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Temperature-Sensitive Inhibition of Development in *Dictyostelium* Due to a Point Mutation in the *piaA* Gene

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The *Dictyostelium* mutant HSB1 is temperature-sensitive for development, undergoing aggregation and fruiting body formation at temperatures below 18°C but not above. *In vivo* G protein-linked adenylyl cyclase activation is defective in HSB1, and the enzyme is not stimulated *in vitro* by GTPγS; stimulation is restored upon addition of wild-type cytosol. Transfection with the gene encoding the cytosolic regulator PIA rescued the mutant. We excluded the possibility that HSB1 cells fail to express PIA and show that the HSB1 *piaA* gene harbors a point mutation, resulting in the amino acid exchange $G^{917}D$. Both wild-type and HSB1 cells were also transfected with the HSB1 *piaA* gene. The $piaA^{HSB1}$ gene product displayed a partial inhibitory effect on wild-type cell development. We hypothesize that PIA couples the heterotrimeric G protein to adenylyl cyclase via two binding sites, one of which is altered in a temperature-sensitive way by the HSB1 mutation. When overexpressed in the wild-type background, PIA^{HSB1} competes with wild-type PIA via the nonmutated binding site, resulting in dominant-negative inhibition of development. Expression of GFP-fused PIA shows that PIA is homogeneously distributed in the cytoplasm of chemotactically moving cells.

Key Words: **adenylyl cyclase; chemotaxis; *Dictyostelium*; G protein; signal transduction; slime molds.**

INTRODUCTION

Cyclic AMP plays a key role in *Dictyostelium* development, acting as chemoattractant, as intracellular second messenger, and as a morphogen (Bonner, 1970; Gerisch, 1987; Johnson *et al.*, 1989; Parent and Devreotes, 1996; Bracco *et al.*, 2000). During growth, *Dictyostelium* cells behave as free-living amoebae, which feed on bacteria by phagocytosis and proliferate by binary fission. Depletion of food gives rise to aggregation centers, which arise spontaneously following pulsatile secretion of cAMP by the central cells within each aggregation territory. The periodic bursts of cAMP attract nearby cells and stimulate them to produce and release additional cAMP, thus relaying the signal distally in the aggregation territory (Alcantara and Monk, 1974; Tomchik and Devreotes, 1981; Gerisch, 1987; van Haastert, 1995). The cAMP pulsatile stimulation also induces repression of growth phase and enhancement of aggregation-specific genes, most notably genes involved in chemotaxis and cell–cell adhesion (Gerisch, 1987; Mann and Firtel, 1989; Bracco *et al.*, 2000). The concerted action of chemotactic cell motility and intercellular adhesion transforms a monolayer of single cells into multicellular three-dimensional aggregates.

Among the intracellular pathways regulated by cAMP, activation of the aggregation-specific adenylyl cyclase (ACA) is crucial both for establishment of the cAMP signal relay system and for its intracellular second messenger function, such as PKA activation and control of gene expression (Williams *et al.*, 1993; Parent and Devreotes, 1996; Anjard *et al.*, 1998). ACA is stimulated by Gβγ-linked activation upon cAMP binding to the membrane receptor cAR1 (Kumagai *et al.*, 1989; Sun and Devreotes, 1991; Wu

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et al., 1995). Activation requires the concomitant activity of at least two cytosolic proteins, CRAC and PIA (Lilly and Devreotes, 1994; Chen *et al.*, 1997). PIA is a 130-kDa component with no known domains, while CRAC contains a pleckstrin homology (PH) domain and binds to membrane PH domain sites which are generated by G$\beta\gamma$ following cAMP activation (Insall *et al.*, 1994; Parent and Devreotes, 1999). Both proteins are essential for ACA activation, but their mechanism of action is unclear.

Mutant HSB1 was described to be specifically defective in ACA activation (Bozzaro *et al.*, 1987a). The mutant was isolated after nitrosoguanidine mutagenization as aggregateless mutant, which could be induced to develop if synergized with wild-type cells. HSB1 cells displayed basal ACA activity and moved chemotactically if stimulated with cAMP diffusing from a microcapillary. ACA stimulation in response to cAMP pulses, however, was absent in the cells, in contrast to guanylyl cyclase, which was normally activated (Bozzaro *et al.*, 1987a). Since the mutant had been obtained by chemical treatment, it was likely to harbor several mutations, and for this reason no further analysis was undertaken.

Recently, we found serendipitously that the phenotype of HSB1 was temperature-sensitive. As will be described further below, HSB1 cells aggregate and form normal fruiting bodies at temperatures below 17°C, whereas they remain unicellular at 23°C, the standard temperature commonly used for studying wild-type cell development. The temperature sensitivity is an indication that the mutant could be specifically defective in one single gene. We have thus reanalyzed the phenotype of the mutant, by assaying the *in vitro* GTPγS activation of ACA. We found that ACA is not activated in HSB1 cell lysates, but activation could be restored by adding wild-type cytosol. By transformation with the wild-type *Pia* gene, the mutant phenotype was completely rescued. We have thus sequenced the HSB1 *piaA* gene and have found a single-point mutation resulting in a $G^{917}D$ substitution. We present evidence that PIA acts as an adapter between G protein and adenylyl cyclase via two binding sites, one of which is altered in a temperature-sensitive way by the mutation. By expressing a GFP–PIA fusion protein, we further show that PIA is distributed homogeneously in the cytoplasm of both resting and chemotactically moving cells, and we present evidence that PIA is strictly required for ACA activation but not for chemotaxis.

MATERIALS AND METHODS

Cell Cultures, Growth, and Developmental Conditions

Cells of the *Dictyostelium discoideum* strains AX2 and HSB1 and transformants expressing *piaA*, *crac*, or *GFP–piaA* were grown axenically in AX2 medium as described (Watts and Ashworth, 1970). Transfected cells were grown with 10 μg/ml G418 selection, unless otherwise indicated. For growth on agar, spores or cells were inoculated on *Escherichia coli B/2* on nutrient agar plates (Bozzaro *et al.*, 1987b). To induce development, cells were harvested by centrifugation at 1000*g* per 3 min and washed threefold in 0.017 mM Na/K Soerensen phosphate buffer, pH 6.0. For development in suspension, cells were resuspended at 1×10^7/ml in Soerensen phosphate buffer and shaken at 23°C and 150 rpm in a climatic cabinet equipped with gyratory shakers (Kühner, Bielefeld, Switzerland). For development on non-nutrient agar (1.5% agar in Soerensen phosphate buffer), starving cells were plated at a density of 5×10^5 cells/cm^2 in 90-mm diameter agar plates and placed in the climatic cabinet. For development at temperatures varying between 10 and 18°C or at 27°C, the plates were incubated in a basket connected to a thermostatic circulator (LKB, Multitemp II).

Chemotaxis Assay

The microcapillary assay (Bozzaro and Roseman, 1983) was used to induce oriented chemotactic motility. Starving cells were incubated on petri dishes, and local stimulation of chemotaxis was obtained by passive diffusion of cAMP from a microcapillary (Femtotips 1; Eppendorf, Hamburg, Germany), filled with a 1.0-mM solution of cAMP in Soerensen phosphate buffer. The microcapillary was positioned with an automated Zeiss micromanipulator (C. Zeiss, Oberkochen, Germany).

In Vitro GTPγS-Stimulated Adenylyl Cyclase Assay

In vitro GTPγS-induced stimulation of adenylyl cyclase was performed as described by Lilly and Devreotes (1994). Briefly, starving AX2 and HSB1 cells were treated with 20-nM cAMP pulses every 6 min for 5 h as described (Bozzaro *et al.*, 1987a). A total of 10^8 cells was pelleted and resuspended in 1 ml of Soerensen phosphate buffer. An equal volume of ice-cold lysis buffer containing 4 mM $MgCl_2$ in 20 mM Tris, pH 8.0, was added. Cells were lysed by passage through a 3-μm pore size Nucleopore membrane in the absence or presence of 30 μM GTPγS, and the lysate was kept on ice for 5 min. An aliquot of 40 μl cell lysate was added to a 40-μl assay mix (20 mM DTT, 1 mM ATP, 2 mM $MgCl_2$ in 10 mM Tris, pH 8.0) and incubated at 20°C for 5 min in a water bath. The reaction was stopped by adding 40 μl 0.1 M EDTA and boiling the samples for 2 min. The total concentration of cyclic AMP in the samples was determined by using the Biotrak cyclic AMP assay kit (Amersham Pharmacia Biotech, Buckinghamshire, England).

Cloning and Sequencing Techniques

To sequence the $piaA^{HSB1}$ gene, genomic DNA from strain HSB1 was extracted and digested with both *Cla*I and *Bam*HI for 6 h at 37°C (Sambrook *et al.*,1989). Total genomic DNA was ligated with pBluescript II SK(−) (Stratagene, La Jolla, CA), using the Amersham ligation system assay. The resulting construct was electroporated into *E. coli* strain MC1061 (Stratagene) by using the Bio-Rad gene pulser, according to the manufacturer's suggested protocol. From a total of 30.000 colonies, after blotting and hybridization with the $piaA^{wt}$ gene, 3 positives (pBPZ-1, -2, and -3) were obtained and sequenced with gene-specific primers using an automated sequencer (ABI 377 PRISM; Perkin Elmer, Norwalk, CT). There is a single *Bam*HI site within the *piaA* gene at nucleotide 702; therefore, to clone the upstream 702-bp fragment, this region was amplified by PCR (GeneAmp PCR system 2004; Perkin Elmer) using CTTGTAATGTCTCTGATCC and AAAAAAACTCCA-

TATAATC as, respectively, 5′ and 3′ primers. The PCR product was ligated in pGEMT (Stratagene) and cloned into *E. coli* strain MC1061, and two independent clones were sequenced. For sequence analysis, the MacVector 7 package software was used.

The full-length $piaA^{HSB1}$ gene was constructed by fusing the 702-bp fragment upstream of the *Bam*HI site, obtained from pMYC-86-6, which bears the $piaA^{wt}$ gene, with the pBPZ-1 vector. The resulting plasmid was called pBPZ-4.

Construction of Expression Vectors and Cell Transformation

To rescue the phenotype of HSB1 mutant, either the pMYC-86-6 vector, containing the $piaA^{wt}$ gene, or the pRHI38 vector, bearing the $crac^{wt}$ gene, was transfected in growing HSB1 cells by using the glycerol shock method (Nellen *et al.*, 1987). Trasformants were selected in AX2 medium plus 10 μg/ml G418 and were called $HSB1^{piawt}$ and $HSB1^{crac}$. After selection, cells were plated on *E. coli* at 23°C to examine their phenotype.

GFP–PIA expression vectors were constructed by fusing the $piaA^{wt}$ or $piaA^{HSB1}$ gene into the pDEXH vector containing the GFP gene (Faix *et al.*, 1992). The $piaA^{wt}$ gene was amplified by PCR from pMYC86-6 using the following primers: 5′-CCATCGATATGACAAGTTC and 3′-GGATCGATTTAATTTAAATCATG. The PCR product was ligated with pGEMT and cloned into dcm^-/dam^- *E. coli*. The resulting construct was digested with *Cla*I, purified, and inserted into the *Cla*I site of pDEXH vector. The resulting plasmid was called PDEXH-*GFP-PiaA*^{wt}. The full-length $piaA^{HSB1}$ sequence was purified from pBPZ-4 by digestion with *Cla*I and inserted in the pDEXH vector. The clone was called PDEXH-*GFP-PiaA*^{HSB1}. PDEXH-*GFP-PiaA*^{WT} and PDEXH-*GFP-Pia*^{HSB1} were electroporated into HSB1 and AX2 cells. Trasformants were selected in AX2 medium plus 20 μg/ml G-418.

Southern, Northern, and Western Blot Analyses

Genomic DNA was extracted and purified by CsCl gradient centrifugation as described in Nellen *et al.* (1987), digested with several enzymes, run onto 0.8% agarose gel, blotted onto Hybond-N membrane (Amersham Pharmacia Biotech), and subjected to Southern assay (Southern, 1975).

RNA isolation, transfer to Hybond-N membrane, and labeling were done as described (Bracco *et al.*, 1997). A ^{32}P-labeled 1.7-kb *piaA* or 2.0-kb *crac* cDNA fragment was used as a probe, and the membranes were then exposed to X-ray films (Kodak, Rochester, NY).

SDS–PAGE electrophoresis of total cell extracts, transfer to nitrocellulose, and immunoblotting were done as described (Bozzaro *et al.*, 1987b). The blot was incubated with a polyclonal antiserum raised against the carboxy terminus of the PIA protein (Chen *et al.*, 1997) and a horseradish peroxidase-conjugated anti-rabbit antibody. The protein bands were visualized by the ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotech) and exposed to Amersham Hyperfilm.

RESULTS

Mutant HSB1 Is Temperature-Sensitive for Aggregation

The mutant HSB1 was isolated as aggregation-deficient mutant, following nitrosoguanidine mutagenization of wild-type AX2 cells (Bozzaro *et al.*, 1987a). Recently, we found that the developmental defect in the mutant is temperature-sensitive. As shown in Fig. 1 (left), when HSB1 cells were incubated on a bacterial lawn at 23°C, they failed to aggregate, whereas they completed development at 13°C. Under the latter conditions, HSB1 cells formed mounds after about 40 h from starvation, compared with 20 h for AX2 cells, whereas postaggregative development proceeded with comparable timing for both strains (Fig. 1, right), suggesting that the critical developmental stage in the mutant is aggregation. To confirm this hypothesis, starving cells were incubated on non-nutrient agar at 13°C for different time periods and subsequently shifted to 23°C. A temperature shift at mound stage, or thereafter, did not affect further development (Fig. 2), whereas shifting from 13 to 23°C at any time before or during aggregation stopped development. If tight mounds, as those shown in Fig. 2A, were disaggregated and the single cells incubated at 23°C, they failed to reaggregate (data not shown). Thus, HSB1 cell aggregation is temperature-sensitive, and this sensitivity is not overcome by putative expression of late-aggregation genes.

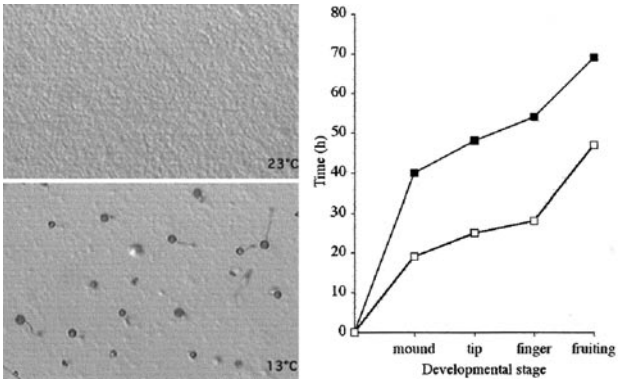

FIG. 1. Development of mutant HSB1 at permissive and nonpermissive temperatures. Starving HSB1 or AX2 cells were plated at a density of 5×10^5 cells/cm^2 on 90-mm diameter agar plates and incubated either at 13 or 23°C. (Left) The morphology of HSB1 cell colonies after 60 h of incubation is shown. At 23°C, cells fail to aggregate, whereas at 13°C, fruiting bodies are formed. (Right) Developmental timing of AX2 (open symbols) or HSB1 (closed symbols) cells incubated at 13°C. Except for aggregation, the time needed to reach the postaggregative stages is comparable for both cell types.

We determined the range of permissive temperature for HSB1 cell aggregation by incubating cells on non-nutrient agar at temperatures varying from 10 to 23°C. Between 10 and 16°C, cells aggregated and formed fruiting bodies, whereas they completely failed to aggregate at or above 18°C. The optimal temperature was 13°C, by which temperature-tight mounds were formed after 35–40 h from starvation, with a linear increase up to 60 h at 16°C. The temporal gap between HSB1 and AX2 cells to form mounds

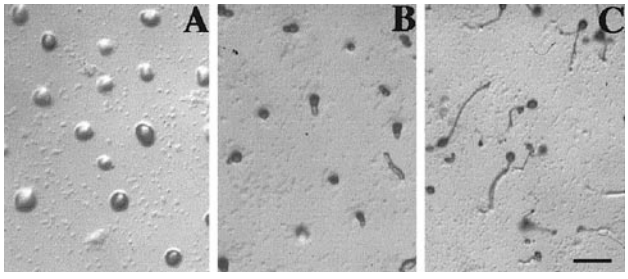

FIG. 2. Development of mutant HSB1 cell aggregates upon shifting from 13 to 23°C. Starving HSB1cells were plated at a density of 5×10^5 cells/cm^2 on 90-mm diameter agar plates and incubated at 13°C. When tight aggregates, as in (A), were formed, the plate was shifted at 23°C, and pictures were taken 5 (B) and 12 h (C) later.

was also closer at 13°C (about 20 h) than at higher temperatures (data not shown). Thus, mutant HSB1 is temperature-sensitive for aggregation, the permissive temperature being 10°C lower than in the wild type, for which the optimum is 23°C.

In Vitro Activation of G Protein-Mediated Adenylyl Cyclase Is Defective in HSB1, and Can Be Restored by Addition of AX2 Cytosol

ACA can be stimulated *in vitro* by treatment of cell lysates with GTPγS, a slowly hydrolysable GTP analog (Lilly and Devreotes, 1994). Addition of GTPγS to HSB1 cell lysates failed to stimulate ACA, whereas a threefold activation was observed in AX2 cell lysates (Fig. 3, left). To test whether the mutant is defective in a cytosolic component

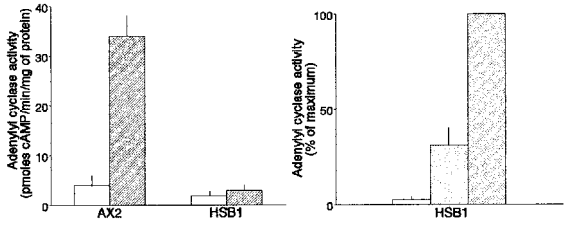

FIG. 3. Adenylyl cyclase activation and reconstitution in HSB1 cell lysates. Starving cells were treated for 5 h with 20 nM cAMP pulses every 6 min. Cell lysates in the presence or absence of 30 μM GTPγS were then prepared by pressing the cells through 3-μm pore size Nucleopore filter, and the lysate was kept on ice for 5 min, followed by further incubation for 5 min at 20°C in the reaction mix assay. The adenylyl cyclase activity was measured by isotope dilution, as described in Materials and Methods. (Left) Adenylyl cyclase activity in AX2 or HSB1 cell lysates in the absence (open bars) or presence (hatched bars) of GTPγS. (Right) GTPγS-stimulated adenylyl cyclase activity in HSB1 cell lysate in the absence (open bars) or presence of 60 μl (dotted bar) or 120 μl (hatched bar) AX2 cytosol.

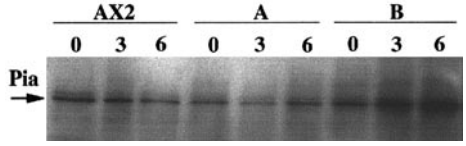

FIG. 4. Immunostaining of total cell protein with an antibody against the PIA protein. Total cell lysates of AX2, HSB1 (A) or HSB1^{piaAwt} (B), at time 0, 3, or 6 of development were separated by SDS–electrophoresis, blotted onto nitrocellulose, and stained with an antibody against the PIA protein.

required for GTPγS-induced ACA activation, cytosol of AX2 cells was added to HSB1 cell lysates following GTPγS treatment. As shown in Fig. 3 (right), under these conditions, activation of ACA by GTPγS was restored, thus indicating that the mutant is defective in a cytosolic component required for G protein-mediated adenylyl cyclase stimulation.

Mutant HSB1 Can Be Rescued by Transformation with the Gene piaA

At least two cytosolic proteins, CRAC and PIA, have been shown to be essential for G protein-mediated ACA activation in *Dictyostelium* cells (Insall *et al.*, 1994; Lilly and Devreotes, 1994; Chen *et al.*, 1997).

The finding that AX2 cytosol was able to reconstitute ACA stimulation in HSB1 cell extracts prompted us to search for the active component, by first testing its possible identity with CRAC or PIA. Thus, HSB1 cells were transfected with an expression vector harboring the resistance to G418 and containing either *crac*^{wt} or *piaA*^{wt} under the control of the actin-15 promoter. G418-resistant cells were examined for overexpression of *crac* or *piaA* mRNA by Northern blots and plated on bacterial lawn to observe their morphology. A wild-type phenotype was obtained from cells transfected with *piaA*^{wt}, whereas cells transfected with *crac*^{wt} failed to aggregate (data not shown). Western blot analysis of total cell lysates from AX2, HSB1, or HSB1 harboring *piaA*^{wt} confirmed a higher expression of wild-type PIA in the transfected cells, while showing at the same time that the HSB1 mutant expressed an endogenous PIA protein of molecular mass and amount comparable with the parent strain AX2 (Fig. 4).

Effects of piaA^{HSB1} Transfection in Mutant and Wild-Type Cells

The Western blot data suggested that the defect in HSB1 mutant is most likely due to a mutation affecting the amino acid sequence of PIA rather than regulation of its expression. It cannot be excluded, however, that the rescuing effect observed by transfecting the cells with *piaA*^{wt} could be due to PIA overexpression bypassing a mutation in a protein different from PIA.

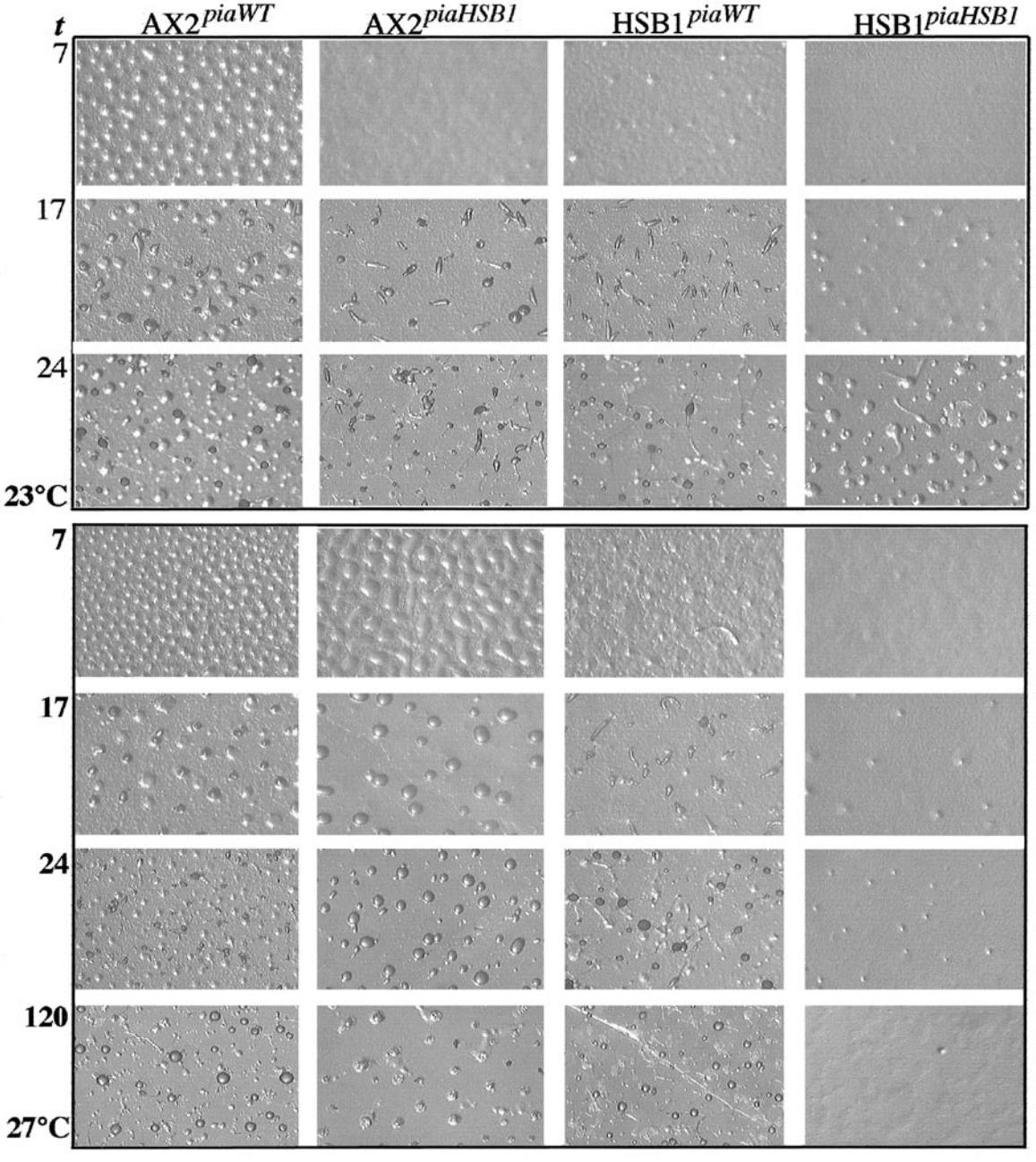

FIG. 5. Development at 23 or 27°C of AX2 or HSB1 expressing the wild-type or HSB1 *piaA* gene. AX2 or HSB1 cells transfected with the $piaA^{wt}$ or $piaA^{HSB1}$ gene were plated on non-nutrient agar at 23 (top) or 27°C (bottom). Photographs were taken at the time in hours indicated on the left.

To discriminate between these possibilities, we cloned the HSB1 *piaA* gene ($piaA^{HSB1}$), constitutively expressed it both in HSB1 or AX2 background, and followed cell development on non-nutrient agar at 23°C. In comparison to AX2 cells (either untreated or overexpressing $piaA^{wt}$), both $AX2^{piaAHSB1}$ and $HSB1^{piaAHSB1}$ cells started to aggregate with a delay varying between 3 and 6 h. Slugs and fruiting bodies were eventually formed in both cell types, but reduced in number compared with controls. The extent of inhibition was much stronger for $HSB1^{piaAHSB1}$, with mounds and fruiting bodies formed after 24 and 36 h, respectively (Fig. 5, top). Clearly, expression of $piaA^{HSB1}$ seems to have two

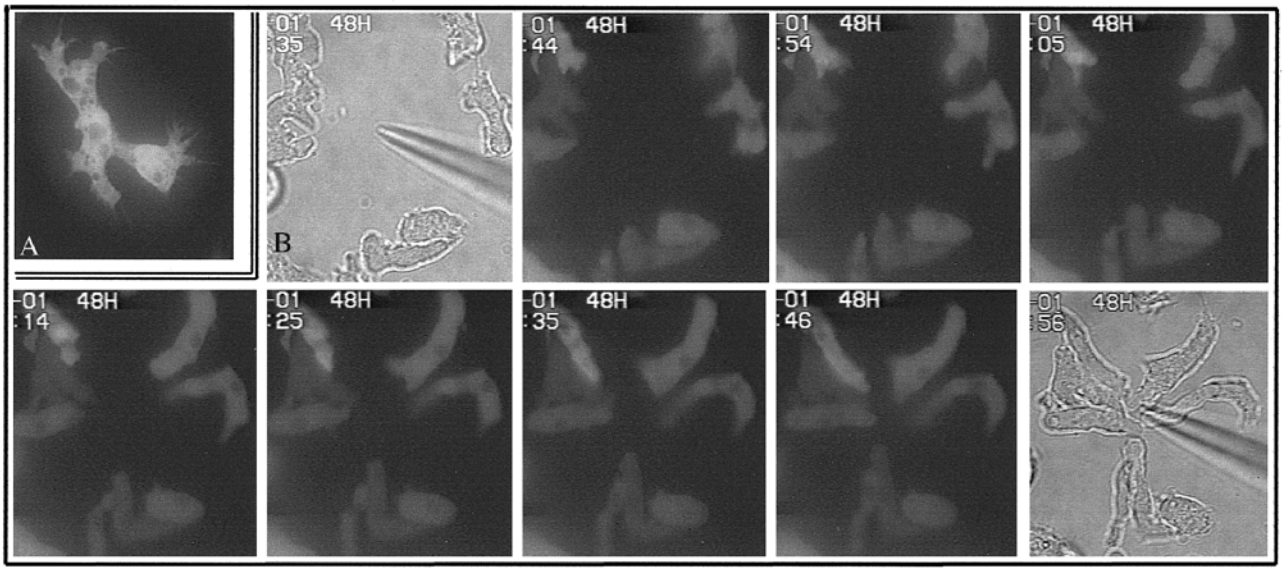

FIG. 6. Localization of GFP-PIA in AX2 cells. Fluorescent images of resting (A) or chemotactically moving (B) AX2 cells expressing GFP–PIA. (A) GFP–PIA is distributed homogeneously in the cytoplasm, in pseudopods and filopodia, but excluded from nucleus or other intracellular vacuoles. (B) No recruitment of GFP–PIA is observed in the leading front of cells moving chemotactically toward a microcapillary filled with cAMP. The image sequence covers a period of 80 s upon positioning of the microcapillary.

opposite effects, as it rescues partially the development of HSB1 cells, inhibiting partially AX2 development.

Since the HSB1 mutation is temperature-sensitive, we tested whether any of the effects of $piaA^{HSB1}$ overexpression in the HSB1 or AX2 background could be abolished by further increasing the temperature from 23 to 27°C. As shown in Fig. 5 (bottom), this was the case for the rescuing effect on HSB1, which was almost totally abolished at 27°C, whereas the inhibitory effect on AX2 development persisted. Thus, $piaA^{HSB1}$ behaves as dominant-negative when overexpressed in the AX2 background, independently of temperature, whereas it displays a weak, temperature-sensitive, stimulatory activity when overexpressed in the HSB1 background.

The $piaA^{HSB1}$ Mutation Results in a $G^{917}D$ Amino Acid Substitution

In order to sequence $piaA^{HSB1}$, genomic DNA was extracted from HSB1 cells, digested with *Bam*HI and *Cla*I, and cloned in *E. coli* strain MC1061. From a total of 30.000 colonies hybridized with the $piaA^{wt}$ gene, 3 independent positives were obtained, subcloned, and sequenced. There is a single *Bam*HI restriction site within the gene, at position 702 bp, and as a consequence, the upstream fragment encoding the N terminus was missing in the three clones. This fragment was PCR cloned and sequenced. A single point mutation was found at position 2781 bp, which results in aspartate (codon GAT) replacing a glycine (codon GGT) at position 917 of the protein sequence. A second point mutation, resulting in a synonymous base change, was found at position 150 bp.

Cellular Localization of PIA during Chemotaxis and cAMP Stimulation

To investigate whether ACA activation by PIA requires translocation of the protein to the membrane and whether this step is affected by the HSB1 mutation, GFP fusion proteins of WT or HSB1 PIA were expressed in AX2 and HSB1 cells. The expression level of each construct in transfected cells was assessed by scanning the cell populations in a FACS scanner. The fusion with GFP did not affect the cellular activity of wild-type or mutated protein, which behaved as their counterparts lacking GFP (data not shown). Consistent with the cytosolic nature of the protein, a homogeneous fluorescent labeling of the cytoplasm was observed in fluorescent images of resting as well as actively chemotacting cells, while nucleus, vacuoles, or other intracellular vesicles were devoid of fluorescence (Fig. 6A). We failed to observe translocation of PIA to the membrane either in cells stimulated with cAMP diffusing from a microcapillary (Fig. 6B) or cells adhering to a substratum and stimulated with a single shot of cAMP (data not shown). It is known that under these conditions rapid and transient recruitment of CRAC or actin to the cell membrane occurs (Westphal *et al.*, 1997; Parent and Devreotes, 1999). It is possible that PIA, in contrast to CRAC, which contains PH domains, does not translocate to the membrane during adenylyl cyclase activation.

DISCUSSION

The mutant HSB1 harbors a point mutation in the gene encoding PIA, which results in a $G^{917}D$ amino acid substitution and is responsible for the thermo-sensitive phenotype of the mutant, namely its inability to aggregate and to develop at temperatures above 17°C. The PIA protein is the second identified cytosolic regulator of adenylyl cyclase ACA in addition to CRAC, and similarly to CRAC, it is essential for ACA activation, it acts downstream of receptor/G protein coupling, and it is not required for receptor/G protein activation of guanylyl cyclase (Bozzaro *et al.*, 1987a; Chen *et al.*, 1997; and this paper). The finding that HSB1 cell aggregates formed at 13°C complete development when shifted at 23°C indicates that PIA is essential for aggregation but not for postaggregative development and favors the hypothesis that PIA is specifically required for ACA activation (see further below).

Consistent with a defect in the PIA protein, constitutive expression of PIA^{WT} rescued HSB1 cells, though the beginning of aggregation was delayed a few hours compared with control AX2 cells. Constitutive expression of PIA^{HSB1} in the HSB1 or AX2 background led to unexpected results: HSB1 cells were able to form a few aggregates and fruiting bodies at 23°C, though many cells failed to aggregate; AX2 cells started to aggregate with a delay of a few hours and gave rise to a lower number of fruiting bodies than control cells. Thus, expression of PIA^{HSB1} appears to partially rescue the HSB1 mutant, while partially inhibiting AX2 cell development. The rescuing effect on HSB1 cells, not however the inhibitory effect on AX2 cell aggregation, was abolished by raising the developmental temperature to 27°C.

These results can be fully explained by assuming that the PIA protein acts as an adapter link between G protein and ACA via two distinct domains, one of which is affected, in a temperature-sensitive way, by the $G^{917}D$ replacement, as shown in the model in Fig. 7. For sake of simplicity, the model assumes that PIA couples directly the G protein with ACA and that the domain affected by the mutation is the ACA-binding site. In our model, a conformational change due to the mutation destabilizes binding, making it more sensitive to thermal motion. At 13°C, binding still occurs and the mutant cells aggregate and develop normally. At higher temperatures, the binding becomes increasingly unstable, with a remnant activity left at 23°C, which becomes evident only if the protein is overexpressed, as occurs in HSB1 cells transfected with pia^{HSB1}; this remnant activity is abolished at 27°C. The HSB1 PIA protein will, however, bind to the G protein at all temperatures via the non-mutated binding domain, thus effectively competing with the endogenous wild-type PIA. Competition results in aggregation to be delayed when PIA^{HSB1} is overexpressed in AX2 cells or, conversely, when PIA^{WT} is overexpressed in HSB1 cells.

The model maintains its validity even if PIA interaction with G protein and/or adenylyl cyclase is mediated by other components, as it is likely since CRAC is as essential as

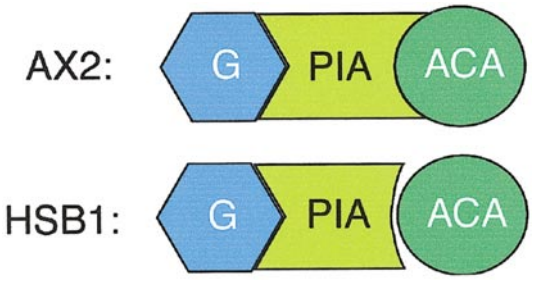

FIG. 7. Model for the function of PIA and the effects of the HSB1 mutation. The PIA protein is required for G protein-mediated ACA activation. PIA is proposed to act as an adapter between the G protein and ACA via two distinct binding sites. The mutation in HSB1 cells affects one binding site only (in the model binding to ACA), making it temperature-sensitive. As a consequence, ACA fails to be activated in HSB1 cells at temperatures above 17°C. Since the binding site to the G protein is unaltered by the mutation, PIA^{HSB1} binds to the G protein. Thus, if PIA^{HSB1} is overexpressed in the AX2 background, it will effectively compete with PIA^{WT}, delaying aggregation of AX2 cells. Conversely, PIA^{WT} rescues the mutant phenotype, when overexpressed in the HSB1 background. See text for details.

PIA for G protein-dependent activation of ACA (Insall *et al.*, 1994), and two additional genes, *erk2* and *AleA*, are somewhat involved in ACA activation (Segall *et al.*, 1995; Insall *et al.*, 1996).

Interestingly, computer analysis with two different algorithms (Chou and Fasman, 1978; Garnier *et al.*, 1996) suggests that the glycine residue in position 917 is at the borderline between a putative α-helix and a turn, and its substitution with aspartate results in a longer α-helix. No known domains are present in the region interested by the mutation nor in the whole of the PIA protein, therefore we are unable to correlate this putative conformational change with structural constraints. We believe, however, to have identified an important region for the activity of PIA and are supported in this conclusion by the high homology found between PIA and the *Schizosaccharomyces pombe* STE16 protein (Chen *et al.*, 1997) in the amino acid stretch containing the G residue and in the two regions flanking this stretch. Further structural analysis is required to determine whether this high homology, and a lower but still significant one with the *Saccharomyces cerevisiae* homologue, defines a novel functional domain.

GFP fusion proteins with both PIA^{WT} or PIA^{HSB1} are as active as their GFP-less counterparts, yet no recruitment to the membrane or to the leading front of cells stimulated with cAMP has been observed. The capillary distribution of PIA in membrane projections suggests that a basic level of the protein might be sufficient for local G protein-dependent ACA activation, without a need for PIA enrichment underneath the membrane. This is plausible if PIA, in contrast (e.g., to CRAC), is strictly required for ACA activation but not for chemotactic motility, as indicated by the

finding that mutant HSB1 cells at 23°C do respond chemotactically to cAMP diffusing from a capillary, but fail to form streams, suggesting that they are capable of responding to, but unable of relaying, cAMP signals (Bozzaro *et al.*, 1987a). A similar phenotype has been described for ACA-minus cells (Pitt *et al.*, 1992).

An intriguing question concerns the roles of PIA and ACA at postaggregative stages. Mounds formed by HSB1 cells at permissive temperature underwent development when shifted at the nonpermissive temperature (this paper). HSB1 cells at nonpermissive temperature were rescued by 20% wild-type cells and completed development (Bozzaro *et al.*, 1987a). These results indicate that PIA, and possibly ACA, are not required after aggregation. A novel adenylyl cyclase activity, named ACB, has been described in migrating and culminating slugs (Kim *et al.*, 1998, Meima and Schaap, 1999). ACB activity is G protein-independent (Kim *et al.*, 1998) and is encoded by the gene *acrA* (Soederbom *et al.*, 1999). *AcrA*-null cells form slugs, however, making it unlikely that ACB replaces ACA in migrating slugs (Soederbom *et al.*, 1999). Deactivating the *acrA* gene in the HSB1 background could help to understand the role of ACA and ACB in the multicellular stage.

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