**Glycosylation of Skp1 Promotes Formation of Skp1–Cullin-1–F-box Protein Complexes in Dictyostelium**

M. Osman Sheikh‡, Yuechi Xu‡, Hanke van der Wel‡, Paul Walden‡, Steven D. Hartson§, and Christopher M. West‡¶

**O2 sensing in diverse protozoa depends on the prolyl 4 hydroxylation of Skp1 and modification of the resulting hydroxyproline with a series of five sugars.** In yeast, plants, and animals, Skp1 is associated with F-box proteins. The Skp1–F-box protein heterodimer can, for many F-box proteins, dock onto cullin-1 en route to assembly of the Skp1–cullin-1–F-box protein–Rbx1 subcomplex of the E3SCFUb ligases. E3SCFUb ligases conjugate Lys48-polyubiquitin chains onto targets bound to the substrate receptor domains of F-box proteins, preparing them for recognition by the 26S proteasome. In the social amoeba Dictyostelium, we found that O2 availability was rate-limiting for the hydroxylation of newly synthesized Skp1. To investigate the effect of reduced hydroxylation, we analyzed knockout mutants of the Skp1 prolyl hydroxylase and each of the Skp1 glycosyltransferases. Proteomic analysis of co-immunoprecipitates showed that wild-type cells able to fully glycosylate Skp1 had a greater abundance of an SCF complex containing the cullin-1 homolog CulE and FbxD, a newly described WD40-type F-box protein, than the complexes that predominate in cells defective in Skp1 hydroxylation or glycosylation. Similarly, the previously described FbxA–Skp1CulA complex was also more abundant in glycosylase-deficient cells.

The CuE interactome also included higher levels of proteasomal regulatory particles when Skp1 was glycosylated, suggesting increased activity consistent with greater association with F-box proteins. Finally, the interactome of FLAG-FbxD was modified when it harbored an F-box mutation that compromised Skp1 binding, consistent with an effect on the abundance of potential substrate proteins. We propose that O2-dependent posttranslational glycosylation of Skp1 promotes association with F-box proteins and their engagement in functional E3SCFUb ligases that regulate O2-dependent developmental progression. *Molecular & Cellular Proteomics* 14: 10.1074/mcp.M114.044560, 66–80, 2015.

Timely protein degradation is a cornerstone of cell cycling and the regulation of numerous physiological and developmental processes. Eukaryotes have evolved an extensive array of polyubiquitination enzymes to tag proteins on a protein-by-protein basis as a recognition marker for degradation in the 26S proteasome. The cullin-RING ubiquitin ligases (CRLs)1 are a prominent subgroup of these enzymes (1) and consist of an E3 architecture that includes a substrate receptor, an adaptor (in most cases), the cullin scaffold, the RING protein, and an exchangeable E2 ubiquitin donor that has been charged with ubiquitin (Ub) by an E1 enzyme. The first discovered and still prototypic example is the CRL1 class (2), also referred to as SCF on account of the names of its founding subunits, Skp1, cullin-1, and F-box proteins (FBPs). The CRL1 (or SCF) complexes utilize FBPs as substrate receptors, Skp1 as the adaptor linking the FBP to the N-terminal region of cullin-1 (Cul1), and Rbx1 as the RING protein that tethers the E2 Ub donor to the Cul1 C-terminal region (see Fig. 2B). CRL1s can be activated by neddylation of Cul1 by a Nedd8-specific E2, which mobilizes Rbx1 to afford rotational flexibility of the E2 and displaces the inhibitor Cand1, permitting docking of the Skp1–FBP heterodimer (3–5). Deneddylation mediated by the eight-subunit COP9 signalosome is required for *in vivo* activity, suggesting that Cand1 serves as a substrate exchange factor to allow for re-equilibration of SCF complexes from preexisting subunits. Each reaction cycle requires the exchange of a new E2-Ub and typically assembles a K48-linked polyUb chain that is recognized by the proteasome. Substrate specificity is conferred by FBPs, a gene family that numbers 69 in humans, 20 in budding yeast.

---

1 The abbreviations used are: CRL, cullin-RING ubiquitin ligase; Ub, ubiquitin; FBP, F-box protein; Cul1, cullin-1; wt, wild type; IP, immunoprecipitation; co-IP, co-immunoprecipitation; CSN, COP9 signalosome; SCF, Skp1–Cul1–FBP complex.
300 in Caenorhabditis elegans, and ~800 in Arabidopsis. Some characterized FBPs can recognize perhaps a dozen or more substrates, and the coding of recognition and the meaning of their control by the same FBP is under intense investigation (6). Recognition is often activated by posttranslational modification of the substrate (often phosphorylation). Regulation of SCF Ub ligases has centered on the neddylation cycle, which potentially influences all seven known CRLs. Regulation of Skp1, investigated in this paper, would be specific to CRLs possessing Skp1, which include CRL1 and possibly the minor class CRL7 (7).

The basic SCF model is thought to be widespread among eukaryotes but has been extensively studied only in fungi/yeasts, plants, and animals. The broad phylogeny represented by protists includes many benign and pathogenic unicellular organisms of great economic, health, and environmental impact. Emerging evidence reveals that Skp1 in some of these groups is subject to a novel form of prolyl 4(trans)-hydroxylation and complex glycosylation (8). The roles of these Skp1 modifications have been most studied in the social amoeba Dictyostelium, which undergoes a starvation-induced developmental program during which individual amoebae chemotactically aggregate into an initial mound that then elongates into a migratory slug. Under appropriate conditions, the slug reorganizes to form a fruiting body consisting of a ball of spores supported by a vertical cellular stalk. The slug-to-slug switch, referred to as culmination, and sporulation are regulated by checkpoints that are sensitive to multiple factors, including O2 (9–11). Functional studies of Dictyostelium Skp1 hydroxylation and glycosylation reveal roles in regulating the O2 dependence of culmination and sporulation (12–14). For example, wild-type (wt) cells require 7% to 10% O2 and phyA requires 18% to 21% O2 in order to achieve 50% spor formation (a quantitative measure of fruiting body formation), whereas glycosylation mutants exhibit a complex pattern of intermediate requirements (13). In addition, at 21% O2, phyA+ cells require an additional 3 to 4 h to complete development relative to their wt counterparts (14). In the apicomplexan Toxoplasma gondii, PhyA is also required for Skp1 glycosylation, and phyA parasites are deficient in proliferation, especially at low O2 (15).

The idea that O2 availability is rate limiting for Skp1 modification was originally based on the observation that the Dictyostelium phyA phenotype mimics that of wt cells in low O2 (9). However, the majority of Skp1 is hydroxylated and glycosylated in wt cells even at low O2 levels where culmination is blocked or delayed. Further analysis of a submerged development model, in which terminal development depended on an atmosphere of 70% to 100% O2 in order to overcome the diffusion barrier posed by the water layer, showed that at atmospheric O2 levels of 5% to 21% where sporulation was blocked, unmodified Skp1 accumulated to a higher level than at permissive O2 levels (10). As Skp1 modifications are thought to be irreversible, this likely resulted from slow hydroxylation of newly synthesized Skp1. To address this in a more physiological setting, we investigated nascent Skp1 directly using metabolic labeling with [35S]Met/Cys and verified that the rate of hydroxylation of newly synthesized Skp1 polypeptide was indeed inversely proportional to O2 levels, which makes PhyA-mediated hydroxylation of Skp1 an excellent candidate for the primary O2 sensor for culmination.

These modifications of Skp1 are of interest as a novel mechanism regulating the SCF ligase. Previously, we showed that hydroxylation and glycosylation of Dictyostelium Skp1 affect its conformation and promote binding to a soluble FBP, guinea pig Fbs1, in studies of purified proteins (16). Here we show that Dictyostelium Skp1 is indeed a subunit of a canonical SCF complex, as expected. The significance of undermodified Skp1 was examined via interactome analysis of Skp1 isoforms that accumulate in modification pathway mutants. Our findings revealed a lower abundance of SCF complexes than in wt cells, suggesting that Skp1 modification may promote SCF assembly and E3[Cys]Ub ligase activities that control timely turnover of select proteins involved in developmental progression.

**EXPERIMENTAL PROCEDURES**

**Metabolic Labeling and Isolation of Skp1**—Cells (gnt1−) were grown axenically in HL-5 medium supplemented with 50 μg/ml streptomycin and 50 μg/ml ampicillin until they achieved a density of 2 × 106 cells per milliliter. Cells were recovered via centrifugation at 1000 × g for 1 min in a 15-ml conical polypropylene tube and resuspended at 102 cells per milliliter in 1 ml of fresh HL-5 pre-equilibrated overnight under an atmosphere of the indicated O2 percentage, with the balance made up of N2. Samples were shaken for 2.5 h under a flowing atmosphere of the same O2 percentage, at which time 0.71 Ci of [35S]Met/Cys (Trans-[35S]-Label, >1000 Ci/mmol; MP Biomedicals, Santa Ana, CA) was introduced. Shaking incubation was continued until cells were recovered via centrifugation, resuspended in degassed PDF buffer (33.3 mM NaH2PO4, 10.6 mM Na2HPO4, 20.1 mM KCl, 5.8 mM MgSO4, pH 5.8) supplemented with 2 mM Na-dithionite, transferred to a 1.5-ml microcentrifuge tube, and centrifuged again. The cell pellet was snap frozen on dry ice and stored at −80 °C.

Cell pellets were resuspended in 110 μl of 8 mM urea, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin); incubated on ice for 15 min and then at 55 °C for 5 min; and clarified via centrifugation at 16,000 × g for 30 min at 4 °C. 50 μl was transferred to a 1.5-ml microtube containing 450 μl of RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.5% (w/v) Na-deoxycholate, 0.1% (w/v) SDS, with protease inhibitors as above). Samples were pre-cleared by the addition of 10 μl of 1 mg/ml normal mouse IgG or 10 μl of preimmune serum (UOK85 or UOK77) and incubated for 1 h on ice, after which we added 50 μl of a 25% slurry of pre-washed (in RIPA buffer) Protein A beads (UltraLink resin; Pierce) or 25 μl of Protein G beads (UltraLink resin; Pierce) and rotated the samples at 4 °C for 1 h. Beads were sedimented for 10 s in a personal microcentrifuge, and the supernatant was transferred to a fresh tube. Total Skp1 was immunoprecipitated by incubation with 5 μg of mAb 4E1 followed by incubation with Protein G resin and centrifugation as described above. HO-Skp1 was similarly isolated via incubation with 5 μg of affinity-purified UOK85 rabbit IgG followed by incubation with Protein A beads and centrifugation as described above. Total Skp1 was alternatively isolated via the addition of 10 μl
of beads covalently modified with 2 mg/ml of affinity-purified UOK77 anti-Skp1 rabbit IgG (described below), incubation at 4 °C for 2 h, and centrifugation. Affinity purification involved adsorption to Skp1 (UOK77) or HO-Skp1 (UOK85) covalently coupled to CNBr-activated Sepharose CL-4B, elution with 0.1 M glycine-HCl (pH 2.8), and immediate neutralization. Beads were washed in two cycles of resuspension in 1 ml of RIPA buffer, centrifugation, transfer of the beads to a fresh tube, and a final RIPA buffer wash, after which the samples were stored at −80 °C. The first supernatant was saved at −80 °C for assay of the total incorporation and protein content. Antibody and bead amounts and incubation times were the minimum required in order to recover maximal Skp1 (typically >75%).

Skp1 was released from the beads via the addition of 50 μl of 1× Laemmli sample buffer and boiling for 3 min, after which it was applied to a 7–20% SDS-PAGE gel. The gel was stained in Coomasie Blue, destained, incubated in H2O for 15 min and then 1× Na salicylate for 60 min, and dried against a sheet of 3MM Whatman filter paper. The gel was autoradiographed against BioMax MR film (Kodak, New Haven, CT), using a Fisher Biotech L-Plus intensifying screen, at −80 °C. The film image was digitized and analyzed densitometrically using ImageJ (version 1.47, National Institutes of Health, Bethesda, MD).

Total protein was determined using a BCA assay (Pierce), and total 35S incorporation was determined via precipitation with trichloroacetic acid, recovery on a GF/C filter, transfer to BioSafe NA scintillation fluid, and quantitation using a Beckman scintillation counter.

**Gel Fractionation of Cytosolic Extracts**—Vegetative (growth-stage) cells were harvested as described above; resuspended in 50 mM Tris-HCl (pH 7.4), 0.25 mM sucrose, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, added just before use); and lysed by means of forced filtration through a 5-μm pore diameter filter as described previously (17). A cytosolic extract was prepared via ultracentrifugation (100,000 × g for 60 min) at 4 °C and chromatographed on a Superose 12 gel filtration column at 22 °C pre-equilibrated in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, and 10% glycerol. Fractions were analyzed via SDS-PAGE and Western blotting for Skp1 using mAb 4E1, and they were quantitated densitometrically using ImageJ (version 1.47, National Institutes of Health, Bethesda, MD).

**CulE Tagging**—The culE 5′-targeting sequence was amplified from Dictyostelium discoideum genomic DNA (Ax3 strain) via polymerase chain reaction (PCR) using primers 5′-AGAGGATCC-TCTAGGACTAATCCGTTTTGCTTT and 5′-AGAAGCTTCTTAATGAAATTTTATATCTGAATATTAGAAAC and ligated into pCR4-TOPO (Invitrogen). The FbxD DNA was excised via digestion with BamHI and SacI (italicized in primer sequences) and ligated into pCR4-TOPO (Invitrogen). The FbxD DNA was excised via digestion with BamHI and SacI (italicized in primer sequences) and ligated into a similarly digested pVS vector (9), which inserted an amino-terminalFLAG tag and directs expression in prespore cells under control of the cotB promoter. Cells were transformed as described above in the presence of 10 μg/ml G-418 (Research Products International, Mount Prospect, IL) in HL-5 medium, further selected with 20 or 120 μg/ml G-418 in HL-5 in gyrating flasks, and cloned as described above. For development, cells grown in HL-5 were washed via centrifugation, and 1.2 × 106 cells in PDF buffer were spread onto 10-cm non-nutrient agar plates. After 13 to 15 h, slugs were scraped off and analyzed for FLAG-FbxD expression via Western blotting using anti-FLAG mAb M2 (Sigma).

**FbxD Overexpression**—The FbxD coding region was amplified from D. discoideum genomic DNA via PCR using primers 5′-AGAGGATCC-TCTAGGACTAATCCGTTTTGCTTT and 5′-AGAAGCTTCTTAATGAAATTTTATATCTGAATATTAGAAAC and ligated into pCR4-TOPO (Invitrogen). The FbxD DNA was excised via digestion with BamHI and SacI (italicized in primer sequences) and ligated into a similarly digested pVS vector (9), which inserted an amino-terminalFLAG tag and directs expression in prespore cells under control of the cotB promoter. Cells were transformed as described above in the presence of 10 μg/ml G-418 (Research Products International, Mount Prospect, IL) in HL-5 medium, further selected with 20 or 120 μg/ml G-418 in HL-5 in gyrating flasks, and cloned as described above. For development, cells grown in HL-5 were washed via centrifugation, and 1.2 × 106 cells in PDF buffer were spread onto 10-cm non-nutrient agar plates. After 13 to 15 h, slugs were scraped off and analyzed for FLAG-FbxD expression via Western blotting using anti-FLAG mAb M2 (Sigma).

**Co-immunoprecipitation**—Aliquots of 3 × 107 slug cells, or 1.5 × 107 exponentially growing vegetative cells in order to match total protein content, were lysed in 250 μl of IP Buffer (0.2% or 1.0% Nonidet P-40 (as indicated), 50 mM HEPES-NaOH, pH 7.4, or 50 mM Tris-HCl, pH 8.0 (as indicated), 50 mM NaCl, 1 mM MgCl₂, 0.1 mM EDTA, and the above-mentioned protease inhibitors) and allowed to incubate on ice for 10 min. Lysates were centrifuged at 16,000 × g for 15 min at 4 °C. For each IP, 50 μl of each S16 supernatant fraction was transferred to packed beads that were washed in IP Buffer. Co-IP of Skp1 was performed using 5 μl of packed pAb UOK77 conjugated to NHS-activated Sepharose beads (GE Healthcare) at 2 mg of protein per milliliter. Beads similarly prepared with total rabbit IgG were used in parallel. Co-IP of CulE-FLAG and FLAG-FbxD was performed using 2.5 μl of packed anti-FLAG M2-EZ view beads (Sigma). CulE-FLAG IPs contained 5 μl EDTA and 10 μm iodoacetamide to inhibit CulE deneddylation by cysteine peptidases. After rotation for 1 h at 4 °C, beads were collected by centrifugation at 2000 × g for 1 min and washed three times with 150 μl of IP buffer, with tubes exchanged after the first wash. Beads were boiled in SDS-PAGE sample buffer for 3 min. The equivalent of 5 × 105 slug cells or 3 × 10⁶ vegetative cells was analyzed via SDS-PAGE and Western blotting using the M2 anti-FLAG mAb (Sigma) or the pan-specific Skp1 mAb 4E1, as well as Alexa-680-conjugated rabbit anti-mouse IgG. Fluorescence was detected using an Odyssey infrared scanner (Li-Cor, Lincoln, NE), and densitometric analysis was performed using ImageJ (version 1.47, National Institutes of Health).
Mass Spectrometry Analysis—For interactome analysis, Cul-E-FLAG, FLAG-FbxD, or Skp1 was immunoprecipitated as described above, scaled up to 6 x 10^7 slug cells (~50 pg of protein per cell) or 3 x 10^7 vegetative stage cells (~100 pg of protein per cell). The method was similar to that described previously (23). The washed Ab beads were eluted with 8 M urea, 50 mM Tris-HCl, pH 8.5. Eluates were separated from their IgG resin pellets via centrifugation, and one-half of each was brought to 80°C for 5 min, then digested with 4 μg/ml sequencing-grade trypsin for 16 h at 37°C. Reactions were stopped by the addition of TFA to 1%, salts were removed by means of solid phase extraction on 100-μl OMIX C18 tips following the manufacturer's instructions (Agilent, Santa Clara, CA), and peptide eluates were lightly dried by means of vacuum centrifugation. Samples were brought to 80°C and analyzed in triplicate in three successive LC-MS/MS analyses on a Thermo Hybrid LTQ Orbitrap-XL mass spectrometer. For LC-MS/MS, 10 μl of peptides were injected onto a 5-cm trap column (3-μm Magic C18 AQ) in a vented column configuration and then subjected to analytical separation on a 40-cm PicoFrit column (75-μm inner diameter; New Objective, Woburn, MA) packed with 3-μm Magic C18 AQ and terminated with an integral fused silica emitter. Peptides were eluted at 250 nl/min in a 1.6%-40% acetonitrile/0.1% formic acid gradient over 110 min, ionized via nanospray, and analyzed using a “Top-6” methodology consisting of one full-range Fourier transform MS scan (nominal resolution of 60,000 full width at half-maximum, 360 to 1400 m/z, using lock mass 445.1200) and up to six data-dependent MS/MS scans performed in the linear ion trap. MS/MS settings included a trigger threshold of 8000 counts, monoisotopic precursor selection, an isolation width of 2 m/z, a normalized collision energy of 35%, an activation Q of 0.25 for 30 ms, and rejection of parent ions that had unassigned charge states or that were previously identified as contaminants on blank gradient runs. Centroided ion masses were extracted using the extract_msn.exe utility in Bioworks 3.3.1 and were used for database searching with Mascot v2.2.04 (Matrix Science, Boston, MA). Searches against the D. discoideum protein list (from dictyBase, May 17, 2012; 12,430 protein sequence models) used the following search parameters: 5 ppm parent ion mass tolerance, 0.6 Da fragment ion tolerance, one missed tryptic cleavage, pyroglutamate cyclization of N-terminal Gin, oxidation of Met, formylation or acetylation of the protein N terminus, and iodoacetamide alkylation of Cys.

Peptide and protein identifications were validated using Scaffold v2.2.00 (Proteome Software, Portland, OR) and the PeptideProphet algorithm (21) using Scaffold’s high-mass-accuracy scoring option. Protein acceptance thresholds were set at a 1% false discovery rate, based upon at least two peptides, each identified at a 1% false discovery rate. In addition, at least two spectral counts had to have been obtained for all three technical replicates of at least one individual analysis. At these settings, three, zero, or zero decoy proteins (i.e. reverse translated peptides) were identified from a population of 282, 527, or 406 total protein identifications from the pooled data shown in supplemental Tables S2–S4 and summarized in Figs. 4–6. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy principles of parsimony. Differences in spectral counts were assessed using Scaffold (unnormalized total spectrum counts, with the minimum spectrum count value set to zero). To ensure a high level of rigor with regard to the protein–protein interactions described, proteins reported in supplemental Tables S2–S4 were excluded from further analysis if their spectral counts from negative-control co-IPs were more than zero and >10% of those observed in the “bait” pulldowns, or if they were ribosomal subunits, chaperones (except for a subgroup in the FLAG-FbxD co-IPs), or intraorganellar or secretory proteins (most members of the latter category were already excluded by the previous criteria).

RESULTS

O2 Level Is Limiting for Skp1 Hydroxylation in Cells—To compare the rates of Skp1 polypeptide synthesis and its prolyl hydroxylation, growth-stage cells were pulse-labeled with [35S]Met/Cys, split in half for separate immunoprecipitations for all Skp1 (pAb UOK77 or mAb 4E1) or the reaction product HO-Skp1 (pAb UOK85), and subjected to autoradiography of the [35S]Skp1 bands. Mutant gnt1– cells were used to prevent glycosylation of HO-Skp1, which blocks recognition by pAb UOK85. pAb UOK85 is highly specific for HO-Skp1 based on Western blotting (13, 16) and ELISA assays (24), and no [35S]Skp1 was detected using nonspecific antibodies (13). The experimental design (Fig. 1A) involved a 2.5-h pre-equilibration of gnt1– cells in 2.5%, 5%, 7.5%, or 21% O2 followed by metabolic pulse-labeling for up to 4 h and isolation of Skp1 isoforms.

At 21% O2, Skp1 polypeptide synthesis was observed to occur at a nearly linear rate after a lag of 30 min (solid blue lines in Figs. 1B and 1C). This is consistent with the estimated Skp1 half-life of 14 to 16 h based on pulse-chase labeling (13), as turnover of labeled Skp1 would be minimal at 4 h. The rate of HO-Skp1 synthesis was also linear, and similar to that of total Skp1 (solid red lines). Thus, in ambient atmosphere, prolyl hydroxylation of Skp1 occurred without a detectable delay, though a small delay could have been missed owing to different recovery efficiencies of the different antibodies.

Skp1 polypeptide synthesis was similar at 2.5% and 21% O2, despite an accompanying global reduction of 35S-dpm in 2.5% O2 into total cellular protein (65% less total [35S]protein after 4 h based on liquid scintillation counting of TCA precipitates). Thus Skp1 synthesis was preserved by selective regulation. In contrast, Skp1 hydroxylation was delayed with a lag phase of 1.5 h and subsequently occurred at about a 30% rate (dashed red line in Fig. 1B). De-hydroxylation of proline is not known to occur at the protein level, so it is likely that the delayed accumulation of HO-Skp1 reflects a reduced rate of prolyl hydroxylation by PhyA owing to limited availability of the O2 substrate and, potentially, the co-substrate α-ketoglutarate, whose levels may also be diminished at low O2 levels. In cells maintained at 5% O2, where total protein synthesis was >90% of that occurring at 21% O2, hydroxylation was initiated without a lag phase relative to polypeptide synthesis, but the rate was only 45% that of the Skp1 polypeptide (Fig. 1C). At 7.5% O2, the rate of hydroxylation rose to about 80% that in the polypeptide synthesis. Thus increasing O2 levels led to proportionately increased rates of Skp1 hydroxylation in cells.

The hydroxylation status of the total Skp1 pool after 6.5 h of exposure to different O2 levels was evaluated via Western
**Glycosylation-dependent Skp1 Interactome**

**A. Experimental time line**

- **O₂ switch**: 2.5 hr
- **(³⁵S)Met/Cys addition**: 0, 0.5, 1.5, 4 hr
- **Sample collection for Westerns (E)**

**B. Immunoprecipitations, 2.5% O₂**

- **21%, total Skp1**: 0.2, 0.4, 0.6, 0.8
- **21%, HO-Skp1**: 0.2, 0.4, 0.6, 0.8
- **21%, H-O-Skp1**: 0.2, 0.4, 0.6, 0.8
- **5%, total Skp1**: 0.2, 0.4, 0.6, 0.8
- **7.5%, total Skp1**: 0.2, 0.4, 0.6, 0.8
- **7.5%, HO-Skp1**: 0.2, 0.4, 0.6, 0.8
- **7.5%, H-O-Skp1**: 0.2, 0.4, 0.6, 0.8

**C. Immunoprecipitations, 5% O₂**

- **21%, total Skp1**: 0.2, 0.4, 0.6, 0.8
- **21%, HO-Skp1**: 0.2, 0.4, 0.6, 0.8
- **21%, H-O-Skp1**: 0.2, 0.4, 0.6, 0.8
- **5%, total Skp1**: 0.2, 0.4, 0.6, 0.8
- **5%, HO-Skp1**: 0.2, 0.4, 0.6, 0.8
- **5%, H-O-Skp1**: 0.2, 0.4, 0.6, 0.8

**D. Immunoprecipitations, 7.5% O₂**

- **7.5%, total Skp1**: 0.2, 0.4, 0.6, 0.8
- **7.5%, HO-Skp1**: 0.2, 0.4, 0.6, 0.8
- **7.5%, H-O-Skp1**: 0.2, 0.4, 0.6, 0.8

**E. Western blots of Skp1 isoforms**

- Unmodified Skp1
- HO-Skp1

---

**Fig. 1. Rate of Skp1 hydroxylation in cells.** A, gmr1/² cells were transferred to fresh medium and shaken under an atmosphere of the indicated O₂ percentage. Metabolic labeling with [³⁵S]Met/Cys commenced at 2.5 h, and cells were harvested at the indicated times and processed for immunoprecipitation using antibodies specific for HO-Skp1 (pAb UOK85) or both HO-Skp1 and Skp1 (pAb UOK77 or mAb 4E1). B–D, time-dependent incorporation of [³⁵S] into HO-Skp1 and total Skp1 in cells held at the indicated O₂ levels. Data in the same panel are from parallel experiments. Maximal incorporation values into total Skp1 and HO-Skp1 pools after 4 h at 2.5% O₂ were adjusted to 1 to account for variations in antibody pulldown efficiency, and the factor was applied to the parallel low-O₂ samples, except for the data in D, which employed the average of factors used for B and C. E, a parallel 4-h sample was subjected to whole cell Western blot analysis using HO-Skp1 (UOK85) and pan-specific (4E1) antibodies to estimate the fraction of Skp1 that was hydroxylated after 6.5 h of exposure to the indicated O₂ level. The fraction of Skp1 that was unmodified at steady state in 21% O₂ was set at 10% based on the ratio of unmodified to glycosylated Skp1 bands in the wild-type strain Ax3. The unmodified fraction at other O₂ levels was derived from the modified fraction via subtraction.

**Blotting (Fig. 1E).** The fraction of hydroxylated Skp1 increased proportionately with increasing O₂ level, rising from 65% at 2.5% O₂ to 70% at 5% O₂, 79% at 7.5% O₂, and 90% at 21% O₂. Considering the 8-h doubling time of cells under these conditions and the ~16-h Skp1 half-life reported at 21% O₂ (13), ~23% unmodified Skp1 is expected at 5% O₂ ([40% due to growth replacement + 20% for protein turnover replacement − 8% for overlap] × 45% hydroxylation efficiency), and is close to the 20% value inferred from the Western blot analysis (Fig. 1E). The properties of nascent Skp1, which is slow to be hydroxylated at limiting O₂ levels, were then investigated through comparison of the interactomes of modified and unmodified Skp1s at 21% O₂ in cells whose Skp1 modifications are controlled genetically.

**Global Assessment of Skp1 Interactions**—To address whether O₂ and other factors that control Skp1 processing might affect its interactions with other macromolecules, we analyzed a series of Skp1 modification pathway mutant strains that result in the accumulation of Skp1 isoforms at the processing step immediately prior to the enzymatic deficiency (Fig. 2A). Fresh S100 soluble fractions of vegetative (growth stage) cells were prepared and immediately fractionated via gel filtration. Based on Western blot analysis of the fractions (Fig. 2C), Skp1 eluted in two broad peaks centered at fractions 14 (peak 1) and 19 (peak 2). Whereas unmodified Skp1 from phyA/² cells eluted mostly in peak 2, fully modified Skp1 from wt (Ax3) cells eluted mainly in peak 1. Increasing modification increased the peak 1:peak 2 ratio (Fig. 2D), as if each modification step contributed incrementally to the formation of larger complexes represented by the earlier eluting peak 1. Peak 2 might represent a simpler complex because of its later elution, but other unknown factors affected elution position, because free Skp1, which is a dimer, eluted at an intermediate position on this column.

**Characterization of SCF Complexes from Dictyostelium Cells**—Skp1 is best known as a subunit of the SCF complex, and the D. discoideum genome encodes many known subunits of the traditional CRL1/SCF complex as defined in yeasts, animals, and plants, as well as other proteins known to regulate CRL1 enzymes (supplemental Table S1). Of the five predicted cullins, three (CulA, CulE, and CulB) are most similar to conventional Cul1s based on sequence analysis using classifications described in Ref. 7 (supplemental Fig. S2). CulE was chosen for further analysis because of its detection in pilot interactome studies of FLAG-FbxD (see below). To minimize effects on CulE expression level, the culE chromosomal locus was tagged with a C-terminal 3×FLAG and BirA recognition sequence to allow for potential biotiny-
glycosylation.

**Fig. 2.** Skp1 modification pathway and global analysis of Skp1 interactions. A, Skp1 is sequentially modified by the indicated enzymes (in blue), resulting in the formation of a pentasaccharide at Pro143. B, model of the SCF complex in the context of the overall E3 Ub ligase, from studies in yeast, plants, and animals. Catalysis involves transfer of Ub from an exchangeable Ub-E2 conjugate to the substrate. Removal of Nedd8 by the COP9 signalosome facilitates binding of Cand1 to Cul1, which inhibits binding of Skp1 to Cul1. C, D, vegetative (growth stage) cells were filter-lysed, and a cytosolic fraction prepared via ultracentrifugation was chromatographed on a Superose 12 gel filtration column. Fractions were analyzed via Western blotting (representative examples are shown in C) followed by densitometry (D). The elution position of free Skp1 from a separate trial is indicated.

**Fig. 3.** Western blot analysis of interaction of CulE with Skp1. Slug cells whose culE locus was tagged with a C-terminal 3 x FLAG tag (CulE-FLAG) in a wild type (Ax3) or phyA− background, or cells whose culE locus was not tagged, were solubilized in 1% Nonidet P-40/pH 7.4 IP buffer and immunoprecipitated with anti-FLAG (M2) (A) or anti-Skp1 (UOK77) (B). The presence of CulE-FLAG and Skp1 was probed via Western blotting with the same antibodies. CulE occurred as the apoprotein and a slower migrating species corresponding to the conjugate with Nedd8 (N8). Skp1 from wt cells migrated more slowly because of glycosylation. C, 20% of the input material. Note that a co-migrating band in phyA− cells interfered with the detection of Skp1 in lane 9.

A. Skp1 modification pathway

- PhyA
- Gnt1
- PgtA-N
- PgtA-C
- Gnt1
- AgtA
- AgtA
- Nedd8
- Ub
- F-box
-substr.
- receptor
- FBP
- Nedd8

B. E3(SCF)ubiquitin ligase model

C. Gel filtration

D. Summary

Glycosylation-dependent Skp1 Interactome

**Table 1.** Summary of Skp1 modification pathway and global analysis of Skp1 interactions.

| Pathway | Skp1 modification | CulE-FLAG | CulE-FLAG/phyA− | CulE-FLAG/phyA− | CulE-FLAG/phyA− | CulE-FLAG/phyA− |
|---------|-------------------|-----------|-----------------|-----------------|-----------------|-----------------|
| N8      | CulE-FLAG        | O5Skp1    | O5Skp1          | O5Skp1          | O5Skp1          | O5Skp1          |

**Fig. 3.** Western blot analysis of interaction of CulE with Skp1. Slug cells whose culE locus was tagged with a C-terminal 3 x FLAG tag (CulE-FLAG) in a wild type (Ax3) or phyA− background, or cells whose culE locus was not tagged, were solubilized in 1% Nonidet P-40/pH 7.4 IP buffer and immunoprecipitated with anti-FLAG (M2) (A) or anti-Skp1 (UOK77) (B). The presence of CulE-FLAG and Skp1 was probed via Western blotting with the same antibodies. CulE occurred as the apoprotein and a slower migrating species corresponding to the conjugate with Nedd8 (N8). Skp1 from wt cells migrated more slowly because of glycosylation. C, 20% of the input material. Note that a co-migrating band in phyA− cells interfered with the detection of Skp1 in lane 9.

The interaction of CulE-FLAG with Skp1 was initially examined via Western blot analysis of co-IPs from slug cells. Co-IP with anti-FLAG (mAb M2) beads was optimized to recover all CulE-FLAG based on the absence of detectable CulE-FLAG remaining in the supernatant (not shown). CulE migrated as two bands corresponding to the apoprotein and the neddylation version as observed in other organisms (26–30), and similar levels were observed in phyA− and phyA+ (wt Ax3) cells (Fig. 3A). As >95% of CulE-FLAG is neddylated when cells are directly analyzed via Western blotting (not shown), the presence of non-neddylated CulE in these co-IP trials was evidently due to post-lysis deneddylation.

Skp1 was also present in the co-IP, and its level was not reproducibly affected by the absence of PhyA, which caused Skp1 to migrate more rapidly owing to the absence of its glycosylation. Comparisons with input levels (Fig. 3C) and with Western blots of the co-IP supernatants (not shown) suggested that <20% Skp1 was associated with CulE under these conditions. This was likely due to partial dissociation associated with post-lysis deneddylation and association of a Skp1 subpopulation with CulA (see below). Co-IP with affinity-purified UOK77 beads recovered similar levels of Skp1 from both strains (Fig. 3B), with about 50% Skp1 captured based on comparison with the unbound fraction and the input (Fig. 3C). These co-IPs also contained CulE-FLAG, with no reproducible differences in the capture of neddylated or deneddylated CulE. Thus a fraction of Skp1 is normally associated with CulE, and the glycosylation status of Skp1 does not appear to influence the extent of association or the activation state of CulE.

The composition of the CulE-FLAG co-IPs was further examined using a proteomics strategy. The relative recoveries of CulE subunits in control versus bait IPs and in wt versus phyA− cells were compared by spectral counting (31). After filtering
to remove contaminants from the CulE-associated proteome (see “Experimental Procedures”), CulE-FLAG was the most abundant protein detected in wt cells based on total spectral counts, and it appeared at similar levels in co-IPs from the same numbers of slug-stage (Fig. 4B) and growth-stage (vegetative) cells (Fig. 4A). Recovery of CulE-FLAG was similar in phyA− and wt cells. Many additional proteins were identified, including expected interactors (see Fig. 2B): the RING protein Rbx1, the Cul1-modifying polypeptide Nedd8, the inhibitory protein Cand1, all eight subunits of the COP9 signalosome (average value shown; see supplemental Fig. S1A for all data), and Skp1. Each of these proteins was similarly represented in mutant and wt vegetative and slug cells, with the Skp1 spectrum counting data confirming the Western blot analyses (Fig. 3). Both Skp1A and Skp1B, which differ by a single amino acid and are expressed at similar levels (32), were similarly represented (not shown). Differences in Nedd8 spectral counts between wt and phyA− cells were not reproducible (compare with Fig. 3), probably because variable deneddylatation occurred in some trials (see above). Subunits of the 19S regulatory particle of proteasomes (PSM) were detected, and substantially higher levels were detected in slug versus vegetative cells (>5-fold) and in wt versus phyA− cells (>5-fold), as depicted by the values averaged over all detected proteasomal subunits (Fig. 4). Similar differences were observed for each of the subunits (supplemental Fig. S1B). All six AAA-

Fig. 4. **Proteomic analysis of CulE interactions.** Vegetative (A) or slug-stage (B) wt or phyA− cells whose culE locus was FLAG-tagged were subjected to co-IP with anti-FLAG (mAb M2) beads, and potential interactors were identified based on mass spectrometric identification of tryptic peptides and exclusion criteria as described in “Experimental Procedures.” Average spectral counts of peptides detected for each protein in triplicate replicates of the single experiment for each panel are plotted in descending order (left to right) of total abundance in phyA− cells. For the protein complexes represented by CSN and PSM (proteasome), average values of detected polypeptides are reported; full data are in supplemental Fig. S1. Protein descriptors and RNA expression patterns are in supplemental Table S1. Full data are shown in supplemental Table S2.
ATPases and both scaffold proteins of the base region, as well as most subunits of the lid region (33), were detected, though only the base region was detected in growth-stage cells. RbrA and RbrB (Ring-between-Ring, most related to Ariadne-like ubiquitin ligases) were abundant CulE interactors, consistent with their presence in complexes with mammalian Cul1 (34). In addition, a number of proteins with unknown functions were detected. This group included three potential F-box proteins, based on sequence prediction algorithms: DDB_G0292312 (referred to as FbxD), DDB_G0281783, and DDB_G0285125 (protein I.D.s at dictyBase). Of these, FbxD was 2- to 5-fold more abundant in wt than in phyA− cells. Seven other proteins, ranging in levels from abundant to rare, were detected at one or both stages, which correlated approximately with levels of expression based on transcriptomic analyses (35) as summarized in supplemental Table S1. The lower abundance species tended to be more prevalent in wt than in phyA− pulldowns. Thus Dictyostelium expresses SCF-type Ub ligases involving CulE, which are related to previously described SCF-type Ub ligases containing the related CuA (36).

The effect of the phyA− background that blocks Skp1 modifications was mainly confined to an increased interaction of CulE with the predicted FBP FbxD and the 19S-regulatory particle of the proteasome. We investigated these effects further by examining the interactomes of Skp1 and FbxD.

The Skp1 Interactome—Because the interaction of FbxD with CulE is expected to be mediated by Skp1, we analyzed the interactome of Skp1 directly using an affinity-purified anti-Skp1 rabbit antiserum (UOK77) coupled to beads. Pilot studies conducted using Ax3 and phyA− strains expressing FbxD-FLAG (see below) indicated that an interaction with Skp1 was detected but inhibited by standard RIPA buffer, higher concentrations of Nonidet P-40, pH extremes, and time. Therefore, samples were not precleared, Nonidet P-40 was varied from 0.2% (most permissive) to 1.0% (lower background), pH ranged from 7.4 to 8.0, and bead washing was minimized. Four independent comparisons of Ax3 and phyA− slug cells were performed, and proteins were identified and filtered as described above. The same exclusion criteria were applied, but an additional requirement was that reported proteins appeared in at least two trials. As no systematic differences were seen across buffer conditions, spectral counts from the four independent co-IPs were pooled together (supplemental Table S2B). Skp1 was an abundant protein in these co-IPs, with the third most spectral counts (Fig. 5A), although it was 19th on the spectral counts list before exclusions were applied. Skp1 was on average 20% more abundant in the co-IPs of phyA− than in those of wt cells, as also observed in Western blots. Analysis of the supernatant fractions indicated that this was due to the greater capture efficiency of Skp1 in phyA− extracts (not shown), rather than to different levels of Skp1, which is consistent with evidence for altered complexes of Skp1 in wt cells (Figs. 2B and 2C).

Fifteen proteins were found to reproducibly associate with Skp1 in a specific fashion (Fig. 5A). Each was less abundant than Skp1, based on the assumption that proteins with higher Mw generate proportionately more spectral counts. The abundance of four proteins was reproducibly different between the wt and phyA− strains (Fig. 5A, asterisks). The three most abundant are predicted to contain an F-box sequence. The first, FbxD, was more abundant in Skp1 co-IPs from wt cells, consistent with its greater association with CulE in wt relative to phyA− cells (Fig. 4). A comparison with mutants that accumulate intermediate glycoforms indicated that full glycosylation is important for increased interaction with FbxD (Fig. 5B). FbxA was, like FbxD, also more enriched in Skp1 co-IPs from wt cells, and this also depended on full modification of Skp1. The third FBP-like protein, JcdI, was, in contrast, more enriched in Skp1 co-IPs from phyA− cells. A fourth potential FBP, DirA, was not differentially enriched. Thus, of the 15 Skp1 interactors reported, 4 of the 8 most abundant are predicted to be FBPs and, of these, 3 are differentially enriched according to Skp1’s modification status. The other four proteins were not differentially enriched. Of the seven less abundant proteins, only one, a predicted Tyr-kinase with ankyrin repeats (DDB-G0278521), was differentially enriched according to Skp1 modification status. CulA, previously observed to associate with the FBP FbxA (36), was also detected, but its abundance was too low to meet our reportability criteria, possibly because of competitive binding with pAb UOK77. CulE and Rbx1 were also detected as expected (Figs. 3 and 4), at similarly low levels.

In an attempt to complement these studies, we examined the interactome of FLAG-Skp1 overexpressed in Ax3 cells using anti-FLAG beads. However, no significant proteins interactors were detected, despite the utility of this construct in yeast (37). Interestingly, we previously noted that FLAG-Skp1 remains unmodified when expressed in Dictyostelium (14), indicating that perturbation of its N terminus interferes with not only its ability to enter SCF complexes, but also its hydroxylation and glycosylation.

The FbxD Interactome—FbxD possesses an F-box-like sequence and seven C-terminal WD40 repeats predicted to generate proportionately more spectral counts. The abundance of four proteins was reproducibly different between the wt and phyA− strains (Fig. 5A, asterisks). The three most abundant are predicted to contain an F-box sequence. The first, FbxD, was more abundant in Skp1 co-IPs from wt cells, consistent with its greater association with CulE in wt relative to phyA− cells (Fig. 4). A comparison with mutants that accumulate intermediate glycoforms indicated that full glycosylation is important for increased interaction with FbxD (Fig. 5B). FbxA was, like FbxD, also more enriched in Skp1 co-IPs from wt cells, and this also depended on full modification of Skp1. The third FBP-like protein, JcdI, was, in contrast, more enriched in Skp1 co-IPs from phyA− cells. A fourth potential FBP, DirA, was not differentially enriched. Thus, of the 15 Skp1 interactors reported, 4 of the 8 most abundant are predicted to be FBPs and, of these, 3 are differentially enriched according to Skp1’s modification status. The other four proteins were not differentially enriched. Of the seven less abundant proteins, only one, a predicted Tyr-kinase with ankyrin repeats (DDB-G0278521), was differentially enriched according to Skp1 modification status. CulA, previously observed to associate with the FBP FbxA (36), was also detected, but its abundance was too low to meet our reportability criteria, possibly because of competitive binding with pAb UOK77. CulE and Rbx1 were also detected as expected (Figs. 3 and 4), at similarly low levels.

In an attempt to complement these studies, we examined the interactome of FLAG-Skp1 overexpressed in Ax3 cells using anti-FLAG beads. However, no significant proteins interactors were detected, despite the utility of this construct in yeast (37). Interestingly, we previously noted that FLAG-Skp1 remains unmodified when expressed in Dictyostelium (14), indicating that perturbation of its N terminus interferes with not only its ability to enter SCF complexes, but also its hydroxylation and glycosylation.
Thus, this mutation did not grossly compromise FbxD expression or subcellular localization, consistent with previous studies showing that such mutations do not unduly distort the tertiary structure of FBP.

In proteomic surveys of the co-IPs, similar levels of FbxD-FLAG were obtained from wt and phyA– cells (Fig. 6A). In contrast, Skp1 recovery with FbxD was 3-fold reduced in phyA– cells, which was consistent with the glycosylation dependence of the reciprocal co-IP (Fig. 5). Co-IPs of FLAG-FbxD(LP-AA) recovered little Skp1 from either strain (AA:LP ratio <<1 in Fig. 6B), confirming the F-box dependence of the interaction. A putative GTPase (DDB_G0283979) bound to FLAG-FbxD in a pattern complementary to that of Skp1, but the small increase in binding to mutant FLAG-FbxD(LP-AA) (Fig. 6B) indicates that the GTPase does not compete with Skp1 binding, unless the GTPase also depends on an intact F-box. CulE and the associated Rbx1 were observed in the FbxD complexes, as expected from the reciprocal co-IP (Fig. 4); the absence of a reproducible difference between wt and phyA– cells might be due to the lower spectral count level.

Fig. 5. Proteomic analysis of Skp1 interactions. Slug-stage cells were subjected to co-IP with affinity-purified anti-Skp1 (UOK77)-coupled beads. Data from four co-IPs conducted under two conditions (pH 7.4 or pH 8.0; 0.2% or 1% Nonidet P-40), each conducted in triplicate, were pooled owing to the absence of systematic differences, and total spectral counts are reported. A, Ax3 and phyA– are compared. Asterisks denote those proteins for which the quantitative difference between wt and phyA– cells was observed in all trials in which the protein was detected. B, comparison of phyA–, gntT–, pgtA–, and agtA– and wt (Ax3) cells. Protein descriptors and RNA expression patterns are in supplemental Table S1. Full data are shown in supplemental Table S3.
from these co-IPs. Notably, CulA and CulB were not detected, suggesting that FbxD is selective for CulE. Aside from predicted chaperone proteins, 25 additional FbxD-associated proteins were identified, and the 20 most abundant are included in Fig. 6. Interestingly, five reproducibly favored binding to FLAG-FbxD when Skp1 was glycosylated, whereas three favored binding when Skp1 was unmodified (Fig. 6A). The latter three are candidates for substrates that are expected to accumulate in association with FbxD in the absence of binding to Skp1, although at this point differential abundance in the extract cannot be excluded. Nevertheless, the \( \geq 2 \)-fold increase observed for 12 proteins in co-IPs from cells expressing FLAG-FbxD(LP-AA) relative to FLAG-FbxD regardless of Skp1 modification status (Fig. 6B) reinforces their candidacy as substrates to be explored in the future.

The FLAG-FbxD co-IPs also contained members of the Hsp20 (HspG, HspL, HspM), Hsp70 (HspH), and Hsp90 (HspD) classes of heat-shock chaperones, and all members of the Cct/TriC class of chaperonins (supplemental Fig. S3). Unlike other Hsp-like proteins, including HspA (Hsp60-like),
Glycosylation-dependent Skp1 Interactome

Fig. 7. Glycosylation dependence of the FbxD interaction with Skp1. Slug extracts (1% Nonidet P-40, pH 7.4) of pathway knockout strains expressing FLAG-FbxD (as in Fig. 6) were subjected to co-IP using anti-Skp1 (A) or anti-FLAG (B) antibodies. Representative Western blots probed with mAb 4E1 (pan-specific toward Skp1 isoforms) or anti-FLAG (M2) are shown. The migration of Skp1 decreased with increasing modification. Bar graphs represent densitometric analysis of Western blots from three independent trials (average values ± S.E.). Intensities were normalized to the maximum signal within each experiment. C, similar analysis of FLAG-FbxD(LP-AA) expressed in wt slug cells.

HspB (Hsc70-like), and HspE-1 (Hsc70-like), these chaperones were not observed in control co-IPs. These chaperones were also not detected in the co-IPs targeting Skp1 or CulE-FLAG, except for the Ccts, which were also observed in the control Skp1 co-IPs and thus excluded. The spectral counts for individual chaperones ranged from very low to up to 153 for individual chaperones. In addition, the levels of co-immunoprecipitated Hsps were reproducibly increased by 2-fold to up to eight Ccts detected. In contrast, minimal FLAG-FbxD(LP-AA) associated with Skp1 (Fig. 7C), confirming its F-box dependence. Thus, formation of the trisaccharide promotes the Skp1–FbxD interaction in cells, which is further enhanced by completion of the pentasaccharide.

**FLAG-FbxD Delays Development in an F-box- and Glycosylation-dependent Fashion**—Previous studies showed that disruption of phyA delays developmental progression, mainly at the culmination stage (transition from slug to fruiting body), by ~3 h at 21% O2 (14). Our novel findings suggested that the underlying mechanism might be an O2- and prolyl-hydroxylation-regulated interaction with FbxD and FbxA. To address this, we analyzed the developmental timing of the FLAG-FbxD overexpression strains. All strains achieved the slug stage of development at about the same time (11 h under the conditions employed here) (Fig. 8). FLAG-FbxD overexpression delayed culmination by about 3 h in wt cells, almost as much as the 4-h delay caused by blocking Skp1 modification. We suggest that overexpression of this FBP upsets the proteostatic network in cells via an as yet uncertain mechanism. An intermediate effect was rendered by the F-box mutant FLAG-FbxD(LP-AA), indicating that inhibition is partially dependent on Skp1 interaction. In contrast, FLAG-FbxD overexpression had no effect on phyA− cells, which is consistent with their acting in the same pathway where Skp1 interaction was already reduced when Skp1 was not modified.

**DISCUSSION**

**Dictyostelium SCF Complexes**—Results presented here confirm bioinformatic findings and limited experimental data that predict that Dictyostelium assembles a canonical family of conventional SCF complexes and corresponding E3SCFUB ligases (Fig. 9). The Dictyostelium SCF repertoire appears to be expanded, however, by the existence of multiple Cul1s, including the previously described CulA, CulE identified here, and possibly CulB (42). Each possesses typical Cul1-like sequence motifs (supplemental Fig. S2). In this work, CulE was associated with the Dictyostelium homolog of Rbx1, with...
relative spectral counts suggesting a stoichiometric relationship. CulE was mostly neddylated, implying its constitutive activation. Skp1 was a prominent interactor of CulE. Three new potential F-box proteins were detected in addition to FbxA (36). One, FbxD, was validated by site-directed mutagenesis and, of the three, preferentially associated with CulE. As CulE was presumably expressed at native levels, these interactions may represent what occurs in the cell. Candidate E2 sequences were not detected, but their association is expected to be labile.

The CulE interactome also included additional proteins that could be classified as (i) regulatory, (ii) function related, (iii) previously observed but unknown, and (iv) unknown. Known regulators included Cand1 and the COP9 signalosome (CSN). All eight subunits of the previously characterized Dictyostelium CSN (43), expected to mediate CulE deneddylation, were found in association with CulE (Fig. 3). CSN5 and CSN2 are essential for Dictyostelium proliferation, but because the CSN may also affect other CRLs, a specific role for CRL1s is not certain. As expected, Dictyostelium expresses other genes expected for CulE neddylation (supplemental Table S1). In other organisms, Cand1 associates only with deneddylated Cul1, suggesting that the CulE–Cand1 interaction might have occurred following post-lysis deneddylation. Based on evidence that yeast and mammalian Cand1 is an exchange factor for Skp1–FBP complexes (3–5), Cand1 binding to CulE is expected to be competitive with Skp1 (see Fig. 2B).

Other CulE interactors were the Ariadne-related Ring-between-Ring protein RbrA and its homolog RbrB. RbrA was previously observed in association with mammalian Cul1, but the significance of this interaction is poorly understood (34). Disruption of the Dictyostelium RbrA gene results in compromised fruiting body formation (44) in a way that resembles effects of Skp1 modification mutants, consistent with a possible functional interaction. Seven additional interacting candidates were identified (Fig. 3), but the significance of these unknown proteins is unclear.

Finally, all 10 subunits of the 19S-proteasome regulatory particle, but none from the 20S-core particle, were detected (supplemental Fig. S1B). Association of the SCF complex with the proteasome has been previously observed (45, 46) and might reflect functional coordination as the polyubiquitinated substrate is transferred for proteolytic degradation.

Role of Skp1 Glycosylation—In contrast to the majority of the CulE interactome, which is largely unaffected by Skp1’s O₂-regulated glycosylation, FbxD and the 19S-proteasome regulatory particle amounts were elevated by Skp1 glycosylation (Fig. 9). FbxD was enriched 2- to 5-fold in wt versus phyA° cells, and the 19S regulatory particle was enriched 5- to 9-fold. As glycosylation appears to occur only on the Skp1

Fig. 9. Summary of Skp1 interactome. Arrows depict interactions detected in the present or previous studies. The tail denotes the bait, and the tip the prey. Black indicates an interaction not affected by the modification status of Skp1. Green indicates an interaction that was greater in wt than in phyA° cells, and red denotes an interaction that was less. Gray indicates a previously described interaction (36) whose dependence on Skp1 modification has not been examined.
component of SCF complexes (8, 13, 14), this posttranslational modification might promote the assembly of or stabilize the SCF/FbxD complex. Analysis of the Skp1 interactome confirmed increased association of FbxD with fully glycosylated Skp1 (2.6-fold) and, to a lesser extent, with FGGn-Skp1, relative to the less modified isoforms (Figs. 5 and 6). A similar effect was observed for another documented FBP, FbxA. FbxA was not observed in the CulE pulldown, but given that FbxA is known to interact with CulA (36), FBP interactions might be Cul1-subtype specific, consistent with direct FBP–Cul1 contacts for some FBPs (47). Increased interaction of glycosylated Skp1 with FbxD was observed even when FLAG-FbxD was ectopically overexpressed in prespore cells, and reciprocal co-IPs confirmed the greater interaction of FbxD with glycosylated Skp1s (Figs. 6 and 7). General promotion of SCF assembly by glycosylation was suggested by gel filtration analysis of cytosolic extracts (Fig. 2). This contrasts with a proposed role for phosphorylation of yeast Skp1, which might inhibit FBP interactions (48). Stimulation of SCF assembly might explain the increased 19S-proteasomal regulatory particle interaction detected in the CulE co-IPs. Proteasomal subunits were not detected in the Skp1 pulldowns, but notably, not all Skp1 was accessible to the antibody, potentially because of steric interference in some complexes.

The mechanism by which FbxD and FbxA interact more efficiently with glycosylated Skp1 remains to be determined. We recently showed that a model soluble FBP, guinea pig Fbs1, binds preferentially to GFGGn-Skp1 and Gm-Skp1 relative to HO-Skp1 and Skp1 in vitro in the absence of other proteins (16). This suggests that glycosylation might affect Skp1 directly to promote its interactions with FBPs. However, the pattern differs from that of FbxD and FbxA, where GGF-GGn-Skp1 binds substantially better than FGGn-Skp1 and Gm-Skp1. Possibly, peripheral sugars of the glycan interact with certain Dictyostelium F-box domains to stabilize the interaction. Alternatively, an additional factor that recognizes the glycan (or is blocked by the glycan) contributes to differential recognition. To this point, 11 other proteins were less abundantly but reproducibly detected in the Skp1 interactome. One was reproducibly differentially affected by Skp1 glycosylation, and thus might also contribute to selective interactions according to Skp1 glycosylation status. Further studies are needed to distinguish whether increased FBP interactions reflect differential affinity or rates of assembly, or selective stability following cell disruption. The decreased interaction of fully glycosylated Skp1 with Jcdl (Fig. 5) raises the possibility of differential effects of glycosylation on substrate receptor and non-receptor subclasses of FBPs, such that glycosylation might affect FBP selectivity in the competitive environment occurring in the cell. FbxD, like almost all known FBPs (except guinea pig Fbs1), is insoluble when expressed in *Escherichia coli* in the absence of Skp1 (data not shown), making direct biochemical analysis challenging.

FBPs are typically insoluble in the absence of Skp1, and both FLAG-FbxD expressed in *phyA* cells and mutant FLAG-FbxD(LP-AA) are soluble in extracts, so how does this occur if they are not binding Skp1? Two of the detected FLAG-FbxD interactors shown in Fig. 6 match the predicted pattern of increased binding to FLAG-FbxD in *phyA* cells and increased binding to mutant FLAG-FbxD(LP-AA) in either background (RasD and a predicted pyrophosphatase). Chaperones are also candidates to maintain FbxD and other FBPs in solution until Skp1 becomes available, as suggested by the increased association of Hsp20, Hsp70, Hsp90, and Cct/TriC proteins with mutant relative to native FLAG-FbxD (supplemental Fig. S3). Although we did not observe an increased association of these chaperones with FLAG-FbxD in *phyA* relative to wt cells, Hsp70 and Cct/TriC proteins have been implicated in the assembly of the related CRL2 E3 Ub ligases (41). In mammalian cells, some FBPs can bind nuclear exportin-1 in place of Skp1 (49). Although the related *Dictyostelium* protein Xpo2 was detected, it was not enriched in the Skp1 co-IPs from *phyA* versus wt cells.

With its C-terminal WD40 repeats, FbxD is likely to bind multiple substrates like other FbxW-type FBPs such as mammalian βTrCP and FbxW7 and yeast Grr1 (6). Many of the FLAG-FbxD interactors are potential substrates owing to their increased association when the F-box was mutated, and a subset of these was increased in *phyA* relative to wt cells (Fig. 6). Prediction of substrates is, however, confounded by potentially different levels of the interacting protein available for binding and potential targeting by other polyubiquitin ligases. Poor evolutionary conservation of FBP sequences precludes prediction of FbxD and other FBP substrates, which are under investigation using orthogonal approaches.

**Role of Skp1 Modification in O2 Sensing**—We propose a novel model in which the activity of certain FBPs such as FbxD and FbxA is regulated by cellular nutritional status. In this model, hydroxylation of newly synthesized Skp1 is controlled by the level of ambient O2 (Fig. 1). Skp1 might also respond to other metabolic factors such as the co-substrate α-ketoglutarate and another measure of the nutritional or metabolic status of the cells (i.e. sugar-nucleotide concentrations to support glycosylation). We propose that O2 levels low enough to inhibit culmination trap newly synthesized Skp1 in its unglycosylated form, and this isoform persists for a period of time inversely proportional to the available O2. Hypoglycosylation of Skp1 is predicted to inhibit its association with developmentally regulated and newly synthesized substrate receptor FBPs such as FbxD and FbxA. Thus, limitations in O2 and other metabolic substrates could regulate the assembly of key E3SCFUb-ligase complexes, which in turn would regulate the polyubiquitination and degradation of proteins that control the progression of *Dictyostelium* development. This model is consistent with (i) data from a submerged O2-restricted model of development, where accumulation of unmodified Skp1 correlated with inability to sporulate (10); (ii)
Glycosylation-dependent Skp1 Interactome

4. Wu, S., Zhu, W., Nhan, T., Toth, J. I., Petroski, M. D., and Wolf, D. A. (2013) Prolyl 4-hydroxylase of Dictyostelium mediates glycosylation-independent and -dependent responses to O2 without affecting Skp1 stability. J. Biol. Chem. 287, 2006–2016

5. Zhang, D., van der Wel, H., Johnson, J. M., and West, C. M. (2012) Skp1 Prolyl 4-hydroxylase of Dictyostelium mediates glycosylation-independent and -dependent responses to O2 without affecting Skp1 stability. J. Biol. Chem. 287, 2006–2016

6. Sheikh, M. O., Schafer, C. M., Powell, J. T., Rodgers, K. K., Mooers, B. H., and West, C. M. (2014) Glycosylation of Skp1 affects its conformation and promotes binding to a model F-box protein. Biochemistry 53, 1657–1669

7. Ketcham, C., Wang, F., Fisher, S. Z., Ern, A., van der Wel, H., Locke, R. D., Sirajud-Doula, K., Matta, K. L., and West, C. M. (2004) Specificity of a soluble UDP-galactose:galactose α1,3galactosyltransferase that modifies the cytoplasmic glycoprotein Skp1 in Dictyostelium. J. Biol. Chem. 279, 29050–29059

8. Zhang, Y., Zhang, P., and West, C. M. (1999) A linking function for the cell-cycle-binding protein SP85 in the spore coat of Dictyostelium discoidium. J. Cell Sci. 112 (Pt 23), 4367–4377

9. Faix, J., Kreppel, L., Shaulsky, G., Schleier, M., and Kimmel, A. R. (2004) A rapid and efficient method to generate multiple gene disruptions in Dictyostelium discoideum using a single selectable marker and the Cre-loxP system. Nucleic Acids Res. 32, e143

10. Pang, K. M., Lynes, M. A., and Knecht, D. A. (1999) Variables controlling the expression level of exogenous genes in Dictyostelium discoideum mutants by extrachromosomal expression of Cre. Eur. J. Cell Biol. 91, 156–160

11. van der Wel, H., Ern, A., and West, C. M. (2005) The Skp1 prolyl 4-hydroxylase from Dictyostelium is related to the hypoxia-inducible factor-class of animal prolyl 4-hydroxylases. J. Biol. Chem. 280, 14645–14655

12. Voruganti, S., Lacroix, J. C., Rogers, C. N., Rogers, J., Matts, R. L., and Hartson, S. D. (2013) The anticancer drug AUY922 generates a proteomics fingerprint that is highly conserved among structurally diverse Hsp90 inhibitors. J. Proteome Res. 12, 3697–3706

13. West, C. M., van der Wel, H., Chinoy, Z., Boons, G.-J., Gauthier, T. J., Taylor, C. M., and Xu, Y. (2014) Generating isoform-specific antibodies: lessons from the nucleocytoplasmic glycoprotein Skp1. In Glycoscience: Biology and Medicine (Taniguchi, N., Endo, T., Hart, G., Seeberger, P., and Wong, C.-H., eds), Springer, New York, in press.

14. Bosu, D. R., Feng, H., Min, K., Kim, Y., Wallenfang, M. R., and Kipreos, E. T. (2010) C. elegans CAND-1 regulates cullin neddylation, cell proliferation and morphogenesis in specific tissues. Dev. Biol. 346, 113–126

15. Read, M. A., Brownell, J. E., Gladysheva, T. B., Hottelet, M., Parent, L. A., Coggins, M. B., Pierce, J. W., Podust, V. N., Luo, R. S., Chau, V., and Palombella, V. J. (2000) Nedd8 modification of Cul-1 activates SCF

16. Palombella, V. J. (2000) Nedd8 modification of Cul-1 activates SCF

17. Ohh, M., Kim, W. Y., Moslehi, J. J., Chen, Y., Chau, V., Read, M. A., and Kaelin, W. G., Jr. (2002) An intact NEDD8 pathway is required for Cullin-dependent ubiquitylation in mammalian cells. EMBO J. 21, 177–182

18. Lopokopulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998) A novel protein modification pathway related to the ubiquitin system. EMBO J. 17, 2208–2214

19. del Pozo, J. C., and Estelle, M. (1999) The Arabidopsis cullin AtCUL1 is modified by the ubiquitin-related protein RUB1. Proc. Natl. Acad. Sci. U.S.A. 96, 15342–15347
30. Pintard, L., Kurz, T., Glaser, S., Willis, J. H., Peter, M., and Bowerman, B. (2003) Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in C. elegans. Curr. Biol. 13, 911–921

31. Lundgren, D. H., Hwang, S. I., Wu, L., and Han, D. K. (2010) Role of spectral counting in quantitative proteomics. Expert Rev. Proteomics 7, 39–53

32. West, C. M., Kozarov, E., and Teng-umnuay, P. (1997) The cytosolic glycoprotein FP21 of Dictyostelium discoideum is encoded by two genes resulting in a polymorphism at a single amino acid position. Gene 200, 1–10

33. Finley, D. (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu. Rev. Biochem. 78, 477–513

34. Kelsall, I. R., Duda, D. M., Olszewski, J. L., Hofmann, K., Knebel, A., Langevin, F., Wood, N., Wightman, M., Schulman, B. A., and Alpi, A. F. (2013) TRIAD1 and HHARI bind to and are activated by distinct neddylated Cullin-RING ligase complexes. EMBO J. 32, 2848–2860

35. Parikh, A., Miranda, E. R., Katoh-Kurasawa, M., Fuller, D., Rot, G., Zagar, L., Sucgang, R., Chen, R., Zupan, B., Loomis, W. F., Kuspa, A., and Shaulsky, G. (2010) Conserved developmental transcriptomes in evolutionarily divergent species. Genome Biol. 11, R35

36. Mohanty, S., Lee, S., Yadava, N., Dealy, M. J., Johnson, R. S., and Firtel, R. A. (2001) Regulated protein degradation controls PKA function and cell-type differentiation in Dictyostelium. Genes Dev. 15, 1435–1448

37. Seol, J. H., Shevchenko, A., Shevchenko, A., and Deshaies, R. J. (2001) Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. Nat. Cell Biol. 3, 384–391

38. Kondo-Okamoto, N., Ohkuni, K., Kitagawa, K., McCaffery, J. M., Shaw, J. M., and Okamoto, K. (2006) The novel F-box protein Mfb1p regulates mitochondrial connectivity and exhibits asymmetric localization in yeast. Mol. Biol. Cell 17, 3756–3767

39. Price, C. T., Al-Quadan, T., Santic, M., Rosenshine, I., and Abu Kwaik, Y. (2011) Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. Science 334, 1553–1557

40. Russell, I. D., Grancell, A. S., and Sorger, P. K. (1999) The unstable F-box protein p58-Ctf13 forms the structural core of the CBF3 kinetochore complex. J. Cell Biol. 145, 933–950

41. Melville, M. W., McClellan, A. J., Meyer, A. S., Darveau, A., and Frydman, J. (2003) The Hsp70 and TRiC/CCT chaperone systems cooperate in vivo to assemble the von Hippel-Lindau tumor suppressor complex. Mol. Cell. Biol. 23, 3141–3151

42. Wang, B., and Kuspa, A. (2002) CulB, a putative ubiquitin ligase subunit, regulates preprokary morphogenesis in Dicyostelium spp. Eukaryot. Cell 1, 126–136

43. Rosel, D., and Kimmel, A. R. (2006) The COP9 signalosome regulates cell proliferation of Dicyostelium discoideum. Eur. J. Cell Biol. 85, 1023–1034

44. Whitney, N., Pearson, L. J., Lunsford, R., McGill, L., Gomer, R. H., and Lindsey, D. F. (2006) A putative Ariadne-like ubiquitin ligase is required for Dicyostelium discoideum development. Eukaryot. Cell 5, 1820–1825

45. Farras, R., Ferrando, A., Jasik, J., Kleinow, T., Okrez, L., Tiburcio, A., Salchert, K., del Pozo, C., Schell, J., and Koncz, C. (2001) SKP1–SnRK protein kinase interactions mediate preprokary binding of a plant SCF ubiquitin ligase. EMBO J. 20, 2742–2752

46. Voloshin, O., Bakhrat, A., Herrmann, S., and Raveh, D. (2012) Transfer of Ho endonuclease and Ufo1 to the proteasome by the Ubl-UbA shuttle protein, Ddi1, analysed by complex formation in vitro. PLoS One 7, e39210

47. Schiman, B. A., Carrano, A. C., Jeffrey, P. D., Bowen, Z., Kinnucan, E. R., Finnin, M. S., Elledge, S. J., Harper, J. W., Pagano, M., and Pavletich, N. P. (2000) Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. Nature 408, 381–386

48. Beltrao, P., Albanese, V., Kenner, L. R., Swaney, D. L., Burlingame, A., Villen, J., Lim, W. A., Fraser, J. S., Frydman, J., and Krogan, N. J. (2012) Systematic functional prioritization of protein posttranslational modifications. Cell 150, 413–425

49. Nelson, D. E., and Laman, H. (2011) A competitive binding mechanism between Skp1 and Exportin 1 (CRM1) controls the localization of a subset of F-box proteins. J. Biol. Chem. 286, 19804–19815

50. Ratcliffe, P. J. (2013) Oxygen sensing and hypoxia signalling pathways in animals: the implications of physiology for cancer. J. Physiol. 591 (Pt 8), 2027–2042