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Prolyl 4-hydroxylase-1 mediates O_2 signaling during development of *Dictyostelium*

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Development in multicellular organisms is subject to both environmental and internal signals. In *Dictyostelium*, starvation induces amoebae to form migratory slugs that translocate from subterranean areas to exposed sites, where they culminate to form sessile fruiting bodies. Culmination, thought to be regulated by anterior tip cells, is selectively suppressed by mild hypoxia by a mechanism that can be partially overridden by another environmental signal, overhead light, or genetic activation of protein kinase A. *Dictyostelium* expresses, in all cells, an O_2-dependent prolyl 4-hydroxylase (P4H1) required for O-glycosylation of Skp1, a subunit of $E3^{SCF}$-Ub-ligases. P4H1-null cells differentiate the basic pre-stalk and pre-spore cell types but exhibit a selectively increased O_2 requirement for culmination, from ~12% to near or above ambient (21%) levels. Overexpression of P4H1 reduces the O_2 requirement to <5%. The requirement for P4H1 can be met by forced expression of the active enzyme in either pre-stalk (anterior) or pre-spore (posterior) cells, or replaced by protein kinase A activation or addition of small numbers of wild-type cells. P4H1-expressing cells accumulate at the anterior end, suggesting that P4H1 enables transcellular signaling by the tip. The evidence provides novel genetic support for the animal-derived O_2-sensor model of prolyl 4-hydroxylase function, in an organism that lacks the canonical HIFα transcriptional factor subunit substrate target that is a feature of animal hypoxic signaling.

KEY WORDS: Prolyl hydroxylase, Hypoxia, Oxygen, *Dictyostelium*, Cytoplasmic glycosylation, Skp1

INTRODUCTION

Like many unicellular eukaryotes, the social soil amoeba *Dictyostelium* forms spores in response to starvation (Kessin, 2001). Solitary amoebae aggregate to form migratory slugs consisting of multiple pre-stalk cell types and pre-spore cells. Sensory mechanisms acting in the anterior pre-stalk A cells guide the slug from its relatively dark, humid and cool subterranean habitat to the soil surface, where it executes a slug-to-fruit switch and undergoes culmination. This yields a sessile fruiting body consisting of spores supported aerially on top of a vertical stalk, which facilitates spore dispersal. The slug-to-fruit switch, thought to be mediated by the anterior tip (Smith and Williams, 1980), is subject to regulation by NH_3, light, O_2 and other external cues by mechanisms that involve at least ten genes (Schindler and Sussman, 1977; Newell et al., 1969; Sandona et al., 1995; Newell and Ross, 1982). Genetic and biochemical studies suggest that an NH_3-sensor/transporter, 2-component His kinases (DhkA, DhkC and DhkK), a cAMP phosphodiesterase (RegA), protein kinase A, an $E3(SCF^{FbxA})$-Ub ligase, transcription factor-like proteins (STATa, STATc, CudA, MybC), and cytoplasmic red-ox proteins are involved in transducing the signals (Harwood et al., 1992; Mohanty et al., 2001; Kirsten et al., 2005; Singleton et al., 2006; Choi et al., 2006; Thomason et al., 2006).

Culmination appears to be exquisitely O_2-dependent in *Dictyostelium*, requiring O_2 at a level of greater than 10% (Sandona et al., 1995), whereas growth and early development are relatively normal, down to 2.5% (normoxia=21%). Many acute and chronic sensors and signaling pathways for O_2 availability have been identified in prokaryotes and eukaryotes. Recent studies have revealed a major role for cytoplasmic/nuclear prolyl 4-hydroxylases (PHDs, HPHs or EGLNs) as mediators of hypoxic sensing in animals (Kaelin, 2005b; Dann and Bruick, 2005). PHDs, encoded by three genes in humans, depend on physiological levels of α-ketoglutarate (αKG) and ascorbate for optimal activity, and are rate-limited atmospheric levels of O_2. PHDs were discovered as destabilizers of HIF(1-3)α, a family of basic helix-loop-helix-type transcription factor subunits that mediate activation of hypoxia-associated genes. The PHDs modify two Pro residues in HIFα. The resulting 4-HyPro residues render HIFα a target for polyubiquitylation by the $E3^{VHL}$Ub-ligase, resulting in its degradation by the 26S-proteasome. Hypoxia limits PHD activity, which spares HIFα, allowing it to dimerize with HIFβ and activate appropriate target gene transcription. PHD may also have other hydroxylation substrates (Kuznetsova et al., 2003). Hypoxia might regulate PHD activity directly via O_2 starvation, or indirectly via reactive oxygen species (Kaelin, 2005a) or effects on other regulatory factors and substrates, such as Krebs cycle intermediates (Briere et al., 2005; Lee et al., 2005).

Dictyostelium possesses an ortholog of the animal HIF1α-type PHDs. P4H1 was discovered as a factor required for the glycosylation of Skp1 (van der Wel et al., 2005), a small cytoplasmic protein known best as an adaptor of the SCF-class of E3-Ub-ligases (Zheng et al., 2002; Willems et al., 2004) that is evolutionarily related to the VHL class described above. The HyPro residue on Skp1 is subsequently capped by an αGlcNAc residue (van der Wel et al., 2002b), which is then extended by a series of four more glycosylation reactions (West et al., 2004), resulting in the formation of a HyPro-linked pentasaccharide (Teng-umnuay et al., 1998). This modification appears to be conserved in selected other protists (West et al., 2004; West et al., 2006). *Dictyostelium* lacks basic helix-loop-helix-type transcriptional factors (Eichinger et al., 2005) such as HIFα, which are the best-known targets of PHDs in mammals. A phenotypic analysis of the P4H1-null strain used to establish the requirement of P4H1 for Skp1 glycosylation (van der Wel et al., 2005) revealed a specific effect on culmination, as occurs in hypoxia.

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Evidence presented here suggests that PHDs mediate an unexpectedly ancient mechanism of O_2 sensing, predating animals, that in *Dictyostelium* regulates culmination non-cell autonomously from the tip of the slug.

MATERIALS AND METHODS

Cell culture and analysis

Strains listed in Table 1 were grown in association with *Klebsiella aerogenes* or axenically in HL-5 medium at 22°C (Sassi et al., 2001). For development, HL-5 grown cells were washed by centrifugation and deposited on Millipore filters, non-nutrient agar or water agar (for migrating slugs). Plates were incubated under overhead (ceiling) fluorescent lighting in humid chambers at 22°C in ambient atmosphere, or in sealed plastic boxes through which prepared mixtures of humidified O_2 and N_2 (Airgas) flowed. Some trials were conducted under strong overhead light (two 40 W fluorescent bulbs at 50 cm) or in the dark. Strain NC-4 slugs and pre-culminants were dissected as described previously (West and McMahon, 1979), lysed by freeze-thaw in 20 mM Tris-HCl (pH 8.0), and centrifuged at 17,000 ***g*** for 30 minutes to yield a soluble S17 fraction. Cellulose stalk tubes and slime sheath were observed using 0.1% Calcofluor White ST (American Cyanamid) in 10 mM potassium phosphate (pH 6.5). *lacZ* expression was analyzed cytochemically as described (Ferguson et al., 1994). RFP-fluorescence was recorded from slugs partially flattened under a supported coverslip. Spore number was determined in a hemacytometer after vortexing filters in 15 ml polyethylene tubes containing 0.5% NP-40, 5 mM EDTA (pH 8.0). Phase contrast and fluorescence images were photographed digitally and processed in Adobe Photoshop to display the image over the full 2^8-step range, without changing contrast. Fluorescence intensity was quantified using the Histogram function in Photoshop.

Expression plasmids

The integrating pVS4-derived plasmid for expressing P4H1-myc under control of the discoidin 1γ promoter (pVS-P4H1) (van der Wel et al., 2005) was modified by removing its discoidin promoter using *Xho*I and *Kpn*I, and replacement by new promoter DNA amplified with primers containing *Xho*I or *Kpn*I sites in their 5′ tails. P4H1 promoter DNA was amplified from genomic DNA using primers 5′-AA*CTCGAG*AACTCTTGTTTCTGCTT-AATTAAATTATTAAACT-3′ and 5′-TT*GGTACC*TAATACCCGAAAG-TAGTTCTTACCACACC-3′ (restriction sites in italics) in a PCR reaction, and cloned into pCR4TOPO (Invitrogen). This includes the entire 740 nt of intergenic region between P4H1 and the stop codon of the adjacent, upstream, forward-oriented gene. *ecmA* promoter DNA, referred to as *ecmA*(FL), was amplified from genomic DNA using 5′-TT*CTCGAG*-TTTTTTTATTTTTTATTTTTTTAACTTTACAAAGC-3′ and 5′-TT*G-GTACC*TTTCAACGTTATAATTTTTAAACTAATGATTTTAG-3′. This includes 1832 nt upstream of the *ecmA* start codon that matches the full-length pDd63 promoter construct described previously (Early et al., 1993). *cotB* promoter DNA was amplified from cotBGal17 (Fosnaugh and Loomis, 1993) using 5′-TT*CTCGAG*GACATTGTGTTATTATTTGTGTGAAAA-AAA-3′ and 5′-TT*GGTACC*TTTATTACTGGTACTTTTACTATATTAA-TGGTATATGTATA-3′, which includes 1714 nt upstream of the *cotB* start codon as described previously. PCR-amplified DNA was identical to corresponding sequences at dictyBase (http://www.dictybase.org). The resulting plasmids are pVSP-P4H1 (*phyA*), pVSE-P4H1 (*ecmA*) and pVSC-P4H1 (*cotB*).

Point mutations were introduced as previously described (van der Wel et al., 2002a). Primers for the D132N mutation, 5′-*GGAT**CC***ATAGAA**-**ATTCAAATAGTAGAATTCAAGATA-3′ and 5′-CTATTTGAAT**T**TCT-AT***GG***ATCC*ATTGAATATAATCAC-3′, resulted in a silent mutation, A387C, which creates a new *Bam*H1 site, and G394A, the desired missense

Table 1. Normal and mutant Dictyostelium strains

Name	Parent	Genotype (reference)
NC-4	Wild-type	
Ax3	NC-4	*axeA/B/C*
Ax3-RFP	Ax3	*act15*::RFP
HW285	Ax3	*phyA*$^+$, *dscB*::*phyA*-myc
HW288	Ax3	*phyA*$^-$(=P4H1), clone 1a (van der Wel et al., 2005)
HW289	Ax3	*phyA*$^-$, clone 1b (van der Wel et al., 2005)
P4H1$^-$-RFP	HW288	*phyA*$^-$, *act15*::RFP
HW401	HW288	*phyA*$^-$, *dscB*::*phyA-myc* (semi-constitutive)
HW402	HW288	*phyA*$^-$, *phyA*::*phyA-myc*
HW403	HW288	*phyA*$^-$, *cotB*::*phyA-myc* (prespore)
HW404	HW288	*phyA*$^-$, *ecmA*(FL)::*phyA-myc* (prestalk)
HW405	HW288	*phyA*$^-$, *pspA*::*lacZ* (prespore)
HW406	HW288	*phyA*$^-$, *ecmA*(construct O)::*lacZ* (PstA) (Early et al., 1993)
HW407	HW288	*phyA*$^-$, *ecmA*(construct S)::*lacZ* (PstA) (Zhukovskaya et al., 2006)
HW408	HW288	*phyA*$^-$, *ecmA0*::*lacZ* (PstA & 0) (Early et al., 1993)
HW409	HW288	*phyA*$^-$, *ecm0*::*lacZ* (Pst0) (Early et al., 1993)
HW410	HW288	*phyA*$^-$, *pkaC*::*pkaC* (Anjard et al., 1992)
HW415	Ax3	*phyA*::RFP
HW421	Ax3	*dscB*::*phyA-myc* (semi-constitutive)
HW422	Ax3	*phyA*::*phyA-myc*
HW423	Ax3	*cotB*::*phyA-myc* (prespore)
HW424	Ax3	*ecmA*(FL)::*phyA-myc* (prestalk)
HW425	Ax3	*pspA*::*lacZ* (prespore)
HW426	Ax3	*ecmA*(construct O)::*lacZ* (PstA) (Early et al., 1993)
HW427	Ax3	*ecmA*(construct S)::*lacZ* (PstA) (Zhukovskaya et al., 2006)
HW428	Ax3	*ecmA0*::*lacZ* (PstA & 0) (Early et al., 1993)
HW429	Ax3	*ecm0*::*lacZ* (Pst0) (Early et al., 1993)
HW430	Ax3	*pkaC*::*pkaC* (Anjard et al., 1992)
HW260	Ax3	*pgtA*$^-$ (van der Wel et al., 2002a)
HW263	HW260	*pgtA*$^-$, *cotB*::*phyA*
HW264	HW260	*pgtA*$^-$, *ecmA*(FL)::*phyA*
HW265	HW260	*pgtA*$^-$, *act15*::*RFP*
HR6	Ax3	*fbxA*$^-$ (Ennis et al., 2000)

A series analogous to HW401-404 was established in HW289 as HW411-414. Independent clones were established and analyzed for other strains. Under genotype is listed promoter::coding region.

mutation. Numbering starts at ATG of the start codon and changed nts are in bold. Primers for the R276A mutation, 5′-TAAT***TTCGAA***CCA-**GCT**ATTGCAATTACAACTTGGATTTATAG-3′ and 5′-TAATTGCA-ATA**AGC**TGG***TTCGAA***ATTACATTGTAAAACTTCAT-3′, resulted in a silent mutation, T819C, which creates a new *Bst*B1 site, and A826G/G827C/A828T, the desired missense mutation.

Gal (*lacZ*)-containing reporter plasmids (Early et al., 1993) and pTX-RFP, derived from pTX-GFP (Levi et al., 2000) by replacement of GFP by RFP from mRFP-mars, were from the *Dictyostelium* Stock Center (http://dictybase.org/StockCenter/StockCenter.html). EcmA(construct S)-Gal (Zhukovskaya et al., 2006) was from J. G. Williams. The A7-Neo vector for overexpression of the catalytic subunit of PKA (Anjard et al., 1992) was from C. Anjard.

The phyA::RFP expression plasmid was created by amplifying the RFP coding region of mRFPmars (Fischer et al., 2006) using 5′-AAG-GTACCAAAATAAAATAAAAAAATGGCATCATCAGAAGATGTTAT-TAAAG-3′ and 5′-AAAGATCTTGCACCTGTTGAATGTCTACCTTCT-3′, by PCR and cloning into pCR4TOPO. The *Kpn*I site in codon T127 of RFP was eliminated by site-directed mutagenesis that did not alter the amino acid sequence, using primers 5′-AAATTAAGAGGTACTAATTTTCCA-TCAGATGGT-3′ and 5′-TGATGGAAAATTAGTACCTCTTAATTTAA-CTTTATAAAT-3′, as above. The coding region was excised with *Kpn*I and *Bgl*II, and cloned into similarly digested pVSP, yielding pVSP-RFP. For expression of labile GFP, the coding region was excised from pV18-I-S65T-GFP (from the Dictyostelium Stock Center) and cloned into pVSP-RFP(T127T) digested with *Sac*I and *Bam*HI, yielding pVSP-lGFP.

Plasmids were electroporated into growing *Dictyostelium*, and G418-resistant cells were selected at 20 or 120 μg/ml G418 to enrich for chromosomally integrated low- or high-level expressors, and cloned on bacteria plates (van der Wel et al., 2005). Strains are listed in Table 1.

anti-P4H1 antiserum

Recombinant P4H1 was purified from *Escherichia coli* as described (van der Wel et al., 2005), mixed with Freund's complete/incomplete adjuvant, and injected into rabbits (21st Century Biochemicals). Preimmune serum and antiserum from the seventh bleed were used at 1:100.

Protein expression analysis

Whole cells ($1\text{-}2\times10^6$) or soluble S17 or S100 fractions were subjected to SDS-PAGE and western blotting (van der Wel et al., 2005). Skp1 was analyzed on 15-20% polyacrylamide gradient gels. Blots were blocked in 3% bovine serum albumin or 5% nonfat dry milk in Tris-buffered saline, and antibodies were prepared in the same solutions. Alexa 680-labeled secondary antibodies (Molecular Probes) were used at 1:10,000, and blots were imaged in a Li-Cor Odyssey infrared scanner.

RESULTS

Mild hypoxia selectively blocks culmination

When starved and spread on an agar or filter surface under standard fluorescent illumination in normoxia (21% O_2), normal cells aggregate, form migratory slugs, and culminate to form fruiting bodies consisting of aerial spores on top of a vertical stalk. Hypoxia was previously reported to exert a selective block at pre-culmination (Sandona et al., 1995). As confirmed here (Fig. 1A), incubation in the range of 2.5-10% O_2 resulted in an arrest of development before culmination. At the lower end of the range (2.5%), development to the pre-culmination stage was also delayed by about 5 hours. Development was blocked immediately in anoxia (100% N_2), but resumed after return to normoxia with a 4-8 hour delay (not shown). Proliferation in axenic media was nearly normal down to 2.5% O_2, but less than one population doubling occurred at 1% (Fig. 1C). Therefore culmination was selectively dependent on O_2 in the life cycle. Hypoxic slugs induced pre-stalk and pre-spore promoter::*lacZ* markers (see below) normally and did not initiate stalk formation (not shown). Hypoxic shift studies showed that the sensitive period preceded culmination by 7-8 hours, i.e. a shift down from 21% to 5-10% O_2 before 12 hours blocked culmination, whereas a shift up from 5-10% to 21% before 12 hours allowed normal developmental timing (Fig. 1B). To quantitate development, detergent-resistant spores were counted and spore numbers were found to correlate with a visual assessment of culmination (Fig. 1D), as in all studies reported below.

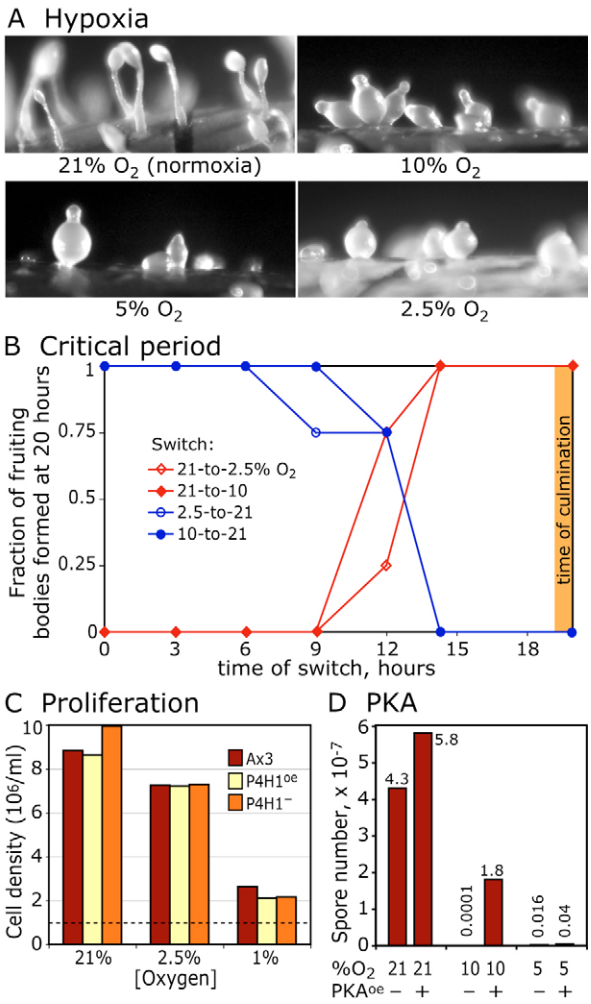

Fig. 1. Hypoxia blocks culmination in the normal *Dictyostelium* strain Ax3. (**A**) Cells were developed for 25 hours on filters in an atmosphere of the indicated percentage of O_2 with N_2. Similar results were obtained in the presence of 1% CO_2 (not shown). (**B**) Timecourse of effect of O_2 shift on culmination visualized qualitatively at 20 hours. (**C**) Effect of hypoxia on cell proliferation. Ax3, P4H1^{oe} (HW285) or P4H1^{-} (HW288) cells (see later) were diluted to 10^6 cells/ml (indicated by dashed line) in growth medium (HL-5) and shaken in the indicated percent of O_2 in N_2. Cells were counted 24 hours later. (**D**) Effect of overexpressing PKAcat on the hypoxic blockade, quantitated by counting detergent-resistant spores after 36 hours. Spore numbers correlated with morphological culmination (not shown). Results are typical of three independent trials.

Protein kinase A (PKA) has been implicated in regulation of the slug-to-fruit switch at culmination (Kirsten et al., 2005). Activation of PKA by overexpression of the catalytic subunit of PKA under its own promoter (PKAcat) partially rescued culmination in 10% O_2 (Fig. 1D). Therefore, mild hypoxia selectively blocks culmination with a crucial time that precedes culmination by 7-8 hours, via a signaling pathway involving PKA.

P4H1-null cells require higher O_2 levels to culminate

Examination of P4H1$^-$ cells, created previously to investigate the requirement of P4H1 for Skp1 glycosylation, revealed a failure to culminate in normoxia similar to the hypoxia arrest described above (Fig. 2A). In most trials, spore numbers ranged from <0.01-1% of normal strain number (Fig. 2B), but variable penetrance was observed (see below). Occasionally, aerial slugs consisting of an elongate mass of undifferentiated cells supported by a delicate, collapsed Calcofluor-positive slime sheath were seen (not shown), suggesting an abortive attempt to bypass the culmination block. Other phases of the life cycle, including proliferation in axenic media (Fig. 1C) or on bacteria (not shown), and development up to the slug stage, appeared normal. Culmination was also relatively normal in a *pgtA*-null mutant (Fig. 2A,B), which lacks a glycosyltransferase that extends the HyPro-dependent glycan on Skp1 past the first sugar, and the O_2 threshold for culmination was indistinguishable from that of normal cells (not shown). Thus the culmination block was not attributable to inability to build HyPro-dependent glycan chains beyond the GlcNAc core.

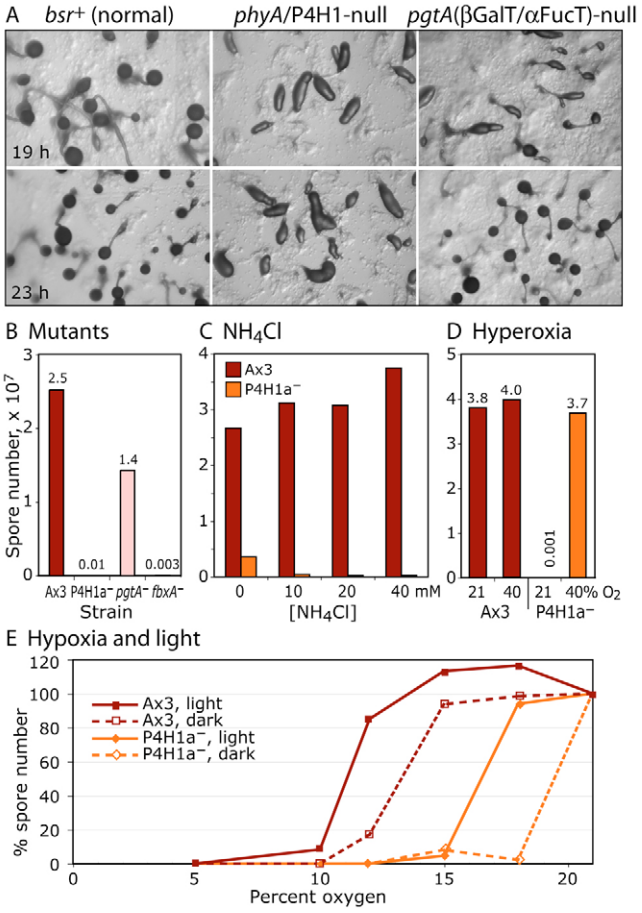

Fig. 2. P4H1-null *Dictyostelium* cells fail to culminate and form spores. (**A**) A normal strain containing randomly integrated P4H1 disruption DNA (*bsr*$^+$), strain P4H1a$^-$ (HW288) and a *pgtA*$^-$ strain (HW260) were starved on non-nutrient agar under standard overhead light in ambient O_2, and photographed after 19 and 23 hours. Normal cells culminate by 19 hours, but neither P4H1a$^-$ nor an independent P4H1$^-$ strain (HW289, not shown) culminated by 96 hours. Similar results were obtained in 24/30 trials (see text and panels C and E). (**B**) Culmination in different strains under ambient conditions was quantified by counting detergent-resistant spores. Spore numbers correlated with morphological appearance. Similar results were obtained in three or more trials, including P4H1$^-$ strains HW288 and HW289. (**C**) Effect of NH_4Cl in MES buffer on strain Ax3 (normal) and P4H1a$^-$ in a trial under ambient conditions in which P4H1a$^-$ exhibited partial culmination breakthrough. (**D**) Effect of incubation in 40% O_2. Similar effects were observed in ten independent trials. (**E**) Interplay of the effects of O_2 and light on culmination, in Ax3 and P4H1a$^-$ cells, based on percent spore numbers relative to values at 21% O_2, which were each within 20% of each other. P4H1a$^-$ required a lower of O_2 than ambient (21%) to culminate in this trial, but nevertheless required 6% more than the parental strain Ax3.

The culmination block was not fully penetrant under standard conditions. In ~20% of the trials (n=30), up to 80% of the normal number of spores was produced with a delay of 3-8 hours. When breakthrough occurred, fruiting bodies typically appeared around the perimeter of the filter or in clusters, suggesting microenvironmental influence and possible positive feedback regulation. Culmination is normally stimulated by light and O_2, and inhibited by NH_3. In a trial in which P4H1$^-$ cells spontaneously culminated, breakthrough was suppressed by NH_4Cl at concentrations that did not affect the parental strain Ax3 (Fig. 2C). When developed at 40-70% O_2, culmination was consistently normal (Fig. 2D) without restoring Skp1 glycosylation (not shown). To investigate the mechanism of breakthrough, cells were developed over a range of O_2 levels. In the dose-response study shown in Fig. 2E, under standard light conditions P4H1$^-$ cells developed normally at 18 and 21% O_2, but not at 15% O_2. In the same trial, parental Ax3 cells required 12% O_2. Therefore the proximity of the elevated O_2 requirement to the ambient O_2 level (21%) may make culmination sensitive to variations in other factors that also influence culmination. To test this idea, parallel cultures were developed in the dark, which normally delays or suppresses culmination. This resulted in a ~3% increase in the level of O_2 required for culmination of both parental and mutant cells (Fig. 2E). Therefore, absence of P4H1 increases the O_2 level required for culmination by 6-10% to a level that makes culmination at ambient conditions sensitive to variations in NH_3, light and other, unknown, factors, which probably explains the variable penetrance. In an attempt to identify other factors that might influence mutant culmination, P4H1$^-$ cells were grown on axenic media or bacteria to different terminal cell densities, developed on different substrata (filter or non-nutrient agar) at different pH values (5.5-7.4) and ionic strength, or in PO_4 or MES buffer. None of these variations rescued culmination at ambient conditions (not shown). To minimize effects of variable penetrance, which was not observed among replicates, all comparisons were done within the same experimental trial. Altogether, the results suggest that O_2, P4H1, light, NH_3 and probably other factors contribute additive signals that influence the slug's decision to culminate and, in the mild hypoxic range (10-21% O_2), the role of O_2 depends on P4H1.

Cell differentiation in mutant slugs

Instead of culminating, mutant slugs appeared to continue migrating based on accumulating slime sheath tracks visible on the filter surface. Slugs consist of ~80% pre-spore cells and ~20% pre-stalk cells (Early et al., 1993). Both normal and P4H1$^-$ slugs accumulated an early pre-spore cell marker, the spore coat protein precursor SP85/PsB (Zhang et al., 1999), with normal kinetics (Fig. 3A). By contrast, SpiA, a late pre-spore marker expressed during culmination (Richardson et al., 1994), was barely detected in P4H1$^-$ cells but expressed normally in a control strain containing randomly

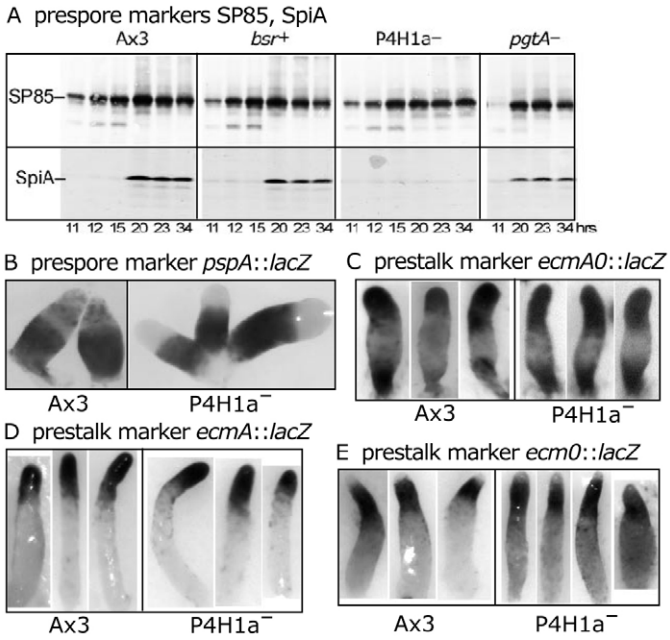

Fig. 3. Effect of P4H1 deletion on developmental marker expression in *Dictyostelium*. (**A**) Western blot analysis for expression of the early pre-spore marker SP85 (mAb 16.1) and the late pre-spore marker SpiA (anti-SpiA) in normal (Ax3 and *bsr*$^+$), P4H1a$^-$ (HW288) and *pgtA*$^-$ cells. Times indicate hours of starvation under ambient conditions. (**B-E**) Microscopic analysis of normal (Ax3) and P4H1a$^-$ cells transfected with various promoter::bacterial β-galactosidase reporter constructs, developed under ambient conditions and processed for β-galactosidase activity, which deposits a dark blue precipitate. Shown are representative examples of pre-culminants or slugs expressing (B) the pre-spore cell marker PspA-Gal (strain HW405), (C) the pre-stalk A and 0 cell marker *ecmA0*::*lacZ* (HW408), (D) the pre-stalk A cell marker *ecmA*::*lacZ* (strain HW406) or (E) the pre-stalk 0 cell marker *ecm0*::*lacZ* (HW409). Anterior tips point upward.

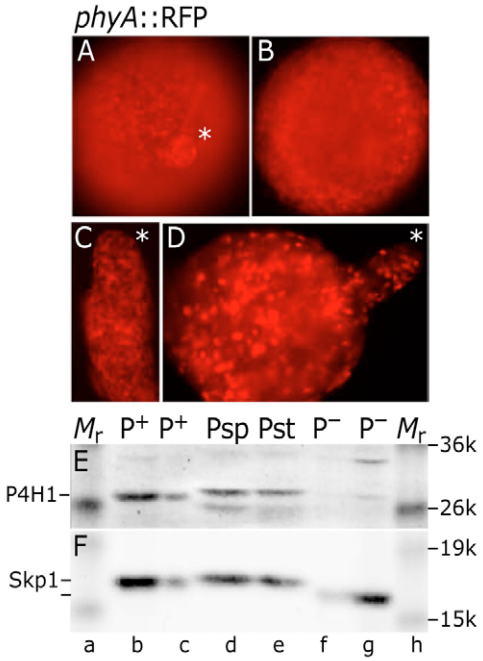

Fig. 4. P4H1 is expressed in *Dictyostelium* tip cells. (**A-D**) Normal (Ax3) cells, transfected with *phyA*::RFP as a reporter for P4H1 expression, were developed to the tipped aggregate (A,B), slug (C) or pre-culmination stage (D) under ambient conditions, placed under a coverslip and photographed for RFP fluorescence. A and B are different planes from the same aggregate. Although not all cells were fluorescent, fluorescence was detected throughout the structures including the tip (denoted by asterisk). (**E**,**F**) Tips (Pst) and central pre-spore (Psp) regions were dissected from normal (strain NC-4) slugs, and 67 μg of S17 fraction protein (lanes d,e) was analyzed by western blotting for P4H1 using anti-P4H1, or for Skp1 using mAb 4E10. Surrounding lanes contain 200 μg (b,g) or 67 μg (c,f) of S100 protein from stationary phase Ax3 (normal) cells (b,c) or P4H1a$^-$ cells (f,g), or M_r standards (a,h). Positions of hydroxylated/glycosylated and unmodified Skp1 are shown. Both panels are from the same blot, and are representative of two experiments.

integrated P4H1-knockout DNA and in *pgtA*$^-$ cells. Microscopic examination in the presence of Calcofluor revealed that no cellulosic stalk tube was formed (not shown). These results are consistent with mutant arrest before culmination.

The position of pre-spore cells was examined in cells transfected with PspA-Gal (*pspA*::*lacZ*), a marker gene expressed specifically in pre-spore cells (Early et al., 1993). P4H1$^-$ and Ax3 pre-culminants exhibited a similar pattern of β-galactosidase expression, confirming a normal pattern of pre-spore cell differentiation (Fig. 3B). However, occasional blue cells in the anterior region of normal pre-culminants, indicative of low-level pre-spore cell mixing, were not observed in mutant slugs.

Pre-stalk cell differentiation was assessed using pre-stalk-specific reporter constructs. EcmA0-Gal, which is expressed throughout the pre-stalk zone, including PstA and Pst0 cells (Early et al., 1993), exhibited a similar pattern in both Ax3 and P4H1$^-$ pre-culminants and slugs (Fig. 3C). EcmA(construct O)-Gal and EcmA(construct S)-Gal, which are similarly expressed in PstA cells, located at the tip, were also expressed normally in the mutant (Fig. 3D and not shown). Ecm0-Gal, specific for pre-stalk 0 cells behind the PstA cells, was also expressed normally (Fig. 3E), although a somewhat higher level of background *lacZ* staining was observed in the pre-spore region of mutant slugs. Therefore, the absence of P4H1 interrupted development after the formation of an apparently normal slug.

P4H1 and Skp1 are expressed in pre-stalk and pre-spore cells

Culmination is regulated in part by the action of anterior tip cells (see Introduction). To address whether tip cells express P4H1, a *phyA*::RFP promoter reporter was stably transfected into normal cells using a multi-copy integrating DNA construct. Cells of clonal isolates exhibited varied fluorescence during growth (not shown). During development, RFP fluorescence was observed in the apical tip (Fig. 4A) and throughout the tipped aggregate (panel B), as well as throughout the slug (panel C) and pre-culminant (panel D), suggesting that the *phyA*/P4H1 gene is expressed in all developing cells. A labile GFP reporter construct was examined to detect expression in a more restricted time frame, but no GFP fluorescence was observed above background (data not shown). To determine if the P4H1 protein was present in tip cells, dissected tips and pre-spore regions were compared by western blot analysis using an anti-P4H1 antiserum developed against full-length recombinant P4H1 purified from *E. coli*. This antiserum

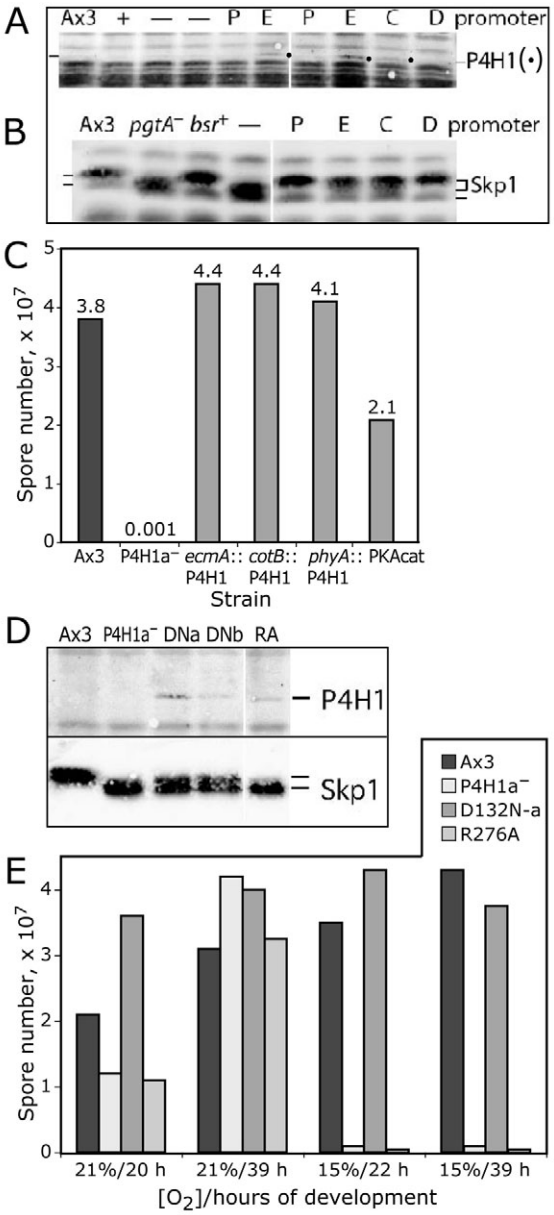

Fig. 5. Rescue of Skp1 hydroxylation and culmination in mutant cells by P4H1 or PKAcat cDNAs. (**A**) Ax3, *bsr*$^+$ (+, normal), P4H1$^-$ clones (–) and P4H1$^-$ clones complemented with P4H1-myc under control of its own promoter (P), the pre-stalk-specific *ecmA*(FL) promoter (E), the pre-spore-specific *cotB* promoter (C) or the discoidin promoter (D), were developed for 16 hours under ambient conditions and analyzed by western blotting with myc-specific mAb 9E10 for P4H1-myc accumulation. The position of P4H1-myc relative to the more intense background bands, which serve as loading controls, is indicated. (**B**) Parallel samples were analyzed by western blotting for Skp1 isoforms using mAb 4E1. Markers indicate high (glycosylated) and low M_r (non-hydroxylated/glycosylated) Skp1 isoforms, and a lower band of unknown identity. Results in A and B are typical of three experiments, including independently complemented HW289-derived cells (not shown). (**C**) Quantitation of culmination under ambient conditions by spore counts. No rescue was observed using an empty vector (not shown). P4H1$^-$ cells transfected with PKAcat (strain HW410) are also shown. (**D**,**E**) Complementation with P4H1-mutants. (D) Fifteen-hour slugs from Ax3, or P4H1a$^-$ expressing the P4H1-myc mutants D132N (DNa and DNb are independent clones) or R276A (RA) under control of the *ecmA*-promoter, were analyzed for P4H1-myc expression as in A, and for Skp1 electrophoretic mobility as in B. (E) Strains were developed at the indicated O_2 level for the indicated time under strong overhead light. P4H1$^-$ cells exhibited breakthrough at 21% O_2 in this trial, but were selectively inhibited relative to Ax3 at 15% O_2. Spore numbers (shown) correlated with morphological appearance (not shown).

recognizes P4H1 in extracts of normal stationary stage cells, but is not reactive, except for a faint presumably cross-reacting band, in P4H1$^-$ cells (panel E). Preimmune serum was negative (data not shown). Similar levels of P4H1 were detected in both regions of the slug, suggesting that the P4H1 protein is constitutively expressed. To address whether P4H1 is active, Skp1 was examined by western blotting. Skp1 was present in similar levels in both regions of the slug, and exhibited a mobility similar to that of normal Skp1 rather than that of Skp1 from P4H1$^-$ cells, which is not hydroxylated and glycosylated (panel F). Altogether, the data strongly suggest that P4H1 is expressed and active in the tip and throughout the slug.

Rescue of culmination by active P4H1 in specific slug cell types

To verify that the genetic defect in P4H1-targeted cells was specific to P4H1, C-terminally tagged P4H1-myc was expressed under control of the semiconstitutive discoidin 1γ promoter. Although below the level of detection by western blotting using anti-myc Ab (Fig. 5A, lane 'D'), expression was sufficient to fully modify Skp1 based on reduced electrophoretic mobility as a result of restored hydroxylation-dependent glycosylation (Fig. 5B). The complemented strain formed fruiting bodies at the normal time and normal numbers of detergent-resistant spores (not shown). Similar results (Fig. 5C) were obtained when P4H1 was expressed under its own promoter (*phyA*::P4H1; lanes 'P' in panels A,B).

To determine if P4H1 is required in a specific cell type, P4H1 was expressed in pre-stalk cells under control of the pan-pre-stalk cell-specific full-length *ecmA* promoter [*ecmA*(FL)], which is similar to the *ecmA0* promoter construct above, or in pre-spore cells using the pre-spore-cell-specific *cotB* promoter. P4H1-myc accumulated in slugs (Fig. 5A, lanes marked 'E' and 'C') at levels higher than achieved under the *phyA* or discoidin promoters, but not earlier in stationary stage cells as expected (not shown). In confirmation of activity, the majority of slug Skp1 glycosylation was rescued in *ecmA*(FL)::P4H1 and *cotB*::P4H1 slugs (Fig. 5B), and not in corresponding stationary stage cells as expected (not shown). The finding that most Skp1 was modified in either strain was surprising considering that these promoters are strictly cell-type specific; this may result from cell-type switching as discussed below. Expression of P4H1-myc in either pre-stalk or pre-spore cells rescued normal culmination and spore numbers (Fig. 5C), showing that ectopically expressed P4H1-myc functionally substitutes for endogenous P4H1.

To determine if complementation required enzyme activity, a point mutation (R276A) was introduced into the *ecmA*(FL)::P4H1 plasmid at a conserved Arg that coordinates the C-5 carboxyl group of αKG and is required for collagen prolyl 4-hydroxylase activity (Myllyharju and Kivirikko, 1997). Both P4H1(R276A)-myc and another point mutant (D132N) at a weakly conserved site were expressed at similar levels (Fig. 5D). The R276A mutant failed to

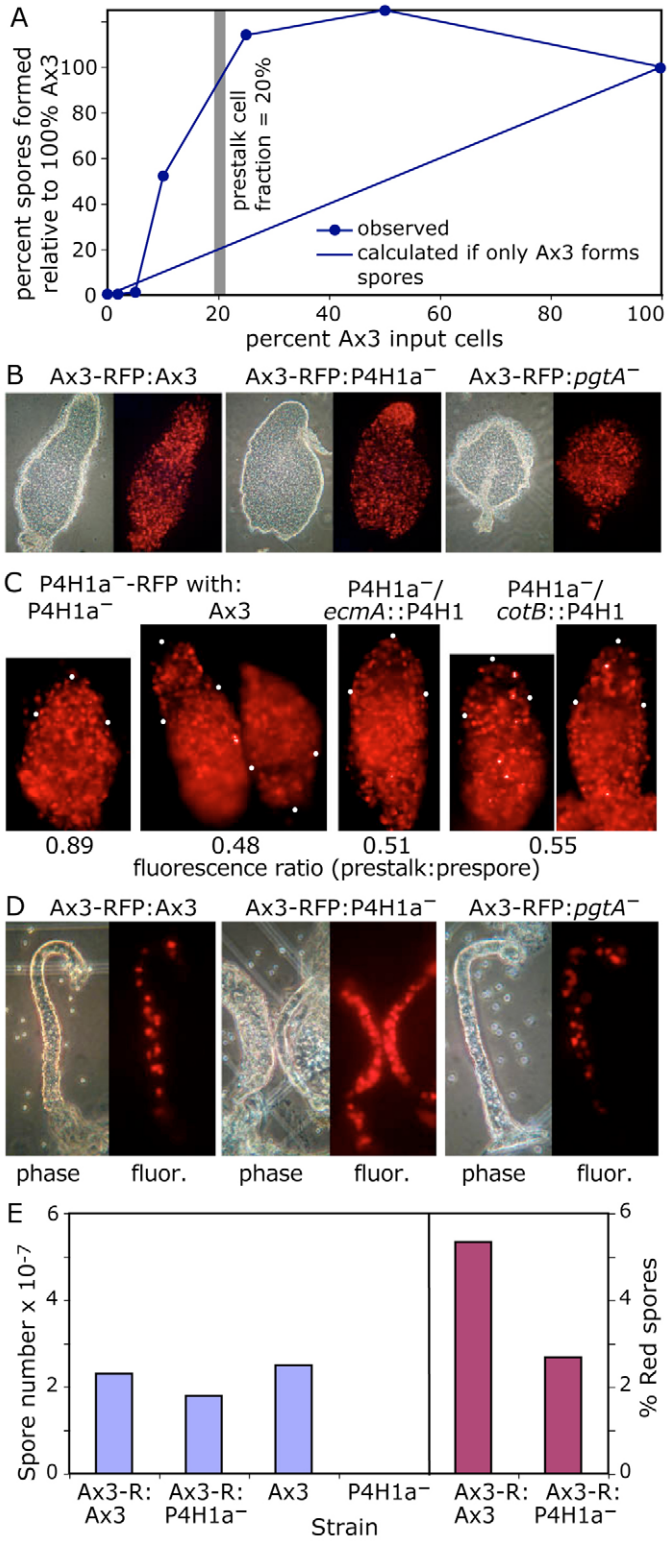

Fig. 6. Rescue of culmination of P4H1⁻ slugs by P4H1-expressing cells, and accumulation of P4H1-expressing cells in the slug tip. (**A**) Various ratios of parental (Ax3) and P4H1a⁻ cells were mixed at time 0, developed under ambient conditions, and harvested to count detergent-resistant spores after 48 hours. The straight line describes the calculated spore number if only Ax3 formed spores independent of an effect from P4H1a⁻ cells. The vertical bar indicates that ~20% of cells are normally pre-stalk cells. (**B**) RFP-expressing normal Ax3 cells were mixed with unlabeled cells, as indicated, in a 7:93 ratio at the start of development. Flattened 16 hour slugs were photographed by phase contrast and for RFP-fluorescence. (**C**) In the reverse experiment, RFP-expressing P4H1⁻ cells were mixed with unlabeled cells, as indicated, in a 3:7 ratio. Numbers below image represent the average ratio of mean intensity in the pre-stalk region (demarcated by three white dots) to that of the pre-spore (remaining) region, for multiple slugs (n=3-6). (**D**) Cultures shown in panel B were allowed to culminate, and stalks were examined. Examples in B and D are typical (n>10). (**E**) Spore numbers were determined in the mixtures shown in D and for Ax3 and P4H1a⁻ cells alone (left panel). Percent of spores that were red in the mixtures is shown in the right panel. Results are representative of two independent experiments.

modify the electrophoretic mobility of Skp1 consistent with the expected absence of P4H1 activity. These cells did not culminate (Fig. 5E), indicating that the prolyl 4-hydroxylase activity of P4H1 is required for complementation. By contrast, the D132N mutant, which partially restored Skp1 hydroxylation/glycosylation consistent with expression in only pre-stalk cells, fully supported culmination. Therefore P4H1 requires its prolyl 4-hydroxylase activity to complement its deficiency.

Rescue of mutant culmination by normal cells or PKA, and accumulation of P4H1-expressing cells in the pre-stalk zone

Normal cells were incorporated into mutant slugs to examine whether the P4H1-deficit affects sending or responding to the culmination signal. Interestingly, as few as 25% Ax3 cells fully rescued culmination and spore formation (Fig. 6A). Mutant spore formation was confirmed by clonal analysis of detergent-resistant cells (not shown). Therefore, normal cells produce a signal that rescues P4H1⁻ cell culmination.

Chimera studies suggest that the culmination signal is provided by pre-stalk cells at the anterior tip (Smith and Williams, 1980). Pre-stalk cells represent about 20% of cells in the slug, similar to the percentage of normal cells that rescue culmination in chimeras. To determine their fate, normal cells were stably transfected with actin15::RFP. Slugs formed from 7% Ax3-RFP and 93% unlabeled P4H1⁻ cells accumulated an excess of Ax3-RFP cells at the anterior tip (Fig. 6B) compared with the rest of the slug. Tip sorting was not an intrinsic property of the marked strain, as sometimes occurs (Ferguson et al., 1994), because Ax3-RFP cells did not preferentially sort to the tip of unlabeled Ax3 or *pgtA*-null slugs. Similar tip accumulation was observed when strain Ax2-expressing calnexin-GFP (gift of M. Clarke, OMRF, Oklahoma City, OK) was mixed with P4H1⁻ cells (not shown), showing that sorting was dominant across strain types. Tip sorting was not as pronounced as when Ax3-RFP cells were mixed with unlabeled *fbxA*-null cells (not shown) (Ennis et al., 2000).

To ensure that differential sorting of strain Ax3 was not the result of RFP expression, labeling was reversed. Mixtures of P4H1⁻ cells transfected with actin15::RFP and unlabeled Ax3 cells at a ratio of 3:7 showed the reverse fluorescence pattern, i.e. less fluorescence in the pre-stalk region (Fig. 6C). This is consistent with Ax3 migrating to the tip and excluding labeled P4H1⁻ cells. No sorting was observed when P4H1⁻-RFP cells were mixed with unlabeled P4H1⁻ cells, as expected. To determine if P4H1⁻ cells complemented with P4H1 behaved as wild-type cells, they were also mixed with 30% P4H1⁻-RFP cells. Like normal Ax3 cells, *cotB*::P4H1 and *ecmA*::P4H1 cells each appeared to exclude P4H1⁻-RFP cells from

the pre-stalk zone, as shown in Fig. 6C. P4H1⁻ cells expressing *cotB*::P4H1 therefore must migrate anteriorly after first expressing P4H1 posteriorly in the pre-spore zone, where *cotB* is active. This behavior is consistent with their signaling culmination from the tip, as inferred from the similar behavior of wild-type cells and when P4H1 is expressed in pre-stalk cells by means of the *ecmA* promoter.

To determine if the tip-associated Ax3-RFP cells differentiate as pre-stalk A (PstA) cells, which are precursors of the cellular stalk, their fate in the fruiting body was examined. Ax3-RFP cells were enriched in the stalk formed from mixtures with P4H1⁻ relative to mixtures formed with unlabeled Ax3 or *pgtA*-null cells (Fig. 6D). In addition, a smaller fraction of spores was fluorescent in chimeras of Ax3-RFP with unlabeled P4H1⁻ cells compared with unlabeled Ax3 cells (Fig. 6E). The differentiation of tip-associated Ax3-RFP cells as stalk cells confirms their conversion to functional PstA cells in the slug, consistent with their proposed ability to signal culmination from the anterior tip.

Overexpression of PKAcat under its own promoter in P4H1⁻ cells partially rescued culmination (Fig. 5C). As in O_2 signaling (Fig. 1D), P4H1 therefore appears to function upstream of PKA, although the possibility that PKA functions as a bypass suppressor cannot be excluded.

P4H1 overexpression reduces the O_2 requirement for culmination

If hypoxia inhibits culmination by limiting hydroxylation of one or more P4H1 substrates, then culmination might be rescued by higher levels of the enzyme protein. Stable transfection of normal Ax3 cells with the pre-stalk-cell-specific *ecmA*(FL)::P4H1 overexpression constructs used in the complementation studies above allowed development-specific expression of P4H1 at a level (Fig. 7A) comparable to that seen in P4H1⁻ cells (Fig. 5A). These cells exhibited near full rescue of culmination and spore formation (75% of normal) at 10% O_2, and partial rescue (33% of normal) at the more stringent 5% O_2 level (Fig. 7B). Stable transfection with pre-spore-cell-specific *cotB*::P4H1, which also resulted in detectable P4H1 expression, yielded partial rescue. Overexpression of P4H1 under its own promoter or the discoidin promoter (not shown) led to negligible rescue, consistent with the low level of expression achieved during development as detected by western blotting (Fig. 7A). The related overexpression strains formed in the P4H1⁻ background, described in Fig. 5, also did not culminate in hypoxia (not shown), indicating an essential role for endogenous P4H1 in rescue. In conclusion, overexpression of P4H1 enabled culmination at an O_2 level below that required by normal slugs.

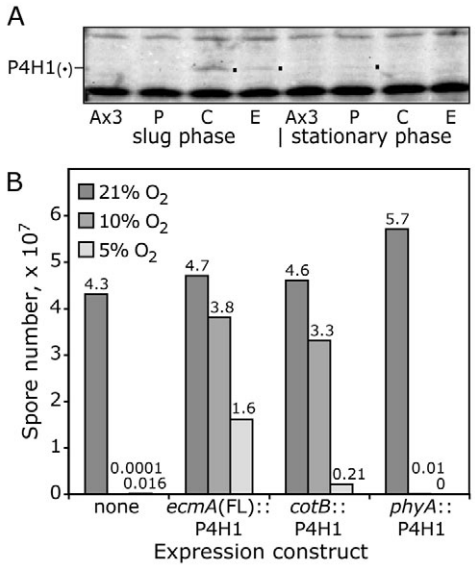

Fig. 7. P4H1 overexpression reduces the O_2 requirement for culmination in *Dictyostelium*. (**A**) Western blot analysis of P4H1-myc levels in clones of strain Ax3 transfected with the promoter expression constructs described in Fig. 5A. (**B**) Effects of overexpressing P4H1 in the Ax3 background on the hypoxic blockade, as determined by counting detergent-resistant spores. Spore numbers correlated with morphological culmination (not shown). Results are typical of three independent trials.

DISCUSSION

Although 2.5% O_2 is sufficient to carry out most growth and developmental processes in *Dictyostelium*, culmination requires levels in excess of 10% (Fig. 1). Disruption of the P4H1 gene increases the O_2 requirement even further, up to 21% (ambient) or more, resulting in essentially a complete block of culmination, with variable penetrance (see below), in normal atmosphere (Fig. 2). By contrast, overexpression of P4H1 in the wild-type background reduces the O_2 requirement to <5% (Fig. 7). Although P4H1 function might require capping of the product HyPro with GlcNAc, as seen in Skp1, subsequent glycosylation mediated by the PgtA β3GalT/α2FucT is inconsequential based on analysis of a *pgtA*-null strain (Fig. 2). These data suggest a model that culmination is uniquely regulated by O_2 via a mechanism that involves the cytoplasmic prolyl 4-hydroxylase P4H1.

Several lines of evidence reinforce the model that P4H1 mediates O_2 signaling of culmination in vivo. Hypoxia and disruption of *phyA* (P4H1) interrupt development at the same stage (Fig. 1A, Fig. 2A). They are each suppressed by activation of PKA (Fig. 1D, Fig. 5C), a master regulator of culmination (Harwood et al., 1992; Kirsten et al., 2005), which suggests they function in the same pathway. P4H1 is expressed throughout the slug before culmination (Fig. 4). P4H1 activity is rate-limited by O_2 in vitro (van der Wel et al., 2005), with a K_m of 40% O_2, and the ability of expressed P4H1 to complement the P4H1 deficiency is dependent on its hydroxylase activity (Fig. 5E). Extra copies of the P4H1 enzyme are expected to yield increased hydroxylation in limiting O_2. These findings offer direct genetic support for the general model, derived from studies in animals, that P4H1 is a physiologically important O_2 sensor (Schofield and Ratcliffe, 2004; Kaelin, 2005b). Further studies are required to differentiate whether P4H1 is regulated by O_2 directly or indirectly, such as via O_2 radicals, α-ketoglutarate (a co-substrate), and/or other Krebs cycle intermediates that competitively inhibit the enzyme (Lee et al., 2005; Koivunen et al., 2007).

The P4H1⁻ defect appears to involve a failure to send rather than respond to a culmination signal, because culmination in ambient O_2 is rescued by co-development with small numbers of normal cells. RFP-tagged normal cells accumulate in the slug tip and become PstA cells (Fig. 6), which are thought to normally regulate the slug-to-fruit switch. Forced expression of P4H1 in either pre-stalk cells (under the *ecmA*-promoter) or pre-spore cells (under the *cotB* promoter) of mutant slugs also rescues culmination. Interestingly, both of the P4H1-expressing cell populations tend to accumulate in the anterior region, as do normal cells (Fig. 6C). In addition, forced expression of P4H1 in pre-stalk cells is more effective than in pre-spore cells for reducing the O_2 dependence of normal cell culmination (Fig. 7). Finally, pre-stalk cell PKA can influence culmination (Harwood et al., 1992). Altogether, the data support the model that P4H1 signals culmination via anterior cells. However, as

sorting of pre-spore cells expressing P4H1 is not absolute and P4H1 is expressed in all slug cells (Fig. 4), a role for pre-spore cell P4H1 in culmination is not excluded.

P4H1 appears to influence culmination jointly with other environmental factors. O_2 and P4H1 function synergistically with overhead light (Newell et al., 1969) to regulate culmination. The O_2 requirement of both normal and mutant cells is decreased by 2-3% when cells are developed in overhead light compared with darkness (Fig. 2E). Previous studies suggested that overhead light attracts the tip aerially, which promotes culmination indirectly (Bonner et al., 1982), although in the present study of stalled pre-culminants, tips appeared to be elevated from the substratum in either light or dark conditions (not shown). The slug-to-fruit switch is also normally regulated by NH_3 (Kirsten et al., 2005), a catabolic by-product of slug metabolism. NH_3 inhibits culmination in P4H1$^-$ cells at lower concentrations than those required to affect normal cells (Fig. 2C), as described for other slugger mutants (Gee et al., 1994), suggesting that O_2 and NH_3 signaling interact. Evidence indicates that NH_3, O_2 and P4H1 each influence culmination via PKA. Genetic manipulations that affect superoxide levels and the cellular redox balance also affect culmination (Garcia et al., 2003; Jeong et al., 2006; Choi et al., 2006) and, as PHD function depends on O_2, reduced Fe^{+2} and ascorbate, which may be affected by superoxides (Kaelin, 2005a; Kaelin, 2005b), these agents might also signal via P4H1. In addition, hyperoxia rescues P4H1$^-$ culmination (Fig. 2D), which suggests a second mechanism by which O_2 regulates culmination. This might involve one of the other four PHD-like genes in the *Dictyostelium* genome (West et al., 2004). The slug-to-fruit switch is evidently regulated by a complex pattern of extracellular signals, and subtle variations, in concert with microenvironmental effects and potential positive feedback interactions between slugs suggested by edge and group effects, may explain the 18-21+% range of O_2 required for culmination in different trials.

A candidate target of P4H1 action is Skp1, the glycosylation of which requires P4H1 in *Dictyostelium* and which was used to monitor P4H1 activity in the mutants. However, Skp1 is glycosylated normally, within the resolution of western blot analysis, in slugs of Ax3, and even HW403 (*cotB*::*phyA* in P4H1$^-$ background) or HW404 (corresponding *ecmA*::*phyA* expression strain), formed in 5-10% O_2 (our unpublished data). In addition, Skp1 is not glycosylated in 40% O_2 (not shown), which overrides inhibition of culmination in P4H1$^-$ cells. Although these results show that hydroxylation of the bulk pool of Skp1 is not regulated in parallel with culmination, a role for Skp1 is not excluded, as a critical subpool (e.g. nascent, nuclear or pre-stalk) might be differentially hydroxylated. Skp1 is the only substrate that accumulates in P4H1$^-$ cells based on an indirect assay with rP4H1 and rGnT1 (our unpublished data). A role for Skp1 is further supported by evidence that CulA and FbxA, proteins that physically associate with Skp1 in SCF-type E3 Ub-ligase complexes (Willems et al., 2004), are also required for multiple developmental steps, including culmination (Mohanty et al., 2001; Nelson et al., 2000; Ennis et al., 2000). However, P4H1$^-$ slugs do not accumulate the E3(SCF^{FbxA})Ub-ligase substrate RegA (not shown), as occurs in *culA*- and *fbxA*-null cells (Mohanty et al., 2001). Yet it is intriguing that the SCF class of E3-Ub-ligases, which contains HyPro-Skp1, is evolutionarily related to the VHL class that recognizes HyPro-HIFα in animals. Additional studies are needed to evaluate the O_2 dependence of the hydroxylation of Skp1, which is encoded by two genes, in the slug tip, and the role of GnT1, which mediates addition of the GlcNAc cap to HyPro-Skp1 (van der Wel et al., 2002b). The best-known substrate of animal PHDs, HIF1α and the $E3^{VHL}$Ub-ligase that recognizes HyPro-HIF1α, are apparently absent from the *Dictyostelium* genome based on bioinformatics studies (not shown), but other potential targets (Kuznetsova et al., 2003) occur.

Hypoxic regulation of culmination may provide a selective advantage to *Dictyostelium* in its normal habitat. Hypoxia encountered by developing cells in confined or water-saturated microenvironments may signal delay of culmination. In this scheme, O_2 synergizes with NH_3 depletion and light, which can override a suboptimal level of O_2 (Fig. 2E). The crucial period for O_2 regulation is many hours in advance of culmination (Fig. 1B), and cells having the highest P4H1 activity tend to migrate to the slug tip (Fig. 6), suggesting complexities that remain to be explored. Another function for O_2 in *Dictyostelium*, cytochrome oxidase subunit VII switching (Sandona et al., 1995), does not involve P4H1 based on DNA microarray studies (L. Eichinger and C.M.W., unpublished). The role of P4H1 in O_2-dependent slug polarization and guidance of slug migration (Sternfeld and Bonner, 1977; Sternfeld and David, 1981) remains to be examined. Because hypoxic regulation is connected with sporulation, *Dictyostelium* offers a unique genetic opportunity to investigate the mechanism of P4H1 action, including its role as a direct O_2 sensor and the identification of upstream and downstream regulatory genes.

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