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Genetics

Developmental signal transduction pathways uncovered by genetic suppressors

(*Dictyostelium discoideum*/saturation mutagenesis/second site suppressors/proteasome/mitogen-activated protein kinase cascade)

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ABSTRACT **We have found conditions for saturation mutagenesis by restriction enzyme mediated integration that result in plasmid tagging of disrupted genes. Using this method we selected for mutations in genes that act at checkpoints downstream of the intercellular signaling system that controls encapsulation in *Dictyostelium discoideum*. One of these genes, *mkcA*, is a member of the mitogen-activating protein kinase cascade family while the other, *regA*, is a novel bipartite gene homologous to response regulators in one part and to cyclic nucleotide phosphodiesterases in the other part. Disruption of either of these genes results in partial suppression of the block to spore formation resulting from the loss of the prestalk genes, *tagB* and *tagC*. The products of the *tag* genes have conserved domains of serine proteases attached to ATP-driven transporters, suggesting that they process and export peptide signals. Together, these genes outline an intercellular communication system that coordinates organismal shape with cellular differentiation during development.**

Development of a well-proportioned multicellular organism relies on concurrent differentiation of individual cells. In *Dictyostelium discoideum*, terminal differentiation into spores and stalk cells is coordinated in both time and space by an intercellular communication system. Two closely related prestalk genes, *tagB* and *tagC*, are necessary to produce a signal that passes from prestalk cells to prespore cells during culmination (ref. 1; G.S. and W.F.L., unpublished data). Null mutations in either gene lead to a cell-autonomous defect in prestalk differentiation such that the signal for sporulation is not produced. Strains carrying these mutations aggregate normally and differentiate into the initial prestalk cells and prespore cells. However, the prestalk cells fail to differentiate further or form the tips that normally appear after mound formation. Therefore, prespore cells never receive the signal to encapsulate. However, sporulation of mutant cells can be induced by developing them in chimeric mixtures where the wild-type cells in the apical prestalk region (PST-A cells) can generate the signal for encapsulation (1).

Both *tagB* and *tagC* encode composite proteins in which a serine–protease homology domain is contiguous with a domain homologous to ATP-driven transporters. Combinations of proteases and peptide transporters have been previously found to function in intercellular communication systems in both yeast and mammals. Processing and secretion of the **a** mating factor in *Saccharomyces cerevisiae* involves two proteases, *AXL1* and *STE23* (2), as well as the transporter, *STE6* (3–5). Major histocompatibility complex (MHC) class I presentation of intracellular epitopes on mammalian plasma membranes involves degradation of cytoplasmic proteins by proteasomes and subsequent transport of the peptides by the TAP-1 and TAP-2 transporters (6–12). The primary sequence of TagB and TagC suggests that they are involved in proteolysis and secretion of a peptide signal from prestalk cells that diffuses to adjacent prespore cells (ref. 1; G.S. and W.F.L., unpublished data), but the nature of the signal itself and the mechanism that receives and integrates it in prespore cells are still unknown.

Second-site suppressor analysis is a powerful genetic method that can be used to recognize components of complex genetic pathways. Commonly, genetic suppressors overcome the consequences of the primary mutation by modifying an interacting or a downstream component in the defective pathway. In *Dictyostelium*, the method of choice for mutagenesis is restriction enzyme mediated integration (REMI) (13), which generates null alleles by insertion of linearized plasmid DNA into restriction sites within genes *in vivo*. Suppressors generated by null mutations are expected to reveal genes that normally function as negative components downstream of the primary defect. To collect all such mutations that can suppress the block to sporulation in *tagB*$^-$ mutants, we developed a method for saturation mutagenesis of nonvital genes. We selected for spores from 10^5 independently transformed cells and found several strains in which the signal transduction mechanism that gates encapsulation of prespore cells is no longer dependent on the signal from prestalk cells.

METHODS

Saturation REMI Mutagenesis and Suppressor Screens. A REMI vector, pBSR1, was made by cloning the blasticidin S resistance cassette (14) into the *Pvu*II site of pGEM-3 (Promega). The *Bgl*II site was removed by site directed mutagenesis and a unique *Bam*HI linker replaced the pGEM-3 multiple cloning site. *tagB*$^-$ cells (1) were grown to 4×10^6 cells per ml in liquid HL-5 medium (15) supplemented with 0.2 μg/ml folic acid and 0.6 μg/ml cyanocobalamin (Sigma). Cells were cooled on ice for 15 min, harvested by centrifugation, and resuspended at 1×10^7 cells per ml in ice-cold electroporation buffer (13). Aliquots (20 ml) of cells were mixed with 10 μg/ml of *Bam*HI-linearized pBSR1 DNA and 30 units/ml of *Dpn*II restriction endonuclease (New England Biolabs) immediately before electroporation. Aliquots (0.8 ml) were electroporated in 0.4-cm gap cuvettes (BTX, San Diego) with a Bio-Rad Gene Pulser set at 1.0 kV and 3 μF and transferred immediately into HL-5. Cells were plated at 3×10^6 per 10 cm plate (Falcon) and incubated for 24 h. Blasticidin S (10 μg/ml) was added and cells were incubated for 6 more days. Drug-resistant cells were collected, counted, and plated at 0.5 to 5.0×10^3 cells per 10-cm plate on SM agar in association with *Klebsiella aerogenes* (15). Plates were screened for mutants with morphologies

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Abbreviations: REMI, restriction enzyme mediated integration; MAP, mitogen-activating protein; MHC, major histocompatibility complex; MDR, multiple drug resistance.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession numbers U60086 (*tagC*), U60168 (*prtC*), U60169 (*mkcA*), and U60170 (*regA*)].

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more advanced than the original *tagB*$^-$ mutant. After 8 days, cells were collected, treated with 0.3% cemulsol NP12 (SFOS, Persan, France) in 20 mM potassium phosphate buffer (pH 6.2), centrifuged, and washed with buffer without detergent. Pellets were resuspended and plated on SM agar in association with *K. aerogenes* (15) to recover detergent-resistant strains.

Nucleic Acid Manipulation. Cloning DNA sequences flanking the insertion sites and recapitulation of mutations by homologous recombination were performed as described (13). Riboprobes were made from the respective plasmids that were linearized with the restriction enzyme used for cloning. cDNA molecules were cloned as described (1). DNA sequencing was performed with an Applied Biosystems Prism 377 DNA sequencer. Purification of nucleic acids, Southern blot analysis, and Northern blot analysis were performed as described (16). RNA *in situ* hybridization was performed as described (17, 18) using cDNA riboprobes as indicated in Fig. 1.

Quantitation of Sporulation. Cells were developed on nitrocellulose filters as described (16). Sporulation efficiency was determined by detergent treatment of 36- to 48-h developed cells and plating on SM agar in association with *K. aerogenes* (15). The number of plaques in the bacterial lawn indicated the number of viable spores.

RESULTS

Saturation REMI Mutagenesis and Selection for Suppressors. Using a plasmid carrying the blasticidin resistance gene, we found that it was possible to generate 10^5 independent transformants by electroporating *Bam*HI-cut plasmid DNA along with the restriction enzyme *Dpn*II into about 10^9 *tagB* null cells. Because each electroporation introduced DNA and enzyme into 8×10^7 cells, we had to carry out 110 electroporations. However, this could be accomplished within a few hours. The cells were then plated and subjected to selection for resistance to blasticidin S. We recovered 10^5 independent drug resistant survivors, indicating that the efficiency of transformation was about $1{:}10^4$. This technique can be used to generate populations with near saturation mutagenesis of the nonvital genes in *Dictyostelium* that could be screened for any desired developmental genes.

A population of 10^5 independent blasticidin S-resistant transformants of the *tagB*$^-$ strain was plated on SM agar in association with *K. aerogenes* to allow each transformant to generate a clone which could develop. The clones were visually screened after a week and 12 were picked because they formed multicellular structures more advanced than the mounds formed by *tagB* null strains. The remainder of the transformants were collected to select for those that could form spores by resuspending them in 0.3% cemulsol which kills cells but not spores. Spores were pelleted from the detergent lysates and plated on SM agar plates in association with *K. aerogenes*. Three to 7 days later, plaques appeared in the bacterial lawns, representing suppressor strains that sporulated and survived the detergent treatment. Six independently derived strains were isolated and examined for the presence of spores by phase contrast microscopy and tested for spore viability following detergent treatment. Genomic DNA fragments flanking the insertion sites in these strains were cloned and used as probes for Southern blot analysis (data not shown). Four of the six sporulation suppressors were found to have suffered an insertion into one gene, *regA*, indicating that the original population carried multiple disruptions of this developmental gene. Each of the other two sporulation suppressors, *mkcA* and STB18, was found only once. Another gene, *prtC*, was found in two independent suppressor strains that were picked by visual inspection of the surviving transformants. However, neither of these strains carrying partial morphological suppressors formed spores. *prtC* is a novel *Dictyostelium* gene homologous to the human C2-proteasome subunit (19) and its sequence was deposited in the GenBank data base. Our recovery of multiple independent insertions in both *regA* and in *prtC* indicate that the mutagenesis procedure resulted in near saturation.

Sporulation in Suppressors of *tagB*. Mutational insertions in *mkcA* or in *regA* were recapitulated by homologous recombination in a fresh *tagB* null host as well as in *tagC* null cells and in wild-type AX4 cells. Sporulation efficiencies of the respective strains are shown in Table 1. To determine the sporulation efficiency of the parental *tagB* null or *tagC* null strains, we developed 10^{10} or 10^9 cells, respectively, and selected for spores following detergent treatment. In neither case did we detect a single viable spore. In the *mkcA*$^-$ *tagB*$^-$ double mutant, about one cell per aggregate (10^5 cells) had encapsulated, and the same ratio was observed when the *mkcA* gene was disrupted in a *tagC* null background (Table 1). Although this sporulation efficiency is low, it represents at least 10^4-fold increase relative to the parental strain. Disruption of *regA* in a *tagB* null or in a *tagC* null background resulted in higher levels of sporulation, up to 30% of the wild-type levels in the case of the *tagB*$^-$ *regA*$^-$ double mutants (Table 1). The number of spores produced by the single *regA*$^-$ or *mkcA*$^-$ mutants were almost indistinguishable from the wild-type number (Table 1). While disruption of *mkcA* results in only partial suppression of the sporulation defect in *tagB*$^-$ or *tagC*$^-$ strains, disruption of *regA* results in very effective suppression. The results presented in Table 1 indicate that in the wild type, both *mkcA* and *regA* function as negative regulators of sporulation. This is consistent with the notion that suppressors generated by insertional mutagenesis should reveal genes that normally function as negative components in the mutated pathway.

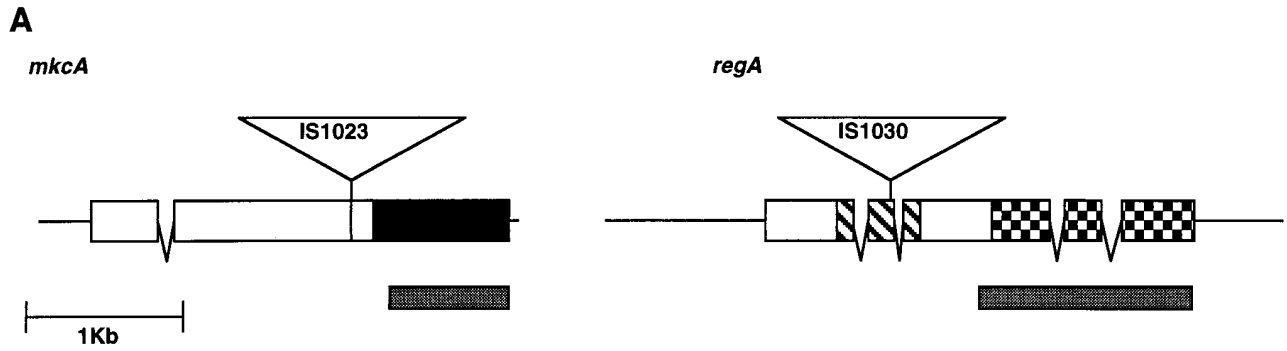
As indicated above, the first step of mutagenesis resulted in near saturation. The second step of selection for spores was dependent on the sporulation efficiency. Therefore, the difference in sporulation efficiency between the *mkcA*$^-$ *tagB*$^-$ double mutants and the *regA*$^-$ *tagB*$^-$ double mutants (Table 1) may account for the difference in the number of independent suppressor strains we found after the detergent selection step.

Sequence Similarity of *mkcA* and *regA* to Signal Transduction Genes. The gene structures of *mkcA* and *regA* are shown in Fig. 1*A*. Genomic and cDNA clones were sequenced and the deduced amino acid sequences were compared with various protein data bases using BLAST (20). Fig. 1*B* shows that *mkcA* is a member of the MAP kinase cascade gene family which includes the yeast *STE20* and the mammalian *PAK65* genes. The predicted carboxy terminus of MkcA contains a kinase homology region (Fig. 1*B*). The amino terminus does not contain previously recognized sequences, and as in *STE20* (21), it may be important for regulating the kinase activity.

In *regA* we found two domains with significant similarity to two distinct gene families. The 5′ end of *regA* encodes a response-regulator domain (Fig. 1 *A* and *C*) similar to those of the bacterial genes *ntrC*, *cheY*, and *pleD* (22, 23). The 3′ end is similar to members of the cyclic nucleotide phosphodiesterase gene family (24) (Fig. 1 *A* and *D*), but is clearly distinct

Table 1. Sporulation efficiency

Genotype	Viable spores per aggregate, 10^5 cells
Wild type	10^5
mkcA$^-$	10^5
regA$^-$	5×10^4
tagB$^-$	0
tagC$^-$	0
tagB$^-$ *mkcA*$^-$	1
tagC$^-$ *mkcA*$^-$	1
tagB$^-$ *regA*$^-$	3×10^4
tagC$^-$ *regA*$^-$	7×10^3



B

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mkcA   570  FDQICKKPTNEMKKLYTFREKVGKGGFGTVYLVRNNMDKSKSRIAIKKLPHVRKKEKKFNVKEIRVLEFTNHPNIITYYNSHMLHDEVWIAMEF
PAK65  256  LRNIVS--VGDPKKKYTRFEKIGQGASGTVYTAMDV--ATGQEVAIKQMNLQQQPKKELIINEILVMRENKNPNIVNYLDSYLVGDELWVVMEY
STE20  607  LNEICS--DGDPSTKYANLVKIGQGASGGVYTAYEI--GTNVSVAIKQMNLEKQPKKELIINEILVMKGSKHPNIVNFIDSYVLKGDLWVIMEY

mkcA   664  MEGGTLAEASNQYPFQESNIAYVAREVLQGLAYLHGIQLVHRDLKSQNIMMTTSGEIKLIDFGLCANLS--KGERIRMCGSPIWMPPEMIQQKQ
PAK65  346  LAGGSLTDVVTETCMDEGQIAAVCRECLQALEFLHSNQVIHRDIKSDNILLGMDGSVKLTDFGFCAQITPEQSKRSTMVGTPYWMAPEVVTRKA
STE20  697  MEGGSLTDVVTHCILTEGQIGAVCRETLSGLEFLHSKGVLHRDIKSDNILLSMEGDIKLTDFGFCAQINELNLKRTTMVGTPYWMAPEVVSRKE

mkcA   756  HGYTCDIWSTAICLLELANRNNK-LRKNPIKTMFMVGSEGIKDPFEDPHKWSDEFHDIINKCLQMDPHKRPSAQELLKHPFI-QLADNKKKMG
PAK65  440  YGPKVDIWSLGIMAIEMIEGEPPYLNENPLRALYLIATNGTPE-LQNPEKLSAIFRDFLNRCLEMDVEKRGSAKELLQHQFL-KIAKPLSSLT
STE20  791  YGPKVDIWSLGIMIIEMIEGEPPYLNETPLRALYLIATNGTPK-LKEPENLSSSLKKFLDWCLCVEPEDRASATELLHDEYITEIAEANSSLA
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C

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regA   155  YSPSKVRILVADDDDVQRKILNNLLKKFHYN-VTLVPNGEIAWEYINKGQQKYDLVLTDVMMPHITGFDLLQRINDHPVHRHIPVILMSG
cheY     1  MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDGVDALNKLQAG--GYGFVISDWNMPNMDGLELLKTIRADGAMSALPVLMVTA
ntrC     1    MPAGSILVADDDTAIRTVLNQALSRAGYE-VRLTGNAATLWRWVSQGEG--DLVITDVVMPDENAFDLLPRIKK--MRPNLPVIVMSA
pleD     1     MSARILVVDDIEANVRLLEAKLTAEYYE-VSTAMDGPTALAMAARICPT--IILLDVMMPGMDGFTVCRKLKDDPTTRHIPVVLI--

regA   244  TAVDYKYANDTIK---IGGQDFLTKPIAKELLKKKIDTV..
cheY    89  EAKK----ENIIAAAQAGASGYVVKPFTPATLEEKLNKIFEKLGM
ntrC    84  QNT----FMTAIRPSERGAYEYLPKPFDLKELITIVGRALA
pleD    83  TALDGR--GDRIQGLESGASDFLTKPIDDVMLFARVRSLTRFKLV..
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D

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regA   433  WEFDVFKYSE----DDLMPLLVDMFENFQLPEIFKIPIEKLQRFIMTVNALYRK-NNRYHNFTHAFDVTQTVYTFLTSFNAAQYLTHLDI
r.PDE  196  WGLDVFKVAELSGNRPLTAVIFRVLQERDLLKTFQIPADTLLRYLLTLEGHY-HSNVAYHNSIHAADVVQSAHVLLGTPALEAVFTDLEV
b.PDE  144  WSFDVFALNEASGEHSLKFMIYELFTRYDLINRFKIPVSCLIAFAEALEVGYSKYKNPYHNLIHAADVTQTVHYIMLHTGIMHWLTELEI

regA   518  FALLISCMCHDLNHPGFNNTFQVNAQTELSLEYNDISVLENHHAMLTFKILRNSECNILEGLNEDQYKELRRSVVQLILATDMQNHFEHT
r.PDE  285  LAAIFACAIHDVDHPGVSNQFLINTNSELALMYNDSSVLENHHLAVGFKLLQGENCDIFQNLSTKQKLSLRRMVIDMVLATDMSKHMSLL
b.PDE  234  LAMVFAAAIHDYEHTGTTNNFHIQTRSDVAILYNDRSVLENHHVSAAYRLMQEEEMNVLINLSKDDWRDLRNLVIEMVLSTDMSGHFQQI

regA   608  NKFQHHLNNLPFDR------NKKEDRQMILNFLIKCGDISNIARPWHLNFEWSLRVSDEFFQQSHYETICGYPVTPFMDKTKTTRARIAA
r.PDE  375  ADLKTMVETKKVTSLGVLLLDNYSDRIQVLQSLVHCADLSNPAKPLPLYRQWTERIMAEFFQQGDRERESGLDISPMCDKHTASVEKSQV
b.PDE  324  KNIRNSLQQPE----GL-------DKAKTMSLILHAADISHPAKSWKLHHRWTMALMEEFFLQGDKEAELGLPFSPLCDRKSTMVAQSQI

regA   693  DFIDFVASPLFQSMAKFLKE----------------------------------------------------SQFLLKVISKNRENWQAYMELQ
r.PDE  466  GFIDYIAHPLWETWADLVHP----------------------------------------------------DAQELLDTLEDNRE-WY-----Q
b.PDE  404  GFIDFIVEPTFSLLTDSTEKIIIPLIEEDSKTKTPSYGASRRSNMKGTTNDGTYSPDYSLASVDLKSFKNSLVDIIQQNKERWK-----E

regA   734  KEGKCNDDDLQFMEDPTILVKSKLPKIDEEENRDKVSSSSSSSTAPLTSTSSSNNETSSS
r.PDE  502  SRVPCSP-PHAIGPD-RFKFELTLEETEEEEEEDERH
b.PDE  488  LAAQGEPDPHKNS-----------DLVNAEEKHAETHS
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FIG. 1. Sequence analysis of *mkcA* and *regA*. (*A*) Sequenced regions of *mkcA* and *regA* genomic DNA are shown. Coding regions are indicated in boxes, and V shapes indicate introns. Plasmid (4.5 kb, not to scale) insertion sites (IS) in the respective mutant strains are indicated as triangles. Solid box in *mkcA* is the kinase homology domain. Thatched boxes in *regA* encode the response regulator homology domain, and checked boxes encode the cyclic nucleotide phosphodiesterase homology domain. Gray boxes under the genes indicate cDNA probes. (*B*) Sequence similarity between the putative protein kinase domain of *mkcA* and those of the mitogen-activating protein (MAP) kinase cascade genes PAK65 (Swiss-Prot Protein Sequence Data Bank no. P35465) and *STE20* (GenBank accession no. L04655). (*C*) Sequence similarity between the putative response regulator domain of *regA* and those of the bacterial response regulators *cheY* [National Center for Biotechnology Information (NCBI) no. 145525], *ntrC* (Swiss-Prot Protein Sequence Data Bank no. P10576), and *pleD* (NCBI no. 1119215). (*D*) Sequence similarity between the putative cyclic nucleotide phosphodiesterase domain of *regA* and those of a rat phosphodiesterase (r.PDE; NCBI no. 436012) and a bovine calmodulin stimulated phosphodiesterase (b.PDE; NCBI no. 533781). Conserved regions are underlined, and amino acid numbers are indicated on the left.

from *pdsA*, the only other published phosphodiesterase gene of *Dictyostelium* (25). Regulation of cyclic nucleotide phosphodiesterase activity by protein serine-threonine kinases has been demonstrated in many systems (26), and regulation of catalytic enzyme activity by a two-component system was found in the bacterial methylesterase CheB (27). It is therefore likely that the structural linkage between the response regulator and the phosphodiesterase domains in *regA* signifies a similar functional relationship.

A phosphodiesterase regulated by a histidine kinase in a two-component system could function in previously unsuspected signal transduction pathways mediated by the internal levels of cyclic nucleotides. Our data do not directly indicate a connection between the roles of *mkcA* and *regA*. They may function independently or be linked in a pathway, but because a correlation has been established between MAP kinase cascades and two-component systems in other eukaryotes (28–32), it is not implausible that *mkcA* and *regA* act as components of a network that functions to gate sporulation.

Effects of *mkcA* and *regA* on Cell Type Differentiation. Null mutations in either *tagB* or *tagC* lead to a cell autonomous defect in prestalk differentiation and a 5-fold reduction in the expression of the prestalk gene *ecmA*. There is no significant effect on expression of the spore coat gene *cotB*, indicating that, although spores are not made, differentiation of prespore cells is essentially normal (1). We compared the levels of *ecmA* and *cotB* gene expression as well as the expression of another prestalk gene, *ecmB*, in the parental strains and in the respective *mkcA* or *regA* null strains. Northern blot analysis revealed that in both *mkcA* nulls and in *regA* nulls the level of prestalk gene expression was markedly reduced whereas the expression of the prespore marker *cotB* was not affected (Fig. 2*A*). A similar pattern was observed in the suppressor double mutants (data not shown). Despite the low level of expression of the prestalk genes, we were able to detect their mRNAs by *in situ* hybridization. In *mkcA* null cells there was no significant difference from the wild-type pattern of accumulation of either *ecmA* or *ecmB* mRNA (Fig. 2*B*). In *regA* null strains the stalks were short and the lower cup structure was compromised as seen by the lack of hybridization with *ecmB* (arrow in Fig. 2*B*). The level of *ecmB* expression in the upper cup was also reduced (Fig. 2*B*). The reduced level of prestalk gene expression in both *mkcA*$^-$ and *regA*$^-$ strains and the defects in stalk formation in *regA*$^-$ strains (Fig. 2) indicate that *mkcA* and *regA* have functions in prestalk differentiation as well as in encapsulation of prespore cells. We therefore determined the cell type specificity and the developmental regulation of the *regA* and *mkcA* gene expression.

Developmental Regulation of *mkcA* and *regA* mRNA Expression. cDNA probes from *mkcA* and from *regA* (Fig. 1*A*) were used to follow the accumulation of the respective mRNA during development of wild-type cells. The 3.0-kb *mkcA* mRNA was expressed at constant levels during growth and throughout development (Fig. 3*A*). The 3.0-kb *regA* mRNA

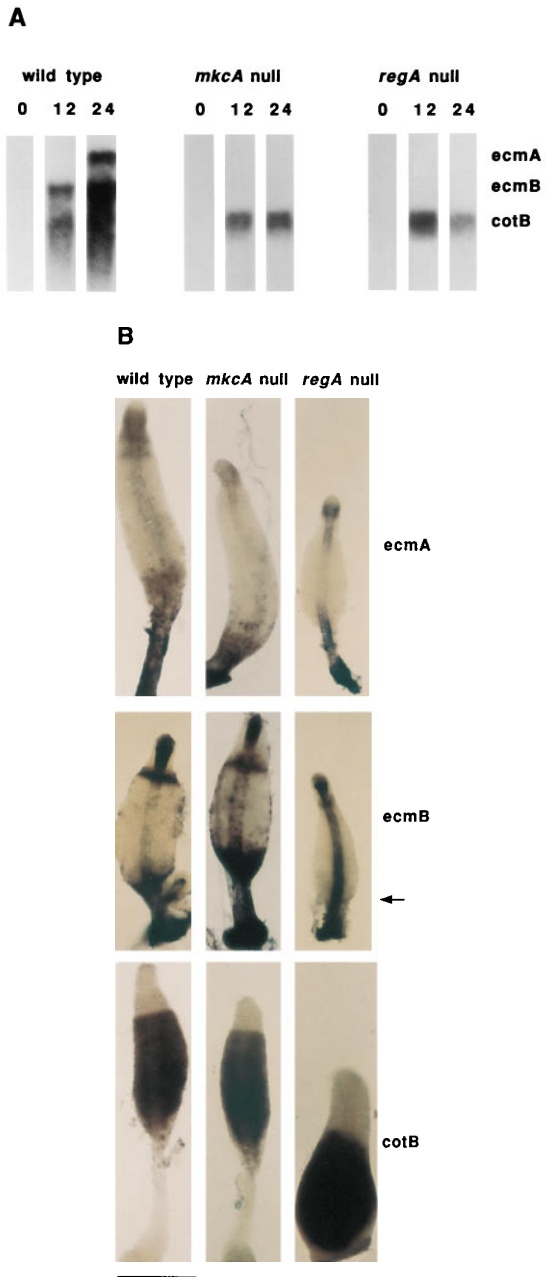

FIG. 2. Expression of cell type-specific genes in *mkcA* null or *regA* null strains. (*A*) RNA from wild-type cells and from mutants was prepared at 0, 12, and 24 h of development as indicated. Northern blots were hybridized with *cotB*, a prespore-specific gene probe and with *ecmA* and *ecmB*, two prestalk-specific gene probes. (*B*) Wild-type or mutant cells were developed to early culmination and subjected to *in situ* RNA hybridization with riboprobes for *ecmA*, *ecmB*, and *cotB* as indicated. Arrow shows the lower cup. (Bar = 0.2 mm.)

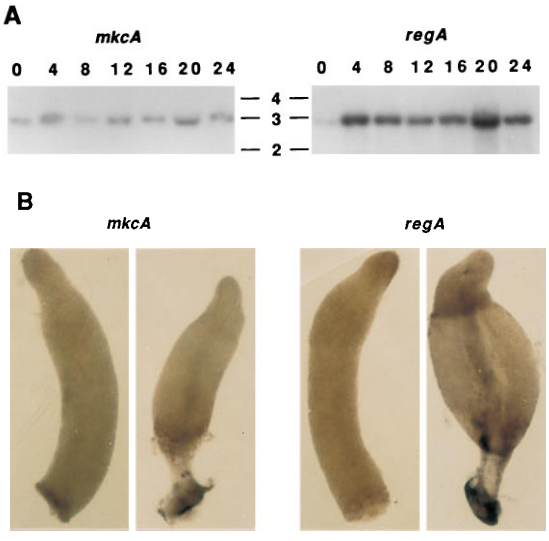

FIG. 3. Regulation and cell type specificity of *mkcA* and *regA* expression. (*A*) RNA was prepared from developing wild-type cells at 4-h intervals and analyzed by Northern blot analysis using cDNA riboprobes for *mkcA* or *regA* (Fig. 1) as indicated. Time (h) is indicated above the lanes; size (kb) is indicated between blots. (*B*) Wild-type cells were developed to the finger stage or to early culmination and subjected to *in situ* RNA hybridization with cDNA riboprobes for *mkcA* or *regA* as indicated. (Bar = 0.2 mm.)

Table 2. Proteases, transporters, and protein kinases in intercellular signaling

	Signal generation				Signal integration	
Organism	Signal source	Peptide precursor	Proteolytic processing	MDR-related transporter	Receiving cell	Signal transduction
Dictyostelium discoideum	Prestalk cells	Unknown	Serine protease domains of TagB and TagC	MDR domains of TagB and TagC	Prespore cells	*mkcA* *regA*
Saccharomyces cerevisiae	**a** cells	pro-**a**-factor (MFA1, MFA2)	AXL1, STE23	STE6	α cells	STE3 receptor STE20–MAP kinase cascade
Mammals	MHC class I positive cells	Cytoplasmic proteins	Proteasome	TAP1–TAP2 heterodimer	Cytotoxic T lymphocytes	T-cell receptor Lck Raf-1 MAP kinase cascade

was weakly expressed in vegetative cells. By 4 h it accumulated to its maximal level which persisted throughout development (Fig. 3*A*). Using RNA *in situ* hybridization to wild-type cells we found that *mkcA* was uniformly expressed throughout the prespore and the prestalk regions at the finger stage as well as during culmination (Fig. 3*B*). *regA* mRNA was uniformly distributed at the finger stage, but during culmination it was enriched in the prestalk upper cup region (Fig. 3*B*). The results presented in Figs. 2 and 3 support the possibility that *mkcA* and *regA* can affect sporulation in a cell autonomous manner and may have an additional function in prestalk cells.

DISCUSSION

mkcA and *regA* were selected as suppressors of the *tagB*$^-$ intercellular signaling defect, implying that they normally function as negative regulators of sporulation to coordinate encapsulation with culmination. The inhibition in prespore cells is lifted when prestalk cells enter culmination and release a signal by a mechanism that depends on *tagB* and *tagC*. This gating mechanism, coupling morphological progression with cellular differentiation and sporulation, was previously implied by the expression pattern of the sporulation-specific gene *spiA* (33).

By primary sequence, *tagB* and *tagC* are likely to act as proteases as well as membrane transporters to facilitate the secretion of a peptide signal from prestalk cells (ref. 1; G.S. and W.F.L., unpublished data). *mkcA* and *regA* are related to protein kinase signal transduction genes. Systems that combine peptide processing and membrane transport in signal generation and kinase cascades in signal transduction have been observed in other organisms. Table 2 demonstrates similarities between molecular strategies in intercellular signaling systems of *Dictyostelium*, yeast, and mammals. The precursor protein for the yeast mating pheromone **a** factor is the gene product of *MFA1* and *MFA2*. The precursor is processed by two proteases, *AXL1* and *STE23* and secreted by the multiple drug resistance (MDR)-related gene product of *STE6* in a cells (2, 5). The peptide interacts with the *STE3* membrane receptor on α cells and the signal is integrated by a MAP kinase cascade that includes the *mkcA* homologue *STE20* (34). Recognition of intracellular antigens by cytotoxic T lymphocytes (CTL) in mammals is mediated by a similar mechanism. Cytoplasmic proteins are processed into small peptides by proteasomes (6) and transported into the pre-Golgi by the MDR-related heterodimeric TAP1–TAP2 transporter. The peptides associate with an MHC class I molecule and are eventually presented on the cell surface (7–12). Ligation of the CTL T-cell receptor with MHC class I-associated peptides leads to activation of a MAP kinase cascade via activation of the Lck and Raf-1 protein kinases (35). Thus, the signaling system we have discovered in *Dictyostelium* is known to function in other eukaryotes and may serve as a general mechanism for peptide-based signaling.

Assuming that *regA* encodes a cAMP phosphodiesterase, then our results indicate that cAMP may be directly involved in triggering encapsulation. A model consistent with all the available genetic and structural evidence can be considered in which signal peptides are generated and exported by the TagB/C complex in prestalk cells as they undergo terminal differentiation. The signal diffuses to adjacent prespore cells where it activates a histidine kinase of the two-component system that includes RegA as the second component. Phosphorylation of RegA inactivates its phosphodiesterase activity leading to an increase in the levels of cAMP such that cAMP-dependent protein kinase (PKA) and other cAMP-dependent processes necessary for encapsulation are activated. Sporulation then follows in short order. This model is supported by the observation that direct addition of the membrane permeable cAMP analog, 8-Bromo-cAMP, triggers encapsulation in dissociated cells where signaling from prestalk cells is precluded (36–38). Moreover, we have found that 8-Bromo-cAMP can trigger encapsulation in *tagB*$^-$ cells where signaling from prestalk cells is genetically precluded (G.S. and W.F.L., unpublished data).

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