  D  G  L  L  P  D  F  G  Q  G  P  P  D  E  K  N  S  S  K  T  L  S  N  E  Q  I  R  1104
4321  TATCTACAACAAAGAAAAGACGAGCCACCTATTGCTATCAGTAGTACCGGAAATGGTGGTAGTGTAAGTAGTACTGGTGGTAGTAGTGGTTTCTTAACTTTTCCTTCTTCAAATTCTTTA
        Y  L  Q  Q  R  K  D  E  P  P  I  A  I  S  S  T  G  N  G  G  S  V  S  S  T  G  G  S  S  G  F  L  T  F  P  S  S  N  S  L  1144
4441  ACTCATCCACCTCAAAGAGATAAACCAACTCAAGAATTCACTCATTTACCTCCAATCACTTCAAATTATAAAGGTATAACTAATACAGGTCAACCACATAAATCATTTGATCAACCATTA
        T  H  P  P  Q  R  D  K  P  T  Q  E  F  T  H  L  P  P  I  T  S  N  Y  K  G  I  T  N  T  G  Q  P  H  K  S  F  D  Q  P  L  1184
4561  GAATTATTTCCAAGACATTCAGCATTTTCTAATAATGGTAATAATGGTAATAATAATAATAATAATAATAATAATTATTAAAGCAAATCAACAACAACAACAACAATCATCATATCAAA
        E  L  F  P  R  H  S  A  F  S  N  N  G  N  N  G  N  N  N  N  N  N  N  N  N  N  I  K  A  N  Q  Q  Q  Q  Q  Q  S  S  Y  Q  1224
4681  CAATCTCAAACACAACAACAACAACAACATATTACATCAACATCAACAACTAATAAATGGATTGACCCATTTGGTGGTTGGGAAACTCAATCATCATTATCACATCCCAACCATCA
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4801  CGTCCACCACCACCTCCACCACCACCACCACAACTACCAGTTAGATCAGAATACGAGATTGATTTCAATGAATTAGAATTTGGTCAAACCATTGGTAAAGGTTTCTTTGGTGAAGTAAAG
        R  P  P  P  P  P  P  P  P  P  Q  L  P  V  R  S  E  Y  E  I  D  F  N  E  L  E  F  G  Q  T  I  G  K  G  F  G  E  V  K  1304
4921  AGAGGTTATTGGAGAGAGACTGATGTTGCCATAAAAATCATCTATCGTGATCAATTCAAAACCAAATCATCATTGGTTATGTTTCAAAATGAAGTTGGAATACTAAGTAAATTAAGACAT
        R  G  Y  W  R  E  T  D  V  A  I  K  I  I  Y  R  D  Q  F  K  T  K  S  S  L  V  M  F  Q  N  E  V  G  I  L  S  K  L  R  H  1344
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        P  N  V  V  Q  F  L  G  A  C  T  A  G  G  E  D  H  H  C  I  V  T  E  W  M  G  G  G  S  L  R  Q  F  L  T  D  H  F  N  L  1384
5161  CTCGAACAAAATCCACATATTCGTTTGAAGTTGGCTTTGGATATTGCAAAAGGAATGAATTATCTACATGGTTGGACTCCACCCATTCTTCATCGTGATTTATCCTCAAGAAACATTTTA
        L  E  Q  N  P  H  I  R  L  K  L  A  L  D  I  A  K  G  M  N  Y  L  H  G  W  T  P  P  I  L  H  R  D  L  S  S  R  N  I  L  1424
5281  TTGGATCACAACATCGATCCAAAGAATCCTGTAGTTTCCTCAAGACAAGATATTAAATGTAAGATCTCTGATTTTGGTCTAAGTAGATTAAAGAAGGAACAAGCCTCTCAAATGACTCAA
        L  D  H  N  I  D  P  K  N  P  V  V  S  S  R  Q  D  I  K  C  K  I  S  D  F  G  L  S  R  L  K  K  E  Q  A  S  Q  M  T  Q  1464
5401  TCGGTTGGTTGTATTCCCTACATGGCACCAGAGGTTTTCAAAGGCGATAGTAATAGTGAAAAGAGTGATGTTTACTCCTATGGCATGGTTTTGTTTGAACTATTAACCTCTGATGAACCT
        S  V  G  C  I  P  Y  M  A  P  E  V  F  K  G  D  S  N  S  E  K  S  D  V  Y  S  Y  G  M  V  L  F  E  L  L  T  S  D  E  P  1504
5521  CAACAAGATATGAAACCAATGAAAATGGCTCACTTGGCTGCTTATGAATCTTATCGTCCTCCAATTCCATTAACTACCTCTTCCAAGTGGAAAGAAATTCTAACTCAATGTTGGGATTCT
        Q  Q  D  M  K  P  M  K  M  A  H  L  A  A  Y  E  S  Y  R  P  P  I  P  L  T  T  S  S  K  W  K  E  I  L  T  Q  C  W  D  S  1544
5641  AATCCTGATAGTCGTCCAACCTTTAAACAAATCATTGTTCATCTCAAAGAAATGGAAGATCAAGGTGTATCTTCTTTTGCATCTGTACCTGTTCAAACTATTGATACCTGGTGTTTATGCT
        N  P  D  S  R  P  T  F  K  Q  I  I  V  H  L  K  E  M  E  D  Q  G  V  S  S  F  A  S  V  P  V  Q  T  I  D  T  G  V  Y  A  1584
5761  TAATTTTTTTTTTATAATTAAAAAAAAAAAAAAAAAAAAACAAAAAAAAAAAAAATAATAATAAATATAATCACTTCAACTTGTAAACAATTTAAATTAATAATAATAATTAATAAATAATGA
        *
5881  TAATTATTGATAAAAAAAAAAAAAAATATTACTGTCGTATATTTAAATCGAAA  5932
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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 [^{32}P]-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.

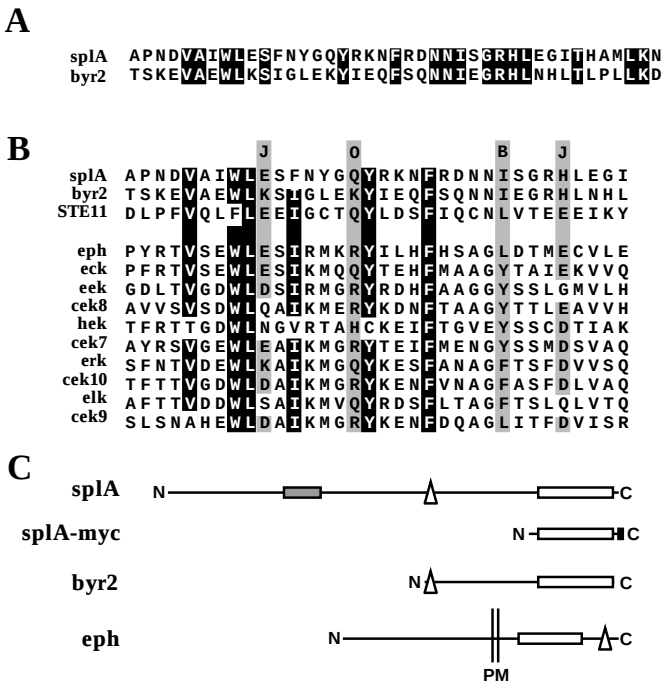

Fig. 2. (A) An alignment of sequences shared between splA and the byr2 kinase of *S. pombe*. (B) The VWLIYF motif is conserved among divergent groups of kinases. For the shaded residues, J indicates charged amino acids, O indicates polar and B indicates hydrophobic. The amino acids were classified according 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, X53431; eph, M18391; eck, M59371; eek, X59290; hek, M83941; erk, D31661; elk, 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 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 asparagine-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.

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

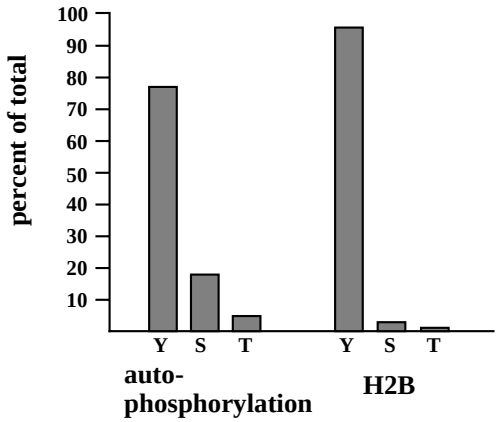

Fig. 3. A truncated 39×10^3 M_r form of the splA kinase with a C-terminal myc epitope tag was expressed in *E. coli* and purified by immunoprecipitation. In vitro kinase reactions were performed with the kinase immunoprecipitate alone or with the addition of histone 2B as a substrate. Phosphoamino acid 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.

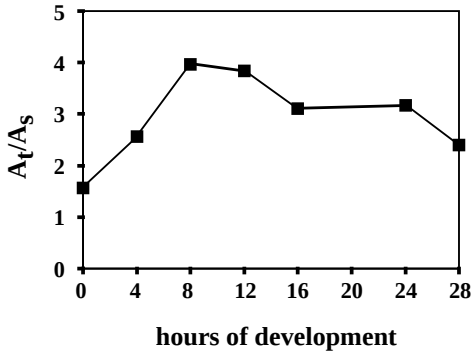

Fig. 4. The relative level of expression of the *splA* message (the target) was determined at time points throughout development by RT-PCR. A homologous control RNA (the standard) was added to each reaction and the level of expression was determined by the ratio of the target to the standard after amplification (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.

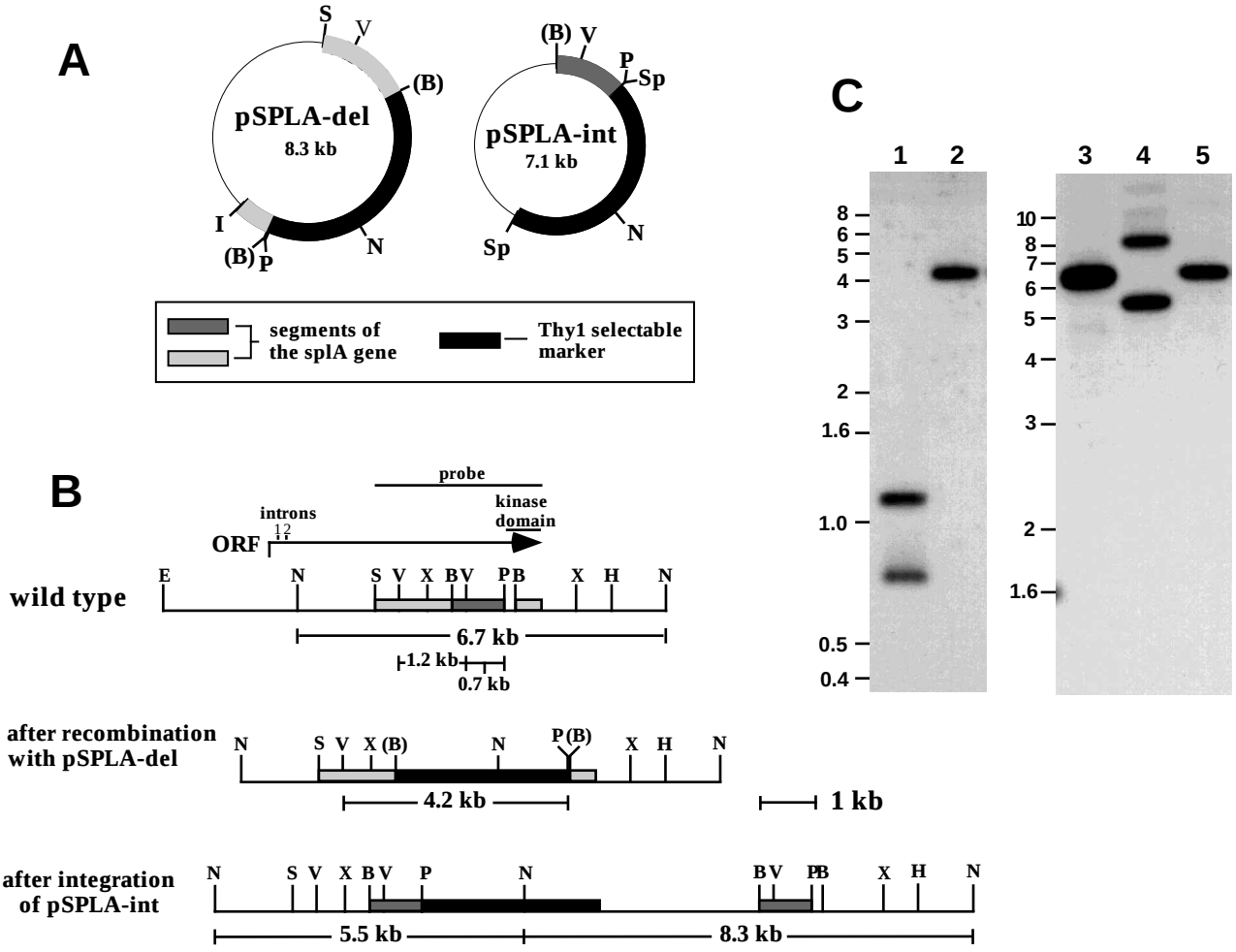

Fig. 5. Disruption of the *splA* gene by homologous recombination. (A) Two plasmids were constructed to disrupt the *splA* gene. The plasmid pSPLA-del was designed to delete 1.3 kb of the *splA* gene. The plasmid pSPLA-int was designed to disrupt the *splA* gene by integrating in a reversible manner. (B) A map of the *splA* locus was derived from genomic Southerns. 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 *Eco*RV and *Pst*I (lane 1) or *Nde*I (lane 3) is shown. Lane 2 shows the *Eco*RV and *Pst*I 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 *Eco*RV site. Lane 4 shows an *Nde*I digest of one of the strains (HS9117) in which a single copy of pSPLA-int, which carries an *Nde*I 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, *Bgl*II; (B), *Bam*HI/*Bgl*II fusion; E, *Spe*I; H, *Hin*dIII; I, *Eco*RI; N, *Nde*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; V, *Eco*RV; X, *Xba*I.

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 (Fig. 5B,C). Since the disruption of the *splA* gene caused a loss of spore viability (described below), we then selected for the homologous ‘loop

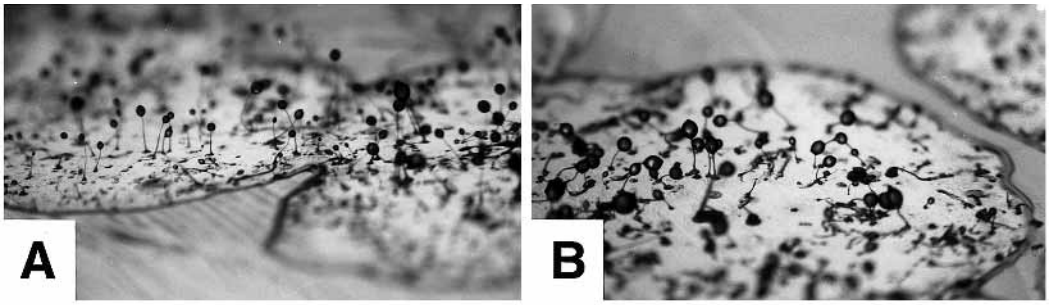

Fig. 6. Strains in which the *splA* gene was disrupted developed into fruiting bodies with minor morphological abnormalities. Cells of the wild-type parental strain JH10 (A) and the *splA*$^-$ strain HS13103 (B) were plated at low density on bacterial lawns so that individual cells would proliferate, clear a plaque in the bacterial lawn and develop. Shown 5 days after plating, the normal fruiting bodies of the wild-type strain have thinner stalks and smaller sori (spore heads) than the mutant strain.

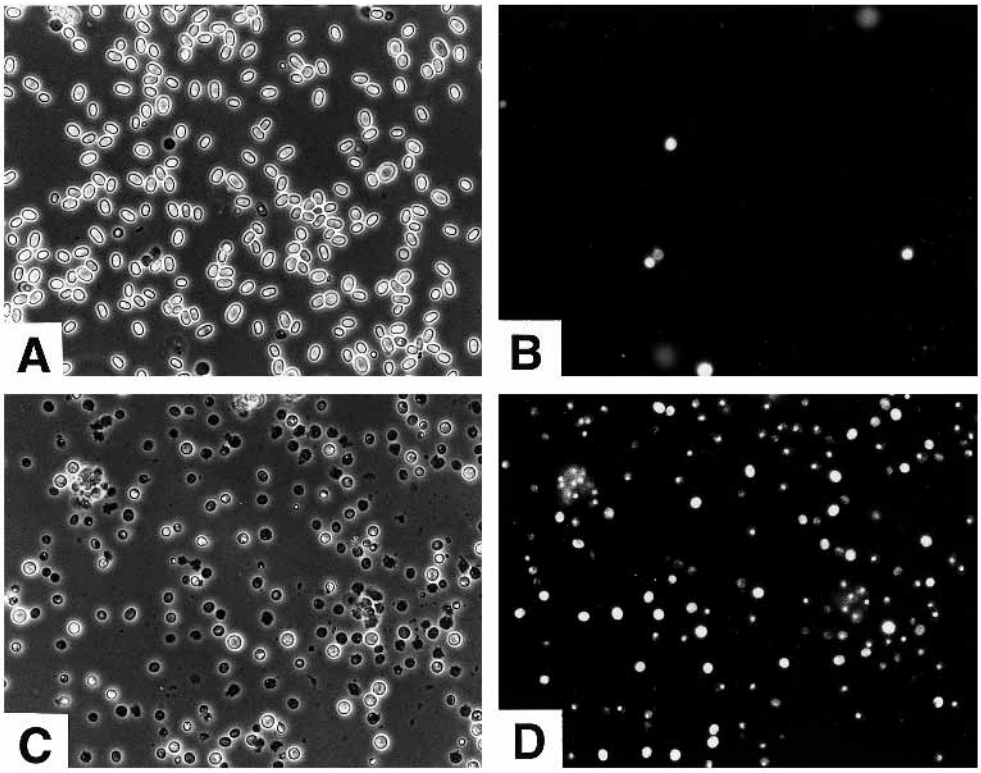

Fig. 7. Strains in which the *splA* gene has been disrupted produce abnormal spores, most of which spontaneously lysed. Wild-type spores of the strain JH10 when viewed by phase-contrast microscopy (A) were oblong and surrounded by a phase bright region indicative of a spore coat. Staining of these spores with the fluorescent vital dye propidium iodide (B) showed that almost all of the spores have intact plasma membranes. The *splA*$^-$ strains such as HS13103 (C) appeared round or irregular in shape and phase dark. Almost all of these *splA*$^-$ spores stained with the vital dye (D) indicating holes in their plasma membranes.

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-phosphotyrosine 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 55×10^3 M_r 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.

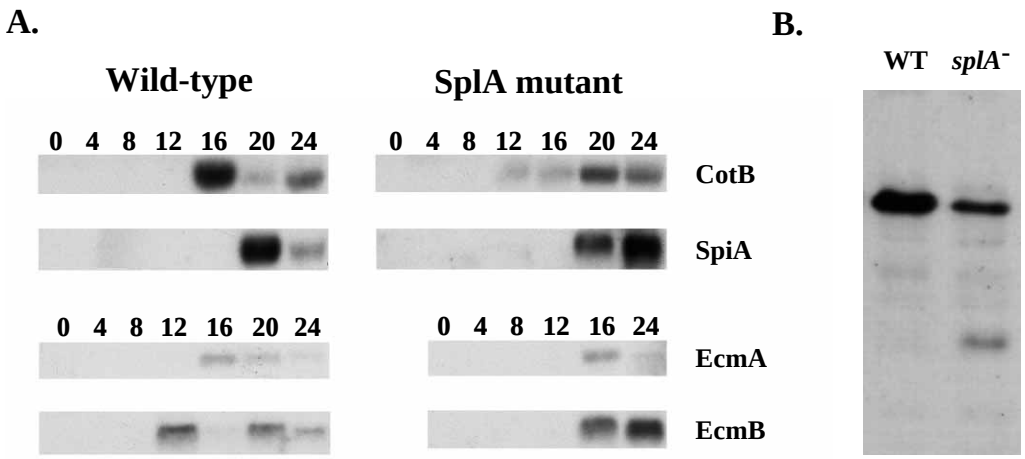

Fig. 8. The *splA*⁻ strains expressed prestalk and prespore markers as well as the spore coat protein spiA. (A) RNA samples were collected at various time following initiation of development from wild-type AX4 and mutant *splA*⁻ cells. Northern blots were probed for *cotB*, *spiA*, *ecmA* and *ecmB* 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.

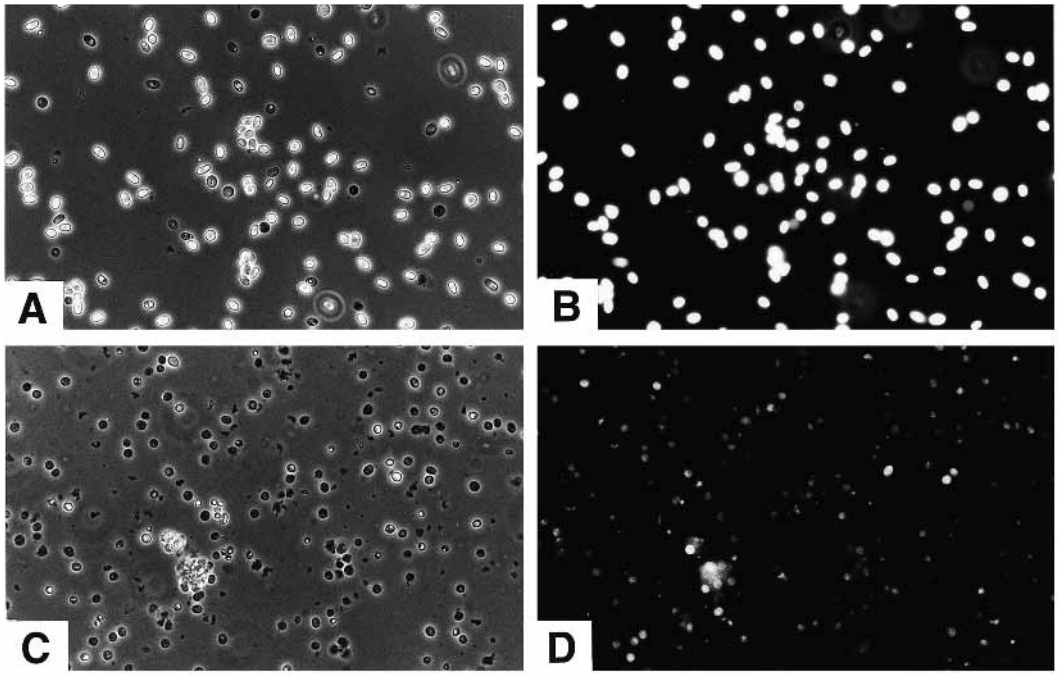

Fig. 9. The *splA*⁻ spores failed to assemble spore coats. Wild-type (JH10) spores (A,B) and *splA*⁻ spores from the strain HS13103 (C,D) were collected from sori and stained with the fluorescent dye calcofluor that binds to cellulose. Phase-contrast microscopy (A,C) shows the differences in spore morphology and calcofluor staining (B,D) shows that few if any of the *splA*⁻ spores assemble a complete spore coat.

We identified a domain of splA with homology to the byr2 kinase of *S. pombe* (Wang et al., 1991). Strains of *S. pombe* with the *byr2* gene deleted are defective in conjugation and sporulation (Wang et al., 1991). Sporulation in *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*⁻ 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*⁻ 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 *cotB* and *spiA*, *splA*⁻ 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,

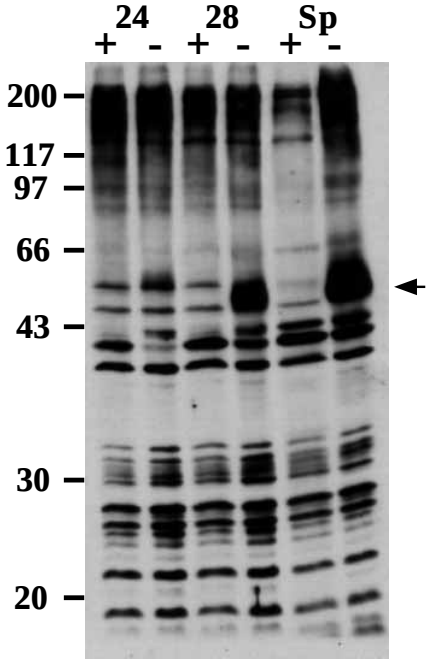

Fig. 10. A polypeptide of approximately 55×10^3 M_r was found to be abnormally tyrosine phosphorylated late during development of *splA*⁻ strains. The wild-type strain JH10 (+) and the *splA*⁻strain HS9117 (–) were developed on non-nutrient agar and gel samples were made before and after the cultures completed fruiting body formation (24 and 28 hours of development). Samples were also made from spore cells (Sp) collected from sori after development was completed. The same amount of total protein from each sample was run on a 12% acrylamide SDS gel, transferred to nitrocellulose and blotted with a monoclonal anti-phosphotyrosine antibody. The arrow indicates a band at approximately 55×10^3 M_r that was consistently found to be phosphorylated in mutant but not in the wild-type strain. Relative molecular mass ($\times10^{-3}$) is shown on the left.

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 (Shaulsky 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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