Prolyl hydroxylation- and glycosylation-dependent functions of Skp1 in O2-regulated development of Dictyostelium

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Abstract

O2 regulates multicellular development of the social amoeba Dictyostelium, suggesting it may serve as an important cue in its native soil environment. Dictyostelium expresses an HIFα-type prolyl 4-hydroxylase (P4H1) whose levels affect the O2-threshold for culmination implicating it as a direct O2-sensor, as in animals. But Dictyostelium lacks HIFα, a mediator of animal prolyl 4-hydroxylase signaling, and P4H1 can hydroxylate Pro143 of Skp1, a subunit of E3SCFubiquitin-ligases. Skp1 hydroxyproline then becomes the target of sequential glycosyltransferase reactions that modulate the O2-signal. Here we show that genetically induced changes in Skp1 levels also affect the O2-threshold, in opposite direction to that of the modification enzymes suggesting that the latter reduce Skp1 activity. Consistent with this, overexpressed Skp1 is poorly hydroxylated and Skp1 is the only P4H1 substrate detectable in extracts. Effects of Pro143 mutations, and of combinations of Skp1 and enzyme level perturbations, are consistent with pathway modulation of Skp1 activity. However, some effects were not mirrored by changes in modification of the bulk Skp1 pool, implicating a Skp1 subpopulation and possibly additional unknown factors. Altered Skp1 levels also affected other developmental transitions in a modification-dependent fashion. Whereas hydroxylation of animal HIF results in its polyubiquitination and proteasomal degradation, Dictyostelium Skp1 levels were little affected by its modification status. These data indicate that Skp1 and possibly E3SCFubiquitin-ligase activity modulate O2-dependent culmination and other developmental processes, and at least partially mediate the action of the hydroxylation/glycosylation pathway in O2-sensing.

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Introduction

When starved, cells of the social amoeba Dictyostelium aggregate and form a migratory slug, which subsequently culminates into a sessile fruiting body composed of tens of thousands of spores supported above a narrow cellular stalk. In the native environment of the soil, this asexual developmental pathway provides a mechanism for normally subterranean, solitary amoebae to synergistically achieve an aerial disposition from which spores may disperse to distant locales to renew proliferation. The slug-to-fruit switch decision critically depends on O2-concentration (Sandona et al., 1995) and other environmental factors such as NH3, light, humidity and warmth, some of which have been shown to signal via protein kinase A (Kirsten et al., 2005). Whereas only 2.5% O2 is required for proliferation, 10–12% O2 is required for development of the normal strain Ax3 past the slug stage if cells reside at an air–water interface (West et al., 2007), and 70% is required for terminal differentiation into stalk and spore cells when cells are submerged (West and Erdos, 1988).

The O2-set point for culmination appears to involve signaling via P4H1, the Dictyostelium ortholog of HIFα prolyl 4-hydroxylase (PHD or HPH) (West et al., 2010), a major O2-sensor of animals (including humans) involved in mid-to-long term responses to hypoxia (Kaelin and Ratcliffe, 2008). Disruption of the phya gene encoding P4H1, or increased P4H1 enzyme activity due to overexpression, causes an increased or decreased O2-requirement for culmination, respectively. However, Dictyostelium, and other protists that possess phya-like genes, lack HIFα, the transcriptional factor subunit that is destabilized by hydroxylation of Pro-residues in its two O2-dependent degradation domains. A known substrate for Dictyostelium P4H1 is Skp1 (van der Wel et al., 2005), a subunit of the SCF-class of E3 Ub-ligases. E3SCFUb-ligases regulate the cell cycle, nutrient sensing, physiology and development in many organisms (Willems et al., 2004), including the latter in Dictyostelium (Ennis et al., 2000; Nelson et al., 2000; Mohanty et al., 2001; Tekinay et al., 2003). Dd-Skp1 is modified at Pro143, which is replaced by Glu in chordate Skp1s. Interestingly, E3SCFUb-ligases are evolutionarily related to the E3VHLUb-ligase which mediates O2-
dependent degradation of animal HIFα in normoxia (Kaelin and Ratcliffe, 2008), suggesting a potentially related signaling mechanism associated with protein stability. Therefore Skp1 is a candidate for mediating the O2-signaling role of P4H1 in Dictyostelium.

Hydroxylated Skp1 is subject to successive further modification by three gene products, resulting in the assembly of a pentasaccharide on the fully processed protein (see Fig. 1A). Disruption of the dual function glycosyltransferase gene pgtA, which results in accumulation of Skp1 whose Hyp is modified by the single sugar GlcNAc, leads to a near wild-type O2-dependence that originally suggested that peripheral glycosylation is not relevant to O2-dependent signaling (West et al., 2007). However, a recent study showed that disruption of agtA (Ercan et al., 2006), required for addition of the final two sugars, results in dependence on high O2 approaching that of P4H1 signaling (Wang et al., 2009). Since Skp1 is the only substrate detected for the PgtA and AgtA glycosyltransferases in biochemical screening studies, Skp1 is implicated as the functional target of P4H1 in O2-signaling as well. However, since animal PHDs appear to have multiple substrates in O2-signaling (Kaelin and Ratcliffe, 2008), and it is challenging to identify PHD targets, further evidence is required to confirm the hypothesized involvement of Skp1 in P4H1 signaling in Dictyostelium.

Wild-type strains of D. discoideum harbor two Skp1 genes, Skp1A and Skp1B, whose amino acid sequences are identical except for a difference at codon 39 (Ser/Ala) in the N-terminal region (West et al., 1997). The axenic strain Ax2 possesses the wild-type complement, and Skp1B, whose amino acid sequences are identical except for a

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### Experimental procedures

**Cell growth and development**

Strains (Supplementary Table 1) were grown axenically in HL-5 medium on orbital shakers. For development, vegetative cells (≤5 × 10⁶ cells/ml) were centrifuged at 1000 g for 2 min, resuspended in ice-cold PDF buffer, centrifuged again, and resuspended in PDF (West et al., 2007). 0.4 ml cells (10⁶/ml) were spread on 47 mm-diameter Millipore filters 60 × 15 mm Petri dishes and incubated in sealed plastic boxes, under overhead room fluorescent lighting at 22 °C, for up to 46 h in the presence of the indicated concentration of flowing O2 with the balance made up with N2. Development was evaluated morphologically and by counting spores in a hemacytometer.

Stationary stage cells were collected at 2–3 × 10⁷/ml. For aggregation stage cells, washed vegetative cells were resuspended at 2 × 10⁷/ml in 2 ml of Agg buffer (0.01 M NaPO₄, 0.01 M KCl, 0.005 M MgCl₂, pH 6.0), and shaken in a flask for 8 h. Slug stage cells were scraped from filters 2–3 h after their initial appearance.

Shaking cells were incubated with 400 μg/ml cycloheximide (unless otherwise stated) from Sigma Chemical Co., diluted from a 50 mg/ml stock solution in DMSO, for the indicated time. Control cultures were incubated in 0.8% DMSO. For metabolic labeling, 5 μCi of [35S]-Met (carrier-free, Amersham) was added 15 min after introduction of cycloheximide and incubated for 2 h. Incorporation into protein was measured by TCA precipitation as before (Sassi et al., 2001).

### Cell extracts and protein analyses

For standard Western blot analysis, cells were collected by centrifugation (1000 g × 1 min), resuspended in ice-cold 50 mM Tris–HCl, pH 8.0, centrifuged at 5000 g × 15 s, and the pellet frozen at −80 °C. For protein determination, pellets were resuspended in ice-cold 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.1% NP40, 0.5 mM

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**Fig. 1.** Specificity of P4H1 and Gnt1. (A) Schematic of the hydroxylation/glycosylation pathway, using Skp1 as a target example (West et al., 2010; AgtA, unpublished data). (B) Soluble extracts (5100 μg) of phyA(P4H1)−/− stationary stage cells were desalted, incubated with rP4H1, rGnt1, and enzyme substrates and cofactors including UDP-[3H]GlcNAc. Controls included parental Ax3 cells (P4H1−/−), bsr−/− cells from a transfection with an empty plasmid, and reactions lacking P4H1. After 2 h, a reaction aliquot was subjected to SDS-PAGE and sliced into bands that were counted for radioactivity. Migration position of Skp1 is indicated. Inset shows low-level P4H1/Gnt1-dependent incorporation at the position of normal cell Skp1. (C) Similar analysis of slug cells.
PMSF, 5 μg/ml each of leupeptin and aprotinin, and assayed using a micro BCA protein Assay Reagent Kit (Pierce), relative to a bovine serum albumin standard. S100 fractions (cytosol) were prepared as described (Wang et al., 2009).

**Western blotting and antibodies**

For SDS-PAGE, cell pellets were resuspended in 1 × Laemmli sample buffer, 20 mM DTT, or cell lysates were mixed with 4 × modified Laemmli sample buffer, 80 mM DTT. After boiling for 1 min, samples were separated on a 7–20% or 15–20%, or 4–12% (NuPAGE Novex Bis–Tris, Invitrogen) acrylamide SDS-PAGE gel at 0.5–1 × 10⁶ cells/lane, and blotted as described (West et al., 2007), or on an iBlot Dry Blotting System (Invitrogen). mAb 4H2, which specifically recognizes non-glycosylated Skp1 (both unmodified and hydroxylated forms), was generated by immunizing mice with a synthetic peptide ([CKNDFTPEEEQ] linked to KLH, as previously described (Wang et al., 2009). mAb 4E1 recognizes all Skp1 isoforms (Kozarov et al., 1995). Affinity-purified anti-actin antibody (from rabbits immunized with a MAP-peptide of SGPS/VHRKCF) was from Sigma (St. Louis, MO). Alexa-680 labeled secondary Abs were from Invitrogen. anti-P4H1 (rabbit) was described previously (West et al., 2007).

Protein levels were quantitated by densitometric analysis of Alexa-680 fluorescence imaged using a Li-Cor Odyssey infrared scanner, over an intensity range validated by analysis of a 2-fold dilution series of cell extracts probed with the same Abs (not shown).

**Skp1 strain constructions**

Skp1A cDNA and Skp1B cDNA (derived from fpaA) were amplified from p48 and p50 plasmids (Sassi et al., 2001), using Skp1A-S (5′-aaGGTACCttagtttccacctttatgttcacacca), by PCR and cloning into pCR4TOPO. The insert was released using SacI and KpnI, and ligated into similarly digested pVSC- (ecm4-promoter) plasmids (West et al., 2007). Plasmids were electrotrans- ported into growing Dictyostelium, and GFP18-resistant cells were selected at 20 or 120 μg/ml G418 to enrich for chromosomally integrated low- or high-level expressers, and cloned on bacteria plates.

Skp1B (fpbA) was disrupted in Ax2 cells by replacing the chromosomal gene with a fpbA fragment containing a floxed-Blasticidin S resistance marker (fbr). A 1067 bp 5′-fragment and a 780 bp 3′-fragment of Skp1B were PCR-amplified from genomic DNA using primers SB1 (5′-gttCCGGCgttattttttatttttgtgtgtattgctttattgg) and SB2 (5′-gaaCTCGAGaatGGATCCtattttttatttttgtgtgtattg), by PCR and cloned into pcRTOPO. The insert was released using SacI and KpnI, and ligated into similarly digested pVSE (ecm4-promoter), and pVSC (cotB-promoter) plasmids (West et al., 2007). Plasmids were electrotransported into growing Dictyostelium, and GFP18-resistant cells were selected at 20 or 120 μg/ml G418 to enrich for chromosomally integrated low- or high-level expressers, and cloned on bacteria plates.

Fragments were separately cloned into pCR4TOPO. A 874 bp fragment was released using SacI and Clal and ligated into similarly digested pcRTOPO (522). Then were separately digested into pcRTOPO (522 + 874) using BglII and PstI. Finally, fbr was ligated into the pcRTOPO(429 + 522 + 874) using NcoI and BamHI.

The Glu143 mutation was generated from the Ala143 construct using site-mutagenesis with primers SAQ-SDM (5′-agagaagactttact-gaagaagaagacaaataagaaaaagaaag), and SAQ-SDM-Rev (5′-atttttctcttctcttgagatgttgttttg) and fbr, which replaced gca (Glu) with gaa.

The mutant constructs were excised using BglII and Clal, treated with Bal 31 exonuclease to remove flanking non-homologous nt, and used to transform strain Ax2 cells by electroporation as above, in the presence of 10 μg/ml Blasticidin S. Gene replacement was confirmed by PCR with two pairs of primers: SA3 (5′-ggtaggatcttattttttttttacctttttcttttc), and SA4 (5′-gaagccctggccagctgaatattagaaataaatataactc) and fBSR-R1 (5′-agataagatggccagcaagacg).

Strains overexpressing both Skp1 and P4H1 were generated by cotransformation of equal amounts of the respective expression plasmids and selected for using G418 as described above. The majority of drug-resistant clones expressed both proteins based on Western blot analysis.

**RFP and GFP expression reporters**

Promoter DNA was amplified from strain Ax3 genomic DNA using PCR reactions containing the following primers: fpaA-sense (5′-ttCTCGAGttttttggttttcctatttttgacttttgacttttg), fpaA-antisense (5′-ttCTCGAGttttttgttttttgttttttgttttttgtttttt), fpaB-sense (5′-ttCTCGAGttttttgttttttgttttttgttttttgacttttg), fpaB-antisense (5′-ttCTCGAGttttttgttttttgttttttgttttttgacttttg). The products of the reactions were gel purified and cloned into pcRTOPO (Invitrogen), excised using Xhol and KpnI, and cloned into similarly digested pVSP-RFPmars (T127T) and pVSP-labGFP (West et al., 2007), using standard procedures. For the fpaA promoter constructs, this resulted in the placement of the phyA-promoter DNA with 366 nt of DNA immediately upstream of the fpaA start ATG, which comprises most of the 441 nt separating fpaA from the reverse-oriented upstream coding sequence (DDB_G0269994) and includes all G/C nt more than 13 nt upstream of the DDB_G0269994. For fpaB, this resulted in inclusion of 2127 nt upstream of the fpaB start codon, which extended 28 nt into the upstream reverse-oriented coding sequence (DDB_G0273387).

**Enzymes and reactions**

HisP4H1 (rP4H1) was expressed in E. coli and purified essentially to homogeneity as described previously (van der Wel et al., 2005).

The Gnt1 ORF was amplified from pTYB1-GnT51 (van der Wel et al., 2002) using the following primers in a standard PCR reaction: GnT51-1s1 (5′-ggagccattcaactgttttttgtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt...
reached 0.4, at which time 1 mM IPTG was added and the culture was incubated for 16 h at 10 °C on a shaker. His-Gnt1 was purified from the S100 fraction on a Ni²⁺-column as for His6P4H1 (van der Wel et al., 2005).

Combined P4H1/Gnt1 reactions were performed on desalted S100 preparations of stationary stage or slug stage cells, in the presence of UDP-[³²P]GlcNAc, and the entire reaction volume was subjected to SDS-PAGE, as described previously (van der Wel et al., 2005). Incorporation of radioactivity was measured by liquid scintillation counting of evenly spaced gel slices created using a razor blade cutter.

**Skp1 purification and mass spectrometry**

FLAG-Skp1A and Skp1B-myc were purified to near homogeneity, from stationary stage and slug stage cells, respectively, and analyzed by MALDI-TOF-MS, as described in the Supplementary material.

**Results**

**Biochemical screen for P4H1 substrates**

To probe for potential P4H1 substrates that accumulate in phyA (P4H1)-null cells, a desalted cytosolic extract was incubated with His6Gnt1 and appropriate cosubstrates and cofactors. The extract also included His6Gnt1 and UDP-[³²P]GlcNAc to transfer [³²P]GlcNAc to the Hyp reaction product, and the entire reaction product was displayed on a 1D SDS-PAGE gel which was then cut into slices to measure radioactivity (Fig. 1B). As expected, Skp1 was strongly labeled. However, no other proteins were detected at more than 0.2% of the level of Skp1 labeling except for a variable, slightly more rapidly migrating peak probably corresponding to a breakdown product of Skp1. About 5% of the level of Skp1 labeled in P4H1− cells was labeled in Ax3 cells (see inset), suggesting the existence of a small steady-state pool of unmodified protein. Similar results were obtained for an extract of slug cells (Fig. 1C). The results suggest that Skp1 is the only substrate of P4H1 that accumulates in P4H1− cells but, since the coupled assay depends on recognition of the P4H1 product by Gnt1, other P4H1 substrates might have been missed. The significance of Skp1 as a potential substrate for P4H1 in O₂-signaling was therefore investigated genetically.

**Genetic modifications of Skp1 loci**

The standard haploid Ax3 strain used in previous studies harbors three Skp1 genes: apa and two identical copies of pab owing to a duplication on chromosome 2 involving about 50 genes (Bloomfield et al., 2008). Skp1A (apaA) and Skp1B (pabB) differ by a single amino acid (S39 vs. A39) as depicted in Fig. 2A. Another standard strain, Ax2, lacks this duplication. The O₂-dependence of culmination was compared by incubating cells in a grded series of O₂ levels, and monitoring development after 2 days based on morphological appearance. Completion of development was quantitated by counting spores. All strains exhibited a typical, sharp dependence on O₂-level to culminate (Fig. 2C). The level required to form 50% of the maximum number of spores (defined as the number formed in 21% O₂) was 7% O₂ for Ax2 compared to 12% for Ax3 (Fig. 2D).

For additional evidence that O₂-dependence is affected by Skp1, the Skp1B (pabB) gene of Ax2 was disrupted by homologous recombination. The Skp1B− strain formed fruiting bodies with extended tenuous stalks (Fig. 2B), and exhibited an even lower requirement for O₂ with a 50% value of only 3% O₂ (Figs. 2C, D). Similar results were obtained for an independent Skp1B− strain (data not shown). Western blot analysis revealed that the steady-state level of total Skp1 was reduced to about 50% in the Skp1B− strain relative to the parental strain Ax2, whereas the level was similar between strains Ax2 and Ax3 (Fig. 2E). Therefore other genetic differences may explain the distinct O₂-requirements for culmination of Ax2 and Ax3. Efforts to disrupt Skp1A (apaA) using a similar approach were unsuccessful. Thus, at a given O₂ level (e.g., 9%), the Skp1B− strain exhibited increased culmination whereas Ax3 exhibited less. As summarized in Table 1 (lines 1, 3 and 5), this suggests that higher Skp1 activity inhibits culmination, whereas higher activity of the hydroxylation/glycosylation pathway, which modifies Skp1, promotes culmination (line 8, from West et al., 2007).

To investigate whether the effect of Skp1 was influenced by its hydroxylation/glycosylation, Pro143 was changed to either Ala or Glu in the chromosomal locus of Skp1A (apaA) of strain Ax2. These substitutions occur naturally in Caenorhabditis elegans SKR2 and human Skp1, respectively. Attempts to similarly modify the Skp1B gene, or the Skp1A gene in the Skp1B− strain, were unsuccessful. The gene replacements were confirmed by PCR, and Western blotting showed a doublet consistent with an equimolar mixture of glycosylated and unmodified Skp1 (data not shown), confirming that both Skp1s are normally expressed (West et al., 1997). The Skp1A3(P143A) and (P143E) strains each exhibited modest but reproducibly higher O₂-requirements for 50% culmination, intermediate between the parental Ax2 strain and Ax3 (Figs. 2C, D). In addition, the Pro143 mutant strains were delayed by several hours in the time to convert from loose to tight aggregates, whereas the time required for the other developmental transitions was not affected (Fig. 2F). The delay of tight aggregate formation is a novel phenotype, but differs from the selective delay in conversion of tipped aggregates to slugs that occurs in hydropia (see Discussion). As previously described for P4H1− strains (West et al., 2007), the above strains exhibited a higher O₂-requirement when developed under darkness compared to overhead light (data not shown), indicating that altered Skp1 levels interact with other signaling pathways as for hydroxylation/glycosylation. The inhibition of culmination seen whether Ala or Glu replaced Pro correlated with inhibition observed in P4H1− cells, that also do not modify Skp1 (West et al., 2007).

**Overexpression of epitope-tagged Skp1s**

O₂-dependent development was then investigated in strains modified by stable transfection with additional copies of Skp1A or Skp1B genes. The previously described strain HW302 (Sassi et al., 2001), in which transgenic Skp1B-myc (C-terminally tagged) is expressed at a level similar to that of endogenous Skp1 in Ax3 (Supplementary Fig. 1), from a tandem array of chromosomally integrated transgenes under control of the semi-constitutive discoidin 1γ (dscC) promoter, exhibited normal dependence on O₂ for culmination (Fig. 3A). In contrast, expression of the same protein under control of the developmental promoter cotb, which directs expression in prespore cells, resulted in an increase in the O₂-requirement to ~18%, approximately the level required for culmination of phyA(P4H1−) cells. The cotb::Skp1B-myc strain expressed 4–5-fold more Skp1B-myc than endogenous Skp1 in slugs, and Skp1B-myc was not detected in vegetative cells confirming promoter specificity (Supplementary Fig. 1). The results are consistent with the above analysis that increased activity of Skp1 increases the level of O₂ required for culmination, and the comparison with HW302 cells suggests that the level and/or timing of expression is important for the effect. Since, at a given level of O₂ (e.g., 12–18%), elevated Skp1 leads to reduced culmination, and Skp1 contributes to the E3SCUb-ligase, Skp1 may normally promote the degradation of a hypothetical activator of culmination.

Previous studies showed that whereas Skp1B-myc expressed under control of the dscC-promoter was predominantly modified by the pentasaccharide (Sassi et al., 2001), Skp1B-myc expressed under control of the cotB-promoter did not appear to be modified based on
gel shift analysis. To address the hydroxylation status of P143, Skp1B-myc was purified essentially to homogeneity from cotB::Skp1B-myc slugs (Supplementary Fig. 2A). MALDI-TOF-MS analysis of the full-length protein was consistent with absence of glycosylation (panel B), and MALDI-TOF-MS of HPLC-separated, endo Lys-C generated peptides detected only the unmodified peptide containing P143 (panels C, D), confirming its unmodified status. Previous Western blot analyses showed that P4H1 is present in both prespore and prestalk cells (West et al., 2007), and activity assays performed on soluble slug extracts revealed P4H1 activity (data not shown) at 33% of the specific activity of that of stationary stage cell extracts (van der Wel et al., 2005). Therefore, P4H1 is present in the slug, but appears to be rate-limiting for the hydroxylation of nascent Skp1B-myc. Thus, the increased O2-requirement resulting from overexpression of Skp1 under control of the cotB-promoter, compared to the dscC-promoter, may depend on less hydroxylation/glycosylation.

A second approach to testing the role of hydroxylation was to examine the effect of overexpressing the Skp1B3(P143A)-myc mutant. When expressed at a level similar to that of normal Skp1B-myc (Supplementary Fig. 1C), Skp1B3-myc exerted a minimal effect on the O2-requirement (Fig. 3B). However, a clone (cotB::Skp1B3.6-myc) that expressed a very high level (Supplementary Fig. 1B) raised the O2-requirement to a threshold that did not, however exceed that of Skp1B-myc. Although its inability to be modified appears to affect the activity of Skp1B3-myc, the possibility that the mutation reduces activity independent of the effect on modification cannot be excluded.

To investigate whether failure to hydroxylate prespore-expressed Skp1B-myc was due to the C-terminal myc-tag or which isoform was expressed, Skp1A with an N-terminal FLAG-tag was expressed in Ax3, as done in yeast studies (e.g., Seol et al., 2001). Unlike the C-terminally-tagged protein, the majority of FLAG-Skp1A was poorly modified even in stationary stage cells when expressed behind the dscC-promoter, based on gel shift analysis (Supplementary Fig. 3A, lanes 4–6). FLAG-Skp1A was also recognized by mAb 4H2 (data not shown), generated by immunization of a mouse with a 13-mer synthetic peptide corresponding to the sequence surrounding Pro143 (Wang et al., 2009), that specifically recognizes unmodified or hydroxylated Skp1 but not glycosylated Skp1 (data not shown), confirming that it is not glycosylated. MALDI-TOF-MS analysis of tryptic peptides of FLAG-Skp1A purified from stationary stage cells showed that the great majority was unhydroxylated (Supplementary Fig. 3B). FLAG-Skp1A was also poorly modified when expressed in slug cells under either a prestalk (ecmA, lanes 7–9) or prespore (cotB, lanes 10–12) promoter, as for Skp1B-myc. Expression of FLAG-Skp1A under control of either promoter had an effect similar to that of cotB::Skp1B-myc in elevating the O2-requirement for culmination (Fig. 3C). In contrast, dscC::FLAG-Skp1A had no effect on the O2-requirement,

Fig. 2. Genetic modifications of chromosomal Skp1 loci affect O2-dependence of culmination. (A) Skp1 isoforms examined in this study. (B) Morphological development of Skp1B− and normal parental strain Ax2 cells on filters after 43 h at 21% or 2.5% O2. (C) Analysis of culmination of Skp1-mutant and normal strains at different O2-levels, based on spore counting after 2 days. The percentage relative to the number formed at 21% O2 (ambient), which was similar (±15%) for all strains, is plotted in this representative trial. (D) The O2 levels required for formation of 50% of the spores produced at 21% O2 averaged from ≥3 independent trials are plotted. Bars represents the range of variation in different trials. (E) Skp1 expression levels relative to actin, based on Western blot analyses of stationary and slug stage cells at 21% O2. The result shown is representative of two independent experiments. (F) Developmental timing at 21% O2. FB = fruiting body. Note: the ‘3’ suffix refers to Pro143 mutants (open symbols used); clonal strains are distinguished as ‘x’ as needed; and genetic backgrounds are indicated after ‘/’. Ax2-derived strains are in shades of red or black; Ax3-derived strains in shades of blue, green (indicating slug overexpression), or orange (semi-constitutive overexpression); P4H1-null strains are denoted by dotted lines whereas P4H1+ strains have thick lines; gatA-null strains denoted by dashed lines.
Table 1
Summary of effects.

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<th>Strain type</th>
<th>Skp1 content</th>
<th>Modification</th>
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- A2x-derived strains
- A3-derived strains
- A3-derived overexpression strains

<table>
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<tr>
<th>Strain type</th>
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<td>phyA+</td>
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- Ax2 and Ax3 strains differ by a 100 kb genome duplication in Ax3 that includes Skp1B.
- Activity of Skp1 overexpression strains as described in the table.
- Data from West et al., 2007.
- Data from Wang et al., 2009.
- Exception as indicated, similar results at either low or high overexpression level of either Skp1A or Skp1B, under control of either prestalk (ecmA) or prespore (cotB) promoters.

Despite the similar level of FLAG-Skp1A that accumulated under the different promoters (Supplementary Figs. 1B, C), the inactivity of dscC::FLAG-Skp1A, which unlike dscC::Skp1B-myc was not glycosylated, suggests that preformed Skp1 is not inactive owing to its average glycosylation status, but inaccessibility to O2-signaling components. The inhibitory activity of tagged Skp1 expressed in spores (summarized in Fig. 3D) correlates with timing of expression components. The inhibitory activity of tagged Skp1 expressed in spores (Figs. 4B, C), even at O2 levels up to 100% (not shown). The few spores produced did depend on O2 in the same way as culmination. As represented in Fig. 4D, the culmination of these and all other (not shown) Skp1 overexpression strains exhibited an elevated O2−requirement, as for tagged Skp1s. In addition, the overexpression strains were delayed in fruiting body formation at 21% O2, as a result of delayed tight aggregate formation (Fig. 4E) as observed also for the Skp1A3(P143A/E) mutants above (Fig. 2F). In comparison, strains expressing tagged Skp1s formed spores at the normal time but tended to be delayed in culmination at 21% O2 (data not shown), as observed for pha−cells (Fig. 4E). Skp1A and Skp1B appeared equally inhibitory, with no correlation observed between the degree of Skp1 overexpression (133–700%; Fig. 5A) and the O2−requirement. Furthermore, similar inhibition was observed whether Skp1 was overexpressed under the prestalk or prespore promoter. Thus overexpression of native Skp1 rendered an effect on culmination very similar to that of the tagged Skp1s (compares lines 9–11, Table 1), confirming the trend that higher Skp1 levels are more inhibitory toward culmination, i.e., elevated O2−are required to culminate. Overexpressed native Skp1 exerted, in addition, two novel functions: inhibition of tight aggregate formation and sporulation.

Glycosylation analysis revealed that, in contrast to the tagged Skp1s, overexpressed native Skp1 exhibited partial glycosylation at 21% O2. Since expressed untagged Skp1 comigrates with endogenous Skp1, this was inferred from increased levels of the primary glycosylated Skp1 band based on densitometric analysis of the ecmA::Skp1 and cotB::Skp1 strains (Figs. 5B, C). Hydroxylation/glycosylation correlates with the ability of overexpressed untagged Skp1 to delay slug formation and inhibit sporulation. In addition, most strains also accumulated unmodified Skp1 (at 21% O2), based on the presence of a more rapidly migrating species in Western blots that was recognized by mAb 4H2. Accumulation of unmodified Skp1 correlates with its ability to inhibit culmination as for the tagged Skp1s. In contrast, overexpression of mutant Skp1A3(P143A) in the Ax3 background caused either no change or a slight decrease in O2−
requirement, and did not delay culmination or inhibit sporulation (Fig. 4C and data not shown; line 12 in Table 1). The minimal effect was potentially due to low activity of the mutant protein as suggested above for Skp1B-myc.

**Genetic interactions between Skp1 and its modification enzymes**

The significance of Skp1 modification was investigated further by testing for genetic interactions between Skp1 level and modification pathway mutants. First, the native Skp1A and Skp1B constructs were expressed in phyA^- and agtA^- backgrounds (see Fig. 1A for pathway genes). Timing of tight aggregate and slug formation was normal, and culmination was delayed slightly as for phyA^- and agtA^- strains (Fig. 6A). In addition, sporulation was normal (example in Fig. 4B, bottom panel). Therefore the inhibitory effects of Skp1 overexpression was contingent upon activity of the hydroxylation/glycosylation pathway, as suggested by the comparison of untagged and tagged Skp1 strains.

In these strains, the O2-level required for culmination approached the O2-requirement of the parental phyA^- and agtA^- strains (Supplementary Fig. 4A, Fig. 6B), which themselves differed by 2–3% O2 (summarized in lines 15–17 and 6–7, Table 1). The observation that O2-requirements were not additive was consistent with their functioning in the same pathway, with maximal signaling achieved either by the absence of inhibitory modification or overexpression of Skp1 to an extent that exceeds the capacity of inhibitory modification. Culmination at higher O2 implies the existence of an unknown bypass pathway that can override Skp1 signaling (West et al., 2010).

To ask if under-modification was important for the inhibitory activity of overexpressed Skp1, strains that overexpressed both P4H1 and Skp1 were created by cotransformation with Skp1B and P4H1 expression plasmids of the same promoter type. Western blotting with anti-P4H1 and mAb 4E1 confirmed that both proteins were overexpressed (Supplementary Fig. 4B). Interestingly, the majority of excess Skp1 accumulated at the position of unmodified Skp1 and reacted with mAb 4H2, consistent with their being unglycosylated. Morphological analysis showed the absence of the delayed tight aggregate formation (Fig. 6A), short fruiting body stature, and reduced sporulation typical of the Skp1 overexpression strains (data not shown). In addition, the O2-requirement for culmination was reduced to levels typical of P4H1-overexpression.
in Ax3 cells lacking overexpressed Skp1 (Fig. 6C, Supplementary Fig. 4B). The epistatic relationship of P4H1 toward Skp1 was consistent with its exerting a dominant inhibitory effect on Skp1 activity, with the biochemical data suggesting that the effect of hydroxylation is mediated by a small subpopulation of Skp1.

To test for an interaction when Skp1 is expressed at subnormal levels, a double Skp1B−/agtA− mutant was created. Independent clones sporulated normally, as expected, and exhibited an O2-threshold for culmination intermediate between that of the single mutants (Fig. 6D), similar to that of the genomic Skp1A3(P143A) mutation (Fig. 2C) in which one of the Skp1s was not glycosylated (compare lines 2 and 3, Table 1). The absence of an epistatic effect of either gene over the other is consistent with opposing effects of Skp1 level and its modification to tune the activity of Skp1 when at near physiological levels in the cell. Repeated attempts to create a phyA−/Skp1B− double mutant were unsuccessful.

Skp1 expression patterns

No differences between Skp1A and Skp1B were observed in the overexpression studies, suggesting that their activities are similar during development. Consistent with this, both proteins are expressed in stationary stage cells (West et al., 1997) and slugs (see above), and RT-PCR studies showed that they are similarly and constitutively co-expressed throughout development (Sassi et al., 2001). However, because the chromosomal loci encoding Skp1A and Skp1B (fpaA and fpaB) appeared to differ in their susceptibility to genetic modification (see above), which might reflect non-redundant activities, their expression patterns in the slug was analyzed. Promoter-RFP and -labile GFP constructs were created by fusing the entire upstream intergenic region from Skp1A (fpaA) and Skp1B (fpaB), excepting a distal A/T-only region for fpaA, to RFP or an unstable form of GFP, and transfected as above for overexpressing Skp1. Clones screened for modest RFP or GFP fluorescence were homogeneously fluorescent along

![Fig. 4. Functional analysis of strains overexpressing native Skp1. (A) Strains overexpressing Skp1A under control of either the ecmA- or cotB-promoter, and the parental strain Ax3, were developed for 43 h at ambient (21%) O2 on filters and photographed. (B) Representative sori from Ax3 or strains expressing Skp1B under the ecmA-promoter in Ax3, or Skp1A under the cotB-promoter in phyA− cells, were squashed under cover slips in the presence of Calcofluor White ST, which labels the cell walls of spores and stalk cells. Panels to the right are at higher magnification to show individual ellipsoid spores. (C) Spore numbers produced by these and representative other strains, as a function of O2-level, are shown. (D) Similar to (C) except that spore numbers are plotted relative to the number produced at 21% O2, to reveal dependence of formation of the spores that did differentiate on O2, which correlated with culmination visualized morphologically. (E) Time dependence of development at 21% O2, as in Fig. 2F. Data are representative of all overexpressing strains (not shown). See Fig. 2 legend for line coding information.]


the length of the slug (Supplementary Fig. 5). Thus no evidence was obtained for differential expression of Skp1A and Skp1B in prestalk and prespore cells of the slug, suggesting that the Skp1 isoforms do not serve prestalk and prespore cell-specific functions.

Stability of Skp1 protein

Since changes in Skp1 levels affect O2-dependence of culmination, we asked if the effects of mutating genes in the hydroxylation/glycosylation pathway affected Skp1 levels. As shown in Fig. 7A, similar levels of Skp1 were detected in slugs from each of the available pathway mutants, indicating that the modification state of Skp1 does not affect its steady-state expression level. In addition, the stability of Skp1 was investigated by incubating cells for varying times in 400 μg/ml cycloheximide, which inhibited incorporation of 35S-Met by over 95% (Supplementary Fig. 6). Skp1 was stable in normal cells with only a slight diminution observed after 80 min of treatment, as expected (Zhou and Howley, 1998), and no differences were observed in mutant cells lacking P4H1 or AgtA (Fig. 7B). Similar results were obtained for stationary stage and aggregation stage cells. The results suggest that overall Skp1 levels are not regulated by the modification pathway.

Discussion

Skp1

Genetic disruption of one of the two loci for Skp1, or stable introduction of multiple transgenic copies of Skp1, yielded strains that expressed over a 10-fold range of steady-state Skp1 levels in slugs of the normal parental strain Ax3. Reduced Skp1 level lowered the O2-dependent culmination of Ax3. Therefore, the Skp1 isoforms do not serve prestalk and prespore cell-specific functions.
requirement for 50% culmination from ~10% to ~3% (Fig. 2), close to the minimal amount required for unicellular growth (West et al., 2007). In comparison, overexpression to ~133% or beyond, under the control of developmental promoters, resulted in an increase to about ~18% (Figs. 3, 4). Similar effects were observed at the highest levels of overexpression, suggesting absence of dominant negative activity. Skp1 was similarly active as its A or B isoforms, or when epitope-tagged, or when expressed under control of a prestalk- or prespore- specific promoter (summarized in Table 1).

Since lower levels of Skp1 promoted and higher levels inhibited culmination at any given level of O2, and Skp1 is associated with polyubiquitination via E3 SCFUb-ligases, it is proposed that Skp1 contributes to the polyubiquitination and proteasomal degradation of a hypothetical activator of culmination. Even at the maximal level of Skp1 activity achieved by this approach, inhibition of development was suppressed by high levels of O2 (~18%). The existence of an override pathway was also inferred from analysis of P4H1 mutants (West et al., 2007). A previously described substrate of the Dictyostelium E3(SCFbxA)Ub-ligase (Mohanty et al., 2001), the cAMP phosphodiesterase RegA, is not a good candidate for culmination regulation by Skp1 since this enzyme inhibits development.

An exception occurred for Skp1 overexpressed under control of the promoter dscC, which resulted in considerable carryover of FLAG-Skp1A or Skp1B-myc into the slug without an effect on the O2-requirement (Fig. 3). This suggests that the modification status or rate of synthesis of Skp1 (dscC is less active in the slug) may be important parameters in addition to the total level of Skp1 in mediating these effects.

The strains overexpressing native (untagged) Skp1 exhibited two additional, novel phenotypes: delayed formation of tipped aggregates for culmination, these defects occurred whether a low or high level of Skp1A or B was overexpressed in prestalk or prespore-specific promoter (summarized in Table 1).

Fig. 6. Genetic interactions. (A) Developmental timing of strains overexpressing native Skp1A or Skp1B, in normal, phyA− or agtA− backgrounds, or in combination with P4H1 overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C) O2-dependence of culmination of Skp1oe/P4H1oe double overexpression, were compared with Ax3, phyA− or agtA− cells, at ambient (21%) O2 as in Fig. 4E. (B) The O2-level required for 50% culmination, based on spore numbers, is plotted for the single gene overexpression strains as in Fig. 2D. Representative raw data are shown in Supplementary Fig. 4A. (C)
Relation of Skp1 activity to hydroxylation/glycosylation pathway signaling in culmination

The range of O₂-requirements exhibited by the Skp1 expression level panel is remarkably similar but inverse to that observed in a P4H1 expression level panel (West et al., 2007), in which phyA⁻/P4H1 cells required ~18% O₂, and P4H1-overexpression strains required 5% O₂ to culminate. Skp1 overexpressed in the slug is poorly hydroxylated, mimicking the phyA⁻ condition. Together, these data suggest that the effects of altered Skp1 levels on O₂-dependent culmination are reflective of normal effects of O₂-regulated modification of Skp1 by P4H1 (see Fig. 7C). Since higher P4H1 levels promote and higher Skp1 levels inhibit culmination at any given O₂ level, we propose that hydroxylation reduces the activity of Skp1. Previous studies showed that Hyp becomes modified by GlcNAc, and subsequently by the addition of up to 4 additional sugars. Reverse genetic analyses showed that the glycosyltransferases that mediate these additions modulate O₂-signaling (Wang et al., 2009), and we propose that the glycosyl-modifications also directly modulate the effect of proline hydroxylation on Skp1 activity.

A direct interaction between Skp1 and the hydroxylation/glycosylation pathway is supported by other mutants and mutant combinations. Substitution of Pro143 with Ala or Glu in the chromosomal locus of Skp1A resulted in a slightly higher O₂-requirement (Fig. 2C), consistent with its inability to be modified.

The mutant strains were monitored for expression levels of Skp1 and evidence for expected effects on its modification status. Generally, endogenous Skp1 is ~90% hydroxylated and glycosylated in the steady state during growth and development (Fig. 1; Sassi et al., 2001).
However, as suggested by that study and shown in Supplementary Fig. 2, prolyl 4-hydroxylation is rate-limiting, even at ambient (21%) O₂-levels, for modification of Skp1B-myc overexpressed in prespore cells. In contrast, Skp1B-myc that is overexpressed during vegetative growth is fully modified (Sassi et al., 2001), though the rates of synthesis have not been directly compared. Similar results were obtained for FLAG-Skp1A expressed in prestalk or prespore cells (Supplementary Fig. 3), except that FLAG-Skp1A is poorly modified even in vegetative and stationary cells. In contrast, while prolyl 4-hydroxylation was also rate-limiting for overexpressed native Skp1A or Skp1B, increased glycosylated Skp1 was detectable (Figs. 5B, C). Surprisingly, co-overexpression of P4H1, which corrected the phenotype, did not appear to increase the fraction of modified Skp1 (Supplementary Fig. 4B). This indicates that a subpopulation of Skp1, too low in abundance to be detected, mediates the effects of P4H1 overexpression, and suggests that accessibility of P4H1 to nascent Skp1 may be important. Alternatively, P4H1 may have other functions in this overexpression setting by signaling via another substrate.

These possibilities, which are not mutually exclusive, are depicted in Fig. 7C. In an attempt to identify an alternative target, soluble extracts from normal and phyA− cells, and stationary cells, were incubated in the presence of recombinant P4H1, Gnt1 and UDP-[³H] GlcNac. No targets other than Skp1 were detected (Fig. 1), though substrates not subsequently modified by Gnt1 GlcNACt would have escaped detection. However, since AgtA, which solely modifies Skp1 and depends on Gnt1 for its biochemical function, modulates the action of P4H1 in O₂-regulated culmination (Wang et al., 2009), Skp1 clearly contributes to P4H1 signaling. Finally, non-enzymatic functions of P4H1 are excluded based on analysis of inactivating active site point mutations (West et al., 2007).

Although altered levels of Skp1 affect the O₂--requirement for culmination, there was no evidence that its modifications affect its level or stability in cells, based on Western blot analysis of the mutant strains treated with cycloheximide (Fig. 7). Thus the role of hydroxylation of Skp1 appears to differ from that of protein HIFα, which is destabilized by becoming a target of E3VHLUb-ligase (Kaelin and Ratcliffe, 2008). However, an effect of hydroxylation in the absence of subsequent glycosylation has not yet been directly analyzed.

Relation of Skp1 activity to hydroxylation/glycosylation pathway activity in aggregation and sporulation

The inhibitory effects of Skp1 overexpression on tipped aggregate formation and sporulation were dependent on its modification. Thus, tipped aggregate formation and sporulation were normal when Skp1 was overexpressed in phyA− or agtA− strains, or when Skp1A3 (P143A) was overexpressed in the normal strain Ax3 (Table 1). FLAG-Skp1A and Skp1B-myc were also not inhibitory. Because tagged Skp1s were similarly overexpressed in slugs, their inactivity was possibly attributable to their lower potential for modification (Supplementary Figs. 2, 3) compared to native Skp1 (Figs. 5B, C). A similar inhibition of tipped aggregate formation occurred when Skp1A of strain Ax2 was mutated to Skp1A3 (P143A/A) (Fig. 2F). A shared trait between these mutant strains is the simultaneous forced expression of both modifiable and unmodified Skp1, suggesting that the ratio is more influential than the absolute level of Skp1. As discussed above, these effects do not correlate with O₂-regulated steps in development, but may portend novel regulatory mechanisms involving hydroxylation-dependent glycosylation or other modes of P4H1 regulation.

These effects of Skp1 on aggregation and sporulation are promoted by the modification pathway, in contrast to the inhibition effect inferred from the analysis of culmination, as summarized in Table 1. The reason for this difference is unclear but may be related to the mechanism of Skp1 action, which is thought to involve association with F-box proteins (Willems et al., 2004). Potentially, each of the effects (slug formation, culmination, sporulation) involves interaction with distinct F-box proteins, potentially synthesized at the specific time that regulation occurs. In addition, F-box proteins, which number around 50 in D. discoideum based on genomic sequence searches (unpublished data), fall into two classes based on the ability to contact cullin in SCF complexes and sensitivity to regulation by Cand1 and NEDDylation (Schmidt et al., 2009). Modification of Pro143 may have differential effects on interactions with individual F-box proteins or classes of F-box proteins or, alternatively, non-SCF functions of Skp1 that have been proposed (Seol et al., 2001; Hermand, 2006).

Developmental significance

The effects of Skp1 overexpression in either of the major cell types of the slug, prestalk (ecmA) and prespore (cotB) cells, were indistinguishable. A similar result was observed in a previous study of P4H1 expression (West et al., 2007). It was noted that in strain mixture experiments, cells expressing higher levels of P4H1 tended, regardless of the promoter used to direct its expression, to migrate to the tip, where prestalk cells direct culmination and possibly also slug formation and sporulation. If Skp1 mediates P4H1-dependent signaling as hypothesized (Fig. 7C), it is predicted that Skp1-underexpressing cells will undergo a similar tip transdifferentiation to regulate development, which would explain the similar activity when initially expressed in either cell type. Although the biological significance has yet to be studied, this phenomenon suggests that development is promoted by cells experiencing the highest O₂-levels, which would be expected to occur at the slug surface at points of minimal radius of curvature such as the tip, but might occur elsewhere. Since the O₂-dependence of development can be controlled by the properties of ~10% of the cells in the strain mixture studies (West et al., 2007), developmental regulation may be ultrasensitive to O₂. This may contribute to the sharp O₂-thresholds within experiments and variations of the precise threshold in independent trials. In addition, ultrasensitivity may help explain why, in instances such as the double P4H1OE/Skp1OE strain, the modification status of the bulk pool of Skp1 does not correspond to expectation based on protein expression alone. As discussed above, Skp1--signaling may be dependent on an efficiently modified subpopulation that is nascent and/or co-compartmentalized with P4H1 and the other enzymes.

As described for P4H1, Skp1 levels appear to be constant throughout the life cycle (Sassi et al., 2001; West et al., 2007), and results here suggest that Skp1 is expressed at similar levels throughout the slug (Supplementary Fig. 5). As also shown here (Fig. 7), Skp1 is a relatively stable protein, though ongoing synthesis replaces at least half of the steady-state pool during development according to prolyl hydroxylase inhibitor studies (Sassi et al., 2001).

Based on our unpublished data that Skp1 is not hydroxylated in vitro after forming a stable complex with FbxA, we reason that cells may continuously monitor O₂, Krebs cycle intermediates (Kivinen et al., 2007), redox sensors and other factors such as sugar-associated signals, via modification of nascently synthesized Skp1 prior to association with coordinately synthesized F-box proteins. With an estimated 50 F-box proteins, the activity of multiple E3³Ub-ligases targeting a potentially larger set of regulatory proteins may be selectively influenced by this ‘timer’ mechanism, to ultimately couple environmental signals reflective of soil microenvironments to appropriate developmental transitions (West et al., 2010). Pulse-chase labeling has the potential to investigate the properties of nascent Skp1 relative to background bulk Skp1 which may not be relevant to signaling.

Forward genetic screens previously implicated cullins and F-box proteins, partners with Skp1 in SCF complexes, in the regulation of multiple developmental transitions in Dictyostelium (Ennis et al., 2000; Nelson et al., 2000; Mohanty et al., 2001; Wang and Kuspa,
Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2010.10.013.

References


