Skp1 Prolyl 4-Hydroxylase of Dictyostelium Mediates Glycosylation-independent and -dependent Responses to O2 without Affecting Skp1 Stability*† §

Dongmei Zhang†‡, Hanke van der Wel†, Jennifer M. Johnson*, and Christopher M. West†**‡

From the †Department of Biochemistry and Molecular Biology and *Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 and the ‡Key Laboratory of Biopesticide and Chemical Biology, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou 350002, China

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1 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, 975 NE 10th St., BRC 417, OUHSC, Oklahoma City, OK 73104. Tel.: 405-271-4147; Fax: 405-271-3910; Email: Cwest2@ouhsc.edu.

Central to autophagy and cellular adaptation in response to environmental stress is the regulation of the von Hippel-Lindau (VHL) tumor suppressor protein by the prolyl 4-hydroxylases (PHDs). However, PHD activity is also dispensable for O2 homeostasis in many aerobic organisms. Dictyostelium-expresses an HIFα-type prolyl 4-hydroxylase (P4H1) that modifies a different protein, Skp1, an adaptor of the SCF E3VHLubiquitin (Ub) ligase and degradation in the 26 S proteasome. Previously thought to be restricted to animals, a homolog (P4H1) of HIFα-type PHDs is expressed in the social amoeba Dictyostelium where it also exhibits characteristics of an O2 sensor for development. Dictyostelium lacks HIFα, and P4H1 modifies a different protein, Skp1, an adaptor of the SCF class of E3-Ub ligases related to the E3VHLUb ligase that targets animal HIFα. Normally, the HO-Skp1 product of the P4H1 reaction is capped by a GlcNac sugar that can be subsequently extended to a pentasaccharide by novel glycosyltransferases. To analyze the role of glycosylation, the Skp1 GlcNac-transferase locus gnt1 was modified with a missense mutation to block catalysis or a stop codon to truncate the protein. Despite the accumulation of the hydroxylated form of Skp1, Skp1 was not destabilized based on metabolic labeling. However, hydroxylation alone allowed for partial correction of the high O2 requirement of P4H1-null cells, therefore revealing both glycosylation-independent and glycosylation-dependent roles for hydroxylation. Genetic complementation of the latter function required an enzymatically active form of Gnt1. Because the effect of the gnt1 deficiency depended on P4H1, and Skp1 was the only protein labeled when the GlcNac-transferase was restored to mutant extracts, Skp1 apparently mediates the cellular functions of both P4H1 and Gnt1. Although Skp1 stability itself is not affected by hydroxylation, its modification may affect the stability of targets of Skp1-dependent Ub ligases.

Cells of aerobic organisms sense O2 and use the information to modulate physiological and developmental processes. Many short and long term mechanisms of O2 sensing have evolved (1–3). Important for humans is the family of prolyl 4-hydroxylases (PHD 1–3)2 that modify the O2-dependent degradation domain of hypoxia-inducible factor α (HIFα), a transcriptional factor subunit that positively controls the expression of many hypoxia-induced genes (3). O2 serves as a substrate for PHD2 to hydroxylate HIFα, which leads to its recognition by the von Hippel-Lindau subunit of the E3VHLUb ligase and subsequent polyubiquitination. Poliubiquitinated HIFα is then degraded by the 26 S proteasome. HIFα accumulates in hypoxia because of low activity of PHD2.

The social soil amoeba Dictyostelium is an important model organism for cell signaling and motility. Dictyostelium, which normally proliferates as solitary amoebae in the soil, undergoes starvation-induced development resulting in formation of a multicellular slug that migrates to the soil surface and culminates into a fruiting body (4–7). Culmination depends on a superphysiological level of O2 (8), suggesting that O2 is an environmental factor that the slug uses to know that it is above ground, in a location suitable for culmination and subsequent dispersal of spores (9).

Dictyostelium expresses an HIFα-like prolyl 4-hydroxylase (P4H1) but lacks an HIFα-like gene based on sequence searches (10). P4H1 was initially discovered as the enzyme required for a novel form of cytoplasmic glycosylation of Skp1 (11). Subsequent to its 4(trans)-hydroxylation, Pro-143 is modified with a residue of GlcNac by a reaction that can be catalyzed in vitro by Gnt1 (12), an αGlcNAcT purified based on this activity from cytosolic extracts of Dictyostelium (13, 14). Recombinant Gnt1

2 The abbreviations used are: PHD, prolyl 4-hydroxylase domain protein; αGlcNAcT, α-N-acetylgalcosaminyltransferase; HIFα, hypoxia-inducible factor-α; Hyp, (4R,2S)-hydroxyproline; Ub, ubiquitin; SCF, complex containing Skp1, cullin-1, and an FBP; FBP, F-box protein; VBC, complex containing von Hippel-Lindau protein, elongin B, and elongin C; nt, nucleotide(s); Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
exhibits similar activity, but the suspected role of Gnt1 in cells has not been confirmed because of difficulty in disrupting the gnt1 locus. GlcNAc-O-Skp1 is subsequently acted on by four additional glycosyltransferase activities encoded by pgtA and agtA (10). Genetic manipulations have suggested that P4H1 behaves as an O2 sensor to control culmination (15). Initial GlcNAc capping of Hyp-143 is sufficient for restoring a near normal O2 requirement (15, 16), and the addition of intermediate sugars by PgtA acts like a second chance mechanism to reverse the effect of hydroxylation until the terminal sugars are added by AgtA (17). Accumulating evidence suggests the importance of other forms of complex cytoplasmic and nuclear glycosylation in eukaryotes (18–20).

Skp1 is best known as a subunit of the SCF class of E3-Ub ligases (21, 22). E3SCFUb ligases are responsible for the polyubiquitination and degradation of potentially hundreds of proteins in the cell, including regulators of the cell cycle, transcription, and vesicle trafficking. This multifunctionality is due to the existence of >50 F-box proteins in humans and Dictyostelium that may be associated with the SCF complex via the Skp1 adaptor. Reverse genetic studies indicate that Skp1 is also involved in O2 regulation (16). Furthermore, genetic interactions with the hydroxylation/glycosylation pathway, together with evidence that Skp1 is the only cellular substrate for these enzymes, suggest that Skp1 is a primary mediator of P4H1 signaling in the cell.

P4H1 appears to be the Dictyostelium ortholog of human PHD2 based on biochemical and functional criteria (10, 12), except that it modifies Skp1 rather than HIFα. With the successful disruption of the gnt1 locus presented here, the predicted role for Gnt1 in Skp1 glycosylation was confirmed and permitted comparison of the effects of prolyl hydroxylation of Skp1 and HIFα. Surprisingly, hydroxylated Skp1 appeared to be as stable as unmodified Skp1. Nevertheless, hydroxylation partially rescued the high O2 requirement of the P4H1 mutant, and Skp1 was the only substrate of Gnt1 that could be detected in gnt1–null cells, suggesting an alternative primordial mechanism of hydroxylation that nonetheless might still affect the stability of clients of Skp1-dependent Ub ligases.

**EXPERIMENTAL PROCEDURES**

**Growth and Development—Dictyostelium discoideum** cells (see Table 1 for list of strains) were typically grown axenically in HL-5 and cloned by growth on SM-agar plates containing *Klebsiella aerogenes* (23). Dictyostelium purpureum (Dp1) was grown on *K. aerogenes*. Development was monitored after incubation in Calcofluor White ST to render cellulose cell walls fluorescent, which was visualized in the DAPI filter channel, as described (15). Spore formation was quantitated by counting in a hemacytometer.

**Antisera—**Antiserum against DpGnt1 was induced by injection of New Zealand White female rabbits with a highly purified preparation of recombinant DdDpHis6Gnt1 (12) mixed with either Freund’s complete (primary injection) or incomplete adjuvant. Antisera obtained after three booster injections reacted specifically at 1:100 dilution with DpGnt1 but not DdDnt1 in extracts using Western blotting. Similarly, antisera were raised against a synthetic Pro peptide that included Skp1 residues 137–149 (centered at Pro-143) that had been conjugated via its N terminus to keyhole limpet hemocyanin as described (17) and to the corresponding Hyp peptide. Final booster injections were conducted with longer peptides extending to residue 151. Antisera from selected animals exhibited desired Skp1 isoform specificity by Western blotting at titers of 1:5000 (see “Results”).

**SDS-PAGE and Western Blotting—**Samples were diluted in concentrated SDS sample buffer to a final concentration of 20 mM dithiothreitol and applied to an Invitrogen NuPAGE® Novex 4–12% Bis-Tris Tris gel using the MES buffer system. Proteins were transferred to a 0.2-μm pore diameter nitrocellulose membrane using an Invitrogen iBlot® dry blotting system. Blots were probed with antisera diluted in 5% nonfat dry milk prepared in Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4), followed by Alexa Fluor 680-conjugated goat anti-mouse IgG (LI-COR Biosciences) also diluted 1:10,000 in the milk solution, and imaged at 700 nm in a LI-COR Biosciences Odyssey infrared scanner. Apparent M, values were estimated using the Benchmark prestained protein ladder from Invitrogen.

**αGlcNAc-transferase Reactions—**αGlcNACT activity was assayed as described previously (13). Briefly, soluble (S100) extracts were prepared from the slug or growth stage, desalted, and incubated with 2 μM recombinant HO-Skp1 (prehydroxylated *in vitro*) in the presence of UDP-[3H]GlcNAc for 2 h. Incorporation of 3H was measured using the SDS-PAGE assay, and the zero time blank value was subtracted. For screening αGlcNACT acceptor substrates, the reactions were amended with highly purified recombinant DpGnt1, and exogenous Skp1 was not added.

**Construction of gnt1 Replacement Vectors—**Three replacement DNA fragments were designed to disrupt gnt1 by homologous recombination via a double-crossover mechanism. The DNAs were constructed using standard PCR and cloning procedures. At most steps, DNAs were purified by electrophoresis on a 1% (w/v) SeaKem GTG-agarose (BioWhittaker) gel, extracted using a freeze-squeeze method, and EtOH-precipitated. The 710-nt upstream targeting DNA was amplified from pTYB1GnT51 or pTYB1GnT51(H104D) (14) using primers GnD and Gn6 (see Fig. 1A, supplemental Table S1, and supplemental Fig. S1) and cloned into pCR4-TOPO (Invitrogen) and into pCR4-TOPO (Invitrogen) to yield pTOPO+1 and pTOPO+1(H104D). To replace a homopolymeric stretch of poly(N), 310-nt of Gnt1 3′-coding DNA was amplified from *D. purpureum* genomic DNA using GnG and Gn4 (see Fig. 1B), cloned into pCR4-TOPO to yield pTOPO+2. Amplification fidelity was confirmed by direct sequencing. The 710-nt targeting DNAs were excised using

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3 Y. Xu and C. M. West, unpublished data.
Role of Prolyl Hydroxylation in Skp1 Function

Spel and Stul and ligated to corresponding sites of pTOPO+2 to yield pTOPO+1+2 and pCR4-TOPO+1(H104D)+2. A 1304-nt downstream targeting DNA was amplified from D. discoideum genomic DNA using primers GnH and Gn2 and cloned into pcRA-TOPO from the pTOPO cloned in reverse orientation into the BglII site of pTOPO resistance cassette from pHygT(plus)/pG7 (a gift from M. Nelson, genomic DNA using primers GnH and Gn2 and cloned in reverse orientation into the BglII site of pTOPO, yielding pTOPO+1+2). The (710+310)-DNA was excised from the pTOPO+1+2 plasmids with Spel and BamHI and cloned into similarly digested pTOPO+4(4xhygr)+5 to yield pTOPO+1+1(H104D)+2+4(4xhygr)+5 (see Fig. 1D). The Y253stop mutation (see Fig. 1D) was introduced into the H104D DNA by site-directed mutagenesis using oligonucleotides GnF and Gn7.

The replacement DNAs were excised with EcoRI and Spel, briefly trimmed with Bal31 exonuclease, and electroporated into D. discoideum strain Ax3 or HW288 (phyA−, where phyA is the Dictyostelium gene encoding P4H1) as described previously (15). Transformants were initially selected at 35 μg/ml hygromycin and then cloned on bacteria plates (11). Oligonucleotides used for PCR trials to confirm the expected recombination events are shown in supplemental Table S1 and Fig. 1A. Strains are listed in Table 1.

Construction of gnt1 Overexpression Strains—Native DpGnt1 cDNA (1035 nt) was PCR-amplified from pDEST527-DdDpGnt1a (chimera a) (12) using GnB and Gn3 (see Fig. 1E, supplemental Table S1, and supplemental Fig. S1) and cloned into pCR4-TOPO to yield pTOPO-DpGnt1. DpGnt1 cDNA was excised using KpnI and BamHI and ligated into similarly digested pVSE (15) to yield pVSE-DpGnt1 (see Fig. 1E). Site-directed mutagenesis using primers GnE and Gn8 was employed to generate pVSE-DpGnt1(D102A) (see Fig. 1F). These vectors were designed to overexpress native or enzymatically inactive DpGnt1 under control of the ecmA promoter in prestalk cells. After electroporation of plasmids into D. discoideum, G418-resistant cells were initially selected at 20 μg/ml G418 and cloned on bacteria plates.

Skp1 Metabolic Labeling and Immunoprecipitation—To estimate the rate of total protein synthesis, logistically growing cells were centrifuged and resuspended at 107/ml in 1 ml of HL-5 medium containing 71 μCi/ml [35S]Met/Cys (carrier-free Trans-label; Amersham Biosciences), 0.1 mM Met, and 0.1 mM Cys and shaken at 22 °C for 40 min. Cells were recovered by centrifugation at 1000 × g for 1 min, resuspended in cold PDF buffer, centrifuged again, and frozen at −80 °C. Incorporation of 35S into protein was determined by trichloroacetic acid precipitation as described (24). To determine incorporation into Skp1 of growing cells, cells were incubated as above in the absence of added unlabeled Met and Cys for 1 h. Incorporation into Skp1 of 15-h developing cells (slugs) was performed by transferring a filter containing the cells into a Petri dish on which had been deposited five 20-μl aliquots of 0.71 mCi/ml [35S]Met/Cys in PDF and incubating for 1 h. For pulse-chase studies, 1-h labeled cells in HL-5 were centrifuged and resuspended in fresh HL-5 containing unlabeled 0.1 mM Met and 0.1 mM Cys. At the indicated times, cells were pelleted and stored at −80 °C, subsequently solubilized in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 8 μM urea, and protease inhibitors (1 mM PMSF, 10 μg/ml apro tinin, and 10 μg/ml leupeptin), and centrifuged at 16,000 × g for 30 min at 4 °C. The supernatant was diluted 10-fold in IP buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and protease inhibitors, precl ered with 10 μg of mouse IgG and 25 μl of a 25% slurry of UltraLink protein G beads (Thermo Scientific), and immunoprecipitated by the sequential addition of 5 μg of mAb 4E1 (1 h on ice) and 25 μl of the UltraLink protein G bead suspension. After incubation for 2 h at 4 °C with gentle mixing, beads were recovered by centrifugation and washed in IP buffer. Pellets were subjected to SDS-PAGE. The washed gel was incubated in 1 M sodium sulfosalicylate for 30 min, dried on Whatman 3MM paper, and autoradiographed using BioMax MR film (Kodak) and a Fisher Biotech L-Plus intensifying screen. The films were scanned and densitometrically analyzed using ImageJ software. A semilogarithmic transformation of the data was fitted by linear regression in Microsoft Excel, based on minimizing the sum of the squares of the error terms.

RESULTS

Disruption of gnt1—Native and recombinant Gnt1 exhibits UDP-GlcNAc:Skp1-Hyp αGlcNAc-transferase activity in vitro. However, efforts to confirm this role in vivo have been thwarted by the inability to disrupt gnt1 using either of two separate linear targeting DNAs expected to homologously recombine via a double-crossover mechanism.4 In the third attempt reported here, the strategy was modified to express an enzymatically inactive protein in case the Gnt1 protein itself is required for viability. In addition, a new 5′-targeting DNA consisting of most of exon 2 of the gnt1 coding sequence and a new 3′-DNA consisting of the downstream intragenic region and the coding sequence of the neighboring gene (Fig. 1C) were selected to guide targeting. A reverse-oriented hygromycin resistance cassette was positioned between with its transcription terminator expected to function for both gnt1 and hygr. In addition, the C-terminal 143 amino acids of Gnt1, which include a non-conserved degenerate 79-amino acid domain highly enriched in Asn residues (polyN in Fig. 1A and supplemental Fig. S1) that interfere with cloning and expression in Escherichia coli (14), were replaced with the corresponding 71-amino acid region of Gnt1 from the related species D. purpureum (25) that lacks the degenerate region (Fig. 1B). The GlcNAcT activity of DpGnt1 was recently shown to be indistinguishable from that of DdGnt1 in vitro (12), and this chimera (referred to as DdDpGnt1.b, or gnt1.0) was active in vivo (see below). The gnt1-coding sequence (within the 5′-DNA) was modified by a point mutation (H104D) expected, based on analysis of recombinant Gnt1 (14), to inactivate the enzyme activity without affecting protein expression. In a second generation strategy (see below), the same disruption DNA was further modified by the introduction of a stop codon at the junction of the Dd and Dp coding regions (codon 253) of the chimera (Fig. 1D) that, based on previous studies (14), resulted in an enzymatically inactive truncated protein when expressed in E. coli.

4 L. Kaplan, Z. A. Wang, H. van der Wel, and C. M. West, unpublished data.
Electroporation of replacement DNA-1 (Fig. 1D) into normal strain Ax3 cells led to the appearance of stable hygromycin-resistant clones in which the gnt1 locus was successfully replaced as expected, based on the size and sequence of PCR products amplified from mutant clone DNA (Fig. 1G, and data not shown). Similar results were obtained using replacement DNA-2. The crossover positions for both disruption DNAs occurred at the 5’/H11032-side of the point mutations, thereby incorporating the mutations into the genomic locus as desired and yielding gnt1.1 and gnt1.3, respectively (Table 1).

To analyze genetic interactions with P4H1, which catalyzes formation of a substrate for Gnt1, the gnt1 replacement DNAs were introduced into the previously described P4H1-null strain HW288 (15). Clones were isolated with the same insertions as above (Fig. 1G) and confirmed to have incorporated the desired point mutations. Replacement DNA-1 was also introduced into the agtA-null strain HW420. Although this DNA integrated in identical fashion based on PCR analysis (Fig. 1G), sequencing revealed the presence of the original H104 codon, indicating that the crossover point for inserting the upstream DNA occurred downstream of H104 position, resulting in expression of the DdDpGnt1.b chimera (gnt1.0) expected to be active. Varied crossover positions also occurred in our previous replacement of the Skp1A gene (16).

To confirm an effect on Gnt1, soluble extracts from vegetative cells were assayed for Gnt1 activity using HO-Skp1 that had been prepared from E. coli transfected with a dual expression plasmid encoding both Skp1 and P4H1 (encoded by phyA).5 Extracts from gnt1.1, gnt1.3, and gnt1.1/phyA− cells exhibited negligible activity (<1%) when compared with parental Ax3 and phyA− cells, as expected (Fig. 2). Extracts of gnt1.0/agtA− cells had near normal Gnt1 activity, consistent with failure to incorporate the H104D mutation because of its downstream crossover point, confirming that the DdDpGnt1.b chimera protein (gnt1.0 allele) was expressed and active.

These replacements of gnt1 were predicted to block modification of Skp1 past the hydroxylation step (see the pathway schematic in Fig. 3A). This was first assessed by analyzing the mobility of Skp1 on SDS-PAGE gels. As shown in Fig. 3B, Western blotting with the pan-specific mAb 4E1 showed that Skp1 from gnt1.1 cells migrated more similarly to that of phyA− and

5 M. O. Sheikh and C. M. West, unpublished data.
**Role of Prolyl Hydroxylation in Skp1 Function**

**TABLE 1**

| Strain | Parental strain | Drug resistance | Genotype | Gnt1 activity* | Ref. |
|--------|----------------|-----------------|----------|----------------|-----|
| Ax3    | NC-4           | None            | *phyA* /gtt1* (normal) | + | 15 |
| HW288  | Ax3            | Blasticidin S   | *phyA*  | + | 39 |
| HW260  | Ax3            | Blasticidin S   | *ggtA* | + | 39 |
| HW261  | Ax3            | Blasticidin S   | *agtA* | + | 32 |
| HW420  | Ax2            | Blasticidin S   | gnt1.1(H104D) | – | This study |
| HW300  | Ax3            | Hygromycin      | gnt1.1(H104D) | – | This study |
| HW301  | Ax3            | Hygromycin      | gnt1.3(H104D/Y253stop) | – | This study |
| HW304  | Ax3            | Hygromycin      | gnt1.3(H104D/Y253stop) | – | This study |
| HW306  | HW500          | G418/hygroycin  | ecmA::DpGnt1/gnt1.1 | (+/++) | This study |
| HW308  | HW501          | G418/hygroycin  | ecmA::DpGnt1/gnt1.3 | (+/++) | This study |
| HW307  | HW500          | G418/hygroycin  | ecmA::DpGnt1(D102A)[gnt1.2]/gnt1.1 | – | This study |
| HW309  | HW501          | G418/hygroycin  | ecmA::DpGnt1(D102A)[gnt1.2]/gnt1.3 | – | This study |
| HW512  | HW288          | Hygromycin      | gnt1.1 phospho* | – | This study |
| HW513  | HW288          | Hygromycin      | gnt1.1 phospho* | – | This study |
| HW515  | HW288          | Hygromycin      | gnt1.3 phospho* | – | This study |
| HW516  | HW288          | Hygromycin      | gnt1.3 phospho* | – | This study |
| HW518  | HW420          | Hygromycin      | gnt1.0/aGtA | + | This study |

*Summarized from Figs. 2 and 3. –, not detected; +, normal activity; (+/+) conditionally high activity in slug only; +/−, slightly less than normal.

**FIGURE 2. Skp1-Hyp GlcNAcT activity in gnt1 mutant strains.** Soluble (5100) extracts were prepared from slugs of normal and mutant strains and desalted, and ~35 μg of protein was incubated with 2 μL recombinant HO-Skp1 in the presence of UDP-[^3H]GlcNAc. Incorporation of ^3H was measured using the SDS-PAGE assay, and the zero time blank value was subtracted. Error bars indicate ± S.E.

The observed m/z of 18,932 indicates the absence of glycosylation. Analysis of peptides generated by endo Lys-C digestion revealed that the most abundant isoform consisted of the Hyp peptide (data not shown). Very minor levels of the fully glycosylated peptide were detected, which might represent contamination from previous purifications on the chromatography columns used or low level processing of Skp1 by residual activity. Because gnt1 is the only Gnt1-like gene in *D. discoideum*, Gnt1 appears to be the sole Skp1:Hyp αGlcNAcT in cells.

**Complementation of gnt1**—To verify that the absence of Gnt1 activity could be attributed to the disruption of gnt1, gnt1.1 and gnt1.3 cells were electroporated with pVE-de- derived expression plasmids, which direct expression of DpGnt1 under control of the prestalk cell-specific promoter *ecmA*. Cells were analyzed for expression of DdDpGnt1 using a new antiserum raised against recombinant DpHis6Gnt1 expressed in and purified from *E. coli*. A reactive band at the expected Mr position for DpGnt1 was detected in slug cells (Fig. 3C), but not vegetative cells (data not shown), as expected according to the strict slug specificity of expression of the *ecmA* promoter. No binding was detected in parental *D. discoideum* cells, indicating species specificity of the antiserum. The expressed protein was enzymatically active as extracts of mutant slugs exhibited about 50-fold increase in Gnt1-like activity relative to normal Ax3 cells (Fig. 2). Finally, the great majority of Skp1 was modified in the gnt1.1 and gnt1.3 slugs expressing DpGnt1, based on loss of the UOK85 epitope and the decreased mobility by SDS-PAGE (Fig. 3C). However, only partial rescue of Skp1 modification was expected as the *ecmA* promoter is active in prestalk cells. The implication that Skp1 of prespore cells, the major cell type of the slug, is also modified suggests possible cell type conversion, which was previously noted when P4H1 was disrupted or overexpressed (15). Glycosylation rescue depended on the enzymatic activity of Gnt1 because overexpression of inactive mutant DpGnt1(D102A) (gnt1.2) did not lead to detectable Skp1 glycosylation (Fig. 3C).

**Gnt1 Contributes to Orderly Morphogenesis**—Clonal growth of gnt1.1 and gnt1.3 cells on bacterial agar plates was normal except that plaque sizes were slightly smaller than those of...
phyA− cells and slightly larger than those of Ax3 cells. Proliferation rates were similar when cells were grown in suspension in HL-5 axenic medium, indicating that the difference was specific to bacterial phagocytosis or digestion. A greater difference was evident after the gnt1 mutant cells developed to form fruiting bodies after exhausting the bacteria. The majority of fruiting bodies formed by gnt1.1 and gnt1.3 cells were deformed with their stalks exhibiting extensive varicosities and frequent bends, in contrast to the predominantly straight and slender profile of the normal stalk (Fig. 4A and data not shown). Microscopic observation in the presence of Calcofluor White ST, a fluorescence brightener whose affinity to cellulose reveals the organization of stalk cell walls and the cellulosic stalk tube proper that envelopes the stalk cells, revealed that mutant stalks lacked the uniform diameter of normal stalks and contained regions of local swelling filled with stalk cells (data not shown). These defects were corrected by overexpression of DpGnt1 under control of the ecmA promoter, which directs expression in prestalk and stalk cells (Fig. 4A). Correction depended on the catalytic activity of DpGnt1 because clones overexpressing the enzymatically inactive D102A mutant remained abnormal. Similar differences were observed in independent gnt1 mutant and Gnt1-complemented clones (data not shown). The morphology of fruiting bodies was more normal when axenically grown cells were developed on filters so, as for proliferation, the effects of gnt1 disruption on stalk formation depended on the environmental context.

Phylogen Hydroxylation Alone Affects the O2 Requirement for Culmination—Previous studies showed that P4H1-null cells exhibited a substantially elevated requirement for O2, ~21%.

D. Zhang, H. van der Wel, and C. M. West, unpublished data.
to culminate, compared with the ≤10% required for the parental Ax3 strain (15). We also reported that disruption of pgtA, such that Skp1 cannot be processed past the GlcNAc-O-Skp1 isoform, was delayed in development but appeared to have a near normal O₂ requirement to culminate. The availability of gnt1 mutant cells made it possible to determine whether hydroxylation per se can rescue or whether GlcNAc capping is also required. Based on morphological analyses (Fig. 4B), gnt1.1 and gnt1.3 cells required ~15% O₂ to culminate, i.e. less than for P4H1-null cells but more than for wild-type (Ax3) cells, which required ≤10% O₂ in this trial. At 10–12% O₂, cells remained organized as slugs that oscillated between a horizontal migratory phase and a stationary state in which the slug rears up into a semi-vertical position, based on time-lapse microscopy. At a low but variable frequency, the vertical hypoxic slugs propelled themselves upward on their slime sheaths to form delicate aerial slugs that do not terminally differentiate (data not shown), as originally described for slugger mutants in normoxia (26) and normal cells in hypoxia (15).
Quantitation of the number of spores produced, which depends on normal culmination, yielded a similar finding. Some mutant terminal differentiation occurred at 12% O2, but 15% was required to generate normal spore numbers (Fig. 4C), and the O2 level required for 50% culmination (~13%) was ~3% higher than for strain Ax3 in the example shown. Spore counting showed that pgTA- cells also required higher O2 than did Ax3 to sporulate, with an O2 level required for 50% culmination indistinguishable from that of gnt1 mutant cells. Because we did not previously observe a difference between Ax3 and pgTA- (17), this comparison was repeated six additional times, always with a difference between the two strains. As reported previously (15), the absolute O2 threshold varies between trials presumably because of other variables such as light level, although the relative order of O2 thresholds between mutants is invariant. Higher resolution was achieved in the present experimental series because of a broader threshold range separating phyA- and Ax3 cells and the closer O2 level increments tested. Plotting the average O2 level required for 50% maximal sporulation over all experiments (Fig. 4E) confirmed that hydroxylation alone caused a substantial decrease in the O2 threshold from the unmodified state (i.e. phyA-) that was indistinguishable from that of the mono-GlcNAcylated state (pgTA-) and confirmed rescue by overexpression of Gnt1 but not mutant Gnt1.2. As reported previously (17), further glycosylation past the GlcNAc addition step to the trisaccharide state (agtA-) reversed the effect (Fig. 4, C and E) before the addition of the terminal αGal residues reduced the O2 threshold to the wild-type level (Ax3). Thus P4H1 contributes both glycosylation-independent and glycosylation-dependent functions to establishing the O2 threshold.

To test whether the reduced O2 threshold (relative to P4H1- cells) was specific to loss of αGlcNAcT activity, the gnt1.1 and gnt1.3 strains that had been complemented by overexpression of the DpGnt1 under the ecmA promoter were analyzed. As shown in Fig. 4, D and E, these strains exhibited an Ax3-like O2 dependence. Rescue depended on the αGlcNAcT activity as similar overexpression (Fig. 3C) of mutant DpGnt1(D102A) (Gnt1.2), which carries an inactivating mutation similar to that in Gnt1.3, did not affect the O2 requirement for culmination and sporulation. Overexpression of active Gnt1 did not reduce the O2 threshold to below the wild-type level, and this was confirmed by testing the overexpression of either active or inactive Gnt1 in the wild-type background (Fig. 4D). Therefore Gnt1 overexpression did not exert a dominant negative effect and, furthermore, Gnt1 did not appear to be rate-limiting for signaling because, unlike for P4H1 (15), overexpression did not drive the O2 threshold to a lower level.

**Skp1 Is the Only Detected Acceptor Substrate of Gnt1 in gnt1.1 Cells**—Because the known function of Gnt1 is to enzymatically modify the product of the P4H1 reaction, we predicted that there would be no consequence of gnt1 mutations in a P4H1- background. This was tested by analyzing the gnt1.1/phyA- and gnt1.3/phyA- double mutants described above. Both mutants exhibited O2 requirements similar to that of phyA- cells (Fig. 4C), indicating that Gnt1 only modifies Hyp products of the P4H1 reaction in cells. A previous study showed that the action of PgtA, whose only detectable substrate in cells is Skp1, can reverse the contribution of P4H1 to O2 signaling (17), suggesting that Gnt1 and P4H1 also signal via Skp1. To explore this model further, the in vivo specificity of Gnt1 was examined by screening an extract of gnt1.1 cells for potential acceptors expected to accumulate in the absence of the enzyme. After the addition of recombinant DpGnt1 and UDP[3H]GlcNAc to extracts of gnt1.1 and Ax3 and allowing the labeling reaction to proceed, the reaction product was displayed on an SDS-PAGE gel, which was analyzed for radioactivity. [3H] Incorporation was observed only in gnt1.1 cells, and the great majority occurred at the M1 position of Skp1 (Fig. 5A). The minimal incorporation seen at higher M1 positions was also observed in the absence of added Gnt1 (panel B) and thus appeared to be nonspecific. Increased incorporation into fractions 14, 20, and 21, which was <0.7% of the incorporation into the Skp1 band, was not reproducible in separate trials with independent gnt1 mutant clones or a phyA-/gnt1- double mutant strain extract supplemented with Gnt1 or Gnt1 + P4H1 (data not shown). The near negligible labeling of Skp1 in parental Ax3 cells supplemented with DpGnt1 (Fig. 5B) indicated that the hydroxylated isofrom did not accumulate in cells under these conditions. The even lower level of incorporation into higher M1 positions of Ax3 extracts probably reflects less contamination from vesicular glycosyltransferases and glycoprotein acceptors in this preparation. These results suggest that Skp1 is the only substrate that accumulates in the absence of Gnt1 and thus is potentially a unique Gnt1 acceptor substrate.

**Analysis of Skp1 Half-life in gnt1.1 Cells**—The Western blot analyses of Fig. 3 show that the steady-state level of Skp1 in growing cells was not affected by hydroxylation in the absence of capping by GlcNAc. This finding differs from that observed for HIFα, whose levels sharply decrease following the hydroxylation events that led to its rapid polyubiquitination. To verify that Skp1 turnover was not affected, growing cells were metabolically labeled with [35S]Cys/Met and incorporation of radiolabel into total protein, as determined by trichloroacetic acid

**FIGURE 5. Gnt1 substrate detection by in vitro complementation.** A, soluble extracts from growing gnt1.1 and normal (Ax3) cells were incubated with UDP[3H]GlcNAc = recombinant DpGnt1. The quenched reaction was subjected to SDS-PAGE, and the apparent M, distribution of [3H] incorporation was determined by liquid scintillation counting of sequential gel slices. B, replot of the same data using a different ordinate scale.
precipitation, or Skp1, as determined by immunoprecipitation and SDS-PAGE, was measured. Western blot analysis showed that 90% of Skp1 was recovered by immunoprecipitation (data not shown), and the autoradiogram shown in Fig. 6 shows the selectivity of the method. A comparison of P4H1-null and gnt1.1 cells showed that similar levels of radiolabel were incorporated into total protein (Fig. 6A) and Skp1 after 1 h of labeling (Fig. 6B). Similar labeling of Skp1 also occurred in slug stage Skp1, normalized to input cell number. D, half-lives of proliferating cell Skp1 were calculated from data shown in panel E. E, results from a pulse-chase study of 1-h labeled proliferating cells are plotted as a function of time. The line was fitted from a linear regression analysis and used to calculated Skp1 half-lives in growing cells. The autoradiogram at the left shows the selectivity of the immunoprecipitation method. Less incorporation was observed into the phyA strain in the example shown, but the fraction of label relative to total incorporation was similar between the two strains.

**DISCUSSION**

**Target Specificity of Gnt1—**Gnt1 is a polypeptide αGlcNAcT that belongs to the lineage of enzymes responsible for initiation of mucin-type O-glycans in eukaryotes. In protists, the initial sugar is typically an αGlcNAc linked to Thr or Ser (27, 28), whereas in animals, the initial sugar is almost always its 4-epimer αGalNAc linked to either of the same hydroxyamino acids (29). A phylogenetic analysis of these sequences suggests that an ancient gene duplication allowed divergent evolution of the version of this enzyme that populates the Golgi apparatus to O-glycosylate cell surface and secretory proteins (30). The product of the other gene retained its apparently primordial location in the cytoplasm where, at least in *Dictyostelium* and probably in *Toxoplasma gondii* (31), it catalyzes the initiation of O-glycosylation of Hyp on Skp1. Here we confirm that this enzyme is solely responsible for Skp1 O-glycosylation at Hyp-143 and present evidence that, in contrast to other enzymes of this gene family, its catalytic activity is devoted to only a single protein target.

The interpretation that Skp1 is the only protein acceptor substrate for Gnt1 derives from both biochemical and functional data. When recombinant Gnt1 was added back to extracts of gnt1.1 cells, expected to accumulate any Gnt1 substrates, the only protein labeled by the radioactive donor substrate above background levels (<0.7%) was Skp1 (Fig. 5). This
finding is consistent with the results of previous similar experiments in which AgtA, PgtA, or P4H1/Gnt1 was added back to the corresponding enzyme mutant (16, 17, 32). In addition, the effect of Gnt1 on the O₂ requirement for culmination depended on P4H1 (Fig. 4C), which most likely indicates that Gnt1 acts on the product of P4H1. Thus Gnt1 is confirmed to modify Hyp during signaling and, because further Gnt1-dependent glycosylation reverses the P4H1 effect (17), P4H1 apparently signals via Skp1. Consistent with this model, genetic manipulations of Skp1 levels have effects on the O₂ dependence of culmination in a manner that is affected by the presence of the target Pro-143 (16). Specificity for Skp1 may involve the previously described bipartite recognition mechanisms surmised for P4H1 and Gnt1 from characterizations of the in vitro reactions using purified proteins (12).

**Roles of P4H1 and Gnt1 in O₂ Signaling**—The ground state O₂ level required for culmination in the absence of Skp1 modification is established by the *phyA* strain and is ~21% O₂ (Fig. 4E). Hydroxylation reduces the threshold to 12–13%, as established by the *gnt1* mutant strains, and this value is not affected further by the addition of GlcNAc alone, as determined in the *pgtA* strain. Thus hydroxylation alone has a substantial effect on O₂ sensitivity. Although GlcNAc addition has little further effect, discrete steps in subsequent elongation of the sugar chain (see Fig. 3A for pathway) differentially modulate the O₂ requirement (10). Therefore hydroxylation renders both glycosylation-independent and glycosylation-dependent effects on cell physiology. Because the effect of catalytically inactive Gnt1 was indistinguishable from that of truncated Gnt1 and because overexpression of inactive or active Gnt1 had no dominant negative effect, the role of Gnt1 may be to simply provide a platform for further glycosylation that affects development.

**Effects of P4H1 and GlcNAc on Skp1**—The comparison of Skp1 and Skp1-OH, from *phyA* and *gnt1.1 or gnt1.3* cells, respectively, revealed no evidence for differences in level (Fig. 3), rate of synthesis, or stability (Fig. 6). A previous study showed a similar result for Skp1 from *phyA*, *agtA*, and normal cells using a cycloheximide treatment method (16). This contrasts with the effect of hydroxylation by the animal ortholog of P4H1, which leads to rapid degradation of HIFα based on recognition by the VHL subunit of the E3VBCUb ligase and subsequent polyubiquitination (3). The difference is consistent with the absence of recognizable subunits of the VBC complex, except for elongin C, in the *Dictyostelium* genome. Future studies are required to evaluate the possibility of indirect effects on the half-life of other proteins via the E3SCFUb ligase of which Skp1, evolutionarily related to elongin C of the E3VBCUb ligase, is a subunit.

The mechanisms of action of the modifications toward Skp1 function are unclear. Because the *gnt1* mutations also affect stalk morphogenesis in normoxia (Fig. 4A), Gnt1 action extends beyond the culmination checkpoint *per se*. Distinct developmental phenotypes appear in other pathway mutants (16), reinforcing the concept that global Skp1 functions, perhaps hydroxylation-dependent but not necessarily O₂-regulated, are affected by Skp1 modification. Skp1 is an adaptor in the multisubunit SCF complex family, with potentially 50 members based on the number of predicted FBP genes, some of which are active in *Dictyostelium* development (33–36). Studies from other organisms indicate that in addition to interacting with F-box proteins and cullins and the modification enzymes discussed here, Skp1 can homodimerize (37) and interact with other proteins such as Sgg1 and Siah-interacting protein (SIP). If the modifications influence local Skp1 conformation, as suggested by related studies in HIFα (38), then disturbances of the kinetics or equilibria of any of these interactions may influence Skp1 activities. An alternative “extrinsic” model in which accessory proteins that recognize discrete modification states is not excluded by these considerations. Conservation of P4H1- and Gnt1-like genes in numerous other protists, including pathogens such as *Acanthamoeba*, *Phytophthora* sp., and the agent for human toxoplasmosis, *T. gondii* (10, 30), suggests that mechanisms operating in *Dictyostelium* will be relevant for O₂ and metabolic sensing in a broad spectrum of unicellular organisms.

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Role of Prolyl Hydroxylation in Skp1 Function

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