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gdt1, a New Signal Transduction Component for Negative Regulation of the Growth–Differentiation Transition in *Dictyostelium discoideum*

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Discoidin I expression was used as a marker to screen for mutants affected in the growth–differentiation transition (GDT) of *Dictyostelium*. By *REMI* mutagenesis we have isolated mutant 2-9, an overexpressor of discoidin I. It displays normal morphogenesis but shows premature entry into the developmental cycle. The disrupted gene was denominated *gdt1*. The mutant phenotype was reconstructed by disruptions in different parts of the gene, suggesting that all had a complete loss of function. *gdt1* was expressed in growing cells; the levels of protein and mRNA appear to increase with cell density and rapidly decrease with the onset of development. *gdt1* encodes a 175-kDa protein with four putative transmembrane domains. In the C terminus, the derived amino acid sequence displays some similarity to the catalytic domain of protein kinases. Mixing experiments demonstrate that the *gdt1*$^-$ phenotype is cell autonomous. Prestarvation factor is secreted at wild-type levels. The response to folate, a negative regulator of discoidin expression, was not impaired in *gdt1* mutants. Cells that lack the G protein α2 display a loss of discoidin expression and do not aggregate. *gdt1*$^-$/*G*α*2*$^-$ double mutants show no aggregation but strong discoidin expression. This suggests that gdt1 is a negative regulator of the GDT downstream of or in a parallel pathway to Gα2.

INTRODUCTION

The process of a cell switching from proliferation to differentiation is of general importance not only for the development of multicellular organisms but also for the initiation of malignant transformation, in which this process is reversed. Similar to most higher eukaryotic cells, extracellular signals control the transition from growth to development in *Dictyostelium discoideum*. The elucidation of these signals and their pathways toward the switch in the genetic program may provide insights into general mechanisms for the initiation of differentiation.

The life cycle of *D. discoideum* consists of two distinct phases: growth and development, which can be easily manipulated in the laboratory. At the growth–differentiation transition (GDT), the discoidin I gene family is among the first to be activated and thus considered an excellent marker for the onset of differentiation (Endl *et al.*, 1996). Transcription of discoidin I is undetectable when cells feed on bacteria. When the food supply decreases, expression is induced and later down-regulated by the cAMP signaling cascade. Discoidin I is a facultative marker for the GDT: expression is not required for development, nor does discoidin expression lead to obligatory development. This is demonstrated by disc^{null} and disc^{over} mutants, which undergo relatively normal development: both display mature fruiting bodies, although disc^{null} cells do not stream but aggregate by random collision (Alexander *et al.*, 1983; Crowley *et al.*, 1985), whereas disc^{over} mutants frequently display a ragged colony shape and broad growth edges (U. Huitl and W. Nellen, unpublished observations). Furthermore, discoidin is continuously expressed at high levels during growth in axenic medium (Blusch *et al.*, 1995).

During exponential growth on bacteria, *Dictyostelium* cells continuously secrete a factor denominated prestarvation factor (PSF) into the extracellular medium (Clarke *et al.*, 1988). Cells measure the concentration of PSF in relation to signals provided by the bacterial food source and thus calculate the density of the population relative to the density of the remaining nutrients (Clarke *et al.*, 1992). Above a threshold level of the PSF:bacteria ratio (approximately three generations before the onset of starvation), low-level discoidin expression is initiated (Rathi *et al.*, 1991). Folate represses

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discoidin expression when added to axenically growing cells (Blusch and Nellen, 1994). Similarly, autoclaved bacteria can repress discoidin (Burdine and Clarke, 1995). When the food source is exhausted and cells stop growing, PSF production declines, and a strong secondary induction of discoidin occurs. This may be mediated by conditioned media factor (CMF), a second secreted factor (Jain *et al.*, 1992), which senses cell density and initiates differentiation or by some other signal. The inducing signaling pathway most likely involves the G-protein $\alpha 2$ (Blusch *et al.*, 1995), pianissmo (Chen *et al.*, 1997; K. Riemann and W. Nellen, unpublished observations), cytosolic regulator of adenylyl cyclase (CRAC), a yet unidentified adenylyl cyclase, and PKA (Endl *et al.*, 1996).

The recently characterized *yakA* gene (Souza *et al.*, 1998), which is also involved in the GDT, may be part of the positive pathway for discoidin and GDT regulation: YakA is required for the shutoff of growth stage genes and the induction of early developmental genes. PufA (Souza *et al.*, 1999) appears to be a translational inhibitor of PKA-C mRNA and should thus serve as a negative regulator of the GDT. PufA is down-regulated by YakA; a disruption of *pufA* can therefore partially rescue the *yakA*$^-$ phenotype.

Itoh *et al*. (1998) have described that overexpression of the calcium-binding protein calfumirin-1 promotes the switch from growth to differentiation possibly by induction of the cAMP receptor 1 gene. This may, however, be a later step in the GDT, because discoidin expression is down-regulated by extracellular cAMP via cAMP receptor 1 ~6 h after the onset of development. Down-regulation by the receptor is independent of G$\alpha 2$ but can be bypassed by Ca^{2+} (Endl *et al.*, 1996).

Several mutants with defects in discoidin regulation have been described (Alexander *et al.*, 1983; Wetterauer *et al.*, 1993). However, these mutants were generated by chemical mutagenesis, and it has not yet been possible to identify the molecular basis of the defect.

To further elucidate GDT signaling, we have used *REMI* (restriction enzyme-mediated integration; Kuspa and Loomis, 1992) to isolate mutants with defects in the expression of the discoidin I genes. Misexpression of discoidin can be monitored by colony blots using a monoclonal anti-discoidin antibody (Wetterauer *et al.*, 1993). Because colony blots are semiquantitative, expression above and below wild-type levels can be detected. From the identified mutants, the disrupted gene can be isolated. We have generated several *REMI* mutants, which displayed over- or underexpression of discoidin I. One of these was identified as a disruption in CRAC (K. Riemann and W. Nellen, unpublished data) and confirmed our previous results that CRAC was involved in the GDT (Endl *et al.*, 1996). This paper describes gdt1, a new signal transduction component, which is a negative regulator of discoidin expression and the GDT in *D. discoideum*.

MATERIALS AND METHODS

Cell Growth

D. discoideum Ax2 and the derived transformants were grown either in AX medium (Watts and Ashworth, 1970) or in suspension with *Klebsiella aerogenes* (KA) as a food source (for details, see Endl *et al.*, 1996). KA was grown for 3 d on standard bacterial medium (SM) agar plates at room temperature and then washed off with 30 ml of phosphate buffer. The resulting growth medium was termed 1× KA suspension.

To obtain single clones, ~50–200 *Dictyostelium* cells were suspended in 100 μl of phosphate buffer (20 mM sodium phosphate, pH 6.0) containing KA and plated on SM plates (Sussman, 1951). Plates were grown at 22°C for 3 d, and single clones were picked and transferred to new KA/SM plates or grown in AX medium with antibiotics (50 μg/ml ampicillin and 100 U/ml penicillin-streptomycin).

Differentiation Conditions

Vegetative cells were harvested from bacterial suspension cultures at a density of $<1 \times 10^6$ cells/ml and washed free of bacteria by differential centrifugation (1200, 1100, and 1000 rpm) in 20 mM phosphate buffer. Cells were resuspended in buffer at 2×10^7 cells/ml and allowed to develop in shaking suspension for 5 h. Axenically growing cells were harvested at 2×10^6 cells/ml, washed with phosphate buffer, resuspended at a density of 2×10^7 cells/ml, and developed in shaking suspension for 5 h.

For monitoring morphological development, cells were harvested from axenic culture at a density of 5×10^6, washed, and resuspended at 1×10^8 cells/ml. Cells (5×10^6) were spotted on phosphate-agar, developed at 22°C as described (Newell *et al.*, 1977), and observed microscopically.

REMI Mutagenesis

REMI mutagenesis was essentially done as described by Kuspa and Loomis (1992). The *ura*$^-$ strain DHI was used as the parent for *REMI* mutagenesis. DHI cells were grown in FM medium (Franke and Kessin, 1977; purchased from Life Technologies, Gaithersburg, MD) supplemented with 20 μg/ml uracil. Twenty micrograms of DIV-2 vector were linearized with *Bam*HI and electroporated into DHI cells together with 100 U of *Bam*HI at 2.5 kV/cm, 3.0 μF. After electroporation, cells were distributed on five Petri dishes, and transformants were selected in FM medium. When clones could be detected on the plates, cells were washed off, counted, and plated in association with KA on SM plates for cloning.

Colony Blot Screen for REMI Mutants

Clones (~0.5 cm diameter) on KA/SM plates were transferred onto nitrocellulose filters and treated as described previously (Wallraff and Gerisch, 1991). Discoidin expression was detected with the monoclonal antibody 80-52-13 (Wetterauer *et al.*, 1993) and a phosphatase-coupled secondary goat anti-mouse antibody (Dianova, Hamburg, Germany). After antibody detection with nitro blue tetrazolium, filters were counterstained with Ponceau S to detect all cellular proteins. Colonies displaying no discoidin expression and colonies showing stronger expression than wild-type cells were picked, recloned, and blotted again to confirm the mutant phenotype and its stability.

Genomic DNA Preparation and Southern Blot Analysis

Genomic DNA was prepared, digested with restriction enzymes as indicated, and blotted onto nylon membranes as described previously (Nellen *et al.*, 1987). Probes were radiolabeled by random priming as specified by the supplier (Stratagene, La Jolla, CA).

Isolation of a 3.7-kb Fragment of the gdt1 Gene from the REMI 2-9 Mutant

A 3.7-kb fragment was recovered from the 2-9 *REMI* mutant by plasmid rescue as described (Kuspa and Loomis, 1992). Genomic DNA from the mutant was digested with *Hin*dIII, circularized by ligation in a diluted solution, and transformed into *Escherichia coli*. A

plasmid (2-9 rescue) containing 3.7 kb of genomic sequence flanking one side of the vector insertion was recovered.

Reconstruction of gdt1 Mutants

A vector (2.9-Bs^R-*Xba*I) was constructed by inserting the Bs^R cassette from vector pUC Bs^R Δ Bam (Sutoh, 1993) into the *Xba*I site within the 3.7-kb fragment from 2-9 rescue. The vector was cut with *Bam*HI and *Bst*XI to generate the 3.7-kb fragment with the Bs^R cassette insert. The mixture of vector and fragment was transformed into Ax2 cells by electroporation (2.5 kV/cm, 3 μF). The resulting gene disruptants (L series) were selected by colony blot.

Similarly, the gdt1 gene was disrupted in the *Hin*dIII site (see Figure 2): the Bs^R cassette was ligated into the pGEM7Z+ vector as a *Hin*dIII–*Xba*I fragment, and then the 3.7-kb *Bam*HI–*Hin*dIII fragment was added. The vector was linearized with *Cla*I and electroporated into Ax2 cells. The gdt1 gene was disrupted by a single-copy integration of the entire vector, and the resulting disruptants (K-series) were screened by colony blot and confirmed by Southern blots with a ^{32}P-labeled 3.7-kb *gdt1* probe.

Two more series of gene disruptants (X and D series) were generated by homologous recombination as indicated in Figure 2. In the X series, the Bs^R cassette and the Psp 72 vetor were inserted into the *Xba*I site. In the D series, the Bs^R cassette and pGem3 vector were inserted into the gene such that 2.6 kb downstream of the *Xba*I site were deleted.

PCR

PCR from genomic DNA was performed in a reaction volume of 50 μl with 1 ng of DNA, 50 pmol of oligonucleotide primers, 25 μM dNTPs, and 2.5 U of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) in 1× PCR buffer (Boehringer Mannheim, Mannheim, Germany). PCR was done for 30 cycles at 94, 40, and 72°C for 1 min each. The first denaturing step was for 2 min; the last extension step was for 5 min.

For inverse PCR, 10 μg of genomic DNA from Ax2 were digested with *Bam*HI and *Bgl*II. After phenol-chloroform extraction, the DNA was dissolved in H_2O and set up for self-ligation in a volume of 100 μl. One microliter of the ligation mix was used for inverse PCR using the 5′ primer CCAATCAATGATAATGATCCTCCC and the 3′ primer AAAGTGAATCCTCGACAAG.

For cloning of the PCR products, the reaction mix was separated on an agarose gel, and the fragment was purified with the JETsorb DNA extraction kit (GenoMed, Beverly Hills, CA) and cloned into the T-cloning vector pUC 57 (MBI Fermentas).

RNA Isolation and Northern Blot Analysis

RNA was prepared and blotted as described previously (Maniak *et al.*, 1989). Antisense in vitro transcripts of the discoidin Iγ gene (Vauti *et al.*, 1990), the gdt1 gene, and the V4 gene (Singleton *et al.*, 1991) were used for hybridization. Blots contained equal amounts of total RNA as measured in a spectrophotometer and confirmed by ethidium bromide staining of rRNA after electrophoresis. Hybridization was performed at 56°C.

λgt11 Library Screening

Infection-competent *E. coli* Y1090 were mixed with 1 μl of a λgt11 library [made from poly(A)$^+$ RNA of vegetative Ax2 cells grown in bacterial suspension; a generous gift from H. Freeze, La Jolla Cancer Research Foundation, La Jolla, CA] at 5 × 10^6 pfu/ml and plated in soft agar on complete medium agar in 11 × 11-cm Petri dishes. Cells were incubated at 37°C until nearly complete lysis. DNA was then transferred to a nylon membrane and hybridized with a ^{32}P-labeled 3.7-kb *gdt1* gene probe. Positive plaques were picked, rescreened, and plaque purified. Inserts were cut out with *Eco*RI and cloned into pGEM 3Z (Promega, Madison, WI).

Expression and Purification of Recombinant D1

Nine hundred sixteen base pairs of the gdt1 gene (341–1257), denominated domain 1 (D1) were amplified from plasmid 2-9 rescue by PCR using the 5′ primer TTCATAGGGAGGATCATTATCATTG and the 3′ primer TGGACCTATTACCAATG. The PCR product was cloned into the pET15b vector (Novagen, Madison, WI). The resulting vector, D1-pET15b, was transformed into *E. coli* BL 21 cells (Novagen) for expressing D1 as a 6x His-tagged recombinant protein. Purification was performed under denaturing conditions (20 mM sodium phosphate, 8 M urea, and imidazole from 10 to 200 mM, pH 7.4) by using the BioLogic fast protein liquid chromatography system (Bio-Rad, Hercules, CA) and the His Trap kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The 36-kDa recombinant D1 eluted at 200 mM imidazol and was further purified by SDS-PAGE. Antisera were generated by immunizing rabbits with the recombinant D1 protein purified on SDS-PAGE (Ausubel *et al.*, 1995). Crude serum after the third boost was used for gdt1 detection on Western blots.

Western Blots

Total protein was prepared by lysing 1–5 × 10^7 *Dictyostelium* cells in 100–500 μl of Laemmli buffer (Laemmli, 1970). Equal amounts of protein were separated on a 12% discontinuous polyacrylamide gel and blotted by semidry transfer (Bjerrum, 1986) for discoidin detection or on a 5–12% gradient SDS-PAGE and blotted by "tank transfer" (Sartorius, Göttingen, Germany) for detection of the gdt1 protein. A discoidin monoclonal antibody (Wetterauer *et al.*, 1993) and a peroxidase-coupled secondary goat anti-mouse antibody (Dianova) were used for detection of discoidin; the polyclonal antiserum against recombinant D1 and a phosphatase-coupled goat anti-rabbit secondary antibody (Dianova) were used for detecting the gdt1 protein.

β-Galactosidase Assays

Cells were harvested at a density of ~1 × 10^6 and collected by centrifugation. Cells were lysed by shock freezing in liquid nitrogen and thawing. Cell debris was pelleted, and β-galactosidase activity was measured in the supernatant by using 2-nitrophenyl-β-D-galactopyranoside as a substrate (Bühl and MacWilliams, 1991). Activity from contaminating *Klebsiella* was negligable.

PSF Measurements

To measure PSF production, Ax2 and *gdt1*$^-$ cells were grown in KA suspension to a density of 5 × 10^6 cells/ml. Residual bacteria and cells were removed by centrifugation, and fresh KA were resuspended in the conditioned buffer. *Dictyostelium* DAG cells, which expressed β-galactosidase under the control of the discoidin Iγ promoter (Wetterauer *et al.*, 1993), were inoculated into the conditioned medium and grown to a maximum density of 10^6 cells/ml. To determine background activity, DAG cells were also grown in fresh medium. β-Galactosidase activity was determined as described above.

RESULTS

Identification of the REMI Mutant 2-9

During vegetative growth on bacteria, discoidin expression in wild-type Ax2 cells is below the detection level. In colony blots, this results in an outer ring, which is stained red by Ponceau S but not by the anti-discoidin antibody. With the onset of development, discoidin expression is induced, resulting in antibody staining of an inner ring of preaggregation cells. Even though transcription is down-regulated in later development, the discoidin protein is stable and can be

detected in late stages. Because colony blots are semiquantitative, overexpression mutants, which display stronger antibody staining than the wild type, can be identified.

The 2-9 mutant was detected in a *REMI* screen as a discoidin overexpressor (Figure 1A). In contrast to wild-type colonies, discoidin protein was found in cells beyond the visible border of the colony, i.e., in growing cells that have sufficient supply of nutrients. In addition, 2-9 mutant cells aggregated close to the growing edge and even on the bacteria lawn. This resulted in a ragged colony shape compared with the smooth edge observed in wild-type clones. (Figure 1B). A similar phenotype was observed in the discoidin overexpression mutants isolated by Alexander *et al.* (1983) (W. Nellen and U. Huitl, unpublished observations).

In wild-type Ax2 cells, discoidin expression is induced approximately three generations before the onset of starvation and then increases precociously (Wetterauer *et al.*, 1995). Cells of the wild type and of a *gdt1*$^-$ mutant (L8, see below) were grown in bacterial suspension culture and harvested at densities of 1×10^6, 2×10^6, and 5×10^6 cells/ml. Expression of discoidin I was monitored on Northern blots using an in vitro transcript of the discoidin Iγ gene as a hybridization probe. Figure 1C shows significant amounts of mRNA at low cell density in the mutant but not in the wild type. Western blots (see Figure 8; our unpublished data) also confirmed that discoidin was significantly increased in growing *gdt1*$^-$ cells compared with the Ax2 wild type.

To further confirm premature induction of discoidin expression, Ax2 and *gdt1*$^-$ cells were grown in *Klebsiella* suspensions of different densities (0.5, 1.5, and 3×) to a titer of 10^6 cells/ml. In wild-type cells, a low supply in nutrients induces discoidin expression at lower cell densities, whereas a high supply shifts expression to higher cell densities. As expected, discoidin expression was only detected at the reduced (0.5×) KA concentration in wild-type cells. For *gdt1*$^-$ cells, strong expression was still observed even at 3× bacterial concentration (Figure 1D). Nevertheless, the amounts of discoidin protein were reduced with increasing density of the food source. The data demonstrated that *gdt1*$^-$ cells could still sense the concentration of the food source but were less sensitive.

It was possible was that *gdt1*$^-$ cells produced more PSF and thus overestimated their own population density. We prepared conditioned buffer from *gdt1*$^-$ and from Ax2 cells and used this to grow DAG cells. Discoidin promoter activity was measured by β-galactosidase assay. As expected, the crude PSF preparation induced β-galactosidase in comparison with fresh buffer. Conditioned medium from high-density cells had a stronger effect than that from lower-density cells (our unpublished results). No significant difference was, however, found between conditioned buffer from Ax2 and *gdt1*$^-$ cells (Figure 1E). This indicated that premature discoidin induction in the mutant strain was not due to an overproduction of PSF.

To see whether the cell cycle with the onset of starvation was affected by the mutation, we monitored cell density in submerged cultures and in suspension cultures after transfer of the cells from bacterial suspension into phosphate buffer. For both *gdt1*$^-$ and Ax2 cells we found that approximately half of the cells completed one round of cell division within the first 2 h in starvation buffer; after that, cell numbers remained constant for at least another 2 h (our unpublished data).

Taken together, the data demonstrate that the mutant prematurely entered the GDT (as defined by discoidin expression) at low cell densities. This was not due to overexpression of PSF but rather to a less-sensitive measuring of the food source. Cell division after the onset of starvation was not changed in the mutant strain compared with wild type.

Reconstruction of the 2-9 Phenotype in Ax2 Cells

Part of *gdt1* was isolated from mutant 2-9 by plasmid rescue using *Hin*dIII-digested genomic DNA. Thus, one flanking genomic sequence of the *gdt1* gene of 3.7 kb could be cloned (Figure 2). The insert was used as a hybridization probe on a genomic Southern blot containing *Hin*dIII-digested DNA from strains DH1, Ax2, 2-9, and a reconstructed *gdt1*$^-$ mutant, L8. The 11.2-kb wild-type fragment in Ax2 DNA, the 6.5-kb fragment in 2-9 cells, and two fragments of 4.5 and 8.1 kb in the reconstructed mutant L8 (see below) agreed with the disruptions depicted in Figure 2 (our unpublished data).

The mutant phenotype was reconstructed in several ways: in the L series, the genomic sequence was disrupted in the *Xba*I site; the K series contained a duplication and a disruption at the genomic *Hin*dIII site; and in the D series 2.6 kb of coding sequence were deleted. X series transformants were similar to the L series in that they were disrupted at the *Xba*I site, but they contained a complete plasmid as an insert. From all transformations, several discoidin overexpressor clones were identified by colony blot and found to be identical in phenotype to the original mutant 2-9. Further analysis by Southern blot confirmed the disruption of *gdt1*. The reconstructed mutants differed from the original in that they were in the Ax2 background and that they carried the blasticidine resistance cassette instead of the *pyr5-6* gene. Furthermore, because the *Bam*HI site and the *Hin*dIII site are located at opposite ends of the 3.7-kb gene fragment, and all disruptions resulted in the same phenotype, this suggested a continuous gene over this stretch of DNA. For most of the further experiments L8, a clone from the L series, was used because it had a short insert and was in the genetic background of the common laboratory strain Ax2.

Sequence Analysis of the gdt1 Gene

Sequence analysis revealed an open reading frame (ORF) over the entire 3.7-kb fragment, but no initiation or termination codon was found. Further gene sequence was obtained by plasmid rescue with *Bgl*II from X series mutants. The fragment contained a 191-bp ORF in frame with the 3.7-kb fragment and started with an ATG. Surprisingly, two other short ORFs, potentially encoding peptides of five and six amino acids, preceded the ORF of *gdt1* (see DISCUSSION).

Several approaches were used to isolate the 3′ end of *gdt1*: cDNA library screening, inverse PCR, and plasmid rescue from D series disruptants using *Sma*I–*Eco*RV digests. All fragments confirmed previous sequence and/or added new data. The continuous ORF was closed with a TAA stop codon 755 bp downstream of the *Hin*dIII site.

Potential AATAAA polyadenylation signals were detected 29, 1003, 1051, 1233, 1380, and 1634 downstream of the stop codon. The last poly(A) signal was probably used, because it agreed with the mRNA size in Northern blots and

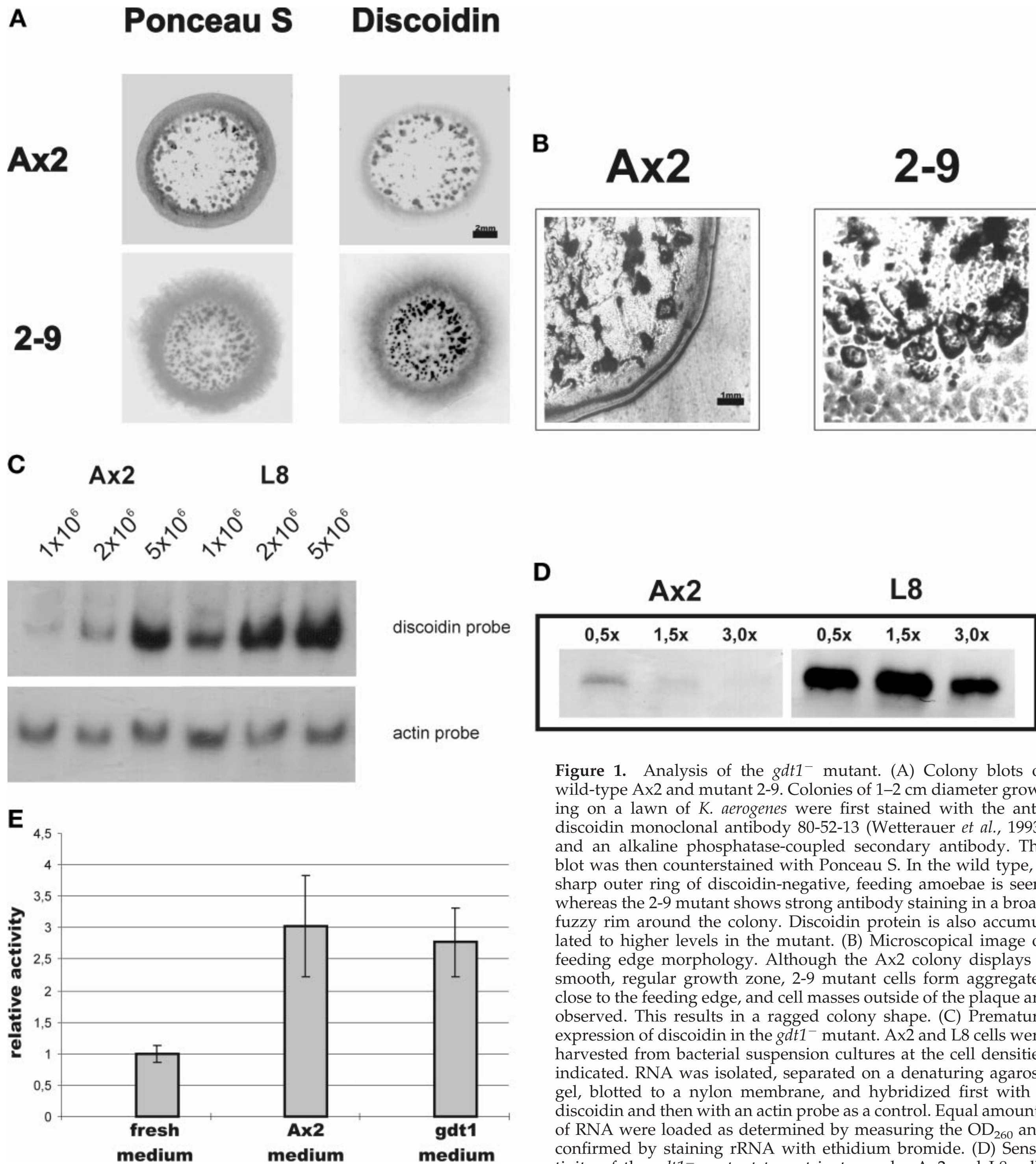

Figure 1. Analysis of the *gdt1*$^-$ mutant. (A) Colony blots of wild-type Ax2 and mutant 2-9. Colonies of 1–2 cm diameter growing on a lawn of *K. aerogenes* were first stained with the anti-discoidin monoclonal antibody 80-52-13 (Wetterauer *et al.*, 1993) and an alkaline phosphatase-coupled secondary antibody. The blot was then counterstained with Ponceau S. In the wild type, a sharp outer ring of discoidin-negative, feeding amoebae is seen, whereas the 2-9 mutant shows strong antibody staining in a broad fuzzy rim around the colony. Discoidin protein is also accumulated to higher levels in the mutant. (B) Microscopical image of feeding edge morphology. Although the Ax2 colony displays a smooth, regular growth zone, 2-9 mutant cells form aggregates close to the feeding edge, and cell masses outside of the plaque are observed. This results in a ragged colony shape. (C) Premature expression of discoidin in the *gdt1*$^-$ mutant. Ax2 and L8 cells were harvested from bacterial suspension cultures at the cell densities indicated. RNA was isolated, separated on a denaturing agarose gel, blotted to a nylon membrane, and hybridized first with a discoidin and then with an actin probe as a control. Equal amounts of RNA were loaded as determined by measuring the OD_{260} and confirmed by staining rRNA with ethidium bromide. (D) Sensitivity of the *gdt1*$^-$ mutant to nutrient supply. Ax2 and L8 cells were grown in 0.5, 1.5, and 3× concentrated KA suspension and harvested at a density of 10^6 cells/ml. Total protein was separated by SDS-PAGE, and discoidin was detected with the specific monoclonal antibody. Equal amounts of protein were loaded. (E) PSF production of Ax2 and *gdt1*$^-$*dt1*$^-$ cells. Ax2 and *gdt1*$^-$ cells were grown in KA suspension to 5×10^6 cells/ml; the conditioned buffer was used to grow DAG cells; and β-galactosidase activity was measured when a density of 10^6 cells/ml was reached. As a control, DAG cells were grown in medium with fresh buffer. β-Galactosidase activity is given relative to the activity in fresh medium (set to 1). The average of three experiments is shown.

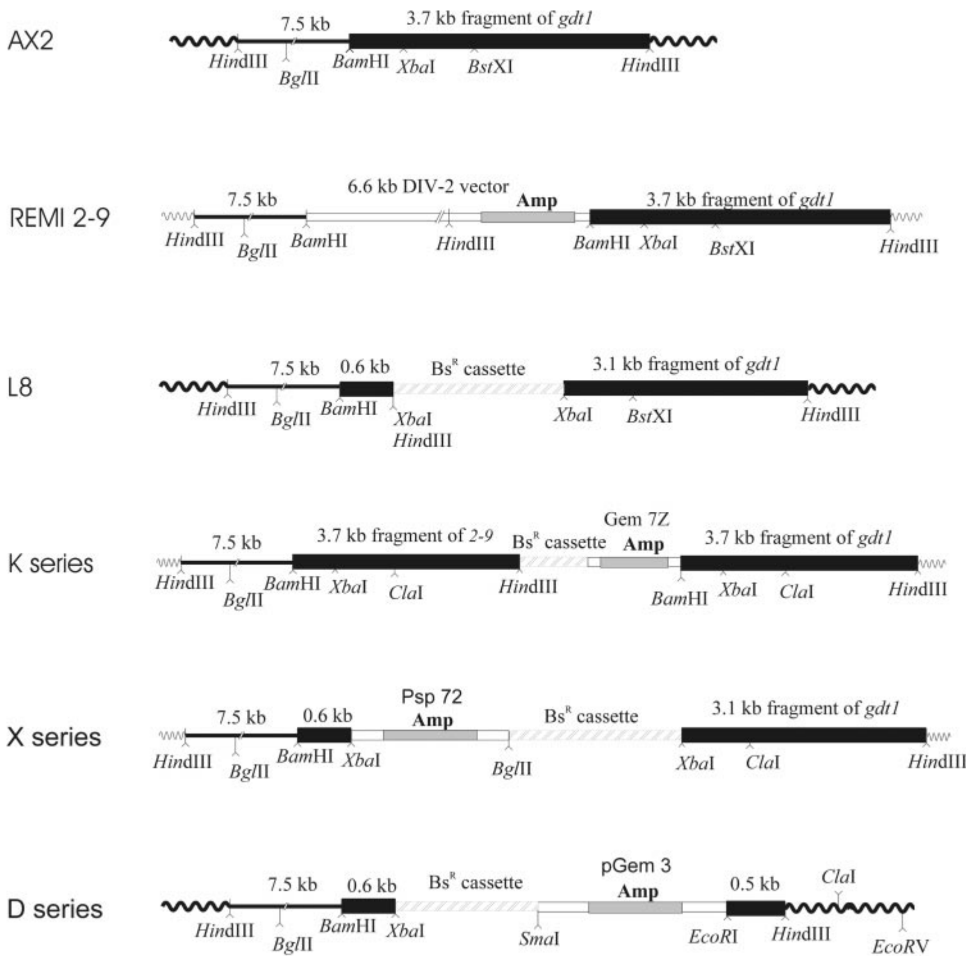

Figure 2. Genomic organization of the *gdt1* gene. *REMI* 2-9 was the original *REMI* mutant generated by insertion of the DIV-2 vector into the *Bam*HI site of the *gdt1* gene (192 bp after the ATG start condon). The right flank of 3.7 kb was isolated by plasmid rescue. The L8 strain was generated by homologous recombination with the 3.7-kb fragment containing the Bs^R cassette inserted into the *Xba*I site. The K series mutants were generated by insertion of a vector containing the 3.7-kb fragment, the Bs^R cassette, and pGem7Z into the *Hin*dIII site. The X series mutants were generated by homologous recombination with the 3.7-kb fragment containing the Bs^R cassette and plasmid Psp72 inserted into the *Xba*I site. In the D series mutants, a 2.6-kb deletion was introduced by replacing the internal *Xba*I–*Eco*RI fragment within the 3.7-kb fragment with a pGem3 vector containing the Bs^R cassette. The 5′ end of gdt1 was isolated by plasmid rescue from an X series mutant. The 3′ end was isolated from a D series mutant and confirmed by a cDNA clone and inverse PCR.

a corresponding fragment was obtained by 3′-rapid amplification of cDNA ends (our unpublished data).

Within the putative 3′ untranslated region, several palindromes were found, which may serve as RNA destabilization elements: a potential stem-loop–destabilizing element (Brown *et al.*, 1996) $_{4922}$UUGGGAC-$_{4948}$GUCCCAA, is located 123 bp after the stop codon, and many UUAUUUAU and CCAA (or UUGG) repeats are found. Preliminary data showed that the 3′ truncated *gdt1* mRNA in the L8 mutant accumulated to higher levels and appeared to be more stable (Figure 3; B. Wetterauer, personal communication).

Overall, almost 12 kb of the gdt1 gene locus have been isolated. These include 4683 bp of the gdt1 coding region, 1895 bp of 3′ flanking region, and 113 bp of 5′ flanking region (European Molecular Biology Laboratory [EMBL] database, accession number AJ000992). Approximately 2.8 kb upstream of the gdt1 gene, an ORF encoding a putative cationic amino acid transporter was detected in the opposite orientation (EMBL database, accession number AJ005263). Still further upstream there was a 1114-bp ORF encoding the 3′ end of a putative glycoprotein with some similarity to csA (EMBL database, accession number AJ005262; Noegel *et al.*, 1986).

The gdt1 Gene Is Expressed in Vegetative Cells Only and Encodes a 175-kDa Membrane Protein with a Putative Kinase Domain

gdt1 is weakly transcribed to an ~7-kb mRNA during growth. With the onset of development, the mRNA was rapidly lost (Figure 3A). Interestingly, the L8 mutant displayed a truncated mRNA of 1.2 kb, which appeared more abundant than the full-size message (Figure 3B), suggesting that the full-length mRNA contained destabilizing elements, which were lost in the shorter message. The expression pattern of *gdt1* was also confirmed by Western blots (Figure 3C) with a polyclonal antiserum directed against the recombinant D1 peptide (see Figure 4A). The 175-kDa gdt1 protein was only detected in Ax2 vegetative cells but not after 5 h of development and not in the L8 mutant.

The *gdt1* gene product contains 1561 amino acids (Figure 4A) and has a calculated pI of 7.15 and a calculated molecular mass of 175,292 Da. Analysis by the EMBL TMpred program predicted four transmembrane domains (TM1–TM4) with the direction of N-i-o-i-o-i-C as indicated in Figure 4A. Western blotting of fractionated cells confirmed the membrane localization of the gdt1 protein (our unpublished results).

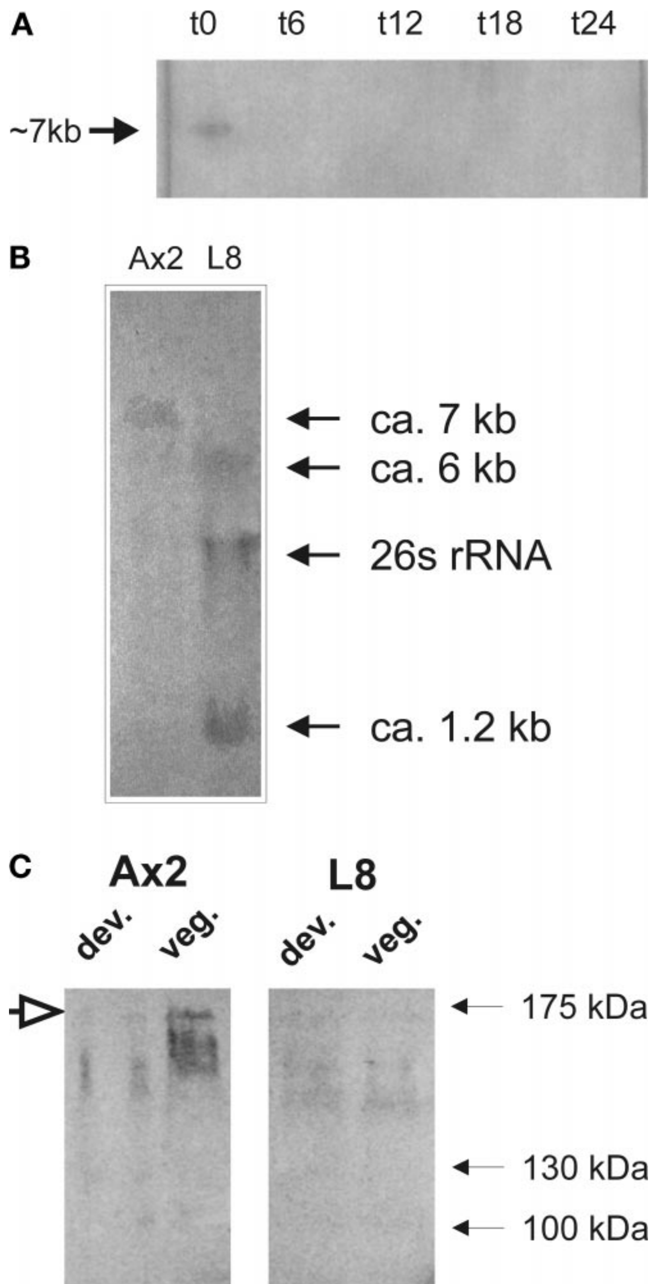

Figure 3. Expression of the *gdt1* gene. (A) Transcription of *gdt1* in Ax2 cells during development. Ax2 cells were harvested from axenic growth at 1×10^6, washed, and plated for filter development. Cells were taken at the indicated times for RNA extraction. Twenty-five micrograms of total RNA were used for each time point, separated on a denaturing gel, blotted, and hybridized with a ^{32}P-labeled 3.7-kb gdt1 gene probe. The blot was exposed for 4 d on a phosphorimager plate. An mRNA of ~7 kb was detected only in growing cells. (B) L8 cells produce a truncated transcript of the *gdt1* gene. RNA was prepared from Ax2 and L8 cells during growth, separated on a denaturing gel, blotted, and hybridized with a ^{32}P-labeled 3.7-kb *gdt1* gene probe. Although Ax2 cells showed the 7-kb message, transcripts of variable size (probably read-through products caused by inefficient termination) with a predominant band at 1.2 kb were detected in L8 cells. Note that the 1.2-kb transcript is more abundant than the 7-kb mRNA. (C) The gdt1 protein is only expressed during growth. Ax2 and L8 cells were grown in bacterial suspension to a density of 1×10^6 cells/ml, harvested, washed, and set up for development. One hundred micrograms of total protein were extracted from growing cells (veg.) and 5 h after development (dev.). Samples were separated on 7.5% SDS-PAGE, blotted, and incubated with the polyclonal antiserum directed against the recombinant D1 peptide. A 175-kDa band was detected in vegetative Ax2 cells but not in L8 cells. The background smear at ~150 kDa may be degradation products of the protein, because it is greatly reduced in the mutant.

Comparisonof gdt1 with different databases indicated similarity of the C-terminal 320 residues with the catalytic domain of protein kinases (Figure 4B). When the repetitive sequence $N_2SN_2SN_{20}SN_2SN_2S_3$, was removed for the analysis (Figure 4A, *) good alignments were found with all 11 subdomains (reviewed by Hanks and Hunter, 1995) in the serine/threonine and tyrosine kinase families; however, some highly conserved signatures of kinases were missing. The best similarity was found with a nonreceptor serine/threonine kinase in *Entamoeba* (ENHPSTK-1), a small 290-amino-acid cytoplasmic protein of the mos family (Lohia and Samuelson, 1994). However, this was mostly due to sequence before the "glycine-rich loop," which is usually not conserved among protein kinase.

The gdt1⁻ Mutant Displays Slow Growth in Bacteria and Accelerated Development

In bacterial suspension culture, L8 mutant cells grew with a generation time of 4.5 ± 0.3 h, whereas for wild-type Ax2 cells the generation time was 3.3 ± 0.3 h. However, when cells were grown in axenic medium, a similar generation time of 8 h was observed for both L8 and Ax2 (our unpublished data). This would be consistent with the assumption that the mutant has a defect in sensing of the bacterial food source.

To further investigate the defect in the developmental process, a timing experiment was performed with the *gdt1*$^-$ mutant and wild-type Ax2 as a control. Development on phosphate-agar plates was monitored microscopically. As shown in Figure 5, L8 cells aggregated earlier than the wild type, whereas the rest of development was normal. In contrast, KP4 cells, which overexpress the PKA catalytic subunit (Anjard *et al.*, 1992), aggregated almost normally but then rapidly passed through tipped aggregates and formed slugs (our unpublished results). We have also monitored earlier stages by timing morphological changes in cells starved in submerged culture. *gdt1*$^-$ cells elongated ~2 h earlier than Ax2 cells under these conditions (our unpublished data). Taken together, this demonstrated that disruption of *gdt1* specifically accelerated the GDT, whereas overexpression of PKA predominantly affected late development.

The gdt1⁻ Mutation Is Cell Autonomous

To see whether gdt1 function was cell autonomous, mixing experiments were performed with L8 and DAG cells, which expressed β-galactosidase under the control of the discoidin Iγ promoter (Wetterauer *et al.*, 1993). *gdt1*$^-$ and DAG cells were grown in bacterial suspension to a density of $<10^6$,

1 MKYRVGNGGIPIYKINVLCVEGTLEIPE**GISFFNVGALFILPGGVL**NSKSSIRFTDLDPY
61 NSKMD**PFNFFPGMMVLGGSLSLIG**EKKRIFQATRIDDYQLQIEDFKKIGSLTNNIYLGSK
121 VTIYSQQISEGQTCSFSFGASNDKINLTSSSSSSLRGTNCLPISKNDKNIIVHFNCKYLL
181 GGSSESFIDSTSTVGSSIYITGDSQVQIENFTLDSIGKTTNKLYNDTKLIFSNDKPNQVI
241 DIIKGENQRFRNSLYIEFSNSVVIKGCAIIDRVKESRAPLIFVSSNVSLSESLIVSKSGS
301 NLIAQYGTEFIKSKLNHYFLIPPLPQPFGLNSPINFPSPFSMDYGFEGNGIYSLSPN**VQS**
361 **INDTFISQLIALNFNFIG**NRSIITGFDNDCYSPCTNTSILFSNLIQYPVDFKINNSTYFY
421 KLNNNNNDNNNNTNNFNLLNINNENNNNDDNNNNNNSQNHYLLNINNNGNSPGRFFTIKD
481 LVASDTVSAYLPNSALVFNNLNATENFSFNGTVSRLDFINSSFNTNLTSTQKFFDETTTT
541 VTNFQNTYIYGSPETTEPPEKVFGSSITPIYYFSKSVLDSLKVETVYPNEPHKMFFNSSV
601 SLSVKLNNMGLKTPIICISSSNGMESKEVQYNPISTKCSFLYVSEKLGNHNIRVTIKNEY
661 SKDSSYYFIIDFPIITVYGQSLFNAGWQMIDPTSTTIIPTPTPTSTSTSTSTSTPTTTNN
721 NQLVNDNNNKINELDGLKFQGGCIKTLGCQLSSNAKYVGGLPNVTASSTLNSLFSWGITS
781 DVQYDPVIIDLFINKSIATLQVQLFFTFYKPIDQYSSPLSVSIQKNPVLVMEPFAGDQPF
841 SKNLTFVYNNTQSLDFLNISFTSRGDIYLTSLAIFSVSDSLPQIIDPITPTLLPIESVKA
901 SK**PAILAIVLSIVLGSLALSIITILIV**KHRKRLSQFLSKSNKDIEYAQNNEIEIKVLPKI
961 TSHSSYPSISILDTISSDSIFNNQIPKNNNRYKFKNQSLNNNNYFNNNNNNNNSNNNNSN
1021 NIIYSNCNSNYSNSNSNNNNNNNNSNSNNNSNSNSNININSNSNSNSNSNSNGNNNYQIY
1081 SNKLESFKIDEISNDTIPIINSTFPDEFQTLEFQKLAFEILKREKRLDFSFRTTNDILTC
1141 CRHYQILKIFSNFPLRFNQSIITFGLINGKAKLGETYYDTLSITNDSTIRFTAFLILPMD
1201 NHSATFTSDHSSFDLGPGETFSIKFSITLHCTTRFFENFSIQINSNNIKEMYTLLKIKVE
1261 SESSTRLDFNDIHFQELIEKYSWEILYRGTVGDKNALLKLIKLKTKNCEEAYRELNIISR
1321 LKHQNILPLIGCVISKDYLCLAFEYPPLGSLDYIISKKKLKMSITQKIRILIDVAKGCKF
1381 LQQSSIIQKTLRARNIFLYDTNENAEVCAKVLDLTSSKTIKGLACNNYIERVDTPINLTR
1441 EISIIRDPKQNNDFNNSNNSNNNNNNNNNNNNNNNSNNSNNSSSLKYNNHSFAVLSYE
1501 LLIDEILVGDTRKFGQEKPSIGLDKIDPNIKNFIHKCWNPIDGFTFNEILKTLKDFIESL
1561 N*

Figure 4. Sequence analysis of the *gdt1* gene product. (A) Amino acid sequence of the *gdt1* gene product. The gdt1 protein is composed of 1561 amino acid residues and contains four putative transmembrane domains (bold letters) in the direction of N-i-o-i-o-i-C (analyzed by the EMBL TMpred program). The putative kinase domain is underlined; the expressed D1 peptide sequence used to generate the antiserum is shown by dotted underlining. (B) Multiple alignment of the putative gdt1 kinase domain with other kinases. The 11 subdomains of protein kinases are shown below the sequence and are separated by colons. Conserved amino acids are indicated by bold letters. MMU76762, murine Fer tyrosine kinase (K. Letwin and T. Pawson, unpublished data); MMECK, a receptor tyrosine kinase implicated in pattern formation in mice (Ganju *et al.*, 1994); CEK4, chicken eph-related receptor tyrosine kinase (Sajjadi *et al.*, 1991); TESK1B, serine/threonine kinase from rat (Toshima *et al.*, 1995); PYK2A, tyrosine kinase from *D. discoideum* (Tan and Spudich, 1992); RTRK, neutrophic receptor tyrosine kinase from *Drosophila melanogaster* (Wilson *et al.*, 1993).

mixed at the indicated ratios, and diluted to 5×10^4 cells/ml in bacterial suspension. Cells were harvested at a density of 1×10^6 cells/ml, and β-galactosidase expression was measured as described (Bühl and MacWilliams, 1991). β-Galactosidase activity, a very sensitive indicator for discoidin promoter activity, increased proportionally to the ratio of DAG cells, indicating that *gdt1⁻* cells do not release an extracellular factor to significantly stimulate the expression of discoidin in the wild-type background (Table 1).

The reverse experiment was done by mixing $gdt1^-$ and Ax2 cells as described above and measuring discoidin protein expression by Western blot (Figure 6). At a cell density of 10^6 cells/ml, discoidin is undetectable in Ax2 cells (in contrast to β-galactosidase activity directed by a discoidin promoter as assayed before). The amount of discoidin protein detected in the blot therefore originates exclusively from the $gdt1^-$ cells. The expression of discoidin I increased proportionally with the ratio of the $gdt1^-$ cells, and no significant inhibition was observed. The data demonstrated that wild-type cells did not produce any extracellular signal that could complement the mutation in L8 cells. The mutant could therefore be considered cell autonomous.

The $gdt1^-$ Mutant Is Not Affected in Sensing of Folate

Folate is known as an extracellular signal that inhibits discoidin expression. In axenic medium, discoidin is expressed at moderate to high levels even at low cell densities. To determine whether the $gdt1^-$ phenotype was due to a loss of sensitivity to folate, cells were grown in axenic medium and treated for various times with 1 mM folate while cell density was kept at or $<10^6$ cells/ml. As shown in Figure 7, discoidin was essentially undetectable in wild-type cells after 19 h of folate treatment. As expected, $gdt1^-$ cells produced considerably higher amounts of discoidin, but they clearly decreased when cells were cultivated in the presence of folate. When $gdt1^-$ samples were diluted 10-fold, the decrease in discoidin expression was also detectable in earlier time points (our unpublished data). The

```
                  10        20        30        40        50        60
                   |         |         |         |         |         |
MMU76762   DVS:LGELLGKGNFGEVYKGTLK-----D-K:TPVAIKTCKEDLPQ:ELKIKFLQEAKILKQY:DHPNIVKL
MMECK      CVA:RQKVIGAGEFGEVYKGTLKASSGKK-E:IPVAIKTLKAGYTE:KQRVDFLSEASIMGQF:SHHNIIPL
CEK4       NIS:IDKVVGAGEFGEVCSGRLKLPS-KK-E:ISVAIKTLKAGYTE:KQRRDFLGEASIMGQF:DHPNIIRL
TESK1B     DFD:CAEKIGAGFFSEVYKVRHR----QSGQ:VMV-LKMNKLPSN-:--RSNTLREVQLMNRL:RHPNILRF
PYK2A      DIQ:FIQKVGEGAFSEVWEGW-W----KGIH:VAI-KKLKIIGDEE:QFKERFIREVQNLKKG:NHQNIVMF
RTRK       DVE:FLEELGEGAFGKVYKGQL-LQPNKT-T:ITVAIKALKENASV:KTQQDFKREIELISDL:KHQNIVCI
GDT1       DIH:FQELIEKYSWEILYRGTV-------GD:KNALLKLIKL--KT:KNCEEAYRELNIISRL:KHQNILPL
Subdomain     :            I             :       II     :      III        :  IV

            70        80        90       100       110       120       130
             |         |         |         |         |         |         |
MMU76762   IGVCTQR:QPVYIIMELVPGGDFLTFLRKRKDE-----:LKLKQLVRFSLDVAAGMLYLESK:NCIHRDLA
MMECK      EGVVSKY:KPMMIITEYMENGALDKFLREKDGE-----:FSVLQLVGMLRGIASGMKYLANM:NYVHRDLA
CEK4       EGVVTKS:KPVMIVTEYMENGSLDSFLRKHDAQ-----:FTVIQLVGMLRGIASGMKYLSDM:GYVHRDLA
TESK1B     MGVCVHQ:GQLHALTEYMNGGTLEQLLSSPEP------:LSWPVRLHLALDIAQGLRYLHAK:GVFHRDLT
PYK2A      IGACYKP:ACI--ITEYMAGGSLYNILHNPNSSTPKVK:YSFPLVLKMATDMALGLLHLHSI:TIVHRDLT
RTRK       LGVVLNK:EPYCMLFEYMANGDLHEFLISNSPTEGK-S:LSQLEFLQIALQISEGMQYLSAH:HYVHRDLA
GDT1       IGCVISK:DYLCLAFEYPPLGSLDYIISKK-----KLK:MSITQKIRILIDVAKGCKFLQQS:SIIQKTLR
Subdomain    IV   :             V                :          VIA           :   VIB

             140       150       160       170       180       190       200
               |         |         |         |         |         |         |
MMU76762   ARNCLV--:-GE--NNTLKISDFGMSR---QE:DGGVYSSSGLKQIPIKWTAPEAL:NYGR-YSSESDVWS
MMECK      ARNILV--:-NS--NLVCKVSDFGLSRVLEDD:PEATYTTSGGK-IPIRWTAPEAI:SYRK-FTSASDVWS
CEK4       ARNILI--:-NS--NLVCKVSDFGLSRVLEDD:PEAAYTTRGGK-IPIRWTSPEAI:AYRK-FTSASDAWS
TESK1B     SKNCLV--:RREDGGFTAVVGDFGLAEKIPVY:REGARKEPLAVVGSPYWMAPEVL:RG-ELYDEKADVFA
PYK2A      SQNILL--:--D-ELGNIKISDFGLSAEKS--:REGSMTMTNGGICNPRWRPPELT:KNLGHYSEKVDVYC
RTRK       ARNCLV--:-NE--GLVVKISDFGLSR--DIY:SSDYYRVQSKSLLPVRWMPSESI:LYGK-FTTESDVWS
GDT1       ARNIFLYD:TNENAEVCAKVLDLTSSKTIKGL:ACNNY--IERVDTPINLT-REIS:II-RDPKQNNDFHS
Subdomain    VIB   :         VII          :        VIII           :    IX      *

               210       220       230       240       250       260       270
                 |         |         |         |         |         |         |
MMU76762   FGILLWETFSLG:VCPYPGMTNQQAREQVE---RGYRMSAPQN:CPEEVFTIMMKCWDYKPENRPKFNDL
MMECK      YGIVMWEVMTYG:ERPYWELSNHEVMKAIN---DGFRLPTPMD:CPSAIYQLMMQCWQQERSRRPKFADI
CEK4       YGIVLWEVMSYG:ERPYWEMSFQDVIKAVD---EGYRLPPPMD:CPAALYQLMLDCWQKDRNNRPKFEQI
TESK1B     FGIVLCE-LI-A:RVP-ADPDYLPRTEDFGLDVPAFRTLVGND:CPLPFLLLAIHCCSMEPSARAPFTEI
PYK2A      FSLVVWEILT-G:EIPFSDLDGSQRSAQ--VAYAGLRPPIPEY:CDPELKLLLTQCWEADPNDRPPFTYI
RTRK       FGVVLWEIYSYG:MQPYYGFSNQEVINLIR---SRQLLSAPEN:CPTAVYSLMIECWHEQSVKRPTFTDI
GDT1       FAVLSYELLIDE:ILVGDTRKFGQEKPSIGLD----------K:IDPNIKNFIHKCW--NPIDGFTFNEI
Subdomain      IX      :             X                :           XI

                 280
                   |
MMU76762   HKEL:TVIKKMIT
MMECK      VSIL:DKLIRAPD
CEK4       VSIL:DKLIRNPS
TESK1B     TQHL:EQILEQLP
PYK2A      VNKL:KEISWNNP
RTRK       SNRL:KTWHEGHF
GDT1       LKTL:KDFIESLN
Subdomain  XI
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Figure 4 (cont).

defect in the mutant was thus not in the folate-sensing pathway.

Disruption of the gdt1 Gene Partially Rescues the Gα2⁻ Mutant Phenotype

Gα2^{-} mutants do not form aggregates and show strongly reduced expression of discoidin when developed after growth on bacteria (Endl *et al.*, 1996). To determine the relationship between Gα2 and gdt1, double mutants were constructed by disruption of *gdt1* in a *Gα2*$^{-}$ background. Successful disruption was confirmed by Southern blot (our unpublished data). Colony blots (Figure 8A) clearly showed that cells with both genes disrupted still did not aggregate but expressed high amounts of discoidin during growth on bacteria and in development. This was confirmed in Western

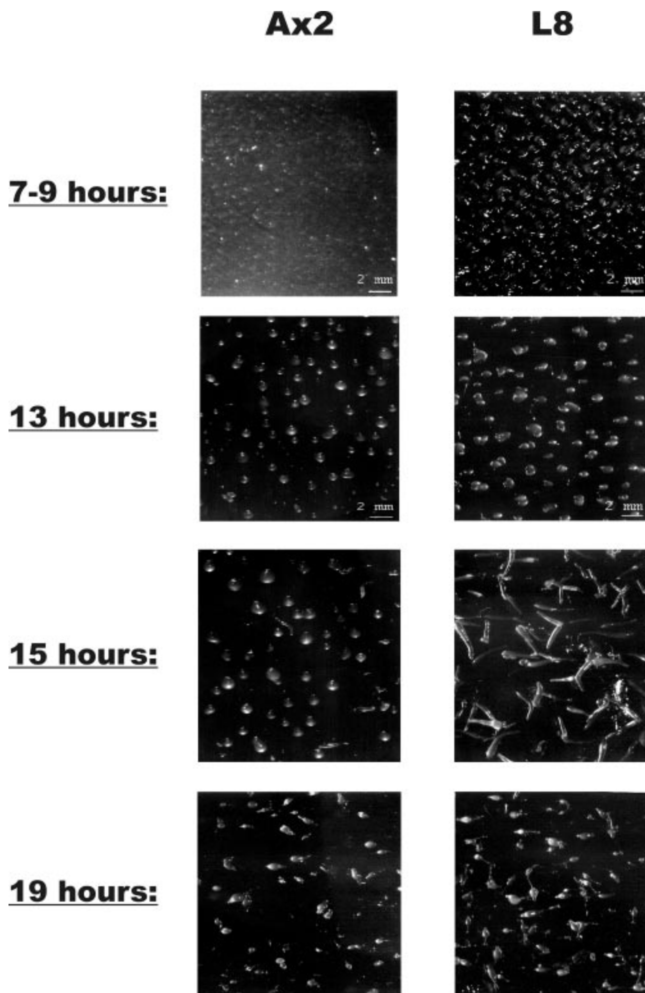

Figure 5. Developmental timing of the *gdt1* mutant. Ax2 and L8 cells were harvested from axenic medium, washed, and spotted at a density of 1×10^8 cells/ml on phosphate-agar. Development at 22°C was monitored with the microscope. At 7 h, L8 cells had aggregated, whereas Ax2 showed faint ripples; at 13 h some tipped aggregates were observed in Ax2, and L8 started to form fingers. At 15 h, tipped aggregates were found in Ax2, whereas all L8 cells were in the finger stage. At 19 h, L8 was in late culmination, whereas Ax2 was only beginning with culmination.

Table 1. *gdt1*$^-$ cells do not influence discoidin expression in *gdt1*$^+$ cells

DAG cells (%)	L8 cells (%)	β-Gal activity (%)
100	0	100
90	10	87
60	40	48
30	70	32
10	90	9
0	100	3

L8 cells and DAG cells (which express β-galactosidase (β-Gal) under the control of the discoidin Iγ promoter) were mixed at the indicated ratios and grown in KA suspension to a density of 1×10^6. Cells were harvested, and β-galactosidase activity was determined by 2-nitrophenyl-β-D-galactopyranoside assay. β-Galactosidase activity is given as the percentage of activity found in 100% DAG cells. The values correspond approximately to the amount of DAG cells in the mixture, indicating that *gdt1*$^-$ cells do not secrete a factor that can enhance discoidin transcription in the tester strain.

blots (Figure 8B), which showed that the double mutant expressed discoidin at similar levels as the gdt1$^-$ mutant. Disruption of *gdt1* could thus partially complement the defect in the *G*α*2*$^-$ mutant, suggesting that the negative regulator gdt1 was downstream of Gα2$^-$ in the same pathway or in a parallel signaling cascade.

DISCUSSION

To further elucidate the signal transduction pathways involved in the GDT, we have screened *REMI* mutants for misregulation of discoidin I expression. A mutant (2-9) was identified because of overexpression of discoidin I and premature aggregation (Figure 1). *gdt1* mutants were different from other signal transduction mutants identified to date: they did not affect aggregation per se, were not sporogenous (our unpublished data), and did not show any obvious effect on the morphology during development or on spore and stalk differentiation. Previously identified mutants displaying rapid aggregation (e.g., rdeA [Abe and Yanagisawa, 1983]) or rapid development (e.g., rdeB, rdeC [Abe and Yanagisawa, 1983] and KP [Anjard *et al.*, 1992]) were all sporogenous and formed abnormal fruiting bodies. This indicated that gdt1 was predominantly involved in the GDT pathway but was not substantial for later development. In agreement with this, *gdt1* disruptants expressed the GDT marker discoidin prematurely at low cell densities.

gdt1 mutants spread out to more abundant resources and switched at lower cell densities from growth to differentiation, probably because of an incorrect interpretation of the cell density:food source ratio. Early aggregation and spreading into the bacterial lawn could be due to a constitutive or enhanced prestarvation response (Clarke *et al.*, 1988). However, the respective PSF signal only results in very-low-level discoidin expression (U. Huitl and W. Nellen, unpublished

Figure 6. *gdt1*$^+$ cells do not influence discoidin overexpression in *gdt1*$^-$ cells. L8 and DAG cells were mixed at the indicated ratios and grown to a density of 1×10^6 cells/ml. Cells were harvested and lysed and proteins were separated on a polyacrylamide gel. After blotting, the filter was incubated with the anti-discoidin antibody and then with an alkaline phosphatase-coupled secondary antibody and stained. At 1×10^6 cells/ml discoidin expression is not detectable in DAG cells (100% DAG lane). Staining in the other lanes thus corresponds to increasing amounts of L8 cells. The increasing amount of discoidin corresponds approximately to increasing amounts of L8 cells, indicating that wild-type cells do not secrete an extracellular factor, which can compensate the overexpression phenotype of the mutant.

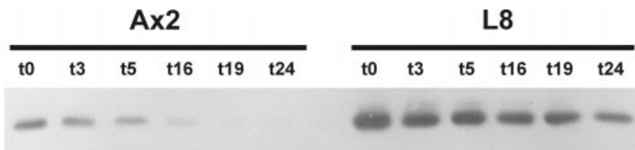

Figure 7. *gdt1*$^-$ cells are not impaired in folate sensing. Ax2 and L8 cells were grown in axenic medium in the presence of 1 mM folate; cell density was kept at or below 10^6 cells/ml. Samples were removed and prepared for Western blots at the times indicated. In wild-type cells, folate reduced discoidin to undetectable levels within 19 h. Even though L8 cells still showed a strong staining after 24 h of growth with folate, the amount of discoidin was significantly reduced, thus indicating that the mutant could sense and respond to the inhibitory signal. Note that staining in L8 is saturated in the first time points.

results; Endl *et al.*, 1996), and we have shown here that PSF production is apparently not enhanced in the mutant. The observation that the mutant is cell autonomous also argues against overproduction of a secreted factor. Although we cannot yet exclude an oversensitivity of *gdt1*$^-$ cells to PSF, we rather assume that the mechanisms to sense the quantity of the food source are impaired although not abolished.

gdt1 RNA and protein are detected in growing cells, and both rapidly disappear with the onset of development. Even though gdt1 protein was not seen in cells at low density (5×10^5; Figure 3C), it is most likely present because even at

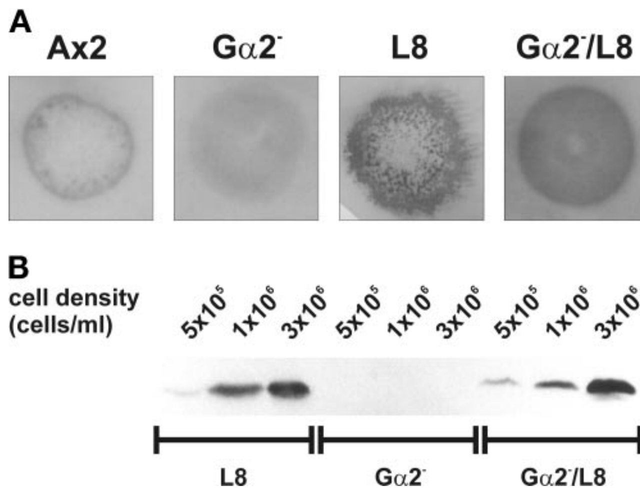

Figure 8. *G*α*2*$^-$/*gdt1*$^-$ cells express high levels of discoidin. (A) Colony blot of Ax2, *G*α*2*$^-$, *gdt1*$^-$, and *G*α*2*$^-$/*gdt1*$^-$ cells. Colonies of Ax2, *G*α*2*$^-$, L8, and *G*α*2*$^-$/L8 cells were stained with the anti-discoidin antibody. The colony blot shows the outer ring staining in Ax2, weak background staining in *G*α*2*$^-$, a strong label in L8, and also a strong signal in *G*α*2*$^-$/L8 double mutant cells. Aggregation and the irregular colony shape are not detected in the double mutant. (B) Western blot of Ax2, *G*α*2*$^-$, *gdt1*$^-$, and *G*α*2*$^-$/*gdt1*$^-$ cells. For Western blot analysis, the double mutant and both single mutants were grown to the cell densities indicated, cells were harvested, and total protein was subjected to electrophoresis, blotted, and stained with the anti-discoidin antibody. The blot demonstrates that discoidin levels in the double mutant are similar to those in L8 cells and that discoidin accumulates with increasing cell density.

lower densities (1×10^5) discoidin overexpression was observed in the disruption mutant. Apparently, gdt1 levels increased with cell density, suggesting that increasing amounts of the protein are required to inhibit increasing competence of the cells to enter the GDT. This was supported by the observation that in the *gdt1* mutants discoidin was not constitutively expressed but precociously accumulated with cell density. We therefore propose that cells gradually acquire developmental competence during growth; this competence is, however, suppressed by increasing amounts of gdt1. Above a certain cell density, suppression is released, and they synchronously enter the GDT.

To confirm that gdt1 was not only a specific inhibitor of discoidin expression but had a general function in the GDT, we examined the expression of the V4 gene (Singleton *et al.*, 1991), which is specifically expressed in vegetative cells and rapidly switched off with the onset of development. V4 expression was strongly reduced in *gdt1*$^-$ cells even at low densities during bacterial growth (our unpublished data).

Suppression of developmental competence may be released by different means; one is obviously the rapid loss of *gdt1* mRNA and protein when cells enter development. In the mRNA, several putative destabilization elements in the unusually long 3′ untranslated region may account for the apparent short half-life of the mRNA (Brown *et al.*, 1996). This assumption is supported by the observation that the truncated 1.2-kb *gdt1* transcript in the L8 mutant appeared more stable than the complete mRNA (Figure 3B; B. Wetterauer, unpublished observations). In the 5′ region, two short upstream ORFs were found, which are often involved in translational regulation (for review, see Geballe and Morris, 1994) and may prevent further translation of gdt1 with the onset of development.

In the N-terminal part, the gdt1 protein revealed no significant similarity to sequences in the databases. However, four putative transmembrane domains were predicted by computer analysis. This was supported by a strong enrichment of gdt1 in the membrane fraction (our unpublished data). No signal peptide for membrane insertion was found in the sequence, unless one assumes that TM1, which is, however, rather far from the N terminus, serves this function. Many polytopic transmembrane proteins such as, e.g., the *Dictyostelium* adenylyl cyclase A (Pitt *et al.*, 1992) do not require signal peptides (Bibi, 1998). The C terminus of the gdt1 protein displayed some similarity to the catalytic domain of protein kinases. However, some of the highly conserved kinase signatures were not found. In the original *REMI* mutant, *gdt1* was disrupted within the second transmembrane domain, the L8 mutant carried a disruption in the intracellular loop between TM2 and TM3, and the K series mutants had an insertion just before the C-terminal kinase-like domain. Because all disruptants displayed the same phenotype, none of them apparently expressed a partially functional protein.

First experiments to elucidate the position of the gdt1 protein in the signal transduction cascades demonstrated that it was apparently not involved in sensing folate, an inhibitor of discoidin expression.

Interestingly, double mutants disrupted in the gene encoding the G-protein α2 and in *gdt1* partially bypassed the defect in *G*α*2*$^-$ mutants: although the cells were still aggregation deficient, they expressed high levels of discoidin. We

have previously shown that Gα2 is part of a positive signaling pathway via CRAC and PKA leading to high discoidin expression (Endl *et al*., 1996; Primpke *et al*., 2000). The data presented here suggest that gdt1 is located downstream of Gα2 and that the positive pathway may function by inactivation of the inhibitory gdt1 protein. Alternatively, gdt1 may be in a parallel, interacting pathway. The idea that discoidin expression is controlled by a network of pathways was already proposed by Alexander *et al*. (1986, 1990). It should be noted that the double mutant also shows that Gα2-mediated signaling apparently splits into two branches: although the GDT (i.e. discoidin expression) can be rescued by *gdt1* disruption, morphological development cannot.

The *REMI* technique and the use of discoidin as a marker for molecular analysis of the GDT has proven to be successful: a screen revealed a signal transduction component, which may be a new type of receptor kinase that responds to food supply. Further experiments are, however, required to examine whether the protein really has kinase activity. In addition, *gdt1* confirmed our previous suggestion that the first steps into differentiation occur in the absence of any visible morphological development (Endl *et al*., 1996).

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