EUKARYOTIC CELL, Feb. 2002, p. 126–136 Vol. 1, No. 1
1535-9778/02/$04.00+0 DOI: 10.1128/EC.1.1.126–136.2002


# CulB, a Putative Ubiquitin Ligase Subunit, Regulates Prestalk Cell Differentiation and Morphogenesis in *Dictyostelium* spp.

Bin Wang[1] and Adam Kuspa[1,2]*

*Verna and Marrs McLean Department of Biochemistry and Molecular Biology[1] and Department of Molecular and Human Genetics,[2] Baylor College of Medicine, Houston, Texas 77030*

Received 23 January 2001/Accepted 1 October 2001

***Dictyostelium*** **amoebae accomplish a starvation-induced developmental process by aggregating into a mound and forming a single fruiting body with terminally differentiated spores and stalk cells.** ***culB*** **was identified as the gene disrupted in a developmental mutant with an aberrant prestalk cell differentiation phenotype. The** ***culB*** **gene product appears to be a homolog of the cullin family of proteins that are known to be involved in ubiquitin-mediated protein degradation. The** ***culB*** **mutants form supernumerary prestalk tips atop each developing mound that result in the formation of multiple small fruiting bodies. The prestalk-specific gene** ***ecmA*** **is expressed precociously in** ***culB*** **mutants, suggesting that prestalk cell differentiation occurs earlier than normal. In addition, when** ***culB*** **mutant cells are mixed with wild-type cells, they display a cell-autonomous propensity to form stalk cells. Thus, CulB appears to ensure that the proper number of prestalk cells differentiate at the appropriate time in development. Activation of cyclic AMP-dependent protein kinase (PKA) by disruption of the regulatory subunit gene (*** ***pkaR*** **) or by overexpression of the catalytic subunit gene (*** ***pkaC*** **) enhances the prestalk/stalk cell differentiation phenotype of the** ***culB*** **mutant. For example,** ***culB*** $^{-}$ ***pkaR*** $^{-}$ **cells form stalk cells without obvious multicellular morphogenesis and are more sensitive to the prestalk O (pstO) cell inducer DIF-1. The sensitized condition of PKA activation reveals that CulB may govern prestalk cell differentiation in** ***Dictyostelium*****, in part by controlling the sensitivity of cells to DIF-1, possibly by regulating the levels of one or more proteins that are rate limiting for prestalk differentiation.**

Development in multicellular organisms is precisely regulated in time and space such that morphogenesis and cell differentiation are coordinated. *Dictyostelium* development is characterized by the chemotactic aggregation of starved cells and the subsequent formation of a multicellular fruiting body composed of the differentiated spores and stalk cells. Two major cell types, prestalk and prespore, initially differentiate within the aggregate in a spatially independent manner. The prestalk cells then sort to the presumptive tip of the aggregate, and subsequently form the tip of an elongated finger-like structure (72, 75, 76). When this finger falls over to form a migrating slug, most of the prestalk cells remain in the anterior region. During terminal-cell differentiation and fruiting body formation, the slug rears up to form a "second finger" and then flattens into a structure resembling a Mexican hat. The culmination of development begins with the formation of a stalk tube that is initiated in the center of this structure by a subpopulation of prestalk cells. The rest of the prestalk cells in the tip migrate into the stalk tube and differentiate into stalk cells. As the stalk forms, it lifts the prespore cells aloft while the prespore cells actively move up it before differentiating into spores within the nascent sorus (64). There is substantial genetic and biochemical evidence for signaling between prestalk and prespore cells and for the coordinated movement of prestalk and prespore cells that is required for spatially and temporally regulated terminal differentiation (see, e.g., references 5, 11, 26, 30, 31, and 56). Recent work has focused on the molecular nature of these signaling events and how they are regulated (13, 15, 21).

One of the key control points for coordinating morphogenesis and cell differentiation in *Dictyostelium* involves the activation of cyclic AMP (cAMP)-dependent protein kinase (PKA) (20, 42). PKA is involved in a wide range of developmental processes in *Dictyostelium*, as has been found in metazoan species. It translates signals from the outside of cells, such as neurotransmitters, peptide transmitters, and growth hormones, into specific phosphorylation of downstream target proteins (69). PKA also plays a critical role in learning and memory in *Drosophila* and mice (2, 16, 59). In *Dictyostelium*, PKA regulates aggregation, prespore and prestalk cell differentiation, morphogenesis, and terminal cell differentiation (reviewed in reference 41). PKA activity is controlled through regulation of the intracellular cAMP concentration. Increased cAMP concentrations activate PKA by binding to its regulatory subunit(s) and releasing an active catalytic subunit(s). Although PKA is essential for *Dictyostelium* development, it is believed that multiple signaling pathways cooperate with PKA to regulate the precise timing of developmental events (5–7, 10, 13, 24, 31, 33, 40, 43, 45, 46, 50, 53, 55, 63, 65).

A number of experiments have shown that increased PKA activity allows *Dictyostelium* cells to develop rapidly, differentiate precociously, or even bypass critical regulatory events throughout development (1, 34, 58, 70, 71). We sought to identify developmental regulators that were more or less independent of PKA control. To do this, we performed a genetic screen for modifiers of the developmental phenotype of a mutant with constitutively active PKA (*pkaR*$^{-}$, a regulatory-subunit null mutant). Since activation of PKA allows cells to bypass many of the regulatory events controlled by cAMP during

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Rm. T321, Houston, TX 77030. Phone: (713) 798-8278. Fax: (713) 796-9438. E-mail: akuspa@bcm.tmc.edu.

early development, we expected to identify genes that function independently or "downstream" of PKA-promoted regulatory events. One mutant from the screen resulted from a null mutation in *culB*, a gene that encodes a protein with a high degree of similarity to the cullins.

Cullins from an evolutionarily conserved family of proteins that were first recognized in *Caenorhabditis elegans* (37). They are involved in a cascade of reactions in which ubiquitin is transferred from a ubiquitin-activating enzyme, E1, to a ubiquitin-conjugating enzyme, E2, and then to a lysine residue in the target protein through an E3-ubiquitin ligase (18, 49, 79, 80). The E3 complex acts as a bridge between the specific target and the appropriate E2, thereby providing specificity to the ubiquitin transfer reaction (54). Multiple rounds of ubiquitination of the initial conjugate lead to polyubiquitination of the target protein, which is subsequently recognized and degraded by the 26S proteosome. Cullins are subunits of some E3 ubiquitin-ligase complexes, such as the anaphase-promoting complex and SCF complex (19, 60, 79, 80). The anaphase-promoting complex is critical for regulating anaphase progression during the cell cycle, while SCF complexes mediate the degradation of a wide array of regulatory proteins in yeast and in mammals, such as the cyclin-dependent kinase inhibitors, transcription factors, and DNA replication initiation proteins (18, 77). Cullin function is fairly well understood in the SCF ubiquitin-ligase complex (reviewed in reference 18). In the SCF complex, the cullin protein Cdc53 interacts with the Skp1 protein. Skp1, in turn, interacts with an F-box protein that confers target specificity. The SCF is brought together with the ubiquitin conjugase E2 through the interaction of Cdc53 with E2.

Recently, ubiquitin-mediated protein degradation has been implicated in the regulation of cell differentiation and morphogenesis in *Dicytostelium*. Cells that are mutant in NosA, a homolog of deubiquitinating enzymes, arrest at the tight-aggregate stage (51). Disruption of Ubc1, a ubiquitin-conjugating enzyme, leads to arrest at the aggregate stage (14). A null mutation in the F-box protein, Mekkα, or overexpression of its F-box domain and WD repeats causes abnormal cell type proportioning (12). Finally, the cullin homolog CulA appears to regulate PKA activity by controlling the level of RegA, a cAMP-specific phosphodiesterase (46). Here, we describe the role of CulB in *Dictyostelium* development and present evidence that CulB regulates prestalk cell differentiation and morphogenesis.

## MATERIALS AND METHODS

**Cell culture, transformation and development.** *Dictyostelium* cells were grown in HL-5 liquid medium or on SM nutrient agar in association with bacteria (61). Ax4 was used as the wild-type strain in these studies. *pkaR*$^-$ cells were generated from Ax4 cells as described previously and used as the parental strain for large-scale mutagenesis (71). Restriction enzyme-mediated integration mutagenesis using *Dpn*II, blasticidin selection, and linearized pBsr1 vector was performed as previously described (3, 38). Approximately 80,000 independent mutants were screened for developmental morphologies that differed from the parental strain. A full description of this screen will be described elsewhere (B. Wang and A. Kuspa, unpublished data). Selected strains were isolated, and their pBSR1 insertions and flanking genomic regions were cloned by plasmid rescue. Recapitulation of the original insertion events by homologous recombination and selection for blasticidin resistance was performed as described previously (3, 39). DNA transformations were performed by electroporation (44) or by calcium phosphate precipitation and glycerol shock (48). For development, cells were washed in phosphate buffer (50 mM $KH_2PO_4$, 50 mM $Na_2HPO_4$ [pH 6.1]) and spread onto Millipore filters or onto 1% nonnutrient agar plates (61).

**cDNA cloning and plasmid construction.** Standard DNA and RNA manipulations were carried out as described previously (52). A cDNA library in the Lambda ACT2 vector (constructed by Sijie Lu) was used to isolate a full-length *culB* cDNA. The 1.6-kb *Xba*I genomic fragment of *culB*, which encodes the N terminus of CulB, was used as a probe to screen the library. Nine positive clones were obtained from $2.5 \times 10^5$ phage. The phage were converted into plasmids as described previously (47). The phage with the longest insert, p8, was thus converted into plasmid pcul8B. The analysis of the pcul8B sequence and the genomic sequence of *culB* confirmed that pcul8B contained the predicted full-length *culB* cDNA. The cDNA insert is 2.5 kb long and is predicted to encode a 772-amino-acid CulB protein. A CulB expression vector was generated by inserting the *culB* cDNA into the pDXA-HY vector, in frame with the upstream actin15 promoter and protein tag sequence (44). Plasmid pCulBsr was constructed by circularizing the *culB Xba*I genomic fragment by ligation with T4 ligase and inserting *Bam*HI-linearized pBsr1 plasmid (a gift from W. F. Loomis) into the *Bam*HI site within the fragment. To generate *culB* insertion mutations, pCulBsr was linearized with *Xba*I and introduced into cells by electroporation.

**Molecular analyses.** Total RNA was prepared from cells developing on Millipore filters. Cells were collected at various times during development, washed, and frozen on dry ice. RNA was purified with TRIZol reagent (Invitrogen) as specified by the manufacturer. For Northern analyses, 10 μg of RNA was loaded into each lane and size fractionated on 1% agarose gels containing 2.2 M formaldehyde. Gel transfer and membrane hybridization to $^{32}$P-labeled DNA probes were performed by standard procedures (52). RNase protection assays were carried out as specified by the manufacturer of the assay (Ambion RPA II). A 360-bp fragment covering the insertion site of *culB* was generated by PCR using primers 5′GAAGGTGGTTTAGCTCCAG3′ and 5′TAATACGACTCACTATAGGGAGGGACCTAACTTCTCTTCC3′. The latter primer includes a T7 promotor at its 5′ end for use in synthesizing the antisense RNA probe used in the RNase protection assays. Southern analyses of genomic DNA were carried out with $^{32}$P-labeled DNA probes as described previously (52).

**Spore assay.** After 48 h of development on filters, the cells were collected into 20 mM potassium phosphate buffer (pH 6.2) and treated with the nonionic detergent NP-40 (0.4%) for 10 min at room temperature. After the detergent treatment, the cells were washed with potassium phosphate buffer twice and disaggregated by trituration with an 18-gauge needle. Ellipsoid and refractile spores were counted by phase-contrast microscopy and plated clonally on SM agar plates with bacteria. The number of resulting colonies was used as an estimate of the number of viable spores in each sample, and this was used to calculate the total number of spores produced from $5 \times 10^7$ input cells. Under these conditions, wild-type cells (Ax4) produce 67% ± 8% detergent-resistant spores and 82% ± 5% of these produce viable colonies on bacterial growth plates. At least three independent determinations were carried out for each strain and are reported as the mean ± standard error of the mean.

**Submerged-monolayer assay.** The submerged-monolayer assay was modified from an assay described previously (29). For stalk cell induction, vegetative cells were washed once with KK2 buffer (16.5 mM $KH_2PO_4$, 3.8 mM $K_2HPO_4$ [pH 6.2]) and three times with stalk buffer (10 mM morpholineethanesulfonic acid [MES], 2 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 200 μg of penicillin-streptomycin per ml [pH 6.2]). The cells were then plated into 24-well tissue culture plates at a density of $10^4$ cells/cm$^2$ and incubated in the presence or absence of differentiation-inducing factor (DIF). After a 48-h incubation, the buffer was removed without disturbing the cells. A Calcofluor solution (0.01%) (25) was added to the wells for 5 min. The Calcofluor solution was then removed, and the cells were observed immediately by microscopy. Only the cells that were vacuolated and stained by Calcofluor were counted as stalk cells (28). Cells were observed with a 32× objective lens, and the number of fluorescent vacuolated cells and the total number of cells were counted. At least 300 cells were counted for each assay, and the percentage of stalk cells formed was calculated by dividing the number of fluorescent vacuolated cells by the total number of cells. For spore cell induction, *pkaR*$^-$ cells and *culB*$^-$ *pkaR*$^-$ cells were washed once with KK2 buffer and three times with spore buffer (10 mM MES, 20 mM NaCl, 20 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and then incubated in spore buffer supplemented with 5 mM cAMP (34).

**Assays for DIF and PKA.** The DIF bioassay was based on the ability of DIF to induce stalk cell differentiation in isolated *pkaR*$^-$ cells in submerged monolayers. Briefly, the monolayer assay to induce stalk cell formation was performed as described above. The supernatants from the different conditions were collected after 48 h of incubation and added to at least three different wells for each assay. Fresh *pkaR*$^-$ cells were diluted 40-fold into the wells to a density of $10^4$ cell/cm$^2$. The number of stalk cells was determined after 48 h of incubation. The percent-

age of stalk cells was calculated as described above. PKA activity measurements were carried out using the SignaTECT PKA assay system (Promega). Cell extracts containing 10 μg of protein were prepared as specified by the manufacturer and were used in reactions with 5 mM cAMP and in the presence or absence of 10 μM PKA-specific inhibitor PKI, which inhibits the *Dictyostelium* enzyme (42). PKA activity is defined as the amount of Kemptide substrate phosphorylated (nanomoles per minute per milligram of protein) in the absence of PKI minus the amount phosphorylated in the presence of PKI.

## RESULTS

**Identification of CulB, a putative cullin homolog.** In an attempt to identify developmental genes that function relatively independently of PKA control, we carried out a screen for genetic modifiers of a mutant with constitutively active PKA (see Materials and Methods). From this screen, we isolated *culB*, a gene that is predicted to encode a cullin homolog. Disruption of the *culB* gene results in cells that form smaller fruiting bodies. By cloning, mapping, and sequencing the disrupted *culB* locus, we found that the mutation resulted from a plasmid insertion in the middle of the *culB* gene and the loss of the 3′ half of the gene along with 8 kb of flanking genomic DNA (data not shown). To test whether the disruption of the *culB* gene or the missing genomic DNA caused the mutant phenotype, we constructed a new targeting vector, pCulBsr, and introduced it into wild-type cells. The mutant phenotype was recapitulated in the homologous recombinants that acquired a simple insertion in *culB*, as confirmed by Southern analysis (Fig. 1A).

The isolation and analysis of genomic and cDNA clones of *culB* revealed a gene that is predicted to encode a 772-amino-acid protein that has 37% identity to the human Cul-1 protein and 40% identity to the *Dictyostelium* CulA protein (Fig. 1B). All of the mutant phenotypes associated with the *culB* mutation that are described below could be rescued when the full-length *culB* cDNA was expressed in *culB*$^-$ cells under the control of the actin 15 promoter, confirming that the phenotypes were caused by the loss of CulB function (data not shown). Expression of the *culB* mRNA could not be detected by Northern analyses of wild-type cells. However, using an RNase protection assay, we found that *culB* was expressed in vegetative cells, and its mRNA level remained constant throughout the first 9 h of development but decreased gradually to the end of the development (Fig. 1C). In *culB*$^-$ cells, the mRNA could not be detected, even by RNase protection assays, confirming that it is a null mutant.

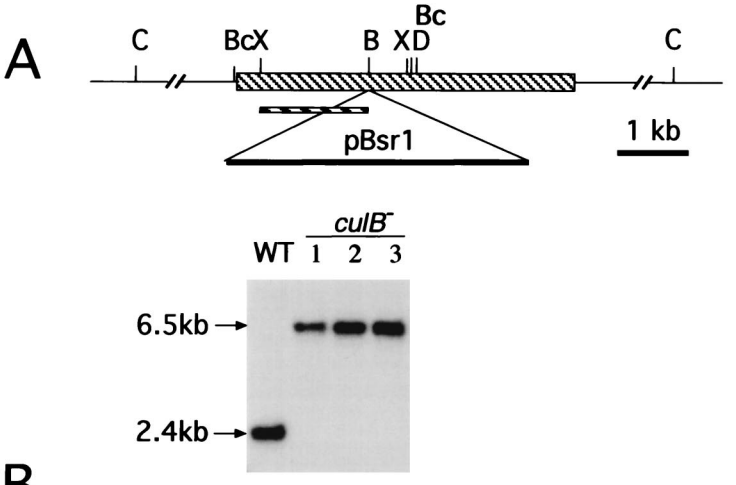
***culB*$^-$ mutants display aberrant prestalk cell differentiation.** *culB*$^-$ cells aggregated on filters in a manner similar to wild-type cells, but multiple tips formed on most of the aggregates, each of which later elongated into a finger structure. These fingers then continued to develop and finally culminated to form small fruiting bodies (Fig. 2A). The *culB*$^-$ cells produced 17% ± 2.8% of the wild-type number of viable spores. The formation of aggregates with supernumerary tips suggested that prestalk cell differentiation was abnormal in the *culB*$^-$ mutant. Therefore we examined the sorting and terminal differentiation of the major cell types in *culB*$^-$ cells by marking them with green fluorescent protein (GFP). Using *ecmA*/*GFP* as a prestalk marker, and *cotB*/*GFP* as a prespore marker, we found that prestalk cells and prespore cells sorted appropriately during the development of *culB*$^-$ cells. Within the aggregates of *culB*$^-$ mutants, although they were multitipped, *ecmA*-positive cells were clearly located in the tips, just as in the wild type (Fig. 2B). In fruiting bodies, *ecmA*-positive cells composed the stalk cell compartments including the upper cup, lower cup, stalk, and basal disk (data not shown). The *cotB*/*GFP*-marked prespore cells remained in the lower half of the aggregate and finger structure and eventually formed spores within the spore head of the fruiting bodies (Fig. 2C).

Examination of the temporal pattern of cell-type-specific gene expression revealed that *ecmA* was expressed precociously in *culB*$^-$ cells (Fig. 3). EcmA mRNA accumulated to detectable levels in *culB*$^-$ cells after 4 h of development, 8 h earlier than it could be detected in wild-type cells. This suggests that the disruption of *culB* results in precocious prestalk cell differentiation. The expression pattern of the prespore gene *cotA* in *culB*$^-$ cells was similar to that in wild-type cells, indicating that prespore cell differentiation is not affected in *culB*$^-$ cells (Fig. 3).

**The propensity of *culB*$^-$ mutants to form stalk cells is cell autonomous.** To test whether *culB*$^-$ cells have a cell autonomous propensity to form stalk cells, we assessed the differentiation of *culB*$^-$ cells in *culB*$^-$/wild-type chimeras. *culB*$^-$ cells carrying an *act15*/GFP construct, which marks all cells, were mixed with unmarked cells in various combinations. In the context of an excess of wild-type cells (9:1 ratio), the *culB*$^-$ cells were found distributed in the stalk, basal disk, upper cup, and lower cup of the fruiting body, suggesting that they have a tendency to form stalk cells (Fig. 4). GFP-marked wild-type cells were distributed in both the stalk region and the spore region when mixed with unmarked wild-type cells (Fig. 4). In control experiments, GFP-marked *culB*$^-$ cells were found distributed throughout the fruiting body when mixed with unmarked *culB*$^-$ cells (1:9 ratio), whereas marked wild-type cells were detected mainly in the spore population when mixed with an excess of *culB*$^-$ cells (data not shown). These experiments indicate that the *culB*$^-$ mutant has a cell-autonomous propensity to form stalk cells placed in the environment of developing wild-type cells.

***culB*$^-$ cells are defective in slug formation and migration.** Prestalk cells within the tips of migrating slugs are thought to control slug phototaxis and thermotaxis (reviewed in references 22 and 23). As further evidence that prestalk cell differentiation is abnormal in *culB* mutants, we found that *culB*$^-$

FIG. 1. Characterization of *culB*. (A) *culB* genomic locus. *pBsr1* is a 4.1-kb plasmid with the selectable *Bsr* cassette that was inserted into the *Bam*HI site of the *culB* gene (hatched rectangle). Genomic DNA from wild-type, *culB*$^-$ (clones 1 and 2) or *culB*$^-$ *pkaR*$^-$ (clone 3) cells was digested with *Bcl*I, separated by agarose gel electrophoresis, blotted, and probed with the *Xba*I-*Bam*HI genomic DNA fragment (hatched bar) in a standard Southern analysis. Restriction enzyme sites: C, *Cla*I; X, *Xba*I: B, *Bam*HI; Bc, *Bcl*I; D, *Dpn*II. (B) Comparison of the predicted amino acid sequences of CulB, CulA, and human Cul1 (hCul1). The amino acid identities between CulB (AF144717), CulA (AF020287), and human Cul1 (U58087) are in black, and amino acid similarities are in highlighted in gray. (C) Expression of *culB*. RNA was extracted from cells at the indicated times after the initiation of development. RNase protection assays carried out on 10 μg of RNA from each sample revealed a single RNA species.


A
C
BcX
Bc
B XD
C
pBsr1
1 kb
culB-
WT 1 2 3
6.5kb
2.4kb
B
CulA -MSMMTSTPT-KRSVKLDDIWPELEEGIYKIITDLNKGFPKQKWIALYTHVYDYCAASQS
hCul1 MSSTRSQNPHGLKQIGLDQIWDDLRAGIQQVYT--RQSMAKSRYMELYTHVYNYCTS---
CulB -----MMNGLGRQDIDFNTIWKNIADQVYKILT----GSQNVSAMFLYEDVYKLCIAQ--
CulA KSSAKVGMPKQQASGANYVGEDLYNRLNLFLKKHMSQLLKLTETKMDEPLLNYYYTEWDR
hCul1 ------------VH--Q-FVGLELYKRLKEFLKNYLTNLLKDGEDLMDESVLKFYTQQWED
CulB ------------P-Q-PYCEPLYENIKKFFEQHVDQILLIILDTKSDTIS-EYLKQWKL
CulA YTSAMKYIN-NIFQYMNRYYIKREIDDGKK-----EVYEIFILSLVLWRDCLFTPLKQRLT
hCul1 YRFSSKVLN-GICAYLNRHWVRRECDEGRK-----GIYEIYSLALVTWRDCLFRPLNKQVT
CulB FFSGCELCNKVIFRYLNLNWINNKKILDKKFGHPPDVYEIQILGLMIWKERLFFKIKDRVL
CulA NSLLDIHESERNGYQINTHLIKGVINGYVSLGLNRE--KPKETILQVYKSGFEELFLTAT
hCul1 NAVLKLIEKERNGETINTRLISGVVQSYVELGLNEDDAFAKGPTLTVYKESFESQFLADT
CulB KCVEILIQKDRDGELVQHQFISQFMESLIKLDSVDK--------DRTLYLIEYEASYLENT
CulA ENYKTNESAKFISENSVADYMKKVETRLNEEVKRVQQQYLHQNTESELIAKCEKVLIEKHV
hCul1 ERFYTRESTEFLQQNPVTEYMKKAEARLLEEQRRVQVVLHESTQDELARKCEQVLIEKHL
CulB RQFYTRESVAFIASSGISSYMKKAETRIDEEEQRSQKYLNSSSHDKMRRLLHSILIEKHK
CulA EVIWNEFQTLLEKDKIPDLTRMYSLLSRIPRGLEPLRTTLEKHVQNVGLQAVSSIATNGV
hCul1 EIFHTEFQNLLDADKNEDLGRMYNLVSRIQDGLGELKKLLETHIHNQGLAAIEKCGEAAL
CulB ELLQSECINYLKDEKLDEIYNMYKLLSRIEGGLAPVLETVQKYIQHVGIDAIKSIPDRNN
CulA IEPKVYIETLLKVFKKYNELVTGAFRSDTGFVASLDKACRRFINENAVTIAAKSSSKSPE
hCul1 NDPKMYVQTVLDVHKKYNALVMSAFNNDAGFVAALDKACGRFINNNAVTKMAQSSSKSPE
CulB PDPKIYVETLLKIYLQFSSIIKKSFNNDVSFITVLDLACHKIFNQNHITRNT---TKSPE
CulA LLARFTDFLLKKSPNNPEESEMEQLLNDVMIVFKYIEDKDVFQDFYSKMLAKRLIHGTST
hCul1 LLARYCDSLLKKSSKNPEEAELEDTLNQVMVVFKYIEDKDVFQKFYAKMLAKRLVHQNSA
CulB LLAKYCDMLLKKGNKQHEEIELEEKLGQIIVLFKYVDDKDVFQKFYSKMLSRRLINGTSV
CulA SEDLEGTMIGKLKSTCGYEYTSKLQRMFTDMSLSRELLDRFNNHIEQVERSSLNHDFSVL
hCul1 SDDAEASMISKLKQACGFEYTSKLQRMFQDIGVSKDLNEQFKKHLTNSE--PLDLDFSIQ
CulB SDDIEKFMITGLKQACGFEYTSKFQRMFNDITLSAETNEEFKNHLIKNSLSIGKIDFSIL
CulA VLATGSWPLQPPSTNFSIPKELQACEQLFQKFYQNQHSGRKLNWLHHLSKGELKTKYLQT
hCul1 VLSSGSWPFQQ-SCTFALPSELERSYQRFTAFYASRHSGRKLTWLYQLSKGELVTNCFKN
CulB VLTSGSWSLHSQTSSFIVPQELTLCISAFQQYYSTQHQGRKLNWLHHLCKAEAKSFFAKK
CulA SKSGYTLQCSTYQIGVLLQFNQYETLTSEEIQESTQLIDSVLKGTLTSLAKSKILLADPP
hCul1 R---YTLQASTFQMAILLQYNTEDAYTVQQLTDSTQIKMDILAQVLQILLKSKLLVLEDE
CulB S---YDFQVTNFQLGILLIFNTQESVSLEEITKFTNLNENELSRTLQSLIEAKILISKKK
CulA -------------------------LPDEEIAKTT--------KFSLNKQFKNKKTKIFIN
hCul1 NAN-------------------------VDEVELKPDT--------LIKLYLGVKNKKLRVNIN
CulB DQNSINNNNNNNNNNNNGEGNNSLVDSANNMQPSSSSSSTQEYTVNSAYSNKRSKVKVS
CulA VPVLTQVKEEIDSIHKTVEEDRKLQIQAAIVRIMKMRKQLAHSGLMTEVISQLQTRFNPK
hCul1 VPMKTEQKQEQETTHKNIEEDRKLLIQAAIVRIMKMRKVLKHQQLLGEVLTQLSSRFKPR
CulB SSLQKETPLQNEETYKGIDEDRKLYLQASIVRIMKARKTMNHVSLIQEVIEHSRLRFQPN
CulA VNIIKKCIDILIEKEYLMRVEGKKDHYSYVA
hCul1 VPVIKKCIDILIEKEYLERVDGEKDTYSYLA
CulB IPMIKKCIEQLIEKEYITRAEGESDRYLYAA
C
Wild-type
culB-
0 3 6 9 12 16 20 24 0 3 6 9 12 16 20 24 hours

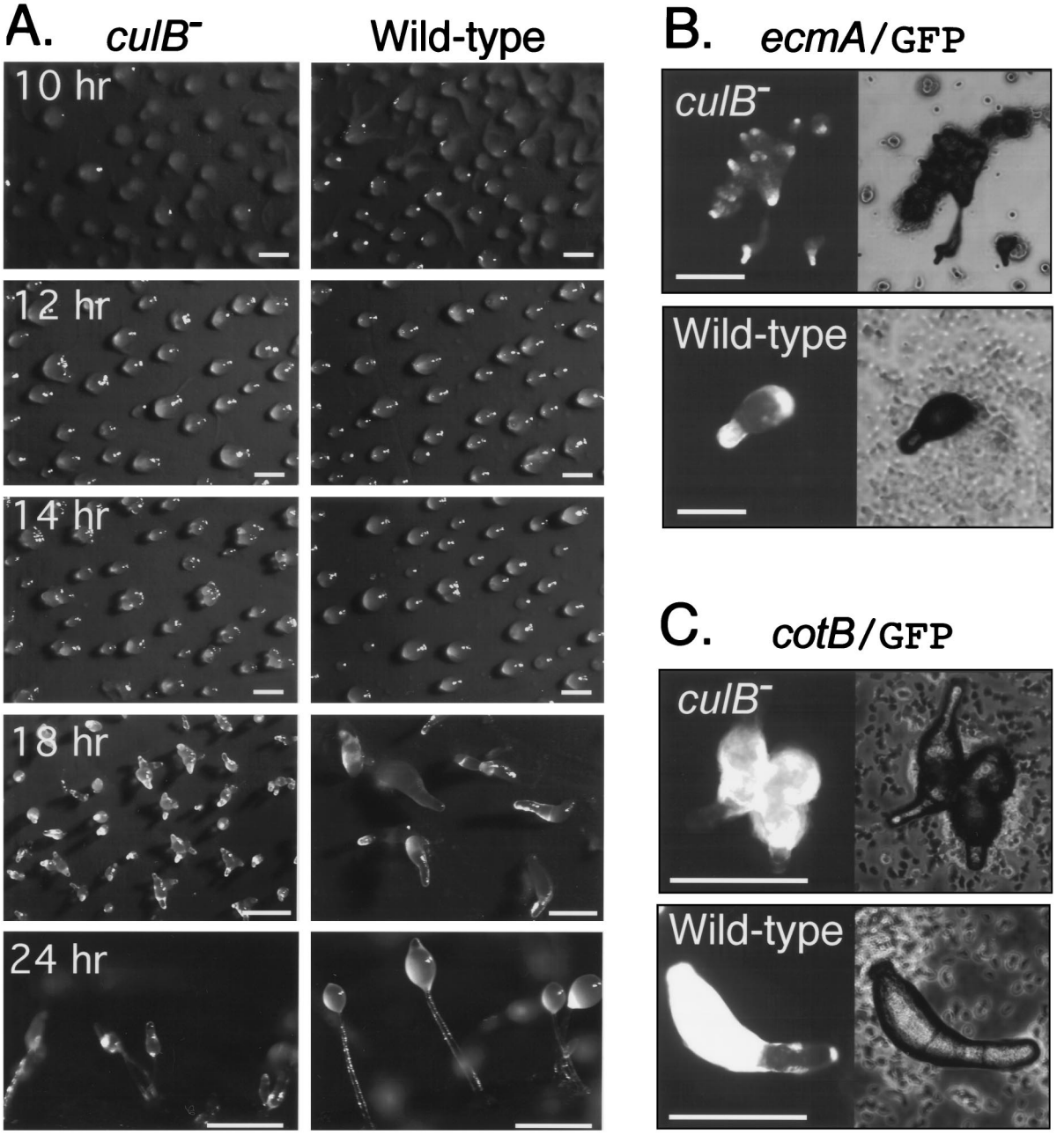



FIG. 2. Abnormal prestalk cell differentiation in *culB*$^-$ cells. (A) Cells were deposited on filters and allowed to develop, and photographs were taken at the indicated times. Bars, 0.5 mm. (B and C) Cells transformed with the reporter plasmids *ecmA*/GFP, a prestalk marker (B), or *cotB*/GFP, a prespore marker (C), were photographed after 18 h of development on filters.

cells were defective in slug formation and migration. When deposited on water agar plates and exposed to unidirectional light, *culB*$^-$ cells had a propensity to form aggregates and culminate rather than to form slugs. When slugs did form, they were smaller than wild-type slugs and not as mobile (Fig. 5). Wild-type slugs migrated about 1 to 2 cm in 24 h, while the *culB*$^-$ slugs all migrated less than 0.5 mm in 24 h.

**Stalk cell differentiation is uncoupled from morphogenesis in *culB*$^-$ *pkaR*$^-$ cells.** It has been shown that PKA is essential for prestalk cell differentiation (reviewed in reference 41). It

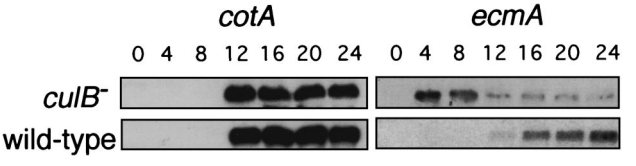



FIG. 3. Cell-type-specific gene expression in *culB*$^-$ mutants. The expression of *cotA* (a prespore gene) and *ecmA* (a prestalk gene) was examined by extracting RNA from cells at the indicated times of development and analyzing them on Northern blots. For each gene probe, the blots were hybridized in the same chamber, and representatives of three independent experiments are shown.

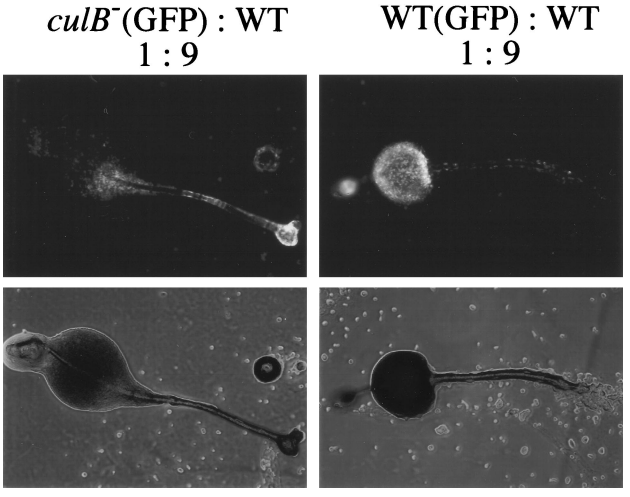



FIG. 4. *culB*$^-$ cells have a propensity to form stalk cells in *culB*$^-$/wild-type chimeras. Cells marked with an actin/GFP reporter plasmid were mixed with unmarked wild-type cells in a 1:9 ratio and allowed to develop into fruiting bodies. The actin/GFP-marked wild-type cells (WT) used as a control were mixed with unmarked wild-type cells.

also has been shown that CulA is required for the degradation of RegA, a cAMP phosphodiesterase that negatively regulates PKA activity, and that the *culA*$^-$ mutant phenotype can be rescued by activating PKA (46). To explore the possible functional relationship between CulB and PKA, we examined double mutants and found that the activation of PKA in *culB*$^-$ cells exacerbated their phenotype. PKA was activated in *culB*$^-$ cells by either overexpressing the catalytic subunit gene (*pkaC*) (by using an actin promoter to drive the expression of *pkaC*) or inactivating the regulatory subunit gene (*pkaR*). The *culB*$^-$ [*act15*/*pkaC*] cells and the *culB*$^-$ *pkaR*$^-$ cells displayed similar phenotypes in all of our assays, and so only the analyses of the *culB*$^-$ *pkaR*$^-$ cells are described below.

During development on filters, the *culB*$^-$ *pkaR*$^-$ cells accumulated into amorphous structures resembling the early stages of aggregation, but they did not form distinct aggregates (Fig. 6A). No spores were detected from cells deposited on filters for up to 48 h (see below). When plated with bacteria to allow growth and development, *culB*$^-$ *pkaR*$^-$ cells failed to aggregate (Fig. 6B). The colony surface remained flat for more than 5 days after the bacteria had been consumed. A circle of accumulated cells often appeared along the edge of the colonies, but they usually disappeared after the colonies expanded further. The control *pkaR*$^-$ cells aggregated and formed spherical structures within the colony, as expected (Fig. 6B). Although the *culB*$^-$ *pkaR*$^-$ cells did not aggregate after growth on bacteria, they did appear to differentiate since *ecmA*/GFP-positive cells were detectable in the colonies (data not shown). In addition, when cells were scraped from *culB*$^-$ *pkaR*$^-$ colonies and observed microscopically, vacuolated stalk-like cells were apparent. Calcofluor staining of these cells revealed cellulose-containing cell walls that are indicative of stalk cells (Fig. 6C). Other aggregation-deficient strains that we tested, such as PkaC-null or CRAC-null cells, do not produce cells that stain with Calcofluor (unpublished observations). However, no refractile ellipsoid spores were detected among *culB*$^-$ *pkaR*$^-$ cells, suggesting that the spore cell differentiation did not occur. We directly tested for viable spores after 48 h of development on filters. Some round, nonrefractile cells survived detergent treatment (~0.05% of input cells), but fewer than 1 in $10^5$ of the cells that were plated retained viability, as evidenced by colony formation on growth plates. These results suggest that the activation of PKA in the absence of CulB causes stalk cell differentiation without multicellular morphogenesis and without appreciable sporulation.

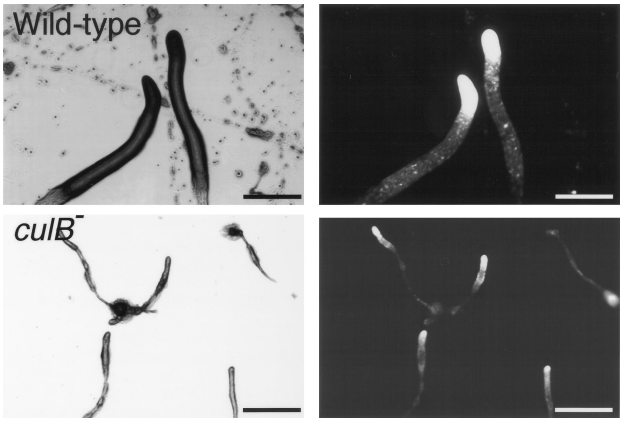



FIG. 5. The *culB*$^-$ mutant is defective in slug formation and migration. Cells were deposited on nonnutrient agar plates and kept in a dark chamber with unidirectional light for 24 h. Both wild-type and *culB*$^-$ mutant slugs, marked with *ecmA*/GFP, were visualized by bright-field (left) and fluorescence (right) microscopy. Bars, 0.25 mm.

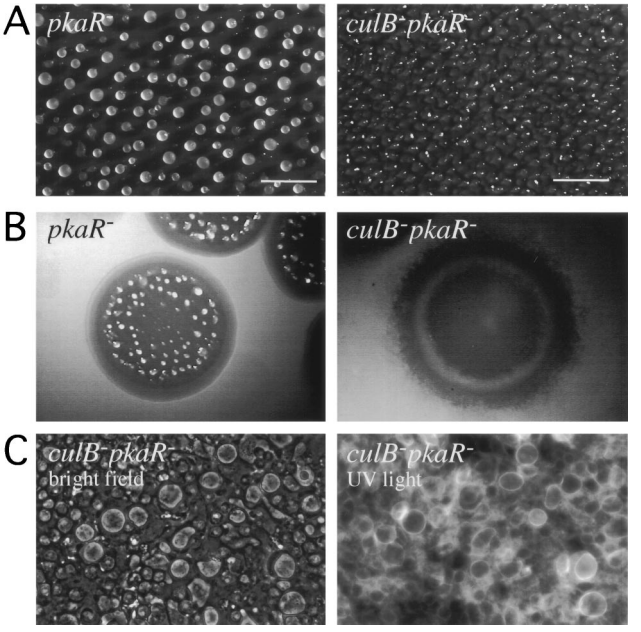



FIG. 6. The *culB*$^-$ *pkaR*$^-$ cells differentiate into stalk cells without obvious morphogenesis. (A) Phenotype of *pkaR* and *culB*$^-$ *pkaR*$^-$ cells after 24 h of development on filters. (B) Colonies of *pkaR* and *culB*$^-$ *pkaR*$^-$ cells photographed after 5 days. (C) *culB*$^-$ *pkaR*$^-$ cells scraped from the colony surface after 5 days and stained with Calcofluor. Bars, 1 mm.

**Prestalk differentiation is precocious and prespore differentiation is absent in *culB*$^-$ *pkaR*$^-$ cells.** To examine the progression of cell differentiation during *culB*$^-$ *pkaR*$^-$ cell devel-

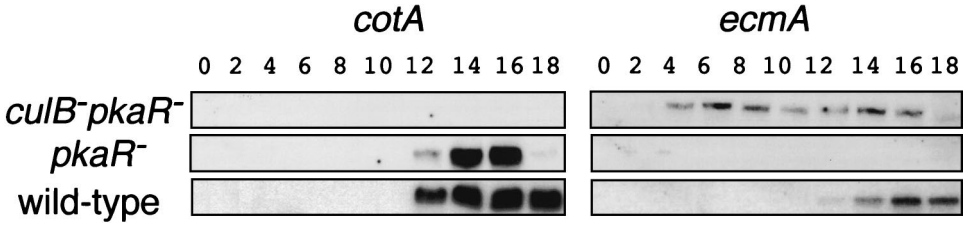



FIG. 7. Cell-type-specific gene expression in *culB*$^-$ *pkaR*$^-$ cells. Expression of the prestalk *ecmA* gene and the prespore *cotA* gene was examined by extracting RNA from cells at the indicated times of development (in hours) and analyzing 10 μg of RNA from each sample on Northern blots. For each gene probe, the blots were hybridized in the same chamber, and representatives of three independent experiments are shown.

opment, we studied the expression of cell-type-specific genes by Northern analysis. The prestalk *ecmA* gene was expressed in wild-type cells 12 h after starvation, as expected, when aggregates had formed. In *culB*$^-$ *pkaR*$^-$ cells, however, *ecmA* expression could be detected as early as 4 h after starvation, as in *culB*$^-$ cells. In the *pkaR*$^-$ control cells, *ecmA* expression could not be detected, and so compared to *pkaR*$^-$ cells, *ecmA* expression was also elevated in *culB*$^-$ *pkaR*$^-$ cells (Fig. 7). Our inability to detect *ecmA* expression by Northern analysis in *pkaR*$^-$ cells is reproducible, but it does not indicate that *ecmA* expression is absent in these cells. When the *pkaR*$^-$ cells carrying an *ecmA*/GFP reporter construct were examined after 36 h of development, about 5 to 10% of the cells were found to be GFP-positive stalk cells, indicating that the *ecmA* promoter is active over the course of development (data not shown). The prespore-specific gene *cotA* was expressed in wild-type cells and *pkaR*$^-$ cells normally after 12 h of starvation (Fig. 7). However, *cotA* was not expressed in *culB*$^-$ *pkaR*$^-$ cells. This is consistent with the observation that *culB*$^-$ *pkaR*$^-$ cells do not form spores and suggests that prespore cell differentiation does not occur in these cells.

***culB*$^-$ *pkaR*$^-$ cells are hypersensitive to DIF-1.** By incubation with cAMP and DIF-1 at low cell density, wild-type cells differentiate into cells with the characteristics of stalk cells in that they express prestalk- and stalk-specific genes, become vacuolated, produce cellulose, and lose viability (68). The addition of cAMP is thought to bring the cells to a DIF-responsive state, after which time they can be induced to form these stalk cells by treatment with DIF-1 (9). When *pkaR*$^-$ cells were incubated under submerged-culture conditions, they formed stalk cells after the addition of >0.2 nM DIF alone, without added cAMP (Fig. 8). These cells displayed a nonlinear response to DIF-1 concentrations between 0.5 and 10 nM and a maximal response to 10 to 100 nM DIF-1, where about half of the cells differentiated. These results with *pkaR*$^-$ cells are consistent with earlier findings (34, 63). However, about 17% of the *culB*$^-$ *pkaR*$^-$ cells formed stalk cells without added DIF-1 or cAMP (Fig. 8). Compared to *pkaR*$^-$ cells, a 5-fold-lower concentration of DIF-1 (0.1 nM) was required to elicit any response from the *culB*$^-$ *pkaR*$^-$ cells in this assay and about 50-fold less DIF-1 (0.2 versus 10 nM) induced a similar maximum percentage of stalk cell formation. The response of *culB*$^-$ *pkaR*$^-$ cells in this assay suggests that they are more sensitive to exogenous DIF-1 than are the control *pkaR*$^-$ cells. In similar DIF-1 assays, wild-type and *culB*$^-$ cells both required cAMP and DIF-1 for the formation of stalk cells and their response to DIF-1 was similar to that of *pkaR*$^-$ cells (data not shown).

If increased cellular synthesis of DIF-1 could account for the response of *culB*$^-$ *pkaR*$^-$ cells in the stalk cell induction assay, measurable DIF-1 should be detectable in the submerged culture media, assuming that excess DIF-1 is not completely dis-

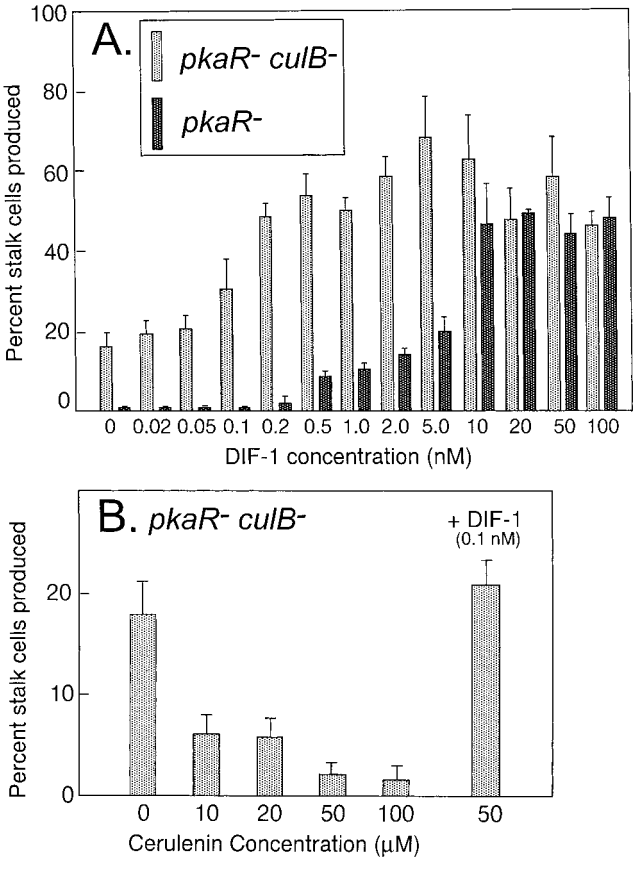



FIG. 8. *culB*$^-$ *pkaR*$^-$ mutants display an increased sensitivity to exogenous DIF in submerged culture. (A) *culB*$^-$ *pkaR*$^-$ cells and *pkaR*$^-$ cells were incubated at low density in submerged culture for 48 h. The percentage of vacuolated stalk cells was determined at different DIF concentrations. The mean and standard error of triplicate determinations are shown. (B) An inhibitor of DIF biosynthesis, cerulenin, inhibits the stalk-like cell differentiation of *culB*$^-$ *pkaR*$^-$ cells in submerged culture. *culB*$^-$ *pkaR*$^-$ cells were incubated for 48 h under the same conditions as in panel A in the presence of cerulenin.

solved in the cell membranes. We tested this by assaying for DIF-1 by its ability to convert *pkaR*$^-$ cells into stalk cells. Supernatants harvested from *culB*$^-$ *pkaR*$^-$ cells did not induce *pkaR*$^-$ cells to form stalk cells significantly more than did the control supernatant (the percentages of *pkaR*$^-$ test cells producing stalk-like cells were 1.5% ± 0.6% and 2.3% ± 0.5% for *pkaR*$^-$ and *culB*$^-$ *pkaR*$^-$ supernatant, respectively, in the absence of DIF after 48 h and 46% ± 2.0% and 43% ± 1.4% for *pkaR*$^-$ and *culB*$^-$ *pkaR*$^-$ supernatant, respectively, in the presence of 100 nM DIF after 48 h). This indicates that *culB*$^-$ *pkaR*$^-$ cells do not secrete large amounts of DIF-1 under these conditions (detection limit, 0.2 nM; Fig. 8A). It is generally accepted that the low-cell-density conditions of this assay prevent cell-cell communication during stalk cell formation since wild-type cells do require exogenous DIF-1 in order to differentiate. Any mutant with altered sensitivity to DIF-1 might challenge this assumption. For example, if 0.2 nM DIF-1 is normally achieved in this assay by mutant or wild-type cells, it would not have been detected in the assay described above, but it might influence stalk cell formation by *culB*$^-$ *pkaR*$^-$ cells. Therefore, we also tested whether *culB*$^-$ *pkaR*$^-$ cells required any DIF-1 that they secrete during the assay to induce their own differentiation. We incubated *culB*$^-$ *pkaR*$^-$ cells in submerged culture monolayers for 48 h and replaced the incubation buffer with fresh buffer every 5 h to remove any soluble DIF-1. About 15% of the *culB*$^-$ *pkaR*$^-$ cells still formed stalk cells. These results, taken together, suggest that secreted DIF-1 cannot account for the stalk cell differentiation of *culB*$^-$ *pkaR*$^-$ cells in the submerged-culture assay.

The assays above for secreted DIF-1 left open the possibility that endogenous DIF-1 that is retained by each cell supplies the DIF-1 needed for *culB*$^-$ *pkaR*$^-$ cells to differentiate into stalk cells. We tested whether the stalk cell differentiation of *culB*$^-$ *pkaR*$^-$ cells was DIF-1 dependent by examining their response to the polyketide synthase inhibitor cerulenin. Cerulenin has been shown to inhibit DIF-1 biosynthesis with a 50% inhibitory concentration of 1 to 2 μM and with maximal inhibition at concentrations of 30 to 100 μM (35). In the presence of 10 μM cerulenin, the formation of stalk cells by *culB*$^-$ *pkaR*$^-$ cells decreased about threefold and ≥50 μM cerulenin reduced the formation of stalk cells to levels roughly equivalent to that observed in the parental *pkaR*$^-$ cells without added DIF-1 (Fig. 8). The addition of DIF-1 together with cerulenin restores normal levels of stalk cell differentiation to the *culB*$^-$ *pkaR*$^-$ cells, demonstrating that the inhibition of stalk cell formation by cerulenin is not due to general cellular toxicity. These results suggest that endogenous DIF-1 synthesis is required for the differentiation of *culB*$^-$ *pkaR*$^-$ cells into stalk cells under these conditions.

## DISCUSSION

We isolated and characterized the *culB* gene and found that it is involved in the regulation of prestalk cell differentiation and morphogenesis. Our results suggest that prestalk cell differentiation is actively suppressed until the proper time during development so that the optimal proportion of stalk and spore cells results. Given the similarity of CulB to known cullin proteins, it may act in a ubiquitin-mediated protein degradation pathway that mediates this suppression, perhaps by limiting the level of a protein, or set of proteins, that promote stalk cell differentiation. There may also be mechanisms to suppress prespore differentiation since prespore cells can be induced to sporulate prematurely by PKA activation (34), but CulB does not appear to be directly involved since prespore cell differentiation is relatively unaffected in the *culB*$^-$ mutant.

The differentiation of most prestalk cells occurs as the aggregate forms, and they are initially intermingled with prespore cells and "undifferentiated" cells, but they can be recognized by their expression of the *ecmA* or *ecmB* genes (reviewed in reference 73). Over the next several hours, many of the prestalk cells sort to the apical tip of the aggregate, and it is thought that once there, they coordinate further morphogenetic movements and differentiation events. The precocious expression of *ecmA*, which marks all prestalk cell subtypes to some extent, indicates that precocious prestalk differentiation is occurring during the development of *culB* mutants. It is possible that the abnormal morphogenesis of both the *culB*$^-$ and *culB*$^-$ *pkaR*$^-$ mutants is caused by this inappropriate prestalk cell differentiation. However, it is not clear whether the defects are due to alterations in the signaling capacity of the prematurely appearing prestalk cells or to an accelerated rate of the appearance of one cell type (prestalk) relative to the other (prespore). The fact that these mutants do not appear to inhibit the development of wild-type cells in chimeras, combined with the fact that they differentiate into all cell types when developing on their own, is suggestive of a cell-autonomous proportioning defect.

The cell type-proportioning defect observed in *culB* mutants is likely to be the cause of the observed morphological defects during development. The relative sizes of the prestalk and prespore regions of the aggregate and slug are thought to be controlled dynamically (reviewed in reference 36). The multiple tips that form in *culB* mutant aggregates "organize" the construction of multiple small fruiting bodies from a single aggregate. The formation of multiple distinct prestalk tips could result from the formation of excess prestalk cells that are less sensitive to the normal mechanisms that control prestalk/prespore ratios or that maintain prestalk cells as a single distinct tissue. However, it is difficult to imagine how the precocious appearance of prestalk cells causes the block in aggregation observed in *culB*$^-$ *pkaR*$^-$ cells. The aggregation deficiency of the *culB*$^-$ *pkaR*$^-$ cells cannot be simply due to the immobility of stalk cells, since mature stalk cells are only observed well after the time cells would normally aggregate. Moreover, the motility of *culB*$^-$ *pkaR*$^-$ cells appears to be relatively normal since they participate in aggregation when they are mixed with wild-type cells (unpublished observations). The *culB*$^-$ cells expressed *ecmA* precociously, 4 h after development, but the morphological defects (e.g., multiple tips) appeared only after aggregation, when, we presume, PKA becomes most active. We expect that in *culB*$^-$ *pkaR*$^-$ cells PKA is active from the start of development, and this could explain why the defects manifest themselves earlier. Similar early phenotypes result when PkaC is expressed in *culB*$^-$ cells, again suggesting that active PKA causes the premature prestalk/stalk cell differentiation that we observe. These results imply that PkaC and CulB act in opposition to each other to control prestalk/stalk cell differentiation and morphogenesis: PkaC promotes prestalk cell differentiation, while CulB suppresses

it. However, this relationship is not a simple one since PKA activation appears to inhibit some aspects of prestalk cell differentiation, as evidenced by the severe reduction in *ecmA* expression in *pkaR*$^-$ cells (Fig. 7).

DIF-1 is one of the signals involved in prestalk differentiation in *Dictyostelium* (reviewed in reference 36). It induces prestalk gene expression and stalk cell formation among individual amoebae in vitro but is not required for these events in vivo (17, 57, 66, 67, 68, 74). Genetic ablation of DIF-1 production has demonstrated the requirement for DIF-1 in the differentiation of the pstO subclass of prestalk cells but not other prestalk cell types (66). Although the correct proportioning of prespore cells and prestalk cell subtypes probably results from the interplay of a number of signaling systems, DIF-1 appears to be a component of the signaling system that controls part of prestalk cell subspecialization. Under submerged low-cell-density conditions, DIF-1 induces stalk cell formation after cells have been incubated with cAMP (9). It is thought that PKA activation promotes DIF-1 induction of stalk cells since *pkaR*$^-$ cells can be induced to form stalk cells by exogenous DIF-1 alone, without the cAMP preincubation (63). The *culB*$^-$ *pkaR*$^-$ cells can differentiate into stalk cells without added cAMP or DIF-1, suggesting a cell-intrinsic propensity of these cells to differentiate into stalk cells. However, in vitro stalk cell differentiation in these cells still appears to depend on DIF-1, since cerulenin inhibits their differentiation. Since *culB*$^-$ *pkaR*$^-$ cells do not appear to overproduce DIF-1 and since these mutants are more sensitive to added DIF-1 than are *pkaR*$^-$ cells, the stalk cell formation of *culB*$^-$ *pkaR*$^-$ cells in submerged culture appears to be mediated by normal levels of endogenous DIF synthesis.

It is well known that growing cells are predisposed to become one cell type or the other during development (e.g., references 27 and 78). A number of physiological factors have been shown to bias cell differentiation, such as cell cycle-dependent differences in cytosolic calcium (8). For instance, vegetative cells grown with or without glucose preferentially differentiate as spores and stalk cells, respectively. Recent evidence suggests that these intrinsic biases are mediated by altered sensitivity to DIF-1 (67). It has been shown recently that a small amount of DIF-1 is produced early in development, about 5 h before tipped aggregates are formed (35). Our data are consistent with the notion that *culB*$^-$ *pkaR*$^-$ cells display a precocious prestalk cell differentiation due to an increased sensitivity to DIF-1 during early aggregation. To the extent that the submerged-culture bioassay accurately reflects differentiation responses in vivo, subnanomolar levels of DIF-1 could mediate the precocious prestalk cell differentiation that we observed. The genetic inference from these experiments is that CulB is normally required to delay prestalk cell differentiation by regulating, in part, the cellular response to DIF-1. Abnormal expression of any component of the DIF-1 response pathway might render cells more sensitive to DIF-1. A DIF-1-binding protein has been identified, and its activity peaks during aggregation when the pstO-prespore cell divergence occurs (32). It is tempting to speculate that this DIF-1-binding protein, or some other effector of the DIF response, is subject to regulation by CulB.

The ubiquitinylation of proteins through the SCF complex appears to play a major role in regulating development in *Dictyostelium*. Null mutations in genes that encode ubiquitin E2 conjugase, Ubc1, and an F-box protein, Mekkα, result in abnormal development (12, 14). Overexpression in wild-type cells of the part of MEKKα that contains the F-box motif and WD40 repeats results in a phenotype that is very similar to that of *culB*$^-$ mutants, a smaller fruiting body with extended spore head (12). The Mekkα-null cells also display a propensity to differentiate into stalk cells when mixed with wild-type cells (12). Thus, one attractive idea is that Mekkα and CulB are components of the same SCF complex that regulates cell differentiation in *Dictyostelium*. A *Dictyostelium* Skp1 homolog has been characterized, but null mutants have resisted isolation, suggesting that it is essential (62; C. West, personal communication). In the future, it will be important to establish whether Mekkα, CulB, and Skp1 function as a complex and to define possible target proteins for such a complex.

CulB is 40% identical to another putative cullin in *Dictyostelium*, CulA. The *culA* mutants form aggregates but fail to produce prespore or prestalk cells efficiently and eventually form large aggregates with multiple protrusions and many vacuolated stalk-like cells (46). Since CulA appears to control the stability of RegA, a cAMP-specific phosphodiesterase, it has been proposed that the *culA* mutant phenotype is caused by reduced intracellular cAMP levels and thus by reduced PKA activity. This is consistent with the observation that *culA* mutants are rescued by overexpressing PkaC (46). Curiously, *culA* mutants can be rescued by expressing CulA specifically in prestalk cells, which suggests that the lack of prespore cells in CulA-null cells is an indirect effect of the lack of prestalk cells. Thus, CulA, like CulB, appears to impinge more directly on prestalk cell differentiation at the cellular level. Finally, the absence of CulA in prestalk cells has effects roughly opposite to the absence of CulB. The simplest interpretation of these data is that CulA and CulB regulate the destruction of proteins that have opposing effects on prestalk cell differentiation. Regulating the relative activities of CulA- and CulB-mediated events would provide another level of control for coordinating cell differentiation and morphogenesis in *Dictyostelium*.

## ACKNOWLEDGMENTS

This work was supported by a grant to A.K. from the National Institutes of Health (GM52359).

We thank Negin Iranfar and Bill Loomis for sequencing *culB* genomic DNA and cDNA, and we thank Sijie Lu for many helpful discussions. We also thank Rick Firtel and Chris West for communicating results prior to publication.

## REFERENCES

1. **Abe, K., and K. Yanagisawa.** 1983. A new class of rapid developing mutants in *Dictyostelium discoideum*: implications for cyclic AMP metabolism and cell differentiation. Dev. Biol. **95:**200–210.
2. **Abel, T., P. V. Nguyen, M. Barad, T. A. Deuel, E. R. Kandel, and R. Bourtchouladze.** 1997. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell **88:**615–626.
3. **Adachi, H., T. Hasebe, K. Yoshinaga, T. Ohta, and K. Sutoh.** 1994. Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. Biochem. Biophys. Res. Commun. **205:**1808–1814.
4. **Anjard, C., S. Pinaud, R. R. Kay, and C. D. Reymond.** 1992. Overexpression of DdPK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*. Development **115:**785–790.
5. **Anjard, C., C. Zeng, W. F. Loomis, and W. Nellen.** 1998. Signal transduction pathways leading to spore differentiation in *Dictyostelium discoideum*. Dev. Biol. **193:**146–155.

6. **Aubry, L., M. Maeda, R. Insall, P. N. Devreotes, and R. A. Firtel.** 1997. The Dictyostelium mitogen-activated protein kinase ERK2 is regulated by ras and cAMP-dependent protein kinase (PKA) and mediates PKA function. J. Biol. Chem. **272:**3883–3886.
7. **Aubry, L., and R. A. Firtel.** 1999. Integration of signaling networks that regulate *Dictyostelium* differentiation. Annu. Rev. Cell Dev. Biol. **15:**469–517.
8. **Azhar, M., P. K. Kennady, G. Pande, M. Espiritu, W. Holloman, D. Brazill, R. H. Gomer, and V. Nanjundiah.** 2001. Cell cycle phase, cellular Ca2+ and development in *Dictyostelium discoideum*. Int. J. Dev. Biol. **45:**405–414.
9. **Berks, M., and R. R. Kay.** 1988. Cyclic AMP is an inhibitor of stalk cell differentiation in *Dictyostelium discoideum*. Dev. Biol. **126:**108–114.
10. **Chang, W. T., P. A. Thomason, J. D. Gross, and P. C. Newell.** 1998. Evidence that the RdeA protein is a component of a multistep phosphorelay modulating rate of development in *Dictyostelium*. EMBO J. **17:**2809–2816.
11. **Chen, T. L., W. A. Wolf, and R. L. Chisholm.** 1998. Cell-type-specific rescue of myosin function during *Dictyostelium* development defines two distinct cell movements required for culmination. Development **125:**3895–3903.
12. **Chung, C. Y., T. B. Reddy, K. Zhou, and R. A. Firtel.** 1998. A novel, putative MEK kinase controls developmental timing and spatial patterning in Dictyostelium and is regulated by ubiquitin-mediated protein degradation. Genes Dev. **12:**3564–3578.
13. **Chung, C. Y., S. Funamoto, and R. A. Firtel.** 2001. Signaling pathways controlling cell polarity and chemotaxis. Trends Biochem. Sci. **26**(9)**:**557–566.
14. **Clark, A., A. Nomura, S. Mohanty, and R. A. Firtel.** 1997. A ubiquitin-conjugating enzyme is essential for developmental transitions in *Dictyostelium*. Mol. Biol. Cell **8:**1989–2002.
15. **Dormann, D., J. Y. Kim, P. N. Devreotes, and C. J. Weijer.** 2001. cAMP receptor affinity controls wave dynamics, geometry and morphogenesis in *Dictyostelium*. J. Cell Sci. **114:**2513–2523.
16. **Drain, P., E. Folkers, and W. G. Quinn.** 1991. cAMP-dependent protein kinase and the disruption of learning in transgenic flies. Neuron **6:**71–82.
17. **Early, V. E., and J. G. Williams.** 1988. A *Dictyostelium* prespore-specific gene is transcriptionally repressed by DIF in vitro. Development **103:**519–524.
18. **Elledge, S. J., and J. W. Harper.** 1998. The role of protein stability in the cell cycle and cancer. Biochim Biophys. Acta **1377:**M61–M70.
19. **Feldman, R. M., C. C. Correll, K. B. Kaplan, and R. J. Deshaies.** 1997. A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell **91:**221–230.
20. **Firtel, R. A.** 1995. Integration of signaling information in controlling cell-fate decisions in *Dictyostelium*. Genes Dev. **9:**1427–1444.
21. **Firtel, R. A., and R. Meili.** 2000. *Dictyostelium*: a model for regulated cell movement during morphogenesis. Curr. Opin. Genet. Dev. **10:**421–427.
22. **Fisher, P. R.** 1997. Genetics of phototaxis in a model eukaryote, *Dictyostelium discoideum*. Bioessays **19:**397–407.
23. **Fisher, P. R.** 1997. Directional movement of migrating slugs, p. 437–452. *In* Y. Maeda, K. Inouye, and I. Takeuchil (ed.), *Dictyostelium*—a model system for cell and developmental biology. Universal Academy Press, Tokyo, Japan.
24. **Gaskins, C., A. M. Clark, L. Aubry, J. E. Segall, and R. A. Firtel.** 1996. The *Dictyostelium* MAP kinase ERK2 regulates multiple, independent developmental pathways. Genes Dev. **10:**118–128.
25. **Giloh, H., and J. W. Sedat.** 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. Science **217:**1252–1255.
26. **Ginsburg, G. T., and A. R. Kimmel.** 1997. Autonomous and nonautonomous regulation of axis formation by antagonistic signaling via 7-span cAMP receptors and GSK3 in *Dictyostelium*. Genes Dev. **11:**2112–2123.
27. **Gomer, R. H., and R. A. Firtel.** 1987. Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell-cycle phase. Science **237:**758–762.
28. **Harrington, B. J., and K. B. Raper.** 1968. Use of a fluorescent brightener to demonstrate cellulose in the cellular slime mold. J. Appl. Microbiol. **16:**106–113.
29. **Harwood, A. J., S. E. Plyte, J. Woodgett, H. Strutt, and R. R. Kay.** 1995. Glycogen synthase kinase 3 regulates cell fate in *Dictyostelium*. Cell **80:**139–148.
30. **Hopper, N. A., C. Anjard, C. D. Reymond, and J. G. Williams.** 1993. Induction of terminal differentiation of *Dictyostelium* by cAMP-dependent protein kinase and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. Development **119:**147–154.
31. **Hopper, N. A., A. J. Harwood, S. Bouzid, M. Veron, and J. G. Williams.** 1993. Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP-dependent protein kinase and evidence for its upstream regulation by ammonia. EMBO J. **12:**2459–2466.
32. **Insall, R., and R. R. Kay.** 1990. A specific DIF binding protein in Dictyostelium. EMBO. J. **9:**3323–3328.
33. **Kawata, T., A. Shevchenko, M. Fukuzawa, K. A. Jermyn, N. F. Totty, N. V. Zhukovskaya, A. E. Sterling, M. Mann, and J. G. Williams.** 1997. SH2 signaling in a lower eukaryote: a STAT protein that regulates stalk cell differentiation in *Dictyostelium*. Cell **89:**909–916.
34. **Kay, R. R.** 1989. Evidence that elevated intracellular cyclic AMP triggers spore maturation in *Dictyostelium*. Development **105:**753–759.
35. **Kay, R. R.** 1998. The biosynthesis of Differentiation-Inducing Factor, a chlorinated signal molecule regulating *Dictyostelium* development. J. Biol. Chem. **273:**2669–2675.
36. **Kay, R. R., P. Flatman, and C. R. L. Thompson.** 1999. DIF signalling and cell fate. Semin. Cell Dev. Biol. **10:**577–585.
37. **Kipreos, E. T., L. E. Lander, J. P. Wing, W. W. He, and E. M. Hedgecock.** 1996. cul-1 is required for cell cycle exit in *C. elegans* and identifies a novel gene family. Cell **85:**829–839.
38. **Kuspa, A., and W. F. Loomis.** 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. Proc. Natl. Acad. Sci. USA **89:**8803–8807.
39. **Kuspa, A., and W. F. Loomis.** 1994. Transformation of *Dictyostelium*—gene disruptions, insertional mutagenesis, and promoter traps. Methods Mol. Genet. **3:**3–21.
40. **Loomis, W. F.** 1996. Genetic networks that regulate development in *Dictyostelium* cells. Microbiol. Rev. **60:**135.
41. **Loomis, W. F.** 1998. Role of PKA in the timing of developmental events in *Dictyostelium* cells. Microbiol. Mol. Biol. Rev. **62:**684.
42. **Mann, S. K. O., W. M. Yonemoto, S. S. Taylor, and R. A. Firtel.** 1992. DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **89:**10701–10705.
43. **Mann, S. K. O., J. M. Brown, C. Briscoe, C. Parent, G. Pitt, P. N. Devreotes, and R. A. Firtel.** 1997. Role of cAMP-dependent protein kinase in controlling aggregation and postaggregative development in *Dictyostelium*. Dev. Biol. **183:**208–221.
44. **Manstein, D. J., H. P. Schuster, P. Morandini, and D. M. Hunt.** 1995. Cloning vectors for the production of proteins in *Dictyostelium discoideum*. Gene **162:**129–134.
45. **Mohanty, S., K. A. Jermyn, A. Early, T. Kawata, L. Aubry, A. Ceccarelli, P. Schaap, J. G. Williams, and R. A. Firtel.** 1999. Evidence that the *Dictyostelium* Dd-STATa protein is a repressor that regulates commitment to stalk cell differentiation and is also required for efficient chemotaxis. Development **126:**3391–3405.
46. **Mohanty, S., S. Lee, N. Yadava, M. J. Dealy, R. S. Johnson, and R. A. Firtel.** 2001. Regulated protein degradation controls PKA function and cell-type differentiation in *Dictyostelium*. Genes Dev. **15:**1435–1448.
47. **Mulligan, J. T., and S. J. Elledge.** 1994. The construction and use of cDNA libraries for genetic selections, p. 65–81. *In* J. R. Johnston (ed.), Molecular genetics of Yeast: a practical approach. Oxford University Press, Oxford, United Kingdom.
48. **Nellen, W., and R. A. Firtel.** 1985. High-copy-number transformants and co-transformation in *Dictyostelium*. Gene **39:**155–163.
49. **Patton, E. E., A. R. Willems, D. Sa, L. Kuras, D. Thomas, K. L. Craig, and M. Tyers.** 1998. Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. Genes Dev. **12:**692–705. (Erratum, **12:**3144.)
50. **Pitt, G. S., N. Milona, J. Borleis, K. C. Lin, R. R. Reed, and P. N. Devreotes.** 1992. Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. Cell **69:**305–315.
51. **Pukatzki, S., N. Tordilla, J. Franke, and R. H. Kessin.** 1998. A novel component involved in ubiquitination is required for development of *Dictyostelium discoideum*. J. Biol. Chem. **273:**24131–24138.
52. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
53. **Saxe, C. L., III, Y. M. Yu, C. Jones, A. Bauman, and C. Haynes.** 1996. The cAMP receptor subtype cAR2 is restricted to a subset of prestalk cells during *Dictyostelium* development and displays unexpected DIF-1 responsiveness. Dev. Biol. **174:**202–213.
54. **Scheffner, M., U. Nuber, and J. M. Huibregtse.** 1995. Protein ubiquitination involving an E1–E2-E3 enzyme ubiquitin thioester cascade. Nature **373:**81–83.
55. **Shaulsky, G., D. Fuller, and W. F. Loomis.** 1998. A cAMP-phosphodiesterase controls PKA-dependent differentiation. Development **125:**691–699.
56. **Shaulsky, G., A. Kuspa, and W. F. Loomis.** 1995. A multidrug resistance transporter serine protease gene is required for prestalk specialization in *Dictyostelium*. Genes Dev. **9:**1111–1122.
57. **Shaulsky, G., and W. F. Loomis.** 1996. Initial cell type divergence in *Dictyostelium* is independent of DIF-1. Dev. Biol. **174:**214–220.
58. **Simon, M. N., O. Pelegrini, M. Veron, and R. R. Kay.** 1992. Mutation of protein kinase-A causes heterochronic development of *Dictyostelium*. Nature **356:**171–172.
59. **Skoulakis, E. M., D. Kalderon, and R. L. Davis.** 1993. Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. Neuron **11:**197–208.
60. **Skowyra, D., K. L. Craig, M. Tyers, S. J. Elledge, and J. W. Harper.** 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell **91:**209–219.
61. **Sussman, M.** 1987. Cultivation and synchronous morphogenesis of *Dictyo-*

*stelium* under controlled experimental conditions. Methods Cell Biol. **28:**9–29.
62. **Tengumnuay, P., H. R. Morris, A. Dell, M. Panico, T. Paxton, and C. M. West.** 1998. The cytoplasmic F-box binding protein SKP1 contains a novel pentasaccharide linked to hydroxyproline in *Dictyostelium*. J. Biol. Chem. **273:**18242–18249.
63. **Thomason, P. A., D. Traynor, G. Cavet, W. T. Chang, A. J. Harwood, and R. R. Kay.** 1998. An intersection of the cAMP/PKA and two-component signal transduction systems in *Dictyostelium*. EMBO J. **17:**2838–2845.
64. **Thomason, P., D. Traynor, and R. R. Kay.** 1999. Taking the plunge-terminal differentiation in *Dictyostelium*. Trends Genet. **15:**15–19.
65. **Thomason, P. A., D. Traynor, J. B. Stock, and R. R. Kay.** 1999. The RdeA-RegA system: a eukaryotic phospho-relay controlling cAMP breakdown. J. Biol. Chem. **274:**27379–27384.
66. **Thompson, C. R., and R. R. Kay.** 2000. The role of DIF-1 signaling in *Dictyostelium* development. Mol. Cell **6:**1509–1514.
67. **Thompson, C. R. L., and R. R. Kay.** 2000. Cell-fate choice in *Dictyostelium*: intrinsic biases modulate sensitivity to DIF signaling. Dev. Biol. **227:**56–64.
68. **Town, C. D., J. D. Gross, and R. R. Kay.** 1976. Cell differentiation without morphogenesis in *Dictyostelium discoideum*. Nature **262:**717–719.
69. **Walsh, D. A., and S. M. Van Patten.** 1994. Multiple pathway signal transduction by the cAMP-dependent protein kinase. FASEB J. **8:**1227–1236.
70. **Wang, B., and A. Kuspa.** 1997. *Dictyostelium* development in the absence of cAMP. Science **277:**251–254.
71. **Wang, B., G. Shaulsky, and A. Kuspa.** 1999. Multiple developmental roles for CRAC, a cytosolic regulator of adenylyl cyclase. Dev. Biol. **208:**1–13.
72. **Williams, J. G.** 1991. Regulation of cellular differentiation during *Dictyostelium* morphogenesis. Curr. Opin. Genet. Dev. **1:**358–362.
73. **Williams, J. G.** 1997. Prestalk and stalk cell heterogeneity in *Dictyostelium*, p. 293–304. *In* Y. Maeda, K. Inouye, and I. Takeuchi (ed.), *Dictyostelium*—a model system for cell and developmental biology. Universal Academy Press, Inc., Tokyo, Japan.
74. **Williams, J. G., A. Ceccarelli, S. McRobbie, H. Mahbubani, R. R. Kay, A. Farly, M. Berks, and K. A. Jermyn.** 1987. Direct induction of *Dictyostelium* prestalk gene expression by D1F provides evidence that D1F is a morphogen. Cell **49:**185–192.
75. **Williams, J. G., K. T. Duffy, D. P. Lane, S. J. Mcrobbie, A. J. Harwood, D. Traynor, R. R. Kay, and K. A. Jermyn.** 1989. Origins of the prestalk-prespore pattern in *Dictyostelium* development. Cell **59:**1157–1163.
76. **Williams, J. G., and K. A. Jermyn.** 1991. Cell sorting and positional differentiation during *Dictyostelium* morphogenesis, p. 261–272. *In* J. Gerhart (ed.), Cell-cell interactions in early development. Wiley-Liss, New York, N.Y.
77. **Winston, J. T., P. Strack, P. Beer-Romero, C. Y. Chu, S. J. Elledge, and J. W. Harper.** 1999. The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. Genes Dev. **13:**270–283.
78. **Wood, S. A., R. R. Ammann, D. A. Brock, L. Li, T. Spann, and R. H. Gomer.** 1996. RtoA links initial cell type choice to the cell cycle in *Dictyostelium*. Development **122:**3677–3685.
79. **Yu, H., J. M. Peters, R. W. King, A. M. Page, P. Hieter, and M. W. Kirschner.** 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science **279:**1219–1222.
80. **Zachariae, W., A. Shevchenko, P. D. Andrews, R. Ciosk, M. Galova, M. J. Stark, M. Mann, and K. Nasmyth.** 1998. Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins. Science **279:**1216–1219.