DGAP1, a homologue of rasGTPase activating proteins that controls growth, cytokinesis, and development in *Dictyostelium discoideum*

Jan Faix*, Werner Dittrich

Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany

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Abstract A protein accumulated in the cortical region of *Dictyostelium discoideum* cells proved to be a homologue of GTPase activating proteins that are responsible for the inactivation of ras in yeast and man. Elimination of this protein, DGAP1, by gene replacement resulted in an increased rate of growth of *D. discoideum* cells on bacterial lawns, and in the formation of aberrant, multi-tipped fruiting bodies. Overexpression of DGAP1 caused the cells to become multi-nucleated since chromosome segregation during mitosis was not reliably followed by cleavage of the cells. These results suggest that in *D. discoideum*, ras or a related small GTP-binding protein is involved in regulating growth based on the phagocytosis of bacteria, and in coupling activities of the cell cortex to the organization of spindle and asters in mitotic cells.

Key words: Cytokinesis; *Dictyostelium*; rasGAP homologue; Phagocytosis

1. Introduction

Members of the ras superfamily of small guanine nucleotide binding proteins act as molecular switches by cycling between an active GTP-bound and an inactive GDP-bound state. They are switched on by guanine nucleotide exchange factors (GEFs) that catalyse the exchange of bound GDP for GTP, and are turned off by proteins that increase their intrinsic GTPase activity (GAPs). In *Dictyostelium discoideum*, a ras-GEF homologue has recently been characterized and shown to be required for cell aggregation [1]. Here we identify a GAP in *D. discoideum* (DGAP1) that shows sequence similarities to ras-related GAPs of other organisms. Elimination of DGAP1 leads to aberrations of multicellular development after the aggregation stage, similar to disturbances of fruiting body formation previously observed in a mutant producing activated rasD-Thr12. Null and overexpressing mutants indicate that DGAP1 is involved in the control of two cell functions related to cytoskeletal activities in *D. discoideum*, the growth of cells on bacteria and mitotic cell division.

2. Materials and methods

Antibody 216-394-1, here designated mAb 394, was raised in BALB/c mice by immunization with a fraction of actin-associated proteins [2]. Screening a *D. discoideum* AX3 λgt11 cDNA library (Clonetech, Palo Alto, CA) with mAb 394 yielded two clones with large inserts. These inserts were excised with *Eco*RI, purified in agarose gels, and cloned into the *Eco*RI site of pIC20R [3]. The inserts were sequenced on both strands using sequence-specific primers and a T_7-polymerase sequencing kit (Pharmacia, Uppsala, Sweden). The predicted amino acid sequence of DGAP1 was analyzed using the MIPSX database, the FASTA program, and aligned with the CLUSTAL program of the Genetics Computer Group software package.

Cells of the *D. discoideum* AX2-214 strain, referred to as wild type, and of transformants were cultivated in shaken suspension at 23°C in nutrient medium as described [4]. To induce development, cells were starved in 17 mM K/Na-phosphate buffer, pH 6.0 and shaken. Growth rates were determined for *D. discoideum* wild type or mutants by transferring cells with a toothpick onto a lawn of *Klebsiella aerogenes* on SM nutrient agar plates. The plates were incubated at 23°C and the radius of colonies was measured every 24 h.

For gene replacement, a *Dgap1* targeting vector was constructed by excising the 1.4 kb blasticidin (*Bsr*) cassette with *Xba*I and *Hin*dIII from plasmid pBsr2 [5]. The overlapping ends were blunted to insert the *Bsr* fragment into the single, blunted *Xho*I site of *Dgap1* cDNA, which is located in the predicted catalytic domain of DGAP1. The 4.0 kb fragment of *Dgap1* cDNA that harbored the *Bsr* cassette was excised with *Eco*RI and used to transform the AX2 strain by the calcium phosphate method. After selection of resistant clones with 10 μg/ml of blasticidin S [5] in nutrient medium for one week, the independent DGAP1-null transformants G3-, G5-, and G10- were identified with mAb 394 by a colony-blot technique [6]. For the rescue of G3- and G5-, and for overexpression of DGAP1 in the AX2 wild-type, a 2.6 kb *Dgap1* cDNA fragment comprising the complete coding region was inserted in sense orientation into the *Eco*RI site of expression vector pDEXRH [7]. In the resulting plasmid, *Dgap1* transcription is driven by the *D. discoideum* actin 15 promoter. Rescued DGAP1-null transformants G3-R, G5-R, and the DGAP1-overexpressing strain G2++ were obtained by selection with 20 μg/ml of G418 for 2 weeks.

For fluorescence labeling, growth-phase cells were fixed with picric acid/formaldehyde, labeled with DGAP1-specific mAb 394 or anti-α-tubulin mAb YL1/2, or stained with DAPI as described [2]. Southern and Northern blots were hybridized in the presence of 50% formamide, and Western blots performed after SDS-PAGE of total cellular proteins in 10% gels. The Western blots were labeled using hybridoma 216-394-1 or 176-2-5 [8] culture supernatant and iodinated sheep anti-mouse IgG (Amersham, Braunschweig, Germany). For quantification of DGAP1 overexpression, antibody ^{125}I-label on the 95 kDa band of DGAP1 was determined with a Fuji Phosphoimager.

3. Results

3.1. Sequence characteristics and expression of DGAP1

Monoclonal antibodies, obtained by injecting mice with a fraction of actin-associated proteins, were used for the screening of a λgt11 library of *D. discoideum* cDNA. One antibody, mAb 394, recognized a cDNA fragment that showed sequence homologies to GAP catalytic domains of other organisms, in particular to the ras-specific Sar1 of *Schizosaccharomyces pombe* and to the human ras-related IQGAP (Fig. 1A). The complete cDNA-derived sequence of DdGAP1 is shown in Fig. 1B; it indicates that the catalytic domain of the *D. discoideum* protein is flanked by sequence strefches with a high probability of coiled-coil formation. With 39 residues interspersed, 8 heptad coiled-coil repeats are located at the N-terminal side of the catalytic domain. At its C-terminal side, the catalytic domain is followed with a distance of 227 residues by 6 heptad repeats (Fig. 2, top panel). In Sar1 and IQGAP, putative coiled-coil regions are flanking the catalytic

*Corresponding author. Fax: (49) (89) 8578-3885.
E-mail: Faix@vms.biochem.mpg.de

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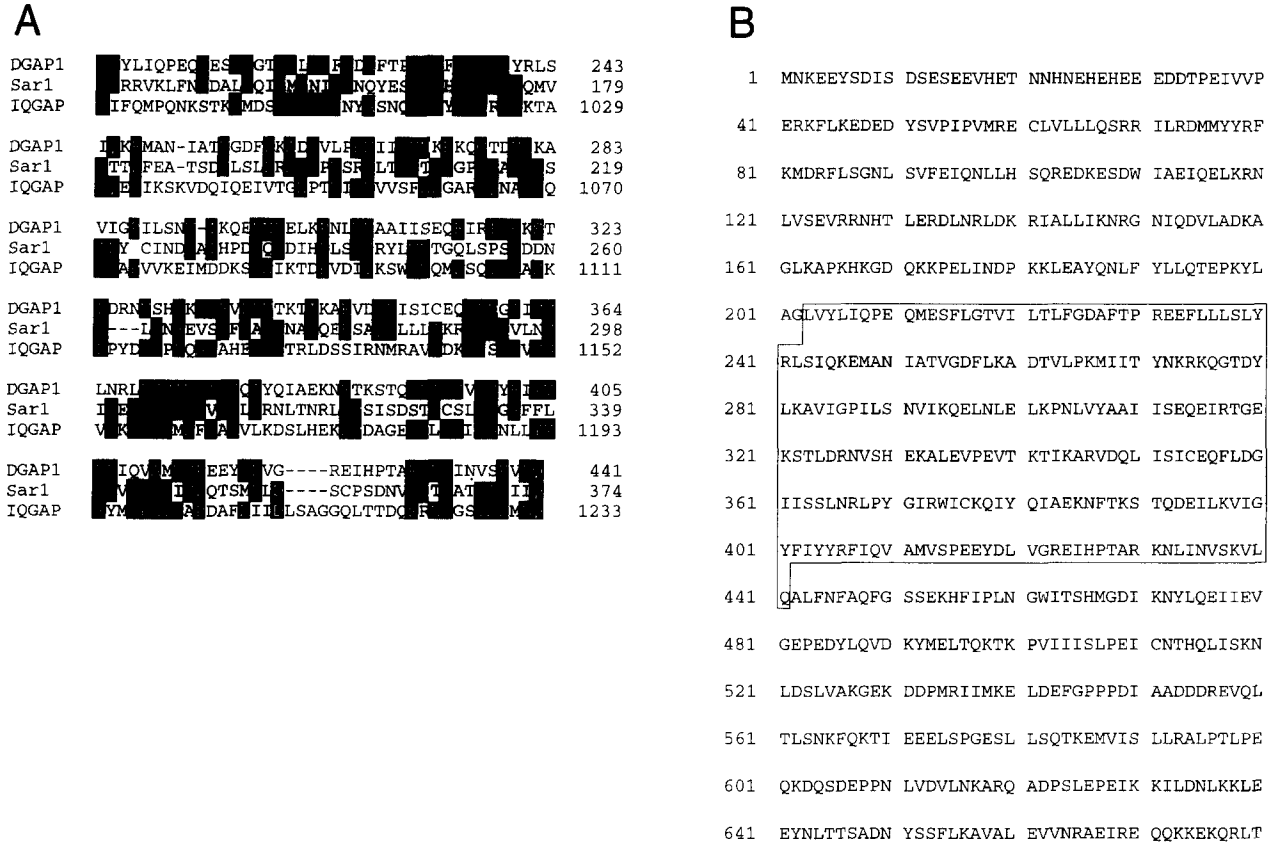

Fig. 1. A: Comparison of the amino acid sequence of the predicted catalytic domain of DGAP1 with corresponding domains of two ras-related GAPs, Sar1 of *S. pombe* [9] and human IQGAP1 [10]. Identical amino acid residues are shown on black ground. B: Complete cDNA-derived amino acid sequence of DGAP1. The boxed region corresponds to the predicted catalytic domain. The sequence of the *Dgap1* gene is available from GenBank/EMBL/DDBJ under accession number L79794.

consensus domain at similar distances as in DGAP1 (Fig. 2, middle and bottom panels).

Southern analysis indicates that under high stringency only single fragments are labeled with a *Dgap1* probe after digestion of genomic DNA from *D. discoideum* with a variety of restriction enzymes (Fig. 3A). This result, indicating a single gene that encodes DGAP1, is in accord with Western blots, in which a single band representing a protein of 95 kDa was labeled in the growth phase and during early developmental stages of *D. discoideum* cells (Fig. 3B). In Northern blots, however, two bands of 3.0 and 2.9 kb were recognized (Fig. 3C). These transcripts might be the result of differential splicing or some other posttranscriptional modification of the RNA. Both the Western and the Northern blots indicate that the DGAP1 gene is constitutively expressed.

3.2. Mutants that lack or overexpress DGAP1

Using a gene replacement vector containing a blasticidin (*Bsr*) resistance cassette, mutants lacking DGAP1 were selected. On Western blots of total cellular proteins from three independent transformant strains (G3-, G5-, and G10-), no labeled band was detected (Fig. 4A). Absence of the protein from the mutant cells was confirmed by immunofluorescence labeling. Whereas in fixed cells of the wild-type, DGAP1 was visualized in the cytoplasm and seen to be strongly enriched in the cell cortex, no antibody label was detected in the mutant cells (Fig. 4B).

A *Dgap1* probe hybridized to Southern blots of genomic DNA from the G3- and G10- strains showed an upshift of the labeled band by 1.4 kb, as predicted from the size of the inserted *Bsr* cassette. The smaller size of the corresponding fragment in the G5- strain indicates that integration of the vector was accompanied in this particular mutant by a deletion (Fig. 4C). Proof for integration of the vector specifically into the *Dgap1* gene was obtained by hybridizing Southern blots with a *Bsr* probe (Fig. 4D). In all three transformants analyzed, the single bands labeled with the *Dgap1* probe were the only ones that contained the *Bsr* cassette. The absence of the *Bsr* label from any other band demonstrates that the vector did not randomly integrate into other regions of the genome.

For the rescue of null mutants and for the overexpression of DGAP1 in the wild type, cells were transfected with a vector carrying the entire coding region of the protein under control of the highly active actin 15 promoter of *D. discoideum*. Strong selection with G418 was used in order to force multiple copies of the vector to integrate into the genome. In four transformants including the G2++ strain later chosen for further analysis (see Section 3.4), at best a threefold increase of DGAP1 over wild type expression was obtained on the protein level, as revealed by quantification of the antibody label on Western blots.

3.3. Increased growth rate and disturbed development in mutants that lack DGAP1

At the border of a colony of *D. discoideum* cells growing on

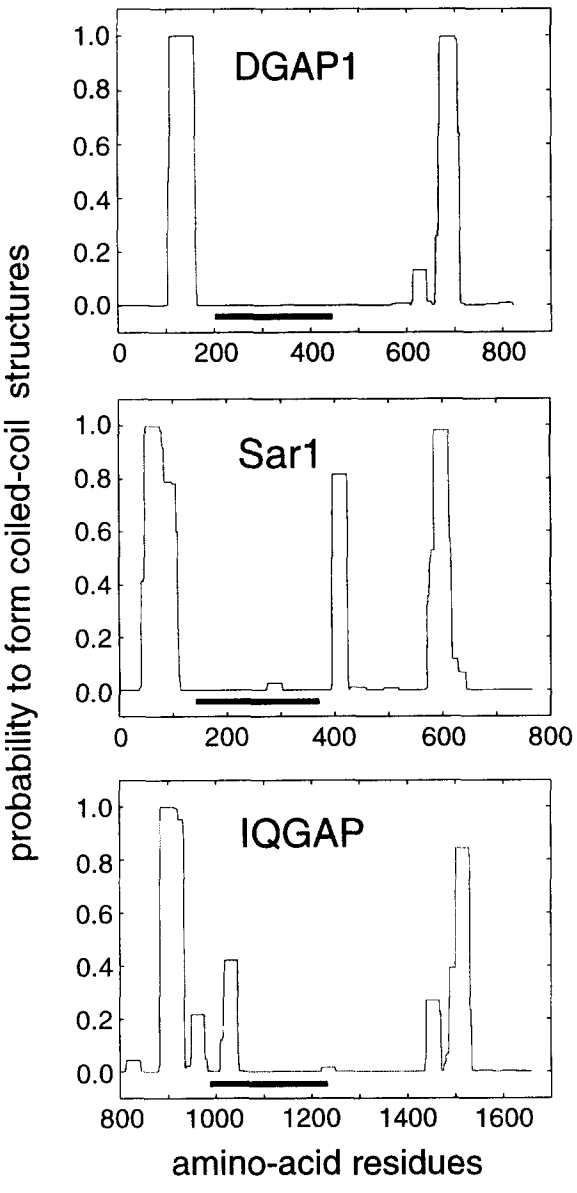

Fig. 2. Coiled-coil structure predictions for DGAP1, Sar1 and for the C-terminal region of IQGAP. Sequences were analyzed by the COILS algorithm, using MTIDK matrix and a window size of 28 [11]. The horizontal bars indicate positions of the consensus catalytic domains.

an agar surface, bacteria are completely used up, and a constant number of bacteria (about 10^3) is required for completion of one cell cycle [12]. Consequently, the increase in size of the colonies with time is a relative measure for the uptake rate of bacteria and also for the growth rate of wild type and mutant cells. The sizes of colonies visualized on blots suggested that DGAP1-null cells grow faster than wild type cells, and that the growth rate can be normalized by rescue of DGAP1 production in the mutant cells (Fig. 5A,B). A negative correlation between the expression of DGAP1 and the rate of growth was established for cultures on agar plates by measuring at intervals the colony diameters of wild-type and DGAP1-null mutants, as well as of rescued and DGAP1-overexpressing mutants (Fig. 5C,D).

The increase in the growth rate of DGAP1-null mutants on bacteria was accompanied by alterations of development at the multicellular stage. The DGAP1-null cells were capable of aggregating, but within the aggregates multiple tips were formed. These tips were broader than those of the wild type, and gave rise to branched multicellular structures (Fig. 5E,F). Eventually, atypical fruiting bodies were formed by DGAP1-null mutants, that had short, thick stalks and often irregularly shaped clusters of spores. These structures contrast to the regular fruiting bodies in the wild-type, where spores are located in a rounded head at the tip of a tapered stalk.

3.4. *A cytokinesis defect in cells overexpressing DGAP1*

Many large cells were detected in cultures of mutants that overexpress DGAP1. The staining of DNA with DAPI displayed many nuclei in these cells, mostly located in their central region (Fig. 6A). By counting the nuclei, mutant cell populations were clearly distinguishable from wild type ones. Whereas in the wild type 93% of the cells contained one or at most two nuclei, 69% of the cells had three or more nuclei in the DGAP1-overexpressing mutant G2++ (Fig. 6B).

Mitosis occurred synchronously in the nuclei of a DGAP1-overexpressor cell. This was shown by the labeling of α-tubulin, which revealed asters of microtubules and spindles of similar length in all nuclei of a multi-nucleated cell, and thus provided evidence that all the nuclei were in the same stage of mitosis (Fig. 6C, left panel).

When DGAP1-null cells were attached to a substrate surface, the tendency to grow into multi-nucleated cells was counterbalanced by irregular fragmentation of the cells into pieces which moved away from each other. This process occurred in the interphase state of the cell cycle (Fig. 6C, middle and right panels) and resembled the traction-mediated cytofission observed in other mutants of *D. discoideum* [2,13,14]. By traction-mediated cytofission, portions of a multi-nucleated cell are pinched off due to the active movement into different directions of multiple leading edges [15].

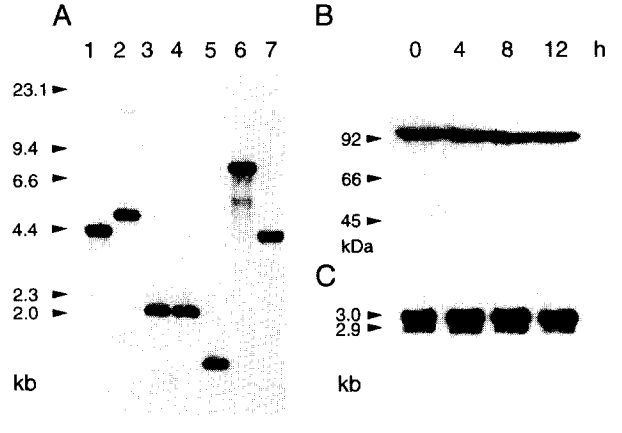

Fig. 3. Southern (A), Western (B), and Northern (C) blots labeled with a *Dgap1* DNA probe or with anti-DGAP1 mAb 394, respectively. The blots indicate the presence of a single *Dgap1* gene in the *D. discoideum* genome, and show its expression during growth and early development. For lanes 1–7 of A, wild type genomic DNA was digested with *Eco*RI, *Hin*dIII, *Eco*RI/*Hin*dIII, *Dra*I, *Dra*I/*Hin*dIII, *Eco*RV, or *Eco*RV/*Hin*dIII, and labeled with a probe encoding residues 180–332 located in the catalytic domain. For B, total proteins from 1×10^6 cells per lane were loaded, and for C, 10 μg of total cellular RNA per lane.

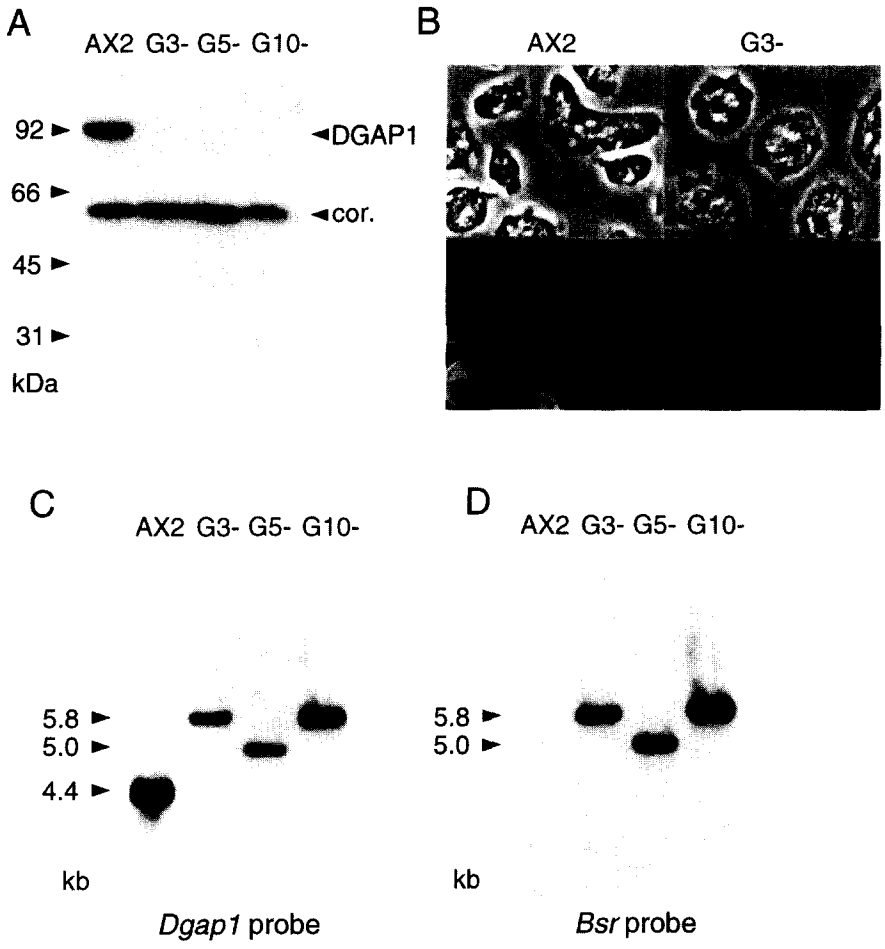

Fig. 4. Analysis of DGAP1-null mutants by Western blots (A), immunofluorescence labeling (B), and Southern blots (C, D). A: Total cellular proteins from wild-type AX2 and from three independent (G3-, G5-, and G10-) transformants were double-labeled after SDS-PAGE with anti-DGAP1 mAb 394 and anti-coronin mAb 176-2-5. MAb 394 labeled the 95 kDa DGAP1 band in the wild type but not in the mutants. MAb 176-2-5 served as a control; it labeled the 55 kDa protein coronin (cor.) in all four strains. B: Immunofluorescence labeling of wild type AX2 and DGAP1-null cells of strain G3-. Top panels: Phase-contrast images. Bottom panels: Indirect fluorescence label with anti-DGAP1 mAb 394 as a first antibody and TRITC-conjugated anti-mouse IgG as a second antibody. Bar: 10 μm. C, D: Southern blots of *Eco*RI digested genomic DNA of wild type and three DGAP1-null mutant strains. The blot in C was labeled with a *Dgap1* probe comprising the entire coding region, in D the same blot was hybridized with a probe representing the blasticidin resistance cassette (*Bsr*).

4. Discussion

Two findings argue for an interaction of DGAP1 with a ras protein of *D. discoideum*: the similarity of the multicellular structures formed in DGAP1-null mutants to the multi-tipped structures typical of mutants that express constitutively activated rasD [16], and the sequence relationship of its catalytic domain to Sar1 [9] and to other ras-related GAPs [10,17,18]. However, like IQGAP, DGAP1 lacks invariant amino acid residues, in particular Phe and Leu in position 404 and 405 which are believed to be crucial for the catalytic activity of RasGAPs [10]. Therefore, the interaction and the stimulation of the GTPase activity of ras by DGAP1 need to be established. If so, the possibility that DGAP1 may react specifically with a subset of the five isoforms of ras identified in *D. discoideum* cells [19] has to be taken into account. The knock-out of DGAP1 does not seem to be completely equivalent to expression of activated rasD, since no increase in growth rate has been reported for the rasD-Thr12 mutant [16]. This is not surprising, since rasD is expressed only during development

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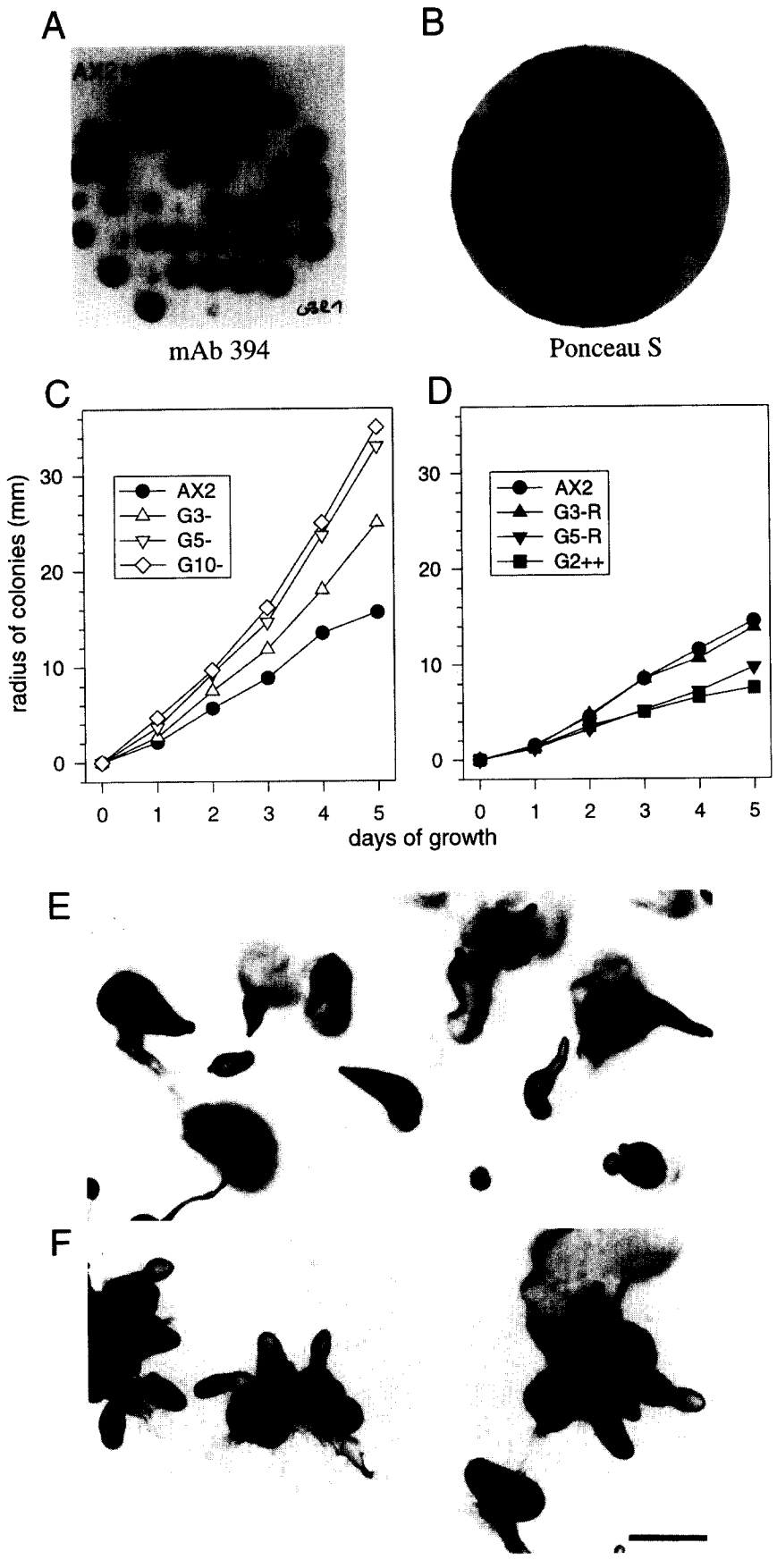
Fig. 5. Phenotypic analysis of DGAP1-null mutants. A, B: Colony blot of wild type and mutants grown on a lawn of bacteria. For A the blot was labeled with anti-DGAP1 mAb 394, for B the same nitrocellulose filter was stained for the transferred cellular proteins. The blot shows the result of an experiment in which *Dgap1* expression was rescued in G3- cells. A control colony of wild type AX2 is indicated on top. The other colonies either remained DGAP1-null, since the efficiency of the rescue was less than 100%, or they became DGAP1-positive by harboring the *Dgap1* construct used for rescuing. Large colony size as visualized in B coincides with the absence of *Dgap1* expression as revealed in A. C, D: Increase in the size of colonies on a bacterial lawn as a measure of growth rate. In C, three DGAP1-null mutants are compared with wild type AX2, as indicated in the inset. In D, two rescued mutants, G3-R and G5-R, and the DGAP1-overexpressing strain G2++ are compared with wild type. E: Early culmination stages of wild type AX2; F: the same of DGAP1-null mutant G3- illustrating the formation of multiple, broad tips in the mutant. Bar: 0.5 mm.


A
B
mAb 394
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C
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radius of colonies (mm)
AX2
G3-
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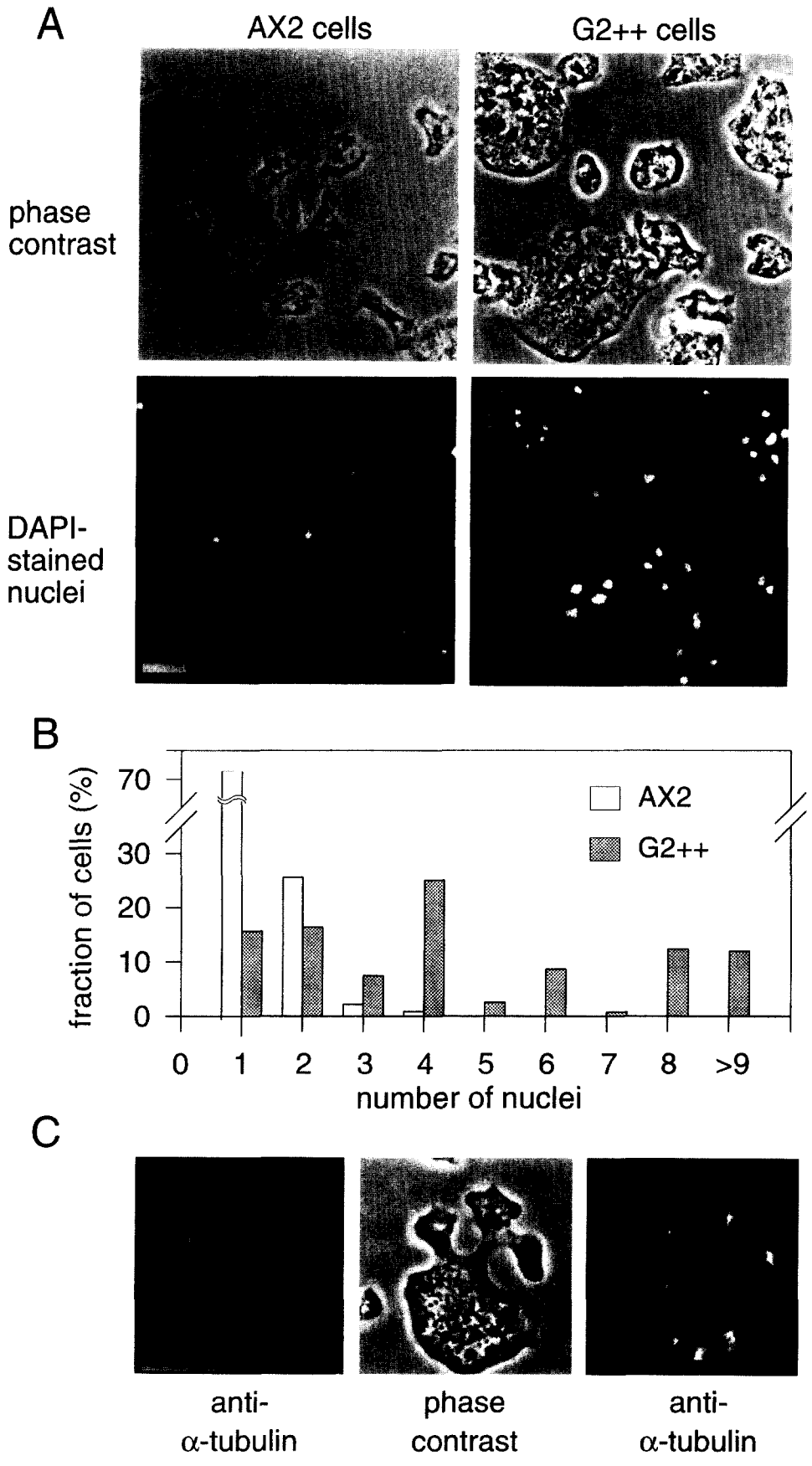
G10-
AX2
G3-R
G5-R
G2++
days of growth
E
F


A
AX2 cells
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70
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Fig. 6. Impairment of cytokinesis in DGAP1-overexpressing cells. A: Phase-contrast images (top panels) and nuclei stained with DAPI (bottom panels) of wild type AX2 and the overexpressor G2++. B: Histogram showing distribution of the number of nuclei in cells of the wild type and of the overexpressing mutant. Nuclei in 200–250 cells were counted for each strain. C: G2++ cells labeled for α-tubulin to reveal organization of the microtubular system in anaphase (left panel) and interphase (right panel) of multi-nucleated DGAP1-overexpressor cells. The phase-contrast image in the middle depicts the same cell as shown on the right, to demonstrate pinching off of mononucleated portions from the cell body, each portion containing a separate system of microtubules around a microtubule organizing center. Bars in A and C: 10 μm.
←

[20], while DGAP1 is already present during growth. By a similar argument, DGAP1 does not seem to have the same specificity for members of the ras superfamily as aimless ras-GEF [1]. Aimless-null cells of *D. discoideum* are severely impaired in chemotaxis and adenylyl cyclase activation. As a consequence, these cells are unable to aggregate. In contrast, overexpression of DGAP1 did not prevent aggregation and fruiting body formation, at least not at the threefold increased level that we maximally obtained.

A surprising finding, raising two questions, is the increased growth rate of DGAP1-null cells on bacteria (Fig. 5). One question is whether the disturbed fruiting body formation in the mutants is sufficient under natural conditions to antagonize an advantage in selection that an increased growth rate is supposed to have. The second question concerns the step that limits in wild type cells the rate of growth on a lawn of bacteria. Is the acceleration observed in DGAP1-null mutants due to an enhancement of the uptake of particles? The uptake of bacteria and yeast particles is known in *D. discoideum* to be based on activities of the actin cytoskeleton [21]. In mammalian cells it is well established that small GTPases, in particular rho, rac, and cdc42, are regulators of actin assembly [22]. DGAP1 is shown in this paper to be accumulated in the cortex of *D. discoideum* cells, this means at a site where rapid assembly, disassembly, and re-organization of the network of actin filaments takes place in a motile cell, and where the actin system is linked to the plasma membrane in a receptor-regulated way.

The deficiency in cytokinesis that is observed in DGAP1-overexpressing mutants points again to an implication of this GAP in the control of cytoskeletal activities. Mutants of *D. discoideum* known to be disturbed in cytokinesis fall into one of two categories. (1) Mutants defective in proteins like myosin II [13,14] and the actin-bundling proteins cortexillin I and II [2], that contribute to the contractile activities or the mechanical properties of the cell cortex. (2) Mutants altered in proteins that play a regulatory role in mitosis. Coronin, a WD40-repeat protein involved in dynamic rearrangements of the actin system [23], racE [24], and DGAP1 belong to this category. These and certainly more regulatory proteins are required in *D. discoideum* cells to guarantee the coordination in time and space of cytoskeletal activities in the cell cortex. Cleavage furrow formation and separation of the cells at the end of mitosis are based on these activities.

An attractive possibility, which will be pursued further, is that DGAP1 participates in the transmission of signals between the microtubular system and the actin system in the cell cortex. In wild type, the assembly of microtubules into the spindle and the asters of mitotic cells appears to dictate the position and time of formation of the cleavage furrow. The production of multi-nucleated cells in DGAP1-overexpressing mutants suggests that the activity of a small GTP-binding protein of the ras superfamily is required to reliably link the cortical activities in mitotic cells to the microtubular system.

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