Isoprenylcysteine Carboxy Methylation Is Essential for Development in *Dictyostelium discoideum*[□]

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Members of the Ras superfamily of small GTPases and the heterotrimeric G protein γ subunit are methylated on their carboxy-terminal cysteine residues by isoprenylcysteine methyltransferase. In *Dictyostelium discoideum*, small GTPase methylation occurs seconds after stimulation of starving cells by cAMP and returns quickly to basal levels, suggesting an important role in cAMP-dependent signaling. Deleting the isoprenylcysteine methyltransferase-encoding gene causes dramatic defects. Starving mutant cells do not propagate cAMP waves in a sustained manner, and they do not aggregate. Motility is rescued when cells are pulsed with exogenous cAMP, or coplated with wild-type cells, but the rescued cells exhibit altered polarity. cAMP-pulsed methyltransferase-deficient cells that have aggregated fail to differentiate, but mutant cells plated in a wild-type background are able to do so. Localization of and signaling by RasG is altered in the mutant. Localization of the heterotrimeric G γ protein subunit was normal, but signaling was altered in mutant cells. These data indicate that isoprenylcysteine methylation is required for intercellular signaling and development in *Dictyostelium*.

INTRODUCTION

Many signaling proteins, including the γ subunit of the heterotrimeric G protein and members of the Ras superfamily of small GTPases, share the carboxy-terminal amino acid sequence –CAAX, where C is cysteine, A is an aliphatic amino acid, and X is any amino acid. After protein synthesis in the cytosol, the C residue is prenylated via a thioether bond with either a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group, depending on the identity of the X-residue and other amino acids near the C terminus (Wright and Philips, 2006). Prenylation targets the protein to the endoplasmic reticulum (Choy *et al.*, 1999) where the –AAX tail is cleaved by the Rce1 protease (Boyartchuk *et al.*, 1997) and the α -carboxy group of cysteine becomes available for methylation by isoprenylcysteine methyltransferase (ICMT) (Clarke *et al.*, 1988).

Early experiments characterizing isoprenylcysteine methylation suggested that ICMT activity is highly regulated. In leukocytes, methylation levels of Rac and G γ rise in response to the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Philips *et al.*, 1993, 1995). Interestingly, methylation is also reversible (Chelsky *et al.*, 1985), but the enzyme activity required for demethylation remains uncharacterized. Isoprenylcysteine methyl-

ation also increases in the presence of guanosine 5'-O-(3-thio)triphosphate (GTP γ S) (Backlund and Aksamit, 1988; Huzoor *et al.*, 1991; Philips *et al.*, 1993), suggesting a link between G protein activation and methylation state. Although the observation that control of methylation/demethylation is finely tuned and linked to G protein activation suggests an important role in the regulation of signal transduction, little is known about the precise function of methylation in cellular signaling pathways.

One clear role for methylation is the subcellular targeting of –CAAX proteins. Ras superfamily small GTPases and other signaling molecules require specific localization for proper function, because they interact and cooperate with partners to propagate signals. In ICMT-deficient mouse fibroblasts, Ras GTPases, which are normally farnesylated, fail to associate with the plasma membrane, whereas the Rho GTPases, typically geranylgeranylated, localize normally (Michaelson *et al.*, 2005). When the –CAAX sequences of N- and K-Ras are replaced with sequences specifying geranylgeranylation, they localize to the membrane independently of ICMT activity. When the –CAAX tails of RhoB and Rac1 are mutated to farnesylation sites, localization of these proteins to the membrane becomes dependent on methylation. These experiments provide further evidence that methylation regulates membrane association of farnesylated, but not geranylgeranylated proteins. In vitro experiments suggest a biochemical basis for this result. The 20-carbon geranylgeranyl group binds reconstituted liposomes, but the 15-carbon farnesyl group does not. Importantly, carboxy methylation of the farnesylated cysteine residue dramatically increases binding to liposomes, but methylation has little effect on the membrane association of the geranylgeranyl group (Silvius and l'Heureux, 1994).

The observation that geranylgeranylated proteins do not require methylation for membrane association but are tar-

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gets of ICMT nonetheless suggests isoprenylcysteine methylation has additional roles. Recent work demonstrated that inhibition of ICMT with the prenylcysteine analogue *N*-acetyl-*S*-farnesylcysteine (AFC) enhances the interaction of Rac1 with its inhibitor RhoGDI in endothelial cells (Papaharalambus *et al.*, 2005). Thus, loss of methylation leads to a reduction in Rac1-mediated signaling. Other evidence comes from experiments in COS cells. A chimeric protein consisting of the membrane targeting domain of CD8 fused to H-Ras is constitutively localized to the plasma membrane, and it has increased activity (Chiu *et al.*, 2004). Importantly, inhibition of ICMT with AFC blocks signaling but not localization, suggesting that methylation regulates the interaction of H-Ras with downstream effectors. Other experiments demonstrate methylation-dependent interaction between K-Ras and microtubules (Chen *et al.*, 2000). Methylation has also been shown to decrease the turnover of geranylgeranylated proteins in mammalian cells, but the functional significance of this is unclear (Backlund, 1997; Bergo *et al.*, 2004). These studies suggest a broader role for isoprenylcysteine methylation in the assembly/disassembly of signaling complexes at the plasma membrane.

Recent investigations into the role of isoprenylcysteine methylation have used ICMT-deficient yeast or cell lines derived from ICMT-deficient mouse embryos. Unlike yeast, *Dictyostelium discoideum* uses a robust G protein-signaling network that is an instructive model for Ras superfamily signaling in other systems. Unlike mammalian cell lines, *Dictyostelium* is genetically tractable; genes can be deleted or modified in a straightforward way. Together, these characteristics suggest *Dictyostelium* to be a useful model organism in which to investigate isoprenylcysteine methylation. Furthermore, its unique life cycle, in which individual, autonomous amoebae aggregate upon starvation, differentiate into specialized cell types, and develop into a multicellular structure that allows the organism to withstand environmental stresses, allows researchers to measure three distinct signaling processes required for *Dictyostelium* development: intracellular communication, cell locomotion, and cellular differentiation, by using quantitative and qualitative methods unavailable in other systems. Previously described algorithms predict the *Dictyostelium* genome encodes at least 53 CAAX proteins that are targets for ICMT, including the sole γ subunit of the heterotrimeric G protein complex, members of the Ras superfamily of small GTPases, and >20 uncharacterized open reading frames (Maurer-Stroh and Eisenhaber, 2005). γ and Ras superfamily small GTPases are involved in signaling pathways that control chemotaxis, differentiation, and development (Wilkins and Insall, 2001; Zhang *et al.*, 2001). Whether methylation is important for GTPase function in this organism, and when methylation is active during the life cycle, is unknown, and it is the target of this study.

MATERIALS AND METHODS

Deletion of *icmA*

The *icmA* deletion construct consisted of DNA fragment I, *bsr*, and DNA fragment II. Primers 5'-ATAAATGGATCCTACGTAAGATGATGTTTTTAAATGAACAGGAAGTGG-3' and 5'-AAATATTGAATTCATTAAGCTTCATGAAATGCACTACTTCTGCTG-3' were used to amplify fragment I, which has a BamHI/SnaBI site at the 5' end and a HindIII/EcoRI site at the 3' end. At the 3' end, fragment I has the first 92 base pairs (bp) of the *icmA* coding sequence. Primers 5'-AAATACAAAGCTTATTGAATTCGGTCTGTATCCAGGTATTCATTG-3' and 5'-ATATAAATAGGTACCTACGATATGTTGTTGTTGTTGATCTTGAGC-3' were used to amplify fragment II, which has a HindIII/EcoRI site at the 5' end and a SnaBI/KpnI site at the 3' end. At the 5' end, fragment II has the last 26 bp of the *icmA* coding sequence. The construct was released from the resultant vector by SnaBI digestion and

used to delete the genomic sequence. A negative result from the polymerase chain reaction (PCR) using 5'-GAGAAAAGAAACTCCAAGAAGCC-3', which is located at the 5' region of deleted *icmA* DNA fragment, and 5'-TCTTCCCATCATTTGAAAACATTC-3', which is located at the 3' region of the deleted *icmA* fragment, confirmed the existence of *icmA* deletion. PCR with 5'-ATTTCATTTATTGACGGAGCA-3' and 5'-AGCCTTCATCAATTTT-TGAGCC-3' primers confirmed that the intergenic DNA between the *icmA* genes remains intact in the deletion.

Cell Growth and Development

Dictyostelium strain AX4 was used in all experiments. Wild-type and *icmA*⁻ cells were cultured on bacterial lawns of *Escherichia coli* B/r as described previously (Fey *et al.*, 1995). Cells grown on bacteria were harvested in PB (20 mM phosphate buffer, pH 6.2), freed of bacteria by centrifugation, and used directly for further experimentation. *icmA*^{-/+} cells and transformants expressing fluorescent fusions proteins were grown in the presence of 10 μ g/ml Geneticin (G-418, Invitrogen, Carlsbad, CA) on the *neo*^r *E. coli* strain B/r-1 (Nellen *et al.*, 1984). To follow development, cells were plated at varying densities on 1–2% agar plates and incubated at 20°C with constant illumination. Cells pulsed with cAMP were prepared by harvesting cells from bacterial lawns in KK2 buffer (20 mM potassium phosphate buffer, pH 6.2), removing bacteria by centrifugation, and resuspending cells at 5 $\times$ 10⁶ cells/ml. They were starved 1 h at 22°C with shaking and then pulsed every 6 min at a final concentration of 50 nM cAMP for 5 h (Devreotes *et al.*, 1987). Hereafter, cells prepared in this way are called "pulsed cells." "Starved cells" have been prepared by suspending vegetative cells in DB buffer (5 mM Na₂HPO₄, 5 mM KH₂PO₄, 1 mM CaCl₂, 2 mM MgCl₂, pH 6.5) and incubating them at 22°C on an orbital shaker for various intervals.

Methylation Assays

Dictyostelium cell extracts were prepared by standard methods (Spudich, 1974). Extracts were labeled by incubating with *S*-adenosyl-[³H-methyl]methionine ([³H]SAM). Labeled extracts were loaded on a 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel and separated by electrophoresis. The gel was sliced into ~18 fractions, and methylation levels were determined as described previously (Volker *et al.*, 1995). The methylation of AFC was determined by heptane-extraction assay (Volker *et al.*, 1995).

Metabolic Labeling

Dictyostelium cells were starved in PB for 8 h with shaking, and then they were labeled with [³H-methyl]methionine at 22°C for 1 h. cAMP (1 μ M) was added to the cells, and at different times, the reaction was stopped in a dry ice-ethanol bath. Frozen samples were boiled for 5 min with 1 volume of 2 $\times$ SDS gel sample buffer and subjected to SDS-PAGE. Levels of protein methylation were determined as described previously (Volker *et al.*, 1995).

icmA Isolation and Genetics

The *icmA* deletion was constructed by replacing the *icmA* genomic DNA fragment (from position 93 to 777) with a Blasticidin S resistant (*bsr*) cassette in the opposite direction (see Supplemental Material). Putative transformants were selected on bacterial lawns containing 25 μ g/ml Blasticidin S, cloned in the presence of Blasticidin, and further characterized. For each clone, ~10⁴–10⁶ cells were resuspended in 25 μ l of 1 $\times$ PCR buffer. Samples were boiled for 5 min, and 5 μ l was used as template for PCR reactions that confirmed the deletion. The deletion was rescued by transforming the *icmA*⁻ cell line with an extrachromosomal G418^r vector by using the genomic fragment between positions 1997 and 2799 or the human cDNA encoding for ICMT.

Northern Analysis

Vegetative cells were harvested and washed with 1 $\times$ PB, resuspended in 1 $\times$ PB, spread onto 1.5% agar plates, and incubated at 22°C. Development was terminated at different time points by harvesting cells and freezing them in liquid nitrogen. Northern analysis was performed using a PCR fragment by these primers 5'-AAAACATTCTCATTTATGGTGGGTAGG-3' and 5'-AGTTGATACTACCAACAAACCAAC-3'. For *car1* expression, a labeled PCR probe specific for *car1* was generated from AX4 genomic DNA by using primers 5'-GTTTCTCTGTTGTTGTTGGGTATTG-3' and 5'-ATTGCTCCATATCGGAACCATATG-3' (Klein *et al.*, 1988). This region does not hybridize to *car2*, 3, and 4. The optical density at 260 nm was used to adjust sample volumes so that all lanes contained equal quantities of material. This was verified by photographing ethidium bromide-stained gels after each electrophoretic run (Supplemental Figure S5).

Cell Autonomy Experiments

Wild type and *icmA*⁻ cells were mixed together at different ratios and allowed to develop on 1–2% agar plates. Then, the cells were monitored by phase contrast and epifluorescence microscopy. After 2 days, several single spore heads were picked. Each spore head was resuspended in KK2 buffer and viewed under the microscope or mixed with bacteria and plated on GYP

plates supplemented with G-418 to confirm the viability of green fluorescent protein (GFP)-expressing spores.

Dark-Field Images

Amoebae were plated on a 1% nonnutrient agar plate that was placed in a dark-field imaging system adapted from the design of Gross *et al.* (1976) and Lee *et al.* (1996). Successive images were collected with a Scion LG-3 frame-grabbing video board and averaged as described previously (Lee *et al.*, 1996).

Dunn Chambers

Freshly harvested cells, and cells pulsed with cAMP were placed in a Dunn chamber and analyzed by time-lapse video microscopy (Zicha *et al.*, 1991). Cell trajectories were analyzed as a function of time and plotted as vectors.

Micropipet Assay

Cells were resuspended in KK2 buffer and plated at 6×10^4 cells/cm² on a coverslip glued to a 1-cm hole in the bottom of a 10-cm Petri dish. An Eppendorf Femtotip filled with 150 μ M cAMP was brought into the field of view using an inverted microscope. In some experiments, a pulse controller was used to deliver short bursts of chemoattractant from the micropipette to follow redistribution of GFP-tagged signaling proteins. Cell responses were followed by time-lapse video microscopy. To quantify the percentage of cells responding to cAMP, membrane fluorescence was measured using the plot profile function in ImageJ (National Institutes of Health, Bethesda, MD). Any cell displaying greater than a 10% increase in fluorescence intensity at any location around the cell periphery was scored as positively responsive.

Under Agarose Assay

Plastic Petri dishes were filled with 1% agarose $\pm$ 3 mM caffeine, and two holes were bored with a glass Pasteur pipette $\sim$ 0.5 cm apart. Cells that were freshly harvested from bacterial lawns and pulsed with cAMP were plated into one well, whereas 150 μ M cAMP was loaded into the other. Individual cells that escaped the well, migrating between the plastic and overlying agarose were recorded with Nomarski optics.

cAMP Binding

Binding assays are described in van Haastert (2006). Briefly, crude membranes were prepared by forcing starved cells through 5- μ m pore Nucleopore membrane twice and then centrifuging at $14,000 \times g$ for 5 min at 4°C. The pellets were washed once with ice-cold PB, pH 6.5, and resuspended in ice-cold PB to 0.71 μ g protein/ μ l. For each binding reaction, the total volume was 100 μ l containing 70 μ l (50 μ g) of crude membrane. For GTP inhibition of cAMP binding, 5 mM dithiothreitol, 10 nM [³H]cAMP (GE TRK498), 10 μ l of H₂O or 1 mM GTP γ S, 10 μ l of H₂O or 1 mM cAMP, and 70 μ l containing 50 μ g of crude membrane in ice-cold PB were incubated for 5 min on ice, and then the mixtures were centrifuged for 2 min at $14,000 \times g$ at 4°C. The pellets were dissolved in 100 μ l of 0.1 M acetic acid with 1% SDS, and then amounts of membrane-bound [³H]cAMP were measured by liquid scintillation counting. cAMP (1 mM) was used to measure nonspecific binding of cAMP. For cAMP stimulation of GTP γ S binding, 10 μ l of 0.1 mM ATP, 0.2 nM [³S]GTP γ S (GE SJ1320), 3 mM MgCl₂, 2 mM PB, 10 μ l of H₂O or 1 mM cAMP, 10 μ l of H₂O or 1 mM GTP, and 70 μ l containing 50 μ g of crude membrane in ice-cold PB were incubated for 30 min on ice, and then the mixtures were centrifuged for 3 min at $14,000 \times g$ at 4°C. The pellets were dissolved in 100 μ l of 0.1 M acetic acid with 1% SDS, and then amounts of membrane-bound [³S]GTP γ S were measured by liquid scintillation counting. GTP (1 mM) was used to measure nonspecific binding of GTP γ S.

cAMP Relay Assay

Vegetative cells were harvested from plates and washed three times with KK2 buffer. They were resuspended in KK2 at 5×10^6 cells/ml, aerated by shaking in a rotary shaker for 1 h at 22°C, and pulsed every 6 min for 5 h with 50 nM cAMP (final concentration). Pulsed cells were then washed twice with KK2, resuspended in KK2 at 1×10^8 cells/ml, and aerated for an additional 10 min. They were then stimulated with 10 μ M 2'-deoxy-cAMP, and at various times, 200- μ l samples were removed and pipetted into perchloric acid-DTT (3.5% and 5 mM, final concentrations). Samples were then neutralized with 50% KHCO₃, and cAMP levels were measured by a cAMP binding assay (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) according to the manufacturer's protocol.

Construction of the N-terminal Tandem Affinity Purification (TAP)-tagged YFP-RasG Fusion Protein and Immunoprecipitation

Dictyostelium RasG coding sequences were PCR amplified and cloned into an integrating protein expression vector, pDdNTAP-NYFP (kindly provided by Dr. Pauline Schaap, School of Life Sciences, University of Dundee, United Kingdom). It contains the *actin15* promoter, TAP tag, yellow fluorescent protein (YFP), 2H3 terminator, in that order; and a Neo resistance cassette

regulated by the *act6* promoter. The RasG PCR product was cloned into this vector as an Xba I-EcoRI fragment. The resulting plasmid was linearized and transformed into *Dictyostelium* wild-type AX4 and *icmA* deletion strains. The integrity of the expressed construct was verified with Western blots by using pan Ras and GFP antisera. Cells were grown with bacteria on GYP plates, harvested, freed of bacteria, resuspended in DB buffer, starved for 1 h, and then pulsed with 50 nM cAMP every 6 min for 5 h. The resulting cells were washed with DB buffer, resuspended in buffer A (100 mM Tris-HCl, pH 8.2, 1 mM EDTA, and 1 mM DTT) with protease inhibitors, and lysed by forcing the cells through a 5- μ m Nucleopore membrane twice. For the *in vitro* labeling assay, 150 μ g of protein from this cellular extract was incubated with 8 μ M unlabeled SAM and 2 μ M [³H]SAM for 1 h, and then an equal volume of 2 $\times$ SDS-PAGE sample buffer was added to the reaction. The sample was boiled for 5 min and loaded onto an SDS-PAGE gel.

RESULTS

Isoprenylcysteine Methyltransferase Activity in *Dictyostelium*

Ras superfamily small GTPase carboxy-methylation activity was initially characterized in *Dictyostelium* cellular extracts by using AFC as substrate, and SAM as methyl donor. The apparent K_M values of 45 μ M for AFC and 1.1 μ M for SAM are similar to those seen with small GTPase-methylating enzymes from other cell types (Volker *et al.*, 1991b). Strong product inhibition by *S*-adenosyl-homocysteine is also typical of ICMTs from other cells (data not shown).

When *Dictyostelium* extracts were incubated with [³H]SAM and then subjected to SDS-PAGE, a distinct peak of carboxy [³H]methyl-labeled proteins was observed with apparent molecular weights of 20–25 kDa, corresponding to the Ras superfamily of methyl accepting proteins (Clarke, 1992) (Figure 1A). Because AFC is an ICMT substrate, it also acts as a competitive inhibitor (Volker *et al.*, 1991a,b). Therefore, it was not surprising that AFC dramatically inhibited carboxy methylation of the 20- to 25-kDa protein region of the gel.

Small GTPases are activated when bound GDP exchanges with GTP, and they are inactivated when the bound GTP is hydrolyzed back to GDP. Nonhydrolysable GTP analogs, such as GTP γ S, lock small GTPases in the active state. In vertebrate tissues, dramatic increases in methylation have been associated with G protein activation (Philips *et al.*, 1993). We obtained similar results with *Dictyostelium*, where addition of GTP γ S led to more than a threefold increase in 20- to 25-kDa protein carboxy methylation (Figure 1B). *Dictyostelium* has two separate life stages. When food is available, cells behave as unicellular amoebae. When food is exhausted, cells undergo a developmental transition during which up to 10⁵ cells aggregate and differentiate to form a fruiting body. Aggregation occurs by chemotaxis toward cAMP. To study the connection between methylation and cAMP signaling, we labeled developing *Dictyostelium* cells with [³H-methyl]methionine *in vivo*. Addition of 1 μ M cAMP to these cells resulted in a transient increase in protein carboxy methylation. Methylation peaked 30 s after addition of cAMP, but it was already close to the peak level by 10 s, and it returned to prestimulus levels by 60 s (Figure 1C). The rapid response time is similar to that observed in neutrophils, which show transient increase in levels of methylation of Rac and G γ in response to the tripeptide fMLP (Philips *et al.*, 1993). Our data show that methylation accompanies signal transduction on the same time scale as cytoskeletal changes (Gerisch and Hess, 1974) and the localization of pleckstrin homology (PH) domains (Parent *et al.*, 1998; Meili *et al.*, 1999), strongly suggesting a central role in modulating responses associated with the early stages of chemotaxis.

Cloning, Deleting, and Rescuing the *icmA* Gene

Information from the *Dictyostelium* Genome Project indicates the gene encoding for ICMT is found on chromosome 2 and

Figure 1. Prenylcysteine carboxy methyltransferase activity in *Dictyostelium* cells and cell-free extracts. (A) AFC is an inhibitor of protein carboxy methylation. AFC inhibition of protein carboxy methylation is localized to the 20- to 25-kDa region of a denaturing gel. (B) AFC inhibits GTP γ S stimulation. GTP γ S can increase the level of methylation by >300%, and AFC can inhibit this increase by >50%. In experiments A and B, *Dictyostelium* cellular extracts were labeled with [3 H]SAM in the presence of AFC, GTP γ S, or both. (C) Transient increase of prenylcysteine carboxy methylation after cAMP stimulation. Cells starved for 1 h were pulsed with cAMP for 5 h and labeled for 1 h with [3 H-methyl]methionine in vivo. cAMP was then added to 1 μ M. The reactions were stopped at the indicated intervals, and proteins were separated by SDS-PAGE. Protein levels were determined as described in *Materials and Methods*. (D) Aliquots containing 150 μ g of protein from extracts of wild-type AX4 or *icmA* deletion (KO) cells overexpressing TAP-tagged YFP-RasG were incubated with SAM. Inset, TAP-YFP-RasG region of the gel, 68 kDa. The inset shows fractions 1–8 at a different scale, demonstrating that this region is methylated in AX4 but not in the mutant.

that a second copy is found in a duplicated region of that chromosome in the AX4 strain used in this study (Eichinger *et al.*, 2005). An *icmA* deletion was constructed by homologous recombination and rescued by transformation with the wild-type allele. The deletion of both copies of the gene was first verified by PCR screening using primers within the portion of the gene targeted for deletion (Figure 2A). Because the *icmA* gene is present in duplicate, homologous recombination could occur in a way that deleted both genes and the intergenic sequence. To confirm the presence of the intergenic sequence, we performed PCR using primers targeted to this region (Figure 2A). Deletion of *icmA* was also confirmed by Southern (data not shown) and Northern analysis (Figure 2B). In wild-type cells *icmA* is induced by starvation and expressed throughout development, with the highest levels occurring ~8 h after starvation. In the mutant, no *icmA* mRNA (Figure 2B) was detectable at any stage of development. Expression levels, although not the timing of expression, were restored in transformants rescued with the wild-type ICMT from *Dictyostelium* (Figure 2B) or the human cDNA sequence (data not shown). When AFC was used as the substrate and [3 H]SAM was used as the methyl donor

in an in vitro assay, no methylating activity was detected in mutant cells. When cellular extracts of mutant cells overexpressing TAP-tagged YFP-RasG were labeled with [3 H]SAM, no methylation was detected for either the TAP-YFP-RasG or endogenous small GTPases (Figure 1D), and no methylation of TAP-YFP-RasG was detected when it was immunoprecipitated by anti-GFP antibody, which confirms our supposition that ICMT is solely responsible for the carboxy methylation of small GTPases in *Dictyostelium*.

The *icmA* Phenotype

As has been observed with other cell types, carboxy methylation of small GTPases is not essential for cell viability (Hrycyna *et al.*, 1991; Bergo *et al.*, 2001). However, the *icmA* deletion strain is severely crippled; it grows slowly on bacterial lawns, it will not grow in shaking axenic culture, and it is difficult to transform. Individual cells fail to make normal pseudopodia and filopodia, but they typically have large flat regions at the cell margin (Figure 3A). Wild-type cells become elongated when pulsed with cAMP, but mutant cells do not.

Figure 2. *icmA* disruption and Northern analysis. (A) PCR products confirming the *icmA* knockout. The primer sequences are described in *Materials and Methods*. (B) Expression profiles of *icmA* and *car1* in wild-type (WT), *icmA*⁻, and *icmA*^{-/-} strains. RNA samples in the first lane (*) of each panel were prepared from vegetative cells that had been pulsed with 50 nM cAMP every 6 min for 5 h. Numbers correspond to hours of starvation on agar plates.

The *icmA*⁻ mutant is also clearly deficient in its ability to develop fruiting bodies when starved. Wild-type cells aggregate within 12 h after plating on nonnutrient agar. However, after 24 h, the majority of the mutant cells have not aggregated, although there are some small mounds of loosely adhering cells, and a few large mounds that seem to be poorly differentiated precursors to multitipped fruiting bodies. However, normal fruiting bodies never formed, and viable spores were never produced (Figure 3, B and D). When cells were pulsed with cAMP and then plated on nonnutrient agar plates, mutant cells again failed to develop normal fruiting bodies. Although mutant cells could form streams at high cell densities, they could not proceed beyond the streaming and loose aggregate stages. The few sparse aggregates that did form could be stained with calcofluor, a cellulose-specific stain, but neither stalk nor spore cells were detected. All of the *icmA*⁻ phenotypes described above were restored by expressing wild-type *icmA* or the human ICMT cDNA sequence under control of the *Dictyostelium* actin15 promoter (data not shown).

The Methylation Defect Interferes with Starvation-induced Changes in Gene Expression

An important event in the transition of *Dictyostelium* cells from independently foraging amoebae to streams of chemo-

taxing cells is the induction of *car1*, a gene that encodes a G protein-coupled seven-transmembrane receptor used to sense extracellular cAMP during the aggregation phase of development (Manahan *et al.*, 2004).

Two features of *car1* regulation in an *icmA*⁻ background stand out. First, *car1* message synthesis is delayed (Figure 2B). We observed weak synthesis beginning at 8 h, and normal levels beginning around 24 h, long after mature sorogen development in wild-type cells. Second, *icmA* null cells exhibit nearly normal *car1* expression after cAMP pulsing for 5 h. *car1* expression is essentially restored in a transformant carrying a wild-type copy of the *icmA* gene. Here, perhaps because the *icmA*⁺ gene is under control of the *act15* promoter, *car1* induction is delayed by ~4 h, but this is sufficient to restore wild-type development. Thus, our results reveal that the aggregation defect of the *icmA*⁻ mutant is in large part due to a failure of *car1* induction.

In sum, the conclusion from these experiments is that a major defect in the *icmA*⁻ strain is the inability to signal autonomously, a block that can be overcome by cAMP pulsing. Because *Dictyostelium* uses cAMP both as the chemotactic signal and as a regulator of transcription (Williams, 2006), cAMP pulsing presumably bypasses signal relay, turning on *car1* transcription directly.

cAMP Wave Propagation

Layers of *Dictyostelium* cells begin to propagate dark-field waves ~3–4 h after starvation (Alcantara and Monk, 1974). Waves reflect cAMP concentrations (Tomchik and Devreotes, 1981), and their onset corresponds to increased synthesis of the cAR1 receptor and other proteins required for cAMP synthesis, release, and modulation. Wave geometry, velocity, amplitude, and frequency are all sensitive manifestations of the signaling system. In *icmA*⁻ cells, the onset of wave propagation was delayed by >3 h (Figure 4A). Wave frequency increased and wave amplitude decreased with time in wild-type cells, whereas for mutant cells, wave frequency and magnitude remained constant. Waves finally died out in the mutant cell monolayer, and aggregation territories never formed. Thus, although a layer of mutant cells is weakly excitable, and wave propagation begins even in the absence of cAMP prepulsing, the *icmA*⁻ cells cannot respond by moving toward the center of the cAMP wave. Clearly, in the absence of small GTPase methylation, the signaling system cannot ramp up, and it eventually terminates prematurely. Because pulsed *icmA*⁻ cells form aggregates soon after plating, we have not found it possible to study wave propagation in monolayers prepared from pulsed cells.

To propagate a cAMP wave, cells need to first detect extracellular cAMP and then produce and release cAMP of their own. When wild-type cells are first pulsed with cAMP for 5 h, and then stimulated, they respond by synthesizing and secreting cAMP into the medium. We assayed total cAMP in the sample as a function of time by pipetting aliquots of the cell suspension directly into perchloric acid (see *Materials and Methods*) (Figure 4B). These results show the mutant relay response is 3 times larger, but the interval over which cAMP is secreted is normal.

The *icmA* Deletion and Chemotaxis

The observation that *icmA*⁻ cells propagate cAMP waves, albeit abnormally, but fail to aggregate raises the question of whether chemotaxis is altered in mutant cells. To address this, we investigated cell movement in a fixed cAMP gradient (Zicha *et al.*, 1991). Wild-type cells washed free of bacteria and placed in a chemotaxis chamber moved up the

Figure 3. The *icmA*⁻ phenotype. (A) Wild-type, *icmA*⁻, and *icmA*^{-/+} cells under 100× Nomarski optics. Bar, 10 μm. (B) Wild-type or *icmA*⁻ cells were washed free of bacteria and plated on 2% agar plates at 2.1×10^5 (a), 8.6×10^5 (b), or 1.7×10^6 (c) cells/cm², and they were allowed to develop for 3 days. Wild-type cells all formed normal fruiting bodies. *icmA*⁻ cells formed a few sparse clumps of cells that failed to develop further. The top panel in each frame illustrates the morphology of sparse clumps; the bottom panel illustrates typical fields of very loosely clumped cells that fail to develop further. (C) Wild-type fruiting bodies at different developmental stages. Cells were grown on plates with bacteria, harvested, washed free of bacteria, and plated on 2% agar plates at 4.2×10^5 cells/cm². (D) *icmA*⁻ cells were inoculated onto heavy streaks of *E. coli* on 2% agar plates and incubated for 3 days. Bars, 100 μm.

gradient at an average speed of $3.8 \mu\text{m s}^{-1}$, and this speed doubled in cells prestimulated with cAMP (Figure 5A). The *icmA* deletion cells never oriented toward cAMP in the absence of prepulsing. More than 99% of the mutant cells failed to respond; they wandered aimlessly over distances of two to three cell diameters (Figure 5A). However, when mutant cells were pulsed with 50 nM cAMP for 5 h, they chemotaxed up the gradient, but they moved more slowly than wild-type cells (average velocity 6.0 vs. $7.8 \mu\text{m s}^{-1}$), and their sense of direction seemed to be less acute (Figure 5A).

We also looked at the ability of mutant and wild-type cells to move toward cAMP pulses delivered from a micropipette tip (Figure 5B). Under these conditions cells are responding to transient gradients that are steep relative to those in a Dunn chamber (Song *et al.*, 1998). Again, wild-type cells moved toward the cAMP pulses whether or not they were pulsed with cAMP, although pulsed cells were more elongated. In the absence of prepulsing, mutant cells showed no response to cAMP, maintaining the round appearance documented in Figure 5B. After a 5-h cAMP prepulse, the *icmA*⁻ cells responded to the pulse from the micropipette, but cell shape was not fully restored. Although mutant cells moved toward cAMP, they failed to elongate. Thus, in both fixed and transient cAMP gradients, *icmA*⁻ cells cannot make the transition from feeding to chemotaxing amoebae. This block

can be partially overcome with pulses of cAMP, which presumably mimic periodic cAMP waves.

To further characterize cell shape and polarization in chemotaxis, we recorded the morphology of cells migrating under agarose up a continuous gradient of cAMP, an assay that allows detailed description of cell shape (Laevsky and Knecht, 2001) (Figure 5C and Supplemental Movie Figure S1). Both wild-type and *icmA*⁻ cells that have been prepulsed with cAMP migrate up a gradient of cAMP, but the mechanics of cell motility differ dramatically. Wild-type cells move with a typical morphology, elongated toward the cAMP source. They often form streams in which cells line up front to back, a process that requires localization of adenylate cyclase in the trailing uropod (Kriebel *et al.*, 2003). Mutant cells neither elongate normally nor do they stream. Instead of elongating in the direction of the gradient, they advance with a broad leading edge that faces the gradient. Instead of inching along the substratum, the cells seem to glide toward the gradient in a manner similar to that of fish keratocytes (Svitkina *et al.*, 1997) or the *Dictyostelium amiB*⁻ mutant strain (Asano *et al.*, 2004). *icmA*⁻ cells fail to align head to tail, and when they do come into contact with one another they usually pull apart quickly. Together, these data demonstrate that loss of isoprenyl cysteine methylation has dramatic effects on *Dictyostelium* polarization, an effect not previously described for ICMT-deficient yeast or murine fibroblasts.

A

B

Figure 4. cAMP wave propagation. (A) Wild-type and *icmA*⁻ cells were plated side by side at the same density on nonnutrient agar, separated by a plastic divider (center of each image). Waves propagate normal to the convex surface of each wave. The left half of each frame is wild type; the right half is *icmA*⁻. The dark bands correspond to peak cAMP concentration, and the lighter bands correspond to cells that are either refractory to cAMP (trailing edge of the expanding wave) or excitable (leading edge). Wild-type cells form typical target and spiral patterns whose frequency increases with time. Signaling in *icmA*⁻ cells is delayed by ~3 h, the wave frequency does not increase with time, and the waves die out before there has been substantial cell movement. By 4:45, wild-type cells have begun to aggregate and wave propagation has died down. Times are in hours and minutes after washed cells were plated on agar. (B) *icmA*⁻ cells respond more vigorously to cAMP stimulation than wild-type cells. Cells starved for 1 h were then pulsed for 5 h with nanomolar cAMP, stimulated with 10 μ M 2'-deoxy-cAMP, and sampled at frequent intervals. Total cAMP was then measured by a protein kinase assay. Each point is the average of four independent reactions. The error bars are $\pm$ SD. In each reaction, cAMP levels were measured with 4×10^6 cells.

This aberrant polarity suggests that chemotaxing *icmA*⁻ cells may exhibit atypical cytoskeletal organization. To test this, we stained starving amoebae with rhodamine-conjugated phalloidin to visualize F-actin (Figure 5D). Wild-type cells starved in shaking suspension and then plated on a coverglass exhibit small foci of phalloidin labeling at regions corresponding to protruding filopodia. *icmA*⁻ cells have large patches of F-actin localized to broad lamellae. We also monitored the localization of a GFP-tagged version of the contractile protein myosin II in motile amoebae. In wild-type cells, GFP-myosin II localizes to the uropod, where actomyosin contraction is responsible for retraction at the rear (Jay *et al.*, 1995). In mutant cells, myosin II is seen around the entire cell periphery (Figure 5E and Supplemental Movie Figure S2), further demonstrating polarity defects.

Cell Mixing Experiments

To determine whether endogenous cAMP waves stimulate chemotaxis and cellular differentiation of *icmA*⁻ cells, we performed cell mixing experiments in which GFP-expressing mutant cells were coplated with wild-type cells. In these experiments, a majority of GFP-expressing *icmA*⁻ cells coaggregate with wild-type cells (Figure 6, A and B), although in each experiment between 20 and 40% of the mutant cells fail to aggregate. As mounds elongate into migrating slugs, GFP-*icmA*⁻ cells segregate to the rear two third of the slug, although in some experiments there is fluorescence signal in the leading tip (Figure 6C). As the slugs crawl, they often deposit a thick slime trail into which many mutant cells are deposited (Figure 6E). These defects are not likely due to overexpression of GFP, because GFP-expressing wild-type cells coaggregate when coplated in a "blank" cell back-

Figure 5. Chemotaxis defects can be rescued with cAMP pulses. (A) Chemotaxis in a Dunn chamber. Each line is a vector whose direction is the direction of movement and whose length corresponds to cell velocity in a stationary cAMP gradient. The y -axis points in the direction of the gradient. The velocity unit is micrometers per minute. In each case, >50 cells were followed. Top left, behavior of wild-type cells without cAMP prepulsing. $n = 30$ cells. Average velocity, $3.8 \mu\text{m}/\text{min}$. Top right, behavior of wild-type cells with cAMP prepulsing. $n = 11$ cells. Average velocity, $7.8 \mu\text{m}/\text{min}$. Bottom left, behavior of *icmA*⁻ cells without cAMP prepulsing. Only 2 of 54 cells were able to move in the gradient. Average velocity, $1.4 \mu\text{m}/\text{min}$. Bottom right, behavior of *icmA*⁻ cells after cAMP prepulsing. $n = 27$ cells. Average velocity, $6.0 \mu\text{m}/\text{min}$. (B) Micropipette chemotaxis assay. Cells for assays in the bottom panel had been pulsed with 50 nM cAMP for 5 h. The micropipette tips were filled with 150 μ M cAMP. (C) Motility under agar. Pulsed cells were plated in an under agar chemotaxis assay and monitored with Nomarski optics. (D) Actin organization. Wild-type and mutant cells were starved with shaking for 6 h, plated on glass coverslips for 1 h, and then fixed and stained with rhodamine-conjugated phalloidin. (E) Myosin II localization in cells migrating under agar. Bars, 10 μm . See Supplemental Material.

ground, are randomly dispersed throughout the migrating slug, and are not shed into slime trails (Figure 6D; data not shown). Although many GFP-*icmA*⁻ cells are excluded from the migrating slug, some remain and participate in culmination. Mutant cells are capable of forming spores, as monitored by epifluorescence microscopy (Figure 6F), and these spores are viable, because new GFP-*icmA*⁻ cultures can be initiated from mixed spore masses. Because cAMP waves are involved in both aggregation and development, it may be that defects in both processes stem from an inability to raise, maintain, and respond to a coherent signal rather than more general defects in chemokinesis and development.

Heterotrimeric G Protein Localization and Signaling

Among the earliest events triggered by cAMP binding to cAR1 are dissociation and activation of the heterotrimeric G proteins complex, whose $G\gamma$ subunit is a target of ICMT and whose activation is central to the control of aggregation and development. Although the observation that *icmA*⁻ cells are

eventually able to propagate cAMP waves (Figure 4A) suggests that the heterotrimeric G protein complex is signaling competent, it does not exclude the possibility that heterotrimeric G protein signaling is aberrant in *icmA*⁻ cells. To characterize the role of methylation in heterotrimeric G protein signaling, we analyzed the localization and signaling of the $G\beta\gamma$ complex in *icmA*⁻ cells. YFP- $G\gamma$ (Figure 7A; Devreotes, unpublished data) and GFP- $G\beta$ (Jin *et al.*, 2000; data not shown) localize to the membrane of both wild-type and mutant cells, suggesting that the complex is in the correct location to signal in response to cAMP.

We also measured cAMP binding to cell surfaces, and cAMP stimulation of GTP γ S binding, in membranes prepared from cells starved in buffer for 6 h without cAMP pulsing, classic methods to study cAMP receptor-G protein interactions. *icmA*⁻ membranes bind only ~34% as much cAMP as membranes prepared from wild-type cells (Table 1), which can be explained by the fact that there is far lower expression of the major cAMP receptor cAR1 in *icmA*⁻ cells at the developmental stage assayed here (Figure 2B). Treat-

Figure 6. Cell mixing experiments. Wild-type and GFP-expressing *icmA*[−] cells or GFP-expressing wild-type cells were mixed together at a ratio of 10:1, and then they were allowed to form fruiting bodies on nonnutrient agar plates. (A and B) GFP-expressing *icmA*[−] cells stream with wild-type cells. (C) GFP-*icmA*[−] cells segregate to the rear of the migrating slug. Arrow, direction of migration. (D) GFP-wild type cells are distributed throughout. (E) GFP-*icmA*[−] cells deposited behind a migrating slug. (F) GFP-*icmA*[−] spores (arrowheads). Single spore bodies were picked, resuspended in KK2 buffer, diluted, and plated on bacterial lawns to confirm the viability of *icmA*[−] spores.

ment with GTP γ S, which leads to dissociation of the heterotrimeric GTPase complex and decreased affinity of cAR1 for cAMP, affects both cell lines similarly, indicating that lack of methylation does not alter the interaction between heterotrimeric G proteins and the major cAMP receptor cAR1. However, although G $\beta\gamma$ seems to be correctly localized in starved cells (Figure 7A), signaling from cAMP receptors to heterotrimeric G proteins is apparently defective in *icmA*[−] cells. In wild-type cells, cAMP stimulates GTP γ S binding by ~50%, whereas there is no stimulation in the *icmA* deletion, suggesting that methylation is required for effective heterotrimer activation. However, because we have shown that methylation is required for RasG localization (Figure 8, A–D), we cannot exclude the involvement of small GTPases in this step, because they are also activated by GTP γ S, and they may not function correctly in *icmA*[−] cells.

On activation, the heterotrimeric G protein complex regulates several signaling pathways leading to cytoskeletal rearrangements and the release of cAMP. One event triggered by cAMP is the redistribution of the lipid kinase phosphatidylinositol 3-kinase (PI3K) from the cytosol to the plasma membrane, an event that has been suggested to depend on signaling through the heterotrimeric G protein complex (Funamoto *et al.*, 2002; Sasaki *et al.*, 2004). Therefore, abnormalities in PI3K relocation in *icmA*[−] cells may reflect deficiencies in heterotrimeric G protein signaling. We have monitored cAMP-stimulated relocation of a protein consisting of the amino-terminal localization domain of PI3K1 fused to GFP (N-terminal PI3K1-GFP) (Funamoto *et al.*, 2002) in wild-type and *icmA*[−] cells. Relocalization of N-terminal PI3K1-GFP is indistinguishable between the two cell types (Figure 7, B and C, and Supplemental Movie

Figure 7. Localization of and signaling by heterotrimeric G proteins. (A) Wild-type and *icmA*[−] cells expressing YFP-G γ were pulsed in shaking culture and plated on coverglasses for examination with confocal optics. (B) Cells expressing N-terminal PI3K-GFP were pulsed, plated on glass coverslips, and then stimulated with short bursts of cAMP from an adjacent micropipette. At least 12 cells were recorded in three separate experiments. (C) Quantification of the proportion of cells that redistribute N-terminal PI3K-GFP in response to cAMP. (D) Activation of ERK2 in response to cAMP. (E) Quantification of maximal ERK2 response. Average of three experiments is shown. Bars, 10 μ m. See Supplemental Material.

Table 1. cAMP receptor activity in mutant and wild-type cells

Cell type	cAMP receptor activity GTP inhibition of cAMP binding		
	cAMP binding w/o GTP γ S (cpm)	cAMP binding with GTP γ S (cpm)	% Inhibition
AX4	5024 $\pm$ 159	1230 $\pm$ 42	75
KO	1700 $\pm$ 65	421 $\pm$ 40	75
	cAMP stimulation of GTP γ S binding		
	GTP γ S binding w/o cAMP (cpm)	GTP γ S binding with cAMP (cpm)	% of Control
AX4	6142 $\pm$ 283	9203 $\pm$ 30	150
KO	6856 $\pm$ 162	7010 $\pm$ 523	102

Specific binding was calculated by subtracting nonspecific background [cAMP binding (372 cpm) or GTP γ S binding (566 cpm)] from total cpm.

Figure S3). In both wild-type and *icmA*⁻ cells N-terminal PI3K1-GFP redistributes to the membrane within 5–6 s of stimulation and returns to the cytoplasm within 15 s.

Another signaling event downstream of heterotrimeric G protein signaling is the activation of extracellular signal-regulated kinase (ERK)2. Regulation of ERK2 activation in *Dictyostelium* is complex, relying on heterotrimeric G protein-dependent and -independent pathways, but maximal activation requires heterotrimeric G protein signaling (Maeda *et al.*, 1996). Both the kinetics and amplitude of ERK2 phosphorylation are similar between wild-type and mutant cells: maximal phosphorylation is seen ~20 s after stimulation in both cell types (Figure 7D) and levels of ERK2 activation in *icmA*⁻ cells are within 10% of that seen in wild-type cells (Figure 7E).

Localization of and Signaling by RasG

The *Dictyostelium* genome encodes numerous members of the Ras superfamily whose signaling is potentially affected in *icmA*⁻ cells. Recent work has shown that RasG, which we have shown to be methylated (Figure 1D), plays important roles in chemotaxis, differentiation, and development (Thiery *et al.*, 1992; Khosla *et al.*, 1996; Tuxworth *et al.*, 1997; Zhang *et al.*, 1999). We have characterized RasG localization and signaling to assess the role of methylation on its function. TAP-YFP-RasG localization in wild-type cells is enhanced at the cell membrane. TAP-YFP-RasG fluorescence is predominantly cytoplasmic in *icmA*⁻ cells, and <20% of cells exhibit any membrane signal (Figure 8A), suggesting

Figure 8. RasG localization and signaling. (A) Pulsed wild-type and *icmA*⁻ cells expressing TAP-YFP RasG were viewed with confocal optics. Fluorescence intensity profiles were obtained using the plot profile function of ImageJ. (B) GFP-CRAC is localized to the membrane of pulsed *icmA*⁻ cells. Wild-type and mutant cells expressing GFP-CRAC were pulsed with cAMP in shaking culture, plated on glass coverslips, and monitored by epifluorescence microscopy. (C) Cells expressing PhdA-GFP stimulated with short bursts of cAMP. (D) Quantification of the proportion of cells that redistribute PhdA-GFP in response to cAMP. Bars, 10 μ m (A and C) and 5 μ m (B). See Supplemental Material.

that defects observed with *icmA*[−] cells may be at least partially dependent upon mislocalization of RasG.

RasG binds to and regulates the activity of PI3K (Sasaki *et al.*, 2004), a lipid kinase responsible for the production of phosphatidylinositol-(3,4)-bisphosphate and phosphatidylinositol-(3,4,5)-trisphosphate, so measuring levels of these lipids provides a readout for RasG function. To examine this step, we have monitored localization of the PH domain-containing proteins cytosolic regulator of adenyl cyclase (CRAC) and PH domain-containing protein (PhdA), which bind to 3-phosphorylated inositol lipids. CRAC-GFP is predominantly cytoplasmic in wild-type cells that have been pulsed for 6 h with cAMP, but ~50% of *icmA*[−] cells exhibit patches of CRAC-GFP (or PhdA-GFP; data not shown) signal at the plasma membrane (Figure 8B). These patches are transient and form at active regions of the plasma membrane. CRAC-mediated activation of adenylate cyclase may lead to the increase in cAMP relay described above (Figure 4B).

Redistribution of PH-domain-containing fusion proteins in response to cAMP is also aberrant in mutant cells. In wild-type cells, PhdA-GFP redistributes from the cytosol to the membrane within 5–6 s of stimulation with cAMP (Funamoto *et al.*, 2001) (Figure 8C). Membrane association is transient, because PhdA-GFP fluorescence returns to the cytoplasm within 15 s. Only ~30% of *icmA*[−] cells exhibit PhdA-GFP relocation in response to cAMP treatment, a nearly threefold reduction compared with wild type cells (Figure 8D and Supplemental Movie Figure S4). Percentages of cells responding are similar in cells with and without PhdA-GFP prepatching. Comparable results are seen with cells expressing CRAC-GFP (data not shown).

DISCUSSION

G proteins mediate cellular responses to extracellular events. Much recent work has focused on elucidating the mechanisms by which guanine exchange factors, GTPase-activating proteins, and other partners regulate G protein activity, but relatively little is known about the roles posttranslational methylation play in regulating signaling. Work presented here demonstrates that isoprenylcysteine methylation of –CAAX proteins is stimulated in response to extracellular signals and that it is required for intercellular communication, differentiation and development of *D. discoideum*.

Initial investigations into –CAAX methylation demonstrated that activity of the methyltransferase depends on extracellular cues. We have found a similar result in *Dictyostelium* amoebae. Methylation of Ras superfamily small GTPases is initiated within 10 s after stimulation with cAMP and returns to baseline levels after 60 s. Interestingly, methylation occurs on a time scale similar to that seen for activation and/or relocation of Ras, PI3K, PTEN and CRAC, suggesting that ICMT is intimately involved in the signaling events required for *Dictyostelium* chemotaxis and development. Indeed, deletion of the *icmA*[−] gene has dramatic effects on the *Dictyostelium* life cycle.

When starved, *icmA*[−] cells propagate cAMP waves, but the waves are initiated late, are chaotic, and terminate without stimulating aggregation. Cells respond to cAMP by changing shape and secreting more cAMP, prerequisites for visualizing waves with darkfield optics, but they do not efficiently migrate toward the chemoattractant source. This defect is at least partially due to an inability to activate development-dependent gene expression. Expression of the cAMP receptor cAR1 is significantly delayed in *icmA*[−] cells,

suggesting a possible role for ICMT in ramping up the signaling capability of cells in response to cAMP waves. With each wave, methylated, signaling competent G proteins may accumulate, thereby amplifying signals required for chemotaxis and development. This effect would be further enhanced by the developmentally dependent increase in ICMT expression described here. In mutant cells, overall levels of G proteins may be reduced, because lack of methylation increases the turnover of geranylgeranylated proteins in mammalian cells (Bergo *et al.*, 2004). Expression of cAR1 is restored with pulsing as is the ability to migrate toward cAMP, presumably because cAMP pulsing bypasses signal relay, turning on *car1* transcription directly.

Although pulsing partially rescues motility, defects remain. *icmA*[−] cells move more slowly than wild-type cells and with less sense of direction, but there is no general inhibition of chemokinesis. This is a surprise, given the important roles that G proteins play in regulation of cell motility. Mutant cells do exhibit unusual polarity, migrating under agarose with a shape similar to that seen with *amiB*[−] cells; the cells extend a broad leading edge facing the cAMP source. Defects in polarity are also seen at the molecular level, because starved *icmA*[−] cells display rich regions of F-actin, aberrant myosin II localization, and patching of PH-domain-containing proteins. Membrane patching of the PH-domain protein CRAC suggests an explanation for the enhanced cAMP relay seen in *icmA*[−] cells. Interestingly, when coplated with wild-type cells, *icmA*[−] cells are able to coaggregate. Many mutant cells participate in streaming and assemble into aggregates, but a significant number of cells are left behind. We speculate that prepolarized cells oriented with their “false fronts” away from the cAMP source are unable to move toward the origin of the cAMP wave. The observation that *icmA*[−] cells display aberrant polarity is also consistent with a role for small GTPase signaling in regulating the symmetry breaking/amplification events that define the front of chemotaxing cells. Previous experiments suggest a role for Ras and Rac proteins in establishing an autocatalytic signaling loop that establishes polarity (Sasaki *et al.*, 2004), and ICMT-dependent regulation of the proteins is likely to affect polarization, although the specific mechanism by which deletion of *icmA* leads to hyperpolarization is not clear.

Mutant cells plated in a wild-type background also regain the ability to differentiate and develop, but again, defects remain. Mutant cells segregate to the rear of the migrating slug and, in some experiments, are left behind in the resulting slime trail. A possible explanation for this result is that regulation of the prespore/prestalk decision is altered in *icmA*[−] cells. Alternatively, defects in cell–cell adhesion could result in exclusion of cells from the migrating slug. These data clearly demonstrate that deletion of *icmA* leads to defects that are not rescued by cAMP pulsing.

Isoprenylcysteine methylation affects both localization of and signaling by GTPases. To address the molecular root of the *Dictyostelium* phenotypes, we monitored localization and activity of Gγ and RasG, whose activation are critical, early events in the cAMP response. TAP-GFP-RasG localization is enhanced in the plasma membrane of wild-type cells, but it is not membrane-associated in *icmA*[−] cells, whereas YFP-Gγ is membrane-associated in both cell types. This result is surprising, because sequence analysis predicts that both proteins are geranylgeranylated and should not require methylation for proper localization. A possible explanation is that the algorithms used to predict prenyl state are not properly tuned for *Dictyostelium*, and that RasG is actually farnesylated. Alternatively, this result could be a novel demonstra-

tion that methylation alters localization of certain geranylgeranylated proteins. Other experiments reveal that signaling by both heterotrimeric G proteins and Ras-family GTPases is altered in *icmA*[−] cells. Although we observed no effects on the interaction between heterotrimeric G proteins and cAMP receptors in mutant cells, the cAMP receptor cAR1 level is reduced, and the ability of the receptor to properly activate the heterotrimeric G protein seems to be defective, although the molecular mechanism underlying this observation is not yet clear. PH-domain protein redistribution to the membrane, triggered by RasG-mediated activation of PI3K, is dramatically reduced in *icmA*[−] cells. Although a significant number have PH-domain proteins prelocalized to the membrane in the absence of cAMP stimulation, few cells redistribute PH-domain proteins in response to cAMP. This suggests that, in the absence of methylation, RasG does not activate PI3K quickly in response to cAMP, but that global levels of 3-phosphorylated phosphoinositides are elevated in the *icmA*[−] cells. A possible explanation is that the global increase of these phosphoinositides, which play roles in many physiological processes, may be a secondary effect of the *icmA* deletion that is necessary for survival of the mutant strain. Whatever the explanation, our results suggest that deletion of *icmA* has diverse effects on cellular signaling pathways in *Dictyostelium*.

Data presented here and in previous studies with yeast and mammalian cells raise many interesting questions. Are all −CAAX proteins methylated in response to the cAMP stimulus, or are some proteins preferentially targeted? Does methylation state correlate with small GTPase activity? How is ICMT activity regulated in response to cAMP? What enzyme is responsible for demethylation? Answers to these questions will significantly advance our understanding of the role of ICMT in cellular signaling.

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