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Suppression of the growth/differentiation transition in *Dictyostelium* development by transient expression of a novel gene, *dia1*

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SUMMARY

In *Dictyostelium discoideum* Ax-2 cells, a specific checkpoint (PS point) from which cells enter the differentiation phase in response to starvation has been specified in the cell cycle. Using the differential display method, we isolated a novel gene, *dia1* (*di*fferentiation-*a*ssociated gene *1*), that is specifically expressed in cells differentiating from the PS point. The *dia1* mRNA has an open reading frame of 1,368 bp and is deduced to code for a 48.6 kDa protein (DIA1). The DIA1 protein is highly serine-rich and the serine residues are predominantly located in the C-terminal region. After the PSORT II search, the protein is predicted to be GPI-anchored at the plasma membrane. Unexpectedly, *dia1* overexpression rather impaired the progression of differentiation, possibly coupled with the reduced expression of early genes such as cAMP receptor1 (*car1*). The inhibitory effect of *dia1* expression on early differentiation was almost completely nullified by externally applied cAMP pulses. In contrast to *dia1* overexpression, antisense RNA-mediated *dia1* inactivation was found to enhance the initial step of cell differentiation, as exemplified by precocious expression of *car1* and other early genes. We discuss the unique structure and function of DIA1 in relation to the cooperative development of cells during the establishment of multicellular organization.

Key word: *dia1*, cAMP-related gene, Differentiation, Cell cycle, PS point, Synergy, *Dictyostelium*

INTRODUCTION

In general, growth and differentiation are mutually exclusive and finely regulated during development. Thus the mechanisms involved in the transition from a proliferation to differentiation state are of prime interest to developmental biologists and in cancer research. *Dictyostelium discoideum* (strain Ax-2) cells grow and multiply by binary fission as long as nutrients are supplied. Starving cells differentiate to acquire aggregation competence, forming multicellular structures by means of chemotaxis to cAMP (Bonner et al., 1969) and EDTA-resistant cohesiveness (Gerisch, 1961). Subsequently, cells differentiate into two cell types in migrating slugs, prestalk and prespore cells. The slug eventually culminates in a fruiting body consisting of a mass of spores and a supporting cellular stalk. The growth and differentiation phases are temporally separated from each other and easily controlled by nutritional conditions. A temperature-shift method for synchronizing the cell-cycle phase has also been established (Maeda, 1986), and a specific checkpoint (referred to as a putative shift point; PS point) has been specified in the cell cycle of Ax-2 cells (Maeda et al., 1989); i.e., Ax-2 cells at any cell-cycle phase enter the differentiation phase by drifting away from the PS point under starvation conditions. Therefore, *Dictyostelium* is particularly useful for elucidating the cellular and molecular mechanisms of the growth/differentiation transition.

The presence of the PS point shows that starvation is necessary but not sufficient for the subsequent cell differentiation, because Ax-2 cells starved at the cell-cycle position far away from the PS point will need a lot of time (about 7 hours in the case of axenic condition) to differentiate from the PS point. Thus it is necessary to discriminate between starvation-specific events and PS point-specific ones; i.e., unification of both the events is required for the initiation of cell differentiation. Recently, Souza et al. (1998) have demonstrated that the overexpression of YakA, a homologue of protein kinase Yak1P in budding yeast, arrests growth and induces differentiation-associated events such as the acquisition of cAMP signaling system even in the presence of nutrients. Thus YakA is most likely involved in starvation-specific signaling as a stress-induced response.

We have already identified several genes (*dia2, dia3, car1)* that are specifically expressed in response to the initial differentiation from the PS point (Abe and Maeda, 1994; Chae et al., 1998; Inazu et al., 1999). The expression of a novel gene, *dia2*, has an essential role in the initiation of cell differentiation, closely relating to the cAMP signaling system (Chae et al., 1998). Recently, *dia3*, which encodes for a mitochondrial gene cluster including *rps4* (ribosomal protein S4), has been shown to be essential for the phase-shift of cells from growth to differentiation (Inazu et al., 1999). The cAMP receptor1 (*car1*) is expressed in starved cells just after the PS

point (Abe and Maeda, 1994) and essential for differentiation (Sun et al., 1990; Sun and Devreotes, 1991). cAMP acting on CAR1 activates a number of rapid intracellular response including guanylyl cyclase (GCA) and adenylyl cyclase (ACA) via heterotrimeric G-proteins, and this activation is achieved by pulsatile cAMP and cell-to-cell communications (Devreotes, 1994; Michael and Loomis, 1998). CAR1-dependent events include receptor phosphorylation and influx of extracellular Ca^{2+} in a G-protein-independent manner (Milne et al., 1995). In this paper, we report another novel gene, *dia1*, which is specifically and temporarily expressed during the transition of Ax-2 cells from growth to differentiation, with reference to the unique structure and somewhat surprising function of the gene product, DIA1 protein.

MATERIALS AND METHODS

Cell culture and developmental conditions

Vegetative cells of *Dictyostelium discoideum* Ax-2 were axenically grown in HL-5 medium (Watts and Ashworth, 1970) supplemented with 1% glucose. Cells that were overexpressing or underexpressing the *dia1* mRNA were shaken in HL-5 medium containing 30 μg/ml of G418. Cells were harvested at the exponential growth phase, washed twice in BSS (Bonner's salt solution; Bonner, 1947) and settled down either in a 24-well titer plate (Falcon, #3047) or on 1.5% non-nutrient agar to allow differentiation. This was followed by incubation at 22°C, as previously described (Inazu et al., 1999). In some experiments, the suspended cultures were shaken at 22°C with externally applied pulses of 50 nM cAMP delivered every 6 minutes for 6 hours. And after 6 hours of cAMP addition, the culture were suspended in BSS to 1×10^6 cells/ml and incubated in a 24-well plate at 22°C.

Synchronization of the cell-cycle phase

Cell synchronization was performed using the temperature-shift method (Maeda, 1986) with a slight modification. Exponentially growing cells ($1.0\text{-}1.5\times10^6$ cells/ml) at 22°C with a doubling time of about 7.6 hours were shifted to 9.4°C, shaken for 14.5 hours and then reshifted to 22°C. Under these conditions, the number of cells doubled within about 2 hour period after a lag phase of about 1 hour. T7 cells, 7 hours after the shift-up from 9.4° to 22°C, were harvested just before the PS point, starved by washing twice in 20 mM phosphate buffer (PB; KH_2PO_4/Na_2HPO_4, pH 6.5), and then shaken at 1×10^7 cells/ml for 2 hours at 150 revs/minute. This yielded T7+2 cells, i.e., newly differentiating cells. T4+2 cells were prepared by starving T4 cells for 2 hours in PB, as starved but not differentiated cells. As another reference, T9 cells obtained by incubating T7 cells for 2 hours in growth (HL-5) medium were used.

Isolation of total RNAs and northern hybridization

Total RNAs were prepared according to the method of Nellen et al. (1987). The total RNA samples were heat treated at 65°C for 15 minutes to denature the RNA and then quickly chilled on ice. The samples (20 μg) were separated on 1.0% formaldehyde agarose gel and transferred on to Hybond N+ (Amersham) by upward capillary transfer. The nucleic acids were immobilized by baking for 2 hours at 80°C and were stored either at room temperature or at 4°C.

Northern hybridization was carried out using the RI or non-RI kit (Amersham). In the case of RI, prehybridization was carried out at 63°C for 2-4 hours in a solution containing 5× Denhardt solution (0.02% Ficoll 400, 0.02% bovine serum albumin (BSA), and 0.02% polyvinylpyrrolidone (PVP), 5× SSPE (43.8 g of NaCl, 6.9 g of $NaH_2PO_4\cdot2H_2O$ and 1.85 g of EDTA; pH 7.4) or 5× SSC (43.8 g of NaCl and 22.05 g of sodium citrate), 0.5% sodium dodecyl sulfate (SDS), and 20-100 mg of denatured salmon sperm DNA per ml. Hybridization was carried out at 63°C for 20 hours in the same solution containing the ^{32}P-labeled cDNA probe by use of the Megaprime™ DNA-labeling system (Amersham). The membranes were washed twice with a washing solution (1× SSC and 0.1% SDS) at room temperature for 15 minutes and then washed twice in 0.1× SSC and 0.1% SDS at 63°C for 5-10 minutes. The membranes were exposed to X-ray films (New A, Konica) for 2-7 days at −80°C. In another experiment using the non-RI kit, prehybridization was carried out at 55°C for 30 minutes in hybridization buffer containing 0.5 M NaCl and 4% blocking agent. Hybridization was carried out at 55°C for 16-20 hours in the same solution containing the alkaline phosphatase-labeled cDNA probe by use of the Alkphos DIRECT DNA-labeling system (Amersham). The membranes were washed twice with the primary wash buffer (2 M urea, 50 mM sodium phosphate pH 7.0, 150 mM NaCl, 10 mM $MgCl_2$ and 0.1% SDS) at 55°C for 10 minutes and then washed twice in secondary wash buffer (50 mM Trizma base, 100 mM NaCl and 2 mM $MgCl_2$) at room temperature for 5 minutes. The membranes were exposed to X-ray films (New A, Konica) for 1-12 hours at room temperature.

Differential display and cDNA sequencing

Differential display was performed by the method of Liang et al. (1994) with the RNAimage kit (GenHunter). DNA-free total RNAs of T7+2 cells, T4+2 cells, and T9 cells were reverse-transcribed with three, one-base anchored oligo(dT) primers (5′-AAGCTTTTTT-TTTTTG-3′; H-T_{11}G, 5′-AAGCTTTTTTTTTTTA-3′; H-T_{11}A and 5′-AAGCTTTTTTTTTTC-3′; H-T_{11}C) that annealed at the start of the poly(A) tails of mRNAs, followed by polymerase chain reaction (PCR) amplification with the anchored primers and six arbitrary primers (5′-AAGCTTTCCTGGA-3′; H-AP25, 5′-AAGCTTGCCAT-GG-3′; H-AP26, 5′-AAGCTTCTGCTGG-3′; H-AP27, 5′-AAGC-TTACGATGC-3′; H-AP28, 5′-AAGCTTAGCAGCA-3′; H-AP29, 5′-AAGCTTCGATCGT-3′; H-AP30). After size fractionation of amplified cDNA fragments on a 6% denaturing polyacrylamide gel electrophoresis and subsequent retrieval, the cDNA fragments of interest were reamplified, cloned into the PCR-TRAP cloning vector (Gene Hunter) and sequenced. cDNA sequencing was carried out using the ABI PRISM™ Dye Terminator cycle sequencing kit (Perkin Elmer) and the ABI Prism 310 Genetic Analyzer (Perkin Elmer). For multiple sequence alignments, the programs of GENETYX-MAX were used.

Plaque hybridization and in vivo excision

The cDNAs encoding full-length mRNAs were screened by the plaque hybridization method from T7+2 cDNA library (Abe and Maeda, 1994) that was constructed in λ-ZAPII (Stratagene). Plaque hybridization was performed as previously described (Chae and Maeda, 1998a,b). A phagemid containing the cloned insert was recloned by in vivo excision of the pBluescript SK(−) phagemid from the λ-ZAPII vector (Stratagene).

Extraction and purification of plasmid DNAs

Extraction and purification of plasmid DNAs were performed using alkaline lysis (Birnboin and Doly, 1979; Birnboin, 1983).

Transformation of cells

The vector constructs with sense or antisense *dia1* were separately introduced into Ax-2 cells by electroporation, as described by Howard et al. (1988). Transformed cells were cloned and selected in HL-5 medium containing 30 μg/ml of G418 in 96- or 384-well titer plates (Falcon). After 5-6 days from the appearance of colonies of transformed cells, the colonies were transferred to 24-well plates. The *dia1*-overexpressing cells and *dia1*-underexpressing cells were incubated by shaken-culture in HL-5 medium containing 30 μg/ml of G418 for 2-3 days.

RESULTS

Specific expression of a novel gene (*dia1*) during transition from growth to differentiation

Using the temperature-shift method for cell synchrony and the differential display method, *dia1* was isolated as one of genes specially expressed in T7+2 cells (just-differentiating cells) from the growth/differentiation checkpoint (PS point; Fig. 1A). After BLAST and FASTA searches, *dia1* had no significant similarity to previously reported genes, and is thus a novel gene. In the development of nonsynchronized Ax-2 cells, the *dia1* mRNA was detectable at 2 hours after starvation and reached a maximum level at 4 hours, followed by rapid decrease (Fig. 1B). Thus the *dia1* expression during development is quite transient and limited to the initial stage of development.

The cDNA of *dia1* has an open reading frame of 1,368bp and is deduced to code for 48.6 kDa protein (DIA1) consisting of 455 amino acids. The DIA1 protein is highly serine-rich (21.3% of the total amino acid residues), and the serine residues are predominantly located in the C-terminal region (350-440 a.a; Fig. 2). A hydropathy profile suggests that this protein has at least two hydrophobic domains. One is located near the N-terminal region (1-21 a.a), which appears to have a cleavable signal peptide and be cleaved during its translocation to the endoplasmic reticulum (ER). The other domain is located at the C terminus (435-451 a.a) and appears to be GPI anchored. Proteins that have a GPI-anchored form have two hydrophobic peptides at N and C termini to be cleaved. DIA1 protein has amino acids that satisfy −1,−3 rule for predicting cleavage by N-terminal signal peptidase and ω, ω+2 rule for predicting cleavage/PI-G addition by C-terminal signal transamidase (Louise et al., 1992).

Overexpression of *dia1* impairs the progression of differentiation

As shown above, *dia1* was expressed only at the initial step of differentiation. Unexpectedly, however, overexpression of *dia1* was found to suppress the progression of differentiation. Ax-2 cells were transformed with a vector (pDNeo2) under the control of actin6 promoter for overexpression by electroporation and selected by 30 μg/ml G418. Several *dia1*^{OE} clones, which strongly express the *dia1* mRNA even at the vegetative growth phase, were isolated. Differences in developmental features among the clones isolated were not observed.

When *dia1*^{OE} and parental Ax-2 cells were separately harvested at the exponential growth phase, washed in BSS and incubated under submerged conditions, they exhibited quite different behaviors. Ax-2 cells were able to form aggregation streams after 8 hours of incubation (Fig. 3A), while *dia1*^{OE} cells were in the process of acquiring aggregation competence (Fig. 3B). The early differentiation of *dia1*^{OE} cells was delayed compared to that of Ax-2 cells. Although *dia1*^{OE} cells eventually formed tight aggregates (Fig. 3D), many single cells, a number of which were round in shape, were seen around these aggregates (Fig. 3D,E).

On agar, starving *dia1*^{OE} cells displayed abnormal morphogenesis. After 5.5 hours of incubation, *dia1*^{OE} cells showed no sign of cell aggregation (Fig. 4B), while Ax-2 cells were able to form aggregation streams (Fig. 4A). Subsequently, *dia1*^{OE} cells formed aggregation streams after 8 hours of incubation (Fig. 4D). Just as in submerged conditions, *dia1*^{OE} cells delayed the initial step of differentiation and some of them could not aggregate. In spite of the delay of development, *dia1*^{OE} cells could form fruiting bodies. Their appearance was normal as compared with Ax-2 (Fig. 4E).

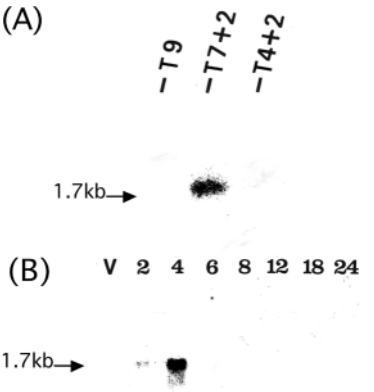

Fig. 1. (A) Specific expression of the *dia1* mRNA in newly differentiating cells (T7+2 cells) from the PS point. Total RNAs were extracted from synchronized T9, T7+2 and T4+2 cells. 10 μg of total RNA for each were size-fractionated and hybridized using the double-stranded *dia1* cDNA probe, as described in Materials and Methods. It is clear that the 1.7 kb of *dia1* transcript is specifically expressed in T7+2 cells starved just before the PS point, but not in undifferentiated cells such as T9 and T4+2 cells. (B) Developmental change of the *dia1* mRNA expression. Non-synchronized Ax-2 cells growing in HL-5 medium were harvested, washed and allowed to develop on 1.5% non-nutrient agar. Northern blotting of *dia1* mRNA was performed, as described in Materials and Methods. Lane V; vegetative growth phase. The numbers indicate incubation times (hours) after starvation. It is evident that the *dia1* expression is transient and limited to the early differentiation phase (t_2-t_4) during the whole course of development.

Essentially the same result was obtained using transformed cells overexpressing the *dia1* gene under the control of actin15 promoter.

Enhanced differentiation by antisense-mediated *dia1* inactivation

To analyze the *dia1* function more precisely, we attempted to disrupt *dia1* by homologous recombination, but did not succeed in obtaining *dia1*-null mutants. The Southern analysis of genomic *dia1* seemed to indicate that Ax-2 cells have two or more copies of *dia1*, thus resulting in a failure to obtain *dia1*-null cells. Therefore, we were obliged to use antisense-mediated gene inactivation. For preparation of transformants expressing the antisense *dia1* RNA, exponentially growing Ax-2 cell were transformed with the antisense construct under the control of actin15 promoter of the pAct15-gal and selected by growth in HL-5 medium containing 30 μg/ml of G418. Among 40 G418-resistant clones obtained, a clone, AS-8 was found to express most strongly the antisense-*dia1* RNA at the vegetative growth phase.

As expected from the inhibitory effect of *dia1* overexpression on differentiation, antisense-mediated gene inactivation of *dia1* enhanced the progression of differentiation. When AS-8 and its parental strain Ax-2 were

separately harvested at the exponential growth phase, washed in BSS and incubated at 2.8×10^5 cell/cm^2 under submerged conditions, AS-8 cells exhibited more rapid aggregation than Ax-2 cells. AS-8 cells acquired aggregation competence and formed small aggregates after 5.5 hours of incubation (Fig. 5C), while Ax-2 cells still remained as non-competent single cells (Fig. 5A). AS-8 cells then formed loose aggregates at 7 hours of incubation (Fig. 5D), but many of Ax-2 cells remained as non-aggregated single cells (Fig. 5B), thus indicating a stimulatory effect of the *dia1* underexpression on the initial step of differentiation. On agar, only slight enhancement of differentiation was noticed in AS-8 cells, as compared with Ax-2 cells (data not shown) and they formed the final structure normally.

Ectopic *dia1* expression affects expression of genes involved in cAMP signaling

To analyze the relation of *dia1* expression to other early genes such as *car1* and *aca*, we examined the expression patterns of these genes in *dia1*-overexpressing cells (*dia1*^{OE}) and *dia1*-underexpressing cells (AS-8; Fig. 6A). Since *mybB* has been demonstrated to encode for a transcription factor and induce the *aca* expression (Otsuka and Haastert, 1998), its expression was also compared.

The forced expression of *dia1* by constitutive Act6 promoter suppressed the expression of *car1* and *aca*. In the case of *car1*, its expression was markedly reduced at 2-4 hours after starvation, as compared with parental Ax-2 cells. The *aca* mRNA was expressed after 4-6 hours of starvation but only weakly. In contrast, *dia1*-underexpressing cells (AS-8) exhibited the precocious expression of *car1* and *aca*. The effect of ectopic *dia1* expression seemed to be more marked on the *car1* expression than on the *aca* expression. As to the *mybB*, AS-8 cells showed a slightly stronger and precocious expression than Ax-2 cells while, in *dia1*^{OE} cells, expression seemed to be only faintly lower, as compared with Ax-2 cells (Fig. 6A).

Developmental defect as observed in *dia1*^{OE} cells is nullified by co-culture with wild-type Ax-2 cells

When starved *dia1*^{OE} and parental Ax-2 cells were mixed at 9:1 ratio and incubated on 1.5% non-nutrient agar, clear synergism was observed between the two. In the mixed culture, abnormal development of *dia1*^{OE} cells, as exemplified by slow aggregation, was almost completely removed by surrounding Ax-2 cells, resulting in normal aggregation. Interestingly, the development of Ax-2 cells was accelerated in the presence of a small number (one-ninth of Ax-2 cells) of AS-8 cells, and began to aggregate at almost the same timing as AS-8 alone. When *dia1*^{OE} and AS-

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AACATATAAATAATGAAATTTAAATTATTTCTTTTAGTATTCTTTGTATTTTTATTACCA    60
            M  K  F  K  L  F  L  L  V  F  F  V  F  L  L  P      16

TATCTATCACAATCATGTGAAAATACTTCTTCATGCCCAACTTTAAATCAAACATGTACA    120
 Y  L  S  Q  S  C  E  N  T  S  S  C  P  T  L  N  Q  T  C  T    36

GGGCAGTGTTCAGCTGGTTTAGTTTGTAATCAAACTTTAAGTTCTTGCCAAGAATATGAA    180
 G  Q  C  S  A  G  L  V  C  N  Q  T  L  S  S  C  Q  E  Y  E    56

AAGTGTGACTCATCACTTCCCTTGGCTGAAGATACATGTTCTCAAGATGGATATATTTGT    240
 K  C  D  S  S  L  P  L  A  E  D  T  C  S  Q  D  G  Y  I  C    76

GTATCAAATGTTTGTCTTCCATTTATGGGAACCCATATCCTTCGTCACAATGTAACAGAA    300
 V  S  N  V  C  L  P  F  M  G  T  H  I  L  R  H  N  V  T  E    96

CTCAAATTGTTTATGTCATTATGTGCGGGAAATATTTGCATGGTTATGCCAGGTTCTCAA    360
 L  K  L  F  M  S  L  C  A  G  N  I  C  M  V  M  P  G  S  Q    116

TGCCAAACAAATTCTCAATGTTCTCCAGACCAATTTTGTAGTACAACCTTTATTGGTACA    420
 C  Q  T  N  S  Q  C  S  P  D  Q  F  C  S  T  T  F  I  G  T    136

ACCTCTACTAGCCCATCAACAATGACATTACCAATGACAATGACATTACCAATGACGATG    480
 T  S  T  S  P  S  T  M  T  L  P  M  T  M  T  L  P  M  T  M    156

ACATTACCAATAACAATGACAATGGGATCTTCATCTGTATGCACCAATAGATTACAATTA    540
 T  L  P  I  T  M  T  M  G  S  S  S  V  C  T  N  R  L  Q  L    176

AACAGTCAATGTTCTACATCTGATTCATGTCAAACCGGTTTAGCATGTATTAATTCAGTT    600
 N  S  Q  C  S  T  S  D  S  C  Q  T  G  L  A  C  I  N  S  V    196

TGTTCACCAATTTTCTCTGGTGCTGAAAACGCAACATGCTTCCCTGGAGCAATAGAACCA    660
 C  S  P  I  F  S  G  A  E  N  A  T  C  F  P  G  A  I  E  P    216

ACCAAAGCAGCATGTGATGTAGGATTATCTTGTTTAAATGGAGCAAATGGTTATAGTTGT    720
 T  K  A  A  C  D  V  G  L  S  C  L  N  G  A  N  G  Y  S  C    236

AAAAGTTATGTTGAAAATCAATCATGCGATCCATCAGATGAATACCCAGTTTGTAATTCA    780
 K  S  Y  V  E  N  Q  S  C  D  P  S  D  E  Y  P  V  C  N  S    256

GATTATCAATCATGTAAATGTAATTCTAAGGGAAAAGGTTCTTGTCAATCCTATTATAAA    840
 D  Y  Q  S  C  K  C  N  S  K  G  K  G  S  C  Q  S  Y  Y  K    276

TTAACACAAGAATGTAAAGATAGTTCAAATAAATTAGTATTGTGTGCAAAATCAAAAAAT    900
 L  T  Q  E  C  K  D  S  S  N  K  L  V  L  C  A  K  S  K  N    296

TCTATTCCATCCTATAAAGATTATGTAACTCAAATTAATTGTCAATCTCAACTTTGTAAT    960
 S  I  P  S  Y  K  D  Y  V  T  Q  I  N  C  Q  S  Q  L  C  N    316

TATTCAAGAGATTGTATTGATCCAAAAGCTAAAGTTTCAACATGTTTTAATGATTTATTT   1020
 Y  S  R  D  C  I  D  P  K  A  K  V  S  T  C  F  N  D  L  F    336

TTAATGTGTCCAAGATATTACCAAGAACCTGAAATTGGTTCAAGTTCATCATCGTCATCA   1080
 L  M  C  P  R  Y  Y  Q  E  P  E  I  G  S  S  S  S  S  S  S    356

TCATCATCATCGTCAGGTTCAAGTAGTAATTCTAACATCTTCATCAACATCATCAACATC   1140
 S  S  S  S  S  G  S  S  S  N  S  N  I  F  I  N  I  I  N  I    376

ATCAACATCATCAGATCTTCAGAATCATCAAATGGTTCAAATAGTAATTCTGTATCATCC   1200
 I  N  I  I  R  S  S  E  S  S  N  G  S  N  S  N  S  V  S  S    396

GAATCATCATCACCATCGTCATCATCAGTTGAATCATCATCTAATTCAAAATCAAATCAT   1260
 E  S  S  S  P  S  S  S  S  S  V  E  S  S  S  N  S  K  S  N  H  416

ACATCATCTGAATCATCATCATCTGATGATGACTTGGGAAATCCATCTTCTTCAAGTATT   1320
 T  S  S  E  S  S  S  S  D  D  D  L  G  N  P  S  S  S  S  I    436

TTATCTGTATCTAAACTTATCATTTTATTAATTTCAATTATTTTATATTGTTTTTAAAAT   1380
 L  S  V  S  K  L  I  I  L  L  I  S  I  I  L  Y  C  F  *      455

GATTTTTTAAAAATAAATTTTAATAATATTTAAATAATAATAAAAAAAAAAAAAAAAA     1440
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Fig. 2. cDNA sequence of *dia1* and deduced amino acid sequence. The *dia1* cDNA was isolated and sequenced, as described in Materials and Methods. This nucleotide sequence is deposited in the DDBJ, EMBL and GenBank databases with Accession No. AB007026. It is of interest to note that the DIA1 protein is highly serine-rich, particularly in the C-terminal region. The putative transmembrane domains are underlined. Open triangles are the amino acids that satisfy the −1,−3 rule for predicting cleavage by N-terminal signal peptidase. Closed triangles are the amino acids that satisfy the ω, ω+2 rule for predicting cleavage/PI-G addition by C-terminal signal transamidase for GPI-anchored proteins (Louise et al., 1992).

8 cells were mixed at 9:1 ratio and incubated, the development of *dia1*^{OE} was completely normal, as in the case of Ax-2.

Externally added cAMP pulses can restore the delayed aggregation and gene expression of *dia1*^{OE} cells to the former state

These results raise the possibility that factors secreted from Ax-2 or AS-8 cells might be responsible for the synergistic effect on *dia1*^{OE} cells. cAMP is the most-likely candidate, because its pulse application to starving cells is known to enhance early gene expression, thus resulting in accelerated aggregation (Mann and Firtel, 1987). To test this, exponentially growing *dia1*^{OE} cells were harvested, washed in BSS and suspended at a density of 1×10^7 cells/ml. The cell suspension was shaken at 22°C with or without 50 nM cAMP pulses at 6 minute intervals for 6 hours, followed by northern analysis using the *car1* cDNA probe. *car1* expression in cAMP-pulsed *dia1*^{OE} cells is markedly increased (Fig. 6B), as compared with that of non-pulsed cells.

As expected from the above result, cAMP pulses restored the delayed aggregation of *dia1*^{OE} cells, i.e., when *dia1*^{OE} cells that had been shaken for 6 hours with or without the cAMP pulses were placed in a 24-well plate at 22°C at a density of 5×10^5 cells/cm^2, it was found that cAMP-pulsed *dia1*^{OE} cells had already acquired aggregation competence (Fig. 7A), while non-pulsed *dia1*^{OE} cells showed no sign of aggregation (Fig. 7B). In addition, the number of single *dia1*^{OE} cells that were not able to join in aggregation was considerably decreased by the cAMP pulses up to the normal level of Ax-2 cells (Fig. 7C). In another experiment, the addition of a high concentration of cAMP (100 μM) as a single shot was found to inhibit the development of both *dia1*^{OE} and Ax-2 cells (data not shown). Thus, cAMP most likely acts as a suppressor of the DIA1 function.

Extracellular Ca^{2+} concentration and the development of *dia1*^{OE} cells

Calcium ion (Ca^{2+}) is believed to be one of key factors required for the onset of *Dictyostelium* development (Itoh et al., 1998; Tanaka et al., 1998). To examine whether or not the *dia1*^{OE} phenotype is related to extracellular Ca^{2+}concentrations $[Ca^{2+}]_e$, we harvested cells at the exponential growth phase, washed in 20 mM sodium/potassium phosphate buffer (pH 6.4), and suspended in Ca^{2+}-EGTA buffer (pH 6.4) prepared as previously described (Itoh et al., 1998). 1 ml of the cell suspension (5×10^5 cells/ml) was plated in a 24-well plate and incubated at 22°C. Ax-2 cells developed normally to form aggregates at 10^{-4} M $[Ca^{2+}]_e$ after 8 hours of incubation. Below 10^{-6} M $[Ca^{2+}]_e$, however, cells never acquired aggregation competence even after 8 hours of incubation. Although Ax-2 cells eventually constructed a tight aggregate even at 10^{-6}-10^{-7} M $[Ca^{2+}]_e$, the number of single cells that had a round shape and appeared unable to aggregate was increased in response to lowering $[Ca^{2+}]_e$. The $[Ca^{2+}]_e$-dependent development as observed in Ax-2 cells, was also noticed in *dia1*^{OE} cells, in a more striking manner: the round-shaped single cells were more prominent than in Ax-2 cells, particularly at 10^{-6} M $[Ca^{2+}]_e$. Thus, it is evident that *dia1*^{OE} cells are more sensitive to lower $[Ca^{2+}]_e$, as compared with parental Ax-2 cells.

DISCUSSION

The novel gene, *dia1*, was found to be specifically expressed in the initial step of differentiation at which cells exit from the PS point of the *Dictyostelium* cell cycle. The *dia1* mRNA is predicted to encode for a structurally unique protein (DIA1: deduced molecular mass, 48.6 kDa) with a relatively high

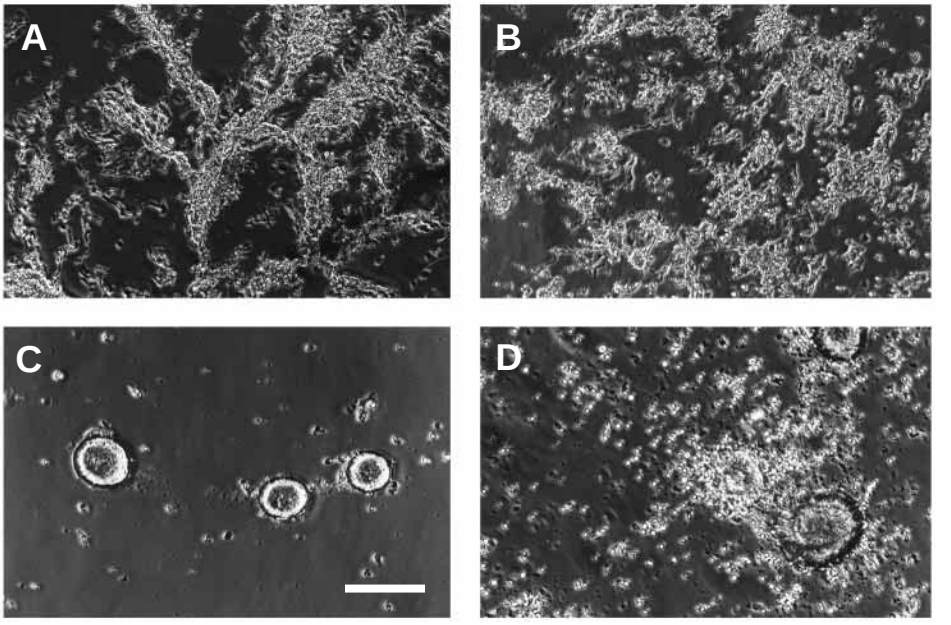

Fig. 3. Development of *dia1*^{OE} (B,D,E) and parental Ax-2 cells (A,C) under submerged conditions. Cells were harvested at the exponential growth phase, washed twice and plated in 24-well titer plates at a density of 5×10^5 cells/cm^2. This was followed by incubation at 22°C for 8 hours (A,B), 24 hours (C-E). At 8 hours of incubation, (A) Ax-2 form aggregation streams, while (B) *dia1*^{OE} cells exhibit delayed aggregation. (D) It is noteworthy that a lot of round-shaped single cells are observed in the outskirts of aggregates in *dia1*^{OE} cells. (E) Higher magnification of D. Bar in C, 200 μm and in E, 100 μm.

content of serine (21.3% of the total amino acid residues). The primary structure of DIA1 protein suggested that it might be glycosylphosphatidylinositol-anchored membrane protein.

In nonsynchronized Ax-2 cells, the expression of *dia1* mRNA was found to be developmentally regulated and quite transient, being limited to the initial step of cell differentiation (Fig. 1B). The expression pattern of *dia1* is also similar to that of the early cAMP receptor 1 (*car1*) gene (Abe and Maeda, 1994). CAR1, a G-protein-linked surface cAMP receptor1, is closely involved in cAMP signaling together with adenylyl cyclase A, ACA, and is essential for *Dictyostelium* development, including cell aggregation; its disruption by homologous recombination or antisense RNA results in a failure of transformed Ax-3 cells to develop (Sun et al., 1990; Sun and Devreotes, 1991). Expression of *car1* (*quit1*) in T7+2 cells (starved just before the PS point) is believed to be one of the earliest events in differentiation (Abe and Maeda, 1994). Surprisingly, however, the present work has shown that forced expression of the *dia1* mRNA suppresses the progression of differentiation, as shown by the delay of aggregate formation, possibly

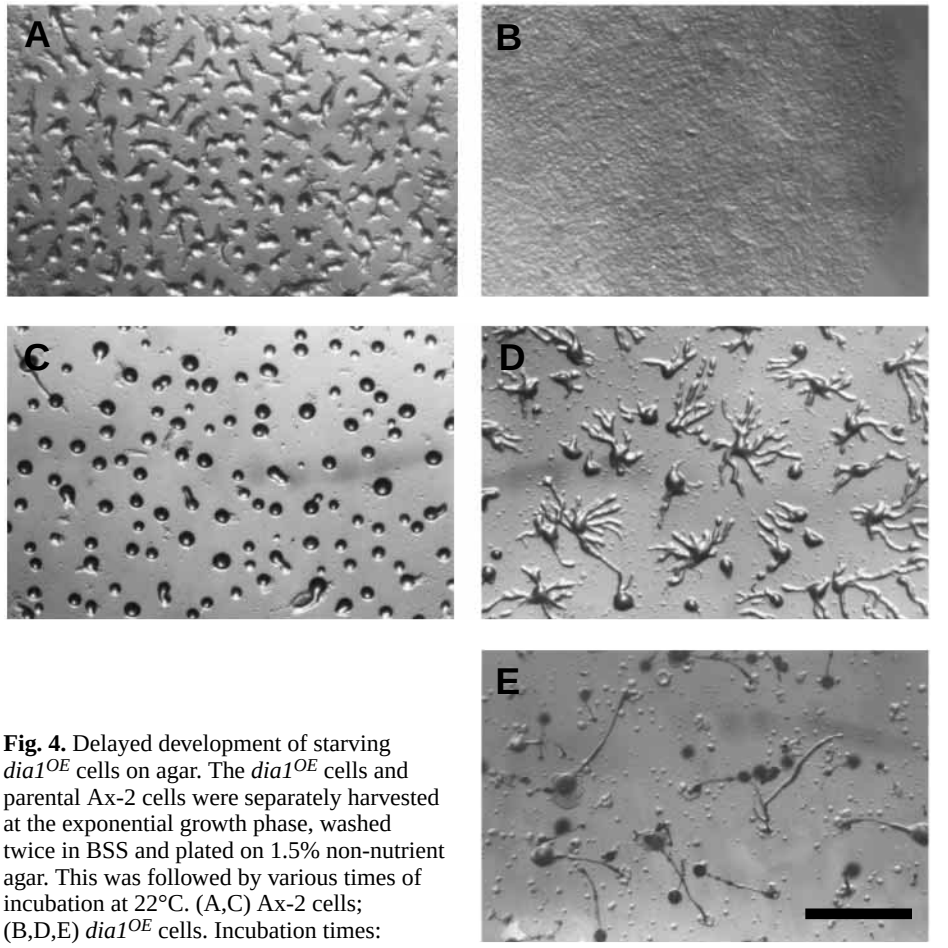

Fig. 4. Delayed development of starving *dia1*^{OE} cells on agar. The *dia1*^{OE} cells and parental Ax-2 cells were separately harvested at the exponential growth phase, washed twice in BSS and plated on 1.5% non-nutrient agar. This was followed by various times of incubation at 22°C. (A,C) Ax-2 cells; (B,D,E) *dia1*^{OE} cells. Incubation times: (A,B) 5.5 hours, (C,D) 7 hours and (E) 24 hours. The *dia1*^{OE} cells exhibit delayed aggregation (B,D), as compared with Ax-2 cells (A,C), but eventually form fruiting bodies after 24 hours of incubation (E). Bar, 200 μm.

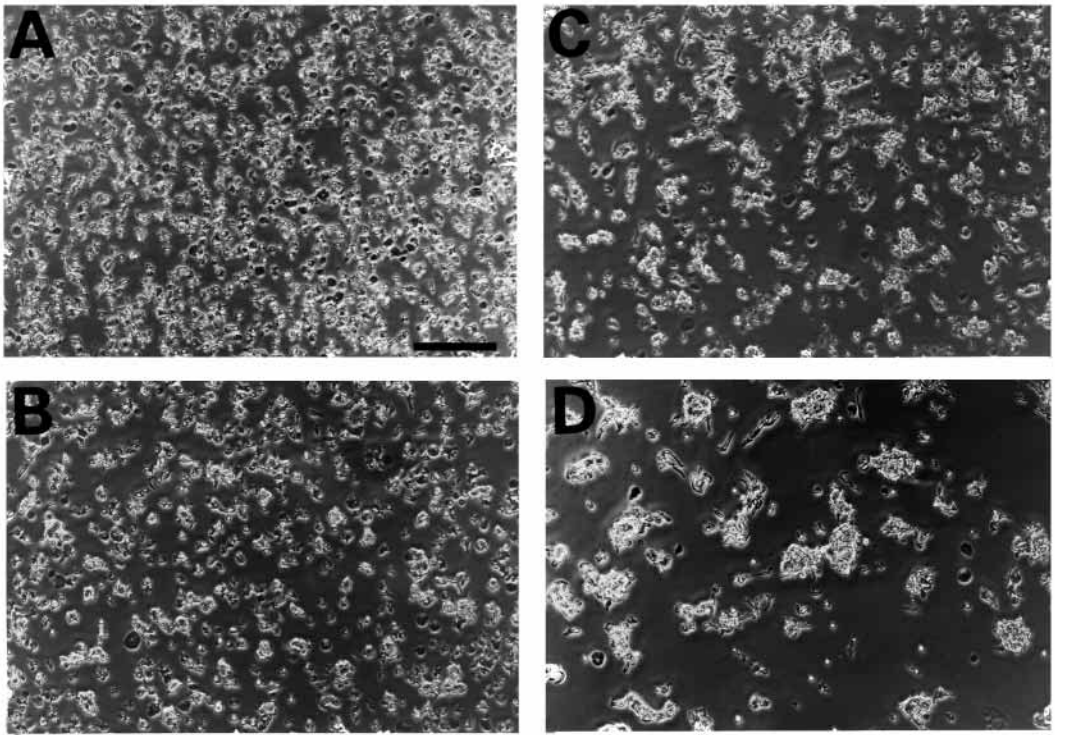

Fig. 5. Antisense-mediated gene inactivation of *dia1* enhances the progression of cell differentiation. AS-8, a *dia1*-underexpressing clone, and parental Ax-2 were separately harvested at the exponential growth phase, washed twice in BSS and suspended at a density of 2.8×10^5 cells/cm^2. This was followed by incubation at 22°C for 5.5 hours (A,C) and 7 hours (B,D). It is clear that AS-8 cells have already acquired aggregation competence at 5.5 hours of incubation (C) and form loose aggregates at 7 hours (D), while that many of Ax-2 cells still remain as non-aggregated single cells even after 7 hours of incubation (B). Bar, 150 μm.

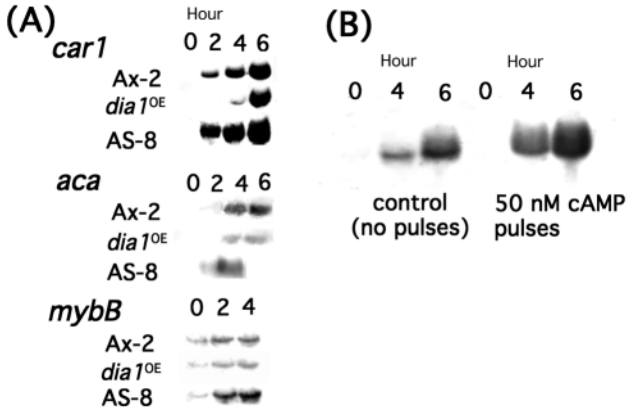

Fig. 6. (A) Gene expression of *car1*, *aca* and *mybB* in *dia1*-overexpressing (*dia1*^{OE}) and -underexpressing (AS-8) cells. At the indicated time after starvation, total RNAs were extracted from *dia1*^{OE}, AS-8 and parental Ax-2 cells. The RNAs (20 μg for each) were size-fractionated and analyzed by northern blots using the cDNA probe of each gene. (B) Induction of the *car1* expression in *dia1*^{OE} cells by cAMP pulses. Exponentially growing *dia1*^{OE} cells were harvested, washed in BSS and suspended at a density of 1×10^7 cells/ml. The cell suspension was shaken at 22°C with or without 50 nM cAMP pulses at 6 minute intervals for 6 hours. At the indicated time, total RNAs were extracted from the cultures. The RNA samples (20 μg) were size-fractionated and analyzed by northern blots using the *car1* probe. It is evident that the *car1* expression in *dia1*^{OE} cells is considerably augmented by the cAMP pulses, particular after 4 hours of incubation.

through inhibition of the expression of *car1* and *aca*. This is consistent with the present fact that underexpression of the *dia1* mRNA by an antisense transcript induces *car1* and *aca* expression precociously and then stimulates cAMP-mediated chemotaxis for cell aggregation. Accordingly, the DIA1 protein seems to be negatively coupled with CAR1- and/or ACA-associated events. Consistent with the above results, expression of *mybB*, which is known to induce the *aca* expression (Otsuka and Haastert, 1998), was also enhanced by *dia1* underexpression and suppressed by *dia1* overexpression. However, it is presently not known if the DIA1 protein is directly related to *mybB* expression, because the observed *dia1* effects on the *mybB* expression are only subtle (Fig. 6A).

Ax-2 cells at any cell-cycle phase have been shown to enter the differentiation phase from the PS point when starved (Maeda et al., 1989). Therefore, to reach the PS point there is a 7 hour difference in timing between synchronized T0 cells (just after the PS point) and T7 cells (just before the PS point). In response to starvation, T7 cells rapidly acquire cAMP signaling systems as well as EDTA-resistant cohesiveness and function as center cells for aggregation (Ohmori and Maeda, 1987; Araki et al., 1997). When starved T0 cells and T7 cells were separately incubated, theoretically there should be a 7 hour difference in developmental time course between the two. Practically, however, the time difference has been shown to be only 3-4 hours (Ohmori and Maeda, 1987). This apparent discrepancy is puzzling. One possibility is that the 7 hour difference is too large to allow all Ax-2 cells at any cell-cycle phase to participate cooperatively in aggregate formation. As regards the function of DIA1, provided that the *dia1* expression transiently suppresses the progression of

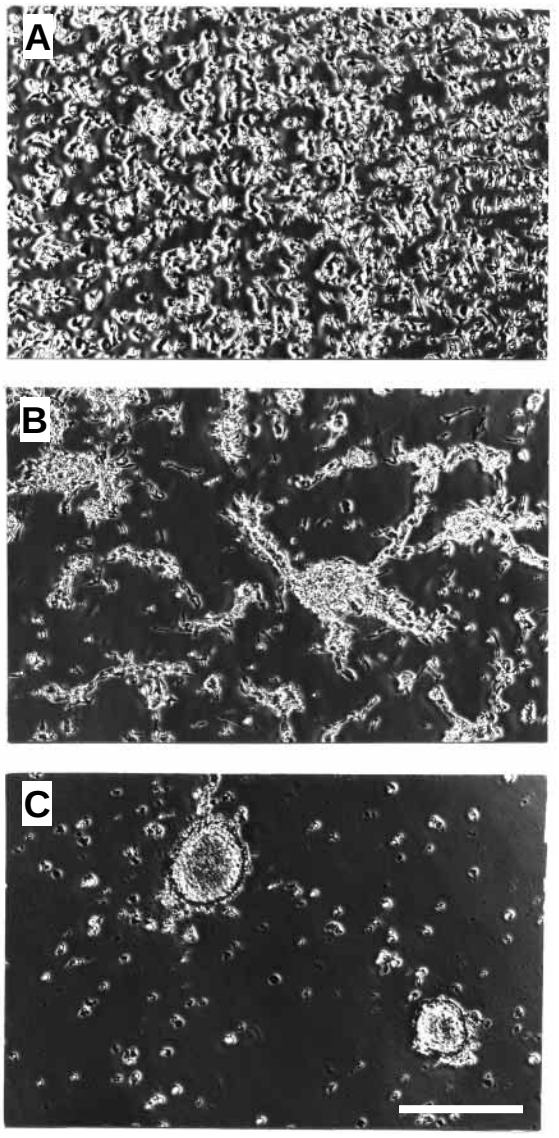

Fig. 7. Effect of cAMP pulses on the development of *dia1*^{OE} cells. The *dia1*^{OE} cells were shaken in BSS for 6 hours with or without externally applied 50 nM cAMP pulses, were placed in 24-well plates at a density of 5×10^5 cells/cm^2 and incubated at 22°C. (A) Development of non-pulsed *dia1*^{OE} cell at 6.5 hours after starvation. Cells show no sign of aggregation. (B) Development of cAMP-pulsed *dia1*^{OE} at 6.5 hours after starvation. A number of aggregation streams are formed. (C) Development of cAMP-pulsed *dia1*^{OE} cells at 24 hours after starvation. Tight aggregates are formed. Bar, 150 μm.

differentiation in T7 cells, it is possible that the time difference (about 7 hours) between T0 and T7 cells may be shortened, thus allowing both the cells to coordinately form a common aggregate. In this connection, it is of interest to note that the developmental defect as observed in the *dia1*-overexpressing cells (*dia1*^{OE} cells) is almost completely canceled by co-culture with wild-type Ax-2 cells. Based on the experimental data shown in Figs 6B and 7, it is possible that a certain level of cAMP secreted from differentiating cells may remove the inhibitory effect of DIA1 and then allow *dia1*^{OE} cells to develop normally.

We have already isolated and identified several genes (*car1*, *caf1*, *dia1*, *dia2*, *dia3* etc.*)* expressed differently during the phase-shift from growth to differentiation (reviewed by Maeda, 1997). The structural and functional characterization of these genes suggest the importance of cAMP, Ca^{2+} and their related events for the growth/differentiation transition of *Dictyostelium* cells. In general, *Dictyostelium* cells cannot differentiate under low $[Ca^{2+}]_e$ conditions around 5×10^{-7} M. The intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) also are greatly affected by externally added EGTA concentrations (Wick et al., 1978). Interestingly, low $[Ca^{2+}]_e$ around 10^{-6}-10^{-7} M had a more severe effect on *dia1*^{OE} cells than on parental Ax-2 cells. This indicates that the DIA1 protein contributes negatively to the proper Ca^{2+} homeostasis in the cells. Since the DIA1 protein is extraordinary serine-rich (Fig. 2) and also may be involved in the intercellular communication as a potent signal transducer, we are now planning to obtain a large amount of purified DIA1 protein and its specific antibody to analyze the precise localization and function of DIA1.

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