O₂ sensing–associated glycosylation exposes the F-box–combining site of the Dictyostelium Skp1 subunit in E3 ubiquitin ligases

Received for publication, July 27, 2017, and in revised form, September 12, 2017. Published, Papers in Press, September 19, 2017, DOI 10.1074/jbc.M117.809160

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Abstract

Skp1 is a conserved protein linking cullin-1 to F-box proteins in SCF (Skp1/Cullin-1/F-box protein) E3 ubiquitin ligases, which modify protein substrates with polyubiquitin chains that typically target them for 26S proteasome-mediated degradation. In Dictyostelium (a social amoeba), Toxoplasma gondii (the agent for human toxoplasmosis), and other protists, Skp1 is regulated by a unique pentasaccharide attached to hydroxylated Pro-143 within its C-terminal F-box–binding domain. Prolyl hydroxylation of Skp1 contributes to O₂-dependent Dictyostelium development, but full glycosylation at that position is required for optimal O₂ sensing. Previous studies have shown that the glycan promotes organization of the F-box–binding region in Skp1 and aids in Skp1’s association with F-box proteins. Here, NMR and MS approaches were used to determine the glycan structure, and then a combination of NMR and molecular dynamics simulations were employed to characterize the impact of the glycan on the conformation and motions of the intrinsically flexible F-box–binding domain of Skp1. Molecular dynamics trajectories of glycosylated Skp1 whose calculated monosaccharide relaxation kinetics and rotational correlation times agreed with the NMR data indicated that the glycan interacts with the loop connecting two α-helices of the F-box–combining site. In these trajectories, the helices separated from one another to create a more accessible and dynamic F-box interface. These results offer an unprecedented view of how a glycan modification influences a disordered region of a full-length protein. The increased sampling of an open Skp1 conformation can explain how glycosylation enhances interactions with F-box proteins in cells. Members of the Skp1/Cullin-1/F-box protein (SCF) family of E3 ubiquitin ligases modify protein substrates with polyubiquitin chains of a type that typically targets them for degradation by the 26S proteasome (1). Skp1 is an adaptor linking the F-box protein (FBP) to the N terminus of cullin-1 (Cul1), and the E2-Ub donor is linked via the RING protein Rbx1 to the other end of Cul1 (1, 2). Substrates are selected by protein–protein interaction domains of FBPs, which number from dozens to hundreds, depending on the species. Alternatively, FBPs may be substrates themselves. The enzyme complex is subject to regulation at multiple levels. Substrate priming, such as by phosphorylation, can promote binding to its cognate FBP (3), which in turn promotes binding of the FBP/Skp1 subcomplex to Cul1. This interaction is regulated by neddylation of Cul1, which inhibits binding of the conserved Cand1 inhibitor protein that dissociates Skp1 from Cul1 (4). Neddylation also activates Rbx1 via a tethering process, and every cycle of Ub addition requires replacement of the spent E2 by a new E2-Ub. Neddylation is mediated by a separate E3 subunit and is removed by the COP9 signalosome, which are under separate regulation (5).

The complex also appears to be regulated at the level of the Skp1/FBP interaction by a novel mechanism in select unicellular eukaryotes (6). Skp1 in the social amoeba Dictyostelium and the human parasite Toxoplasma is modified near its C-terminal FBP-combining region by an O₂-dependent prolyl hydroxylase, PhyA (7, 8). The resulting hydroxyproline (Hyp) is subject to glycosylation by the sequential action of multiple sugar nucleotide glycosyltransferases (see Fig. 1A) that result, ultimately, in assembly of a pentasaccharide (6, 9). Genetic and biochemical

This work was supported in part by National Institutes of Health Grants R01 GM037539, U01 CA207824, P41 GM103390, and P41 RR018502. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains supplemental Tables S1–S8 and Figs. S1–S11. 1 Both authors contributed equally to this work. 2 To whom correspondence should be addressed: 120 E. Green St., Davison Life Sciences A310, Athens, GA 30602. Tel.: 706-542-4259; E-mail: westcm@uga.edu.

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studies indicate that Skp1 prolyl hydroxylation and glycosylation promote, in additive fashion, O_{2}^{-}-dependent development in *Dictyostelium*, enabling conversion of the slug to a fruiting body (10). Interactome studies with various subunits of the SCF complex confirm its existence in *Dictyostelium* and show, by analysis of mutants that interrupt the hydroxylation/glycosylation pathway, that the Skp1/FBP interaction is promoted by full glycosylation (11). The effect was partially recapitulated in an *in vitro* analysis of the interaction between *Dictyostelium* Skp1 and an FBP that has the unusual property of being soluble in the absence of Skp1, guinea pig Fbs1 (12). Fbs1 is unlikely to recognize the glycan directly, because Skp1 is not apparently glycosylated in animals. Because Pro-143 lies in the vicinity of the FBP-combining site of Skp1, this finding suggests that the glycan affects Fbs1 binding via an indirect, perhaps conformational, effect on Skp1. The Skp1/FBP interface might also be subject to regulation in yeast and mammalian cells (13–15), which apparently lack the Skp1 hydroxylation/glycosylation pathway.

Small angle X-ray scattering and circular dichroism studies indicate that unmodified *Dictyostelium* Skp1 is partially disordered, which is supported by NMR-based solution studies and NMR analysis and molecular dynamics (MD) simulations of human Skp1 (16, 17). Glycosylation renders intrinsic changes in Skp1 folding, including increased α-helical content based on CD, and less disorder and an extended envelope based on SAXS analyses (11). Attempts to characterize Skp1 structures directly by crystallography have been unsuccessful, except when in complexes with FBPs (18, 19). The MD simulations of human Skp1 suggest greater mobility near the distal end of the C-terminal FBP interaction region, relative to the more stable N-terminal Cul1-binding region. Although glycans are typically thought to be relatively disordered and, therefore, to be freely rotating in solution at protein surfaces, there is precedent for glycans interacting with protein surfaces and influencing their conformation (20). Given the effects of the Skp1 glycan on both its structure and its interactome and function in cells, we sought to investigate its effect on Skp1 at a more granular level.

In this study, NMR was employed to identify the previously unknown linkage of the non-reducing terminal Gal (αGal2), confirm other linkages, and, together with MD simulations, investigate the organization and dynamics of the fully formed pentasaccharide and its relation to and influence on the Skp1 polypeptide. The studies indicate that the Skp1 glycan adopts a stable conformation that affects Skp1 organization in a way that can explain previous SAXS findings, resulting in a structural model that explains how glycosylation promotes binding to FBPs. The insights afforded by this model will open new avenues to evaluate the contribution of glycosylation to cellular proteostasis and its impact on proteostasis, including infectivity of those that cause disease in plants, humans, and other animals.

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**Results**

**Primary structure of the oligosaccharide component of the Skp1 glycoprotein**

The Skp1 glycoprotein was purified from ~2.4 × 10^{12} *Dictyostelium* cells as described under “Experimental procedures,” yielding ~2 mg (100 nmol) of the native molecule. Because no method was known for release of the oligosaccharide from its hydroxyproline linkage, peptides were prepared using endo-

Lys-C, and the glycopeptide was purified using HPLC (Fig. 1B). It is worth noting that some contaminating peptides remained after preparative purification and that a major loss (~90%) of the glycopeptide occurred during the final HPLC step, which involved the use of trifluoroacetic acid. Less than 10 nmol was recovered using the preparation isolated directly from *Dictyostelium*.

As outlined in Fig. 2A, this quantity was sufficient for partial analysis. Mass spectrometry (MALDI-TOF-MS) of a portion of the intact glycopeptide sample indicated an oligosaccharide having three hexoses, one deoxyhexose, and one N-acetylgalactosamine residue (Fig. 1C), attached at a hydroxyproline that was present in the NDFTP§EEEEQIK sequence of Skp1. A small contamination with a peptide having the sequence ENEWCEDK was observed. 1H NMR of the glycopeptide sample revealed four α-anomeric (H1) signals (Fig. 1D). One, later established as the αGlCNAC H1, showed heterogeneity in its frequency, due to either conformational or structural heterogeneity of the peptide component. An H1 signal of a β-linked sugar was not assigned due to overlap of peptide H α signals upfield of the HOD peak, but it was assigned later using recombinant glycopeptide and 2D NMR experiments.

With the recent availability of multimilligram quantities of recombinant Skp1 glycoprotein modified with the GlcNAc at Hyp143 (12), purified PgtA (βGalT/αFucT) and the AgtA α-galactosyltransferase were used to sequentially add the remaining four sugar residues (outlined in Fig. 2B, *upper arm*). The final two Gal residues, αGal1 and αGal2, were derived from UDP-Gal or UDP-[1-^{13}C]Gal. It is important to note that enzymatic transfer of both the βGal residue and αGal2 required the intact Skp1 glycoprotein (*i.e.* they did not occur using glycopeptide substrates) (21, 22).

Following incorporation of two [1-^{13}C]Gal residues, the Skp1 glycoprotein product was digested with Pronase, and to facilitate glycopeptide purification, the Pronase digest was amended with 1% of material from a parallel reaction labeled with [3H]Gal. A neutral pH method was developed for isolation using C_{18}-SepPak and Carbograph cartridges, which gave a 71% yield based on tritium recovery, the predominant glycopeptide product having two [1-^{13}C]Gal residues. MALDI-TOF-MS analysis revealed enrichment of four major Pronase glycopeptides, which corresponded to the sequences TP§EEEEQ, FTP§EEEEQ, DFTP§EEEEQ, and NDFTP§EEEEQ (Fig. 2, compare C and D, where § corresponds to the [1-^{13}C]Gal-labeled pentasaccharide attached to a hydroxyproline residue, as indicated in Fig. 1A. The reason for incomplete Pronase digestion is unknown. Each glycopeptide contained a ~2 m/z mass increment due to the presence of two [1-^{13}C]Gal residues, as com-

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pared with an additional sample using unlabeled, conventional UDP-Gal (data not shown).

A 1D $^1$H NMR spectrum of the [1-13C]Gal-containing glycopeptide preparation revealed six anomeric signals in the region downfield of the HOD peak where H1 signals of α-linked sugars commonly reside (Fig. 3A and supplemental Table S1). Four of these corresponded to two 1-13C–labeled Gal residue H1 signals, each split further due to the one-bond $J_{C,H}$ coupling of nearly 170 Hz, whereas two other H1 signals were typical of unlabeled sugars. For the 13C-labeled Gal residues, the large

Figure 1. The Skp1 glycosylation pathway and isolation of the Skp1 glycopeptide from native GGFGGn-Skp1. A, schematic of the Dictyostelium Skp1 hydroxylation/glycosylation pathway. A prolyl hydroxylase and five sequentially acting glycosyltransferase activities present in three proteins modify Pro-143 with a linear pentasaccharide. The linkage assignments anticipate results from the present study. B, final HPLC purification step of the endo-Lys-C–generated glycopeptide from native Skp1 isolated directly from Dictyostelium. The desired glycopeptide (NDFTP§ EEEEQIRK) eluted at about 5.9 ml, following a minor (based on $A_{215}$) contaminating peptide (ENEWCEDK), distinguished by its m/z value in MALDI-TOF-MS (not shown), and strong absorbance at 280 and 263 nm due to its Trp content. The glycopeptide showed minor absorbance at 263 nm due to its Phe content. AU = absorbance units.

C, composition of fraction 31 based on MALDI-TOF-TOF-MS in positive-ion mode, which revealed a predominant species at m/z 2486.3, corresponding to the [M + H]$^+$ ion of the intact glycopeptide, and minor fragment ions attributed to loss of sugar residues resulting from in source decay. D, 1D $^1$H NMR spectrum of the glycopeptide pool (fractions 30–33). The peak marked with an X is not reproducible and does not couple to any other protons in the gCOSY experiment in Fig. 4B.
$^{1}J_{C,H}$ coupling collapsed (Fig. 3B) when carbon decoupling was carried out during the directly detected free induction decay, enabling these H1 signals to be assigned to the αGal residues. It is also worth noting that due to peptide heterogeneity, the αFuc and αGlcNAc H1 signals showed some frequency heterogeneity (Fig. 3, A and B). Nearly identical 1D NMR results were observed for the authentic glycopeptide mixture isolated from Skp1 (Fig. 1D) and the recombinant glycopeptide mixture, with minor differences being attributed to differences in their attached peptide(s). For the recombinant sample, a fifth anomic H1 signal (near 4.67 ppm), partially obscured near the HOD peak in 1D spectra, was only revealed in 2D experiments (see below).

Two-dimensional $^1$H–$^1$H gradient-enhanced correlation spectroscopy (gCOSY) experiments (Fig. 4, A and B) were performed, either containing [1-$^{13}$C]Gal with $^{13}$C decoupling in the directly detected dimension (Fig. 4A) or without the $^{13}$C labeling using the native glycopeptide (Fig. 4B). The H1 and H2 chemical shift values of the glycopeptide from the enzymatically generated sample closely matched that of the glycopeptide

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**Figure 2.** Overall strategy and isolation of Pronase-generated glycopeptides from recombinant GGFFGn-Skp1. A and B, outline of sample preparation and analyses performed on native (A) or recombinant (B) GGFFGn-Skp1. C, G*G*FGGn-Skp1 was prepared by recombinant by co-expression in E. coli with PhyA and Gnt1, purification, and ex vivo glycosylation with PgtA and AgtA. The AgtA reaction was conducted in the presence of UDP-[1-$^{13}$C]Gal, designated by asterisks. Glycopeptides were prepared from G*G*FGGn-Skp1 by Pronase digestion and analyzed by MALDI-TOF-MS in positive-ion mode. § denotes glycan (pentasaccharide). D, same, after glycopeptide enrichment over C18 and Carbograph cartridges. Peaks assigned to [M + Na]$^+$ ions of Skp1 glycopeptides, based on m/z values that were +2 units relative to values obtained for similarly purified glycopeptides from unlabeled GGFFGn-Skp1 (data not shown), due to labeling with two molecules of [1-$^{13}$C]Gal, and matching theoretical expectation, are in red boldface type. All peaks whose m/z values were +2 relative to expected values are labeled in red.
**Glycan-induced conformation change**

from native Skp1 (supplemental Table S2), suggesting that ex vivo glycosylation was faithful. Assignments of the H1, H2 cross-peaks in the gCOSY experiment were made in conjunction with the purely absorptive, in-phase gradient total correlation spectroscopy (gTOCSY) experiments utilizing zero-quantum dephasing and the 13C decoupling in the direct dimension. Resonance assignments are marked and correspond to residues of the Skp1 pentasaccharide. The βGal-H1 is near 4.67 ppm and is not marked due to partial overlap of peptide H-α signals. Chemical shifts are relative to internal acetone at 25 °C (δ = 2.225 ppm). Heterogeneity in αFuc H1 and αGlcNAc H1 signals is attributed to peptide heterogeneity.

However, the 1H-13C gradient-enhanced heteronuclear multiple bond correlation (gHMBC) experiment was performed, using a range of delays for different experiments. The spectrum shown in Fig. 4D was optimized for a 3.5-Hz J coupling between 13C and 1H. A large, unique interresidue 3J-C,H from the internal αGal C1 to the Fuc H3 was observed, and an additional interresidue 3J-C,H was seen from the terminal αGal (αGal2) C1 across the glycosidic bond to the internal αGal (αGal1) H3, which clearly indicated 1-3 linkages between both sugar pairs. The H3 of the external αGal2 had a chemical shift (0.002 ppm) slightly different from that of the H2 of the internal αGal1, so both the interresidue and intraresidue cross-peaks could be clearly discriminated. As indicated in the legend to Fig. 4D, two additional cross-peaks remained unassigned. They are possibly three-bond intraresidue couplings between C1 and H5 of the αGal residues but could not be unambiguously assigned because, in the gTOCSY experiment, magnetization did not extend beyond the H4 multiplet; thus, the H5 frequencies for these spin systems could not be independently assigned in this glycopeptide sample. These experiments established directly through bond the non-reducing terminal trisaccharide as α-D-Galp-(1-3)-α-D-Galp-(1-3)-α-L-Fucp-(1-R), R being the remaining β-D-Gal and α-D-GlcNac residues. It is also significant to note that corroborative data exists for the internal Galα1-3Fuc linkage through studies of the enzymatic product from the action of AgtA on p-nitrophenyl-Fuc, based on chromatographic co-elution with one of three synthetic standards (23). Both αGal linkages were susceptible to the action of green coffee bean α-galactosidase on the glycopeptide, and the terminal αGal2 was selectively released by Xanthomonas manihotis α-1,3,6-galactosidase. Thus, in the in vitro enzymatic synthesis using AgtA, both αGal residues were transferred to Fuc-Gal-GlcNAc-Skp1 in a manner consistent with the in vivo biosynthetic product (24).

The glycan structure was determined to be linear based on MS/MS and MS² analysis of a permethylated derivative of the glycopeptide. Dissociation of the unlabeled, doubly charged sodium adduct of a permethylated Skp1 glycopeptide precursor ion (m/z 1226; Fig. 5), yielded a tetrascarbohydrate product ion of m/z 841 (Hex-Hex-deoxyHex-Hex), derived from the non-reducing end of the glycopeptide by fragmentation at the glycosidic linkage between a hexose and the core HexNAc residue. A further step of dissociation (MS³) yielded product ions of 619 and 637 (Hex-Hex-deoxyHex; Fig. 5B), with cleavages on either side of the glycosidic oxygen (supplemental Fig. S1, B and C). Isolation of the m/z 619 product ion and further dissociation (MS⁴; Fig. 5C) yielded a product ion characteristic of a Hex-Hex disaccharide (m/z 463). The deoxyHex is an α-L-Fuc residue, based on previous metabolic labeling and in vitro biosynthetic studies (25) and the NMR studies described above. Also evident in the MS³ spectrum of the isolated m/z 841 ion (Fig. 5B) were cross-ring cleavages on the hexose at the reducing end, yielding product ions of m/z 679 and 723. These product ions contained 2- and 3-carbon fragments derived from the reducing end and are characteristic of a 2-linked fucose residue (supplemental Fig. S1, D and E).

Corroborative evidence for the Fucα1-2Gal linkage is based on exoglycosidase treatment and preferences of the next glycosyltransferase in the pathway, AgtA (23, 26).
shifts were also compared with those of authentic chemically synthesized Fucα1–2Galβ1–3GlcNAcα1–4Hyp (27) and are in agreement. They are also in agreement with those of the CASPER database of all Fuc-Gal regioisomers (28) (supplemental Table S3). This database assigns NMR chemical shifts based on experimental data obtained from constit-

![A. 1H–1H-gCOSY: [1-13C]-αGal labeled glycopeptide](image1)

![B. 1H–1H-gCOSY: native glycopeptide](image2)

![C. 1H–1H-gTOCSY: [1-13C]-αGal labeled glycopeptide](image3)

![D. 1H–13C-gHMBC: [1-13C]-αGal labeled glycopeptide](image4)
GlcNAc-H3 (see Figs. 6 and 7 and corroborative evidence for the primary structure, but NOE data internal appropriate cross-peaks are shown. Artifacts from peaks negative in amplitude are due nuclear Overhauser effects were observed between the intact Skp1 glycoprotein, described below, where interresidue cross-peaks were observed between the terminal residue having a 2-linked Fuc than to any other regioisomers with a different Fuc linkage to βGal.

The final linkage, that of the βGal to the core αGlcNAc, is based on the specificity of the fucosyltransferase activity for Galβ1–3GlcNAc substrates (25). Additional corroborative evidence for the primary structure (linkages) was observed in NMR experiments using specific sugar isotopic labeling with [1-13C]Gal-labeled recombinant Skp1 with carbon decoupling in the direct dimension but not in the indirect dimension, so the 13C decoupling in the directly detected dimension. The gTOCSY experiment did not enable specific H5 -couplings, but they were not assigned because the strongest NOEs are not necessarily between protons directly across the glycosidic linkage. Taken together, the data from multiple experiments indicate that the primary structure of the oligosaccharide attached at the Hyp residue is α-β-Galp-(1–3)-α-β-Galp-(1–3)-α-1-L-Fucp-(1–2)-β-β-Galp-(1–3)-α-β-GlcNAc-(1–4)-β-Hyp.

**NMR linkage analysis of the glycopeptide attached to full-length Skp1**

The glycan was analyzed while still attached to the full-length protein, to explore conformational preferences and, considering its potential instability during purification, to confirm the glycopeptide linkage assignments. For this approach (see Fig. 2B (lower arm) for overall strategy), αGal1 and αGal2 were not unambiguous because the strongest NOEs are not necessarily between protons directly across the glycosidic linkage. Taken together, the data from multiple experiments indicate that the primary structure of the oligosaccharide attached at the Hyp residue is α-β-Galp-(1–3)-α-β-Galp-(1–3)-α-1-L-Fucp-(1–2)-β-β-Galp-(1–3)-α-β-GlcNAc-(1–4)-β-Hyp.

**Figure 4. Two-dimensional NMR spectra of [1-13C]Gal-labeled recombinant and native Skp1 glycopeptides.** A, a 1H–1H gCOSY experiment was conducted on [1-13C]Gal-labeled recombinant Skp1 with carbon decoupling in the direct dimension but not in the indirect dimension, so the 13C αGal1-H1,H2 cross-peaks appear in the indirect dimension as a pair with a near 170-Hz split. H1/H2 cross-peaks for each sugar are labeled. B, similar analysis of the native endo-Lys-C–generated (unlabeled) glycopeptide from *Dictostelium*. C, a 1H–1H gTOCSY experiment was conducted on the [1-13C]Gal-labeled recombinantly derived glycopeptide with zero-quantum dephasing and 100-ms Hartmann–Hahn matching time, with 13C decoupling in the directly detected dimension. The appropriate cross-peaks are shown. Artifacts from peaks negative in amplitude are labeled with X. D, 1H–13C gHMBC spectrum of the [1-13C]Gal-labeled recombinantly derived glycopeptide. Unique interresidue cross-peaks were observed between the terminal αGal2-C1 across the glycosidic bond to the internal αGal1-H3 and between the internal αGal-H1 and the αFuc-H3, which clearly demonstrates the presence of 1→3 linkages between both sugar pairs. Asterisks indicate possible intraresidue C1, H5 three-bond J-couplings, but they were not assigned because the gTOCSY experiment did not enable specific H5 resonances to be assigned for Gal or Fuc residues of glycopeptide samples.
uniformly labeled with $^{13}$C|Gal on recombinant $^{15}$N-labeled Skp1. Twelve peaks were resolved in the $^1$H–$^{13}$C heteronuclear single quantum correlation (HSQC) spectrum that account for all of the expected $^{13}$C atoms (supplemental Fig. S2, red). Peaks were first provisionally assigned by comparison with predicted chemical shifts calculated by the CASPER program (supplemental Fig. S3A). Among potential αGal-αGal-OMe disaccharides, Galα1, 3Galα1-OMe afforded the best fit based on summing all differ-
ntences for each cross-peak (Δppm) in both dimensions (supplementary Fig. S3B, blue bars). Of all 12 potential regioisomers of αGal-αGal-αFuc-OMe, Galα1,3Galα1,3Fuc-OMe was the best scoring match (supplementary Fig. S3B, green bars). This provisional assignment was in accord with the determination of the 13C-glycopeptide above.

To assess the connectivity of the 1H–13C peaks, a 1H–13C HSQC-TOCSY experiment, which correlates all protons within spin systems that contain 13C nuclei, was performed (supplemental Fig. S2, black). Each 1H–13C cross-peak exhibits a connectivity consistent with the CASPER predictions, validating the initial peak assignments.

To independently assess the linkage of αGal2 to αGal1, a 1H–13C HSQC-NOESY experiment was performed (Fig. 6B). A strong NOE peak is observed between αGal2-C1 and αGal1-H3, as expected for a Galα1,3Gal linkage. Interestingly, another NOE cross-peak is observed between αGal2-C2 and αGal1-H4. Whereas this might suggest a misassigned linkage, it instead indicates a conformational proximity of αGal2-H1 to both αGal1-H3 and H4 via through-space magnetization transfer. To address this, an AMBER 12 (31) minimized model of the Skp1 pentasaccharide was generated with the GlyCAM carbohydrate builder (32) using the sequence Galα1,3Galα1, 3Fucα1,2Galβ1,3GlcNAc1-OMe. In the best energy-minimized structure, H3 and H4 of αGal1 are equidistant (2.5 Å) from αGal2-H1, providing a reasonable explanation for NOEs between αGal2-H1 and both αGal1-H3 and -H4. This explanation was examined further by performing a molecular dynamics simulation of the glycans attached to Hyp (see below), with a similar interpretation. Together, these analyses confirm the Galα1,3Galα1,3Fucα1- assignment for the Pronase glycopeptide and exclude the possibility that they were the result of rearrangements during its isolation.

Further studies were performed in which only atoms C1–C6 of the first sugar, αGlcNAc, were uniformly labeled with 13C in recombinant 15N-labeled GGFFGn-Skp1. An 1H–13C HSQC spectrum (supplemental Fig. S4A) was initially assigned using CASPER as described above and validated by a 1H–13C HSQC-TOCSY experiment to assess connectivity between the 13C atoms (supplemental Fig. S4B). The internal organization of the glycan was further investigated in an 1H–13C HSQC-NOESY experiment on 13C-GGFFGnα-Skp1 (Fig. 7). The cross-peak labeled βGal-H1/GlcNAc-H3 is the expected NOE across the glycosidic bond between the Galβ1,3GlcNAc. Strong NOE cross-peaks are present between GlcNAc-H2 and Fuc-H5, GlcNAc-H2 and Fuc-H6, and GlcNAc-H4 and Fuc-H6, based on expected chemical shift values for FucH5 of 3.93–4.25 and for FucH6 of 1.21–1.22 (from carbStructDatabase and CASPER) (78). The GlcNAc-H2/Fuc-H5 NOE was previously observed in LNF-1 and LND-1, which each contain the same Fuc-Gal-GlcNAc trisaccharide (33, 34). These NOEs are consistent with the assignment of the internal glycosidic linkages and provide conformational information on the core trisaccharide suggesting that its conformation is similar to that of the free glycan.

In a regular TOCSY experiment of the same Skp1 construct, the spectrum (supplemental Fig. S5A, bottom) shows mostly glycan peaks, presumably due to the loss of signals from the broader protein resonances during the 60-ms TOCSY mixing time. Signals for the fucosyl residue are indicated, and H5 and H6 are indicated in the 1H–13C HSQC-NOESY experiment (supplemental Fig. S5A, top), confirming the assignments in Fig. 7. An expansion of the TOCSY spectrum (supplemental Fig. S5B) shows signals from all five sugar residues. The expected [13C]GlcNAc H1-H2 cross-peaks are absent, probably due to the increased line width of a residue constrained by interactions with the protein surface. However, the three galactosyl residues and the fucosyl residue show signals consistent with the glycopeptide assignments described above. The spectrum also shows ROE (rotating frame NOE) signals of opposite sign (red peaks in boxes); these are sometimes observed in TOCSY spectra of higher-molecular weight compounds. The ROEs between the αGal-H1 and αFuc-H3, and between the αFuc-H1 and βGal-H2, support those linkages assigned previously.

### Tabular Data

**Table 1**

| Glycan-induced conformation change |
|------------------------------------|

**Table 1:** Relaxation kinetics of the glycan and polypeptide of Skp1

Summary of [13C- and 15N-derived rotational correlation times for the glycan and polypeptide, respectively. Lipari–Szabo model-free order parameters (S) for the terminal galactose residues calculated using ModelFree (Palmer group) are also reported.

| Glycan | α | τ_anis | S^a |
|--------|---|-------|-----|
| Skp1   | 0.43 ± 0.04 | 34.4 ± 7.3 | 19.5 ± 2.2 |
| GGFFGn-Skp1 | 0.45 ± 0.10 | 31.1 ± 4.9 | 17.8 ± 2.1 |

| Glycan | α | τ_anis | S^a |
|--------|---|-------|-----|
| αGal2  | 2.1 ± 0.4 | 13.9 ± 5.9 | 2.6 ± 0.8 |
| αGal1  | 2.2 ± 0.4 | 20.6 ± 5.4 | 3.1 ± 0.4 |
| αGlcNAc | 1.3 ± 0.1 | 18.0 ± 2.2 | 3.1 ± 0.2 |

| Glycan | α | τ_anis | S^a |
|--------|---|-------|-----|
| Free monosaccharides^d | | 0.11 ± 0.01 |

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^a From Refs. 38–40.
^b Average of C1 to C5 values (supplemental Figs. S6 and S7) reported.
^c Average of C1 to C5 (except for C2) values (supplemental Fig. S6) reported.
^d Calculated using ModelFree (Palmer Lab) (59, 60).

**Organization and mobility of the protein-linked glycan**

To help understand how the glycan interacts with the Skp1 polypeptide, 15N and 13C spin relaxation experiments were performed to measure the molecular tumbling of αGal2 and αGal1 relative to Skp1. T1 relaxation (longitudinal or spin-lattice relaxation) describes the z component of the magnetization vector coming into thermodynamic equilibrium with its surroundings, whereas T2 relaxation (transverse or spin-spin relaxation) describes the decay of the x,y component of the magnetization vector due to spin-spin interactions with other nuclei, resulting in the loss of phase coherence (35, 36). Relaxation rates (R1 and R2, reciprocals of T1 and T2) were calculated from resolved 1H–15N cross-peaks in a well-organized region of Skp1 (supplemental Table S4). The rotational correlation time (τ_anis time taken to rotate approximately 1 radian) for the GGF-Gn-Skp1 polypeptide derived from 13N spin relaxation experiments and calculated using the ModelFree computer program (37, 38) was 17.8 ± 2.1 ns, which is close to the prediction for a dimer as shown by SAXS analysis and chemical cross-linking studies (12). Relaxation rate data for each position of each U-[1-13C]-labeled αGal residue are shown in supplemental Fig. S6 (A–D), and averaging of values around each ring yielded estimates for αGal1 and αGal2 of 3.1 ± 0.4 and 2.6 ± 0.8 ns, respec-
Glycan-induced conformation change

For free monosaccharides, $\tau_e$ estimates are on the order of $10^{-11}$ to $10^{-12}$ s (39–41). Therefore, the $\tau_e$ values measured for the Skp1 glycan suggest that its motion, relative to untethered carbohydrates, is inhibited by being covalently linked to the protein, yet still faster than the ordered part of the protein that was chosen for reference.

ModelFree was also used to calculate Lipari–Szabo model-free squared generalized order parameters ($S^2$) for the Gal residues, which provides a description of the spatial restriction of motion where values closer to 0 indicate flexibility and a value of 1 describes a completely rigid molecule (37, 38). The values in the range of 0.24–0.38 (Table 1) confirm the relative mobility of the glycan.

To gain further information about motions within the glycan itself, relaxation rates were calculated for each labeled position of the proximal sugar, GlcNAc, of the $^{13}$C-GlcNAc–labeled version of GGFGGn*-Skp1 described above (supplemental Fig. S7 and Table 1). The average $\tau_e$ for $^{13}$C-GlcNAc calculated from $R_1$ and $R_2$ using ModelFree was 3.1 ± 0.2 ns, similar to values calculated for the terminal Gal residues. These results indicate that the proximal GlcNAc and distal Gal residues exhibit similar motion. If this is due to restrained rotations within the glycan, then the motions of the non-reducing terminus might be ascribable to rotations around the core GlcNAc-Hyp linkage, puckering of the Hyp pyrrolidine ring, or motions of the polypeptide itself.

To help interpret the significance of the motions indicated by the relaxation measurements, a fully solvated 500-ns MD simulation was performed of the Skp1 pentasaccharide linked to Hyp. The average structure (Fig. 8) is close to the gas-phase energy-minimized structure obtained from GLYCAM-Web (described above), and consistent with the three NOE contacts between the GlcNAc and Fuc residues and between aGal1 and aGal2. In addition, the simulation predicts a hydrogen bond linking the 6-OH moieties of the GlcNAc and aGal1 almost 50% of the time, which would restrict relative motions between the core and distal end of the pentasaccharide. Restricted glycan internal rotation leads to the prediction that NMR relaxation and rotational parameters should be similar for the core GlcNAc and distal aGal residues, as observed (Table 1). Therefore, the glycan is inferred to be well-organized according to the average image shown in Fig. 8, and the high degree of mobility of the glycan relative to ordered regions of the polypeptide, inferred from the NMR data, results from motions and flexions in the polypeptide chain N-terminal to glycosylated Pro-143.

Structural effects of glycosylation on Skp1 in silico

MD simulations were performed on four Skp1 isoforms to understand how modifications affect the structural dynamics of the protein, namely the unmodified protein containing Pro, the hydroxylated isoform containing Hyp, the Hyp isoform linked to a single GlcNAc (Gn) unit, and a final model containing the complete glycan (GGFGGn). To enhance the conformational search, six independent trajectories were performed for 250 ns each. All simulations began from the same starting conformation of the protein. Based on an assessment of the Cα 2D root mean square deviation (supplemental Fig. S8) as well as visual inspection (Fig. 9A), the trajectories failed to converge over the 250-ns periods. This suggests that no individual simulation fully sampled conformational space. To identify trajectories of the GGFGGn isoforms that best match experiments, relaxation rates of the glycan ($R_1/R_2$) were computed (see supplemental material) for aGal2, aGal1, and GlcNAc from each of the 250-ns trajectories and compared with the experimental values described previously.

The NMR data excluded three simulations of the fully glycosylated isoform (see supplemental Results and Tables S6 and S7), indicating a difference in glycan mobility between the six simulations. Indeed, those simulations most comparable...
with the experiment consistently demonstrated interactions between the glycan and the protein. Three hydrogen bonds attained >50% occupancy across the three simulations (Fig. 10 (A and B) and supplemental Table S8), and their persistence, once formed (Fig. 10, C and D), indicated that they are stable. The conformation of the glycan was similar to that of the free

**Figure 9. MD simulations of Skp1 polypeptide isoform conformations.** A, graphical representations represent each of six trajectories per group for the Pro (blue), Hyp (purple), GlcNAc (pink), and GGFGGn isoforms (red or orange). The three selected trajectories of the GGFGGn isoform (orange) are at the right (columns 4–6). B, distance from the protein core to the final residue of H8 (Asn-153) and solvent-accessible surface area (SASA) of the F-box–binding site (residues 93–162). Data are expressed as mean ± S.D. (error bars) between trajectories (*, p ≤ 0.05 compared with all other groups. C, a single frame is overlaid every 20 ns from the simulations of the selected (orange) and excluded (red) GGFGGn isoform. The glycan was present in the simulations but is not depicted.
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![Figure 10. MD simulation of the conformation of GGFGGn-Skp1. A, the average structure over the three selected simulations for the GGFFGn isoform of Skp1 is displayed, and helices 6–8 are labeled. B, hydrogen bonds that are maintained for >40% of the simulations are depicted and colored according to the atom to which the hydrogen is connected. C and D, diatomic distances between the Gal1 O2 atom and Asn-139 backbone nitrogen atom (C) and between the Fuc O2 atom and the Glu-129 OE1 atom (D) over time are plotted for each of the three selected trajectories. Additional hydrogen bond time courses are shown in supplemental Fig. S10. E, surface representation of the image in A.](image-url)

glycan attached to Hyp (supplemental Table S7). These interactions appear to shift the conformational equilibrium toward one in which the terminal helix (H8) is extended away from the protein core, as compared with the other isoforms in which H8 is retracted (Figs. 9C and 10A). This extension correlated with an increased solvent-accessible surface area of H5–H8 (Fig. 9B), suggesting enhanced availability of the FBP-binding site.

To assess in silico whether glycosylation might impact binding to the FBP, a single trajectory was generated (250 ns) of the fully glycosylated Skp1 bound to the FBP Skp2, initiated from the crystal structure of the unglycosylated co-complex (18). The complex remained stable for the duration of the trajectory (Fig. 11). The described hydrogen bonds between the protein and glycan were not observed during this simulation; however, additional replicates would be required to form conclusions regarding interactions within the glycoprotein.

**Discussion**

Glycosylation was shown previously to exert marked effects on the organization of the Skp1 protein. The NMR studies presented here define the covalent structure of the pentasaccharide and characterize its motions on Skp1. Knowledge of the internal linkages of the glycan enabled a series of molecular dynamics simulations of the overall conformation of the glycan alone and when linked to the Skp1 protein. The simulations that satisfied NMR-derived properties of glycan motions indicate that the glycan associates with an intrinsically flexible region of the protein, resulting in a conformational change in the polypeptide that opens a volume between helix-7 and helix-8 associated with binding to F-box proteins.

**The Skp1 glycan sequence**

Previous studies suggested that the pentasaccharide consists of Galα1,3Fucα1,2Galβ1,3GlcNAcα1- linked to 25,4R-hydroxyproline, modified by an αGal at an unknown position. However, these linkages were assigned based on indirect evidence, such as exoglycosidase sensitivity and in vitro characterization of the glycosyltransferase substrate preferences and product characterizations (12, 23–27). The NMR-based data presented here confirm the Fucα1,2Gal- and the Galα1,3Fuc linkages in the isolated glycopeptide, based on HMBC analysis and chemical shift comparisons with an authentic standard (Fig. 4D and supplemental Table S3). The HMBC analysis also established that the terminal sugar, αGal2, forms a Galα1,3Gal linkage with αGal1 (Fig. 4D). The MS" analysis of the permethylated glycopeptide confirmed that the pentasaccharide is linear, and cross-ring cleavages confirmed the Fucα1,2Gal-linkage (Fig. 5 and supplemental Fig. S1). Significantly, supportive evidence for each of these linkages and for the Galβ1,3GlcNAc linkage was obtained based on NOESY and ROESY signals at the level of the intact glycoprotein (Fig. 7 and supplemental Figs. S3 and S5). Furthermore, some of the features were confirmed on the native glycoprotein isolated from amoebae (Figs. 1D and 4B). Confirmation at the protein level is important because of the instability of the glycopeptide encountered during this study. Thus, both catalytic activities of AgtA modify the 3-position of the acceptor sugar. These results
provided the following sequence for the MD simulations: Gal\(1\rightarrow3\text{Gal}\)/H\(\beta\)1,3Gal\(1\rightarrow3\text{Fuc}\)/H\(\beta\)1,2Gal\(1\rightarrow2\text{GlcNAc}\)/H\(\beta\)1–4(trans)Hyp.

**Conformation of the Skp1 glycan**

The proximal (\(\alpha\)GlcNAc) and distal (\(\alpha\)Gal) residues exhibit similar relaxation coefficients and rotational correlation times, suggesting coordinated movements on Skp1 (Table 1). The NOEs observed between GlcNAc-H2 and Fuc-H5 and -H6 (Fig. 7), together with the modeling (Fig. 8), suggest that the core trisaccharide, analogous to the blood group H type 1 structure, adopts the same conformation as reported in an unrelated oligosaccharide (33, 34). The NOE between Gal2 and Gal1 (Fig. 6) and a hydrogen bond that persisted 40–50% of the time between the GlcNAc and Gal1 in the 500-ns simulation of the free glycan (Fig. 8) indicate that the Gal\(1\rightarrow3\text{Gal}\)/H\(\beta\)1-disaccharide adopts a relatively stable orientation toward, while not disturbing, the underlying trisaccharide organization. The observations are consistent with the lowest-energy conformation predicted by MD simulation (Fig. 8), which indicated approximately 15% oscillation (one S.D.) around the minimum-energy conformation for each glycosidic linkage (supplemental Table S7).

**Influence of the glycan on Skp1 organization**

An ongoing NMR characterization of *Dictyostelium* Skp1 shows that residues 126–162, which comprise H7, a loop, and H8, exhibit partial disorder relative to their organization in complexes with FBPs, based on chemical shift index, \(^{15}\text{N}(\text{H})\) NOE, and \(^{15}\text{N}\) NOESY-HSQC studies, consistent with recent studies on human Skp1 (16, 17). Comparison with Gn-Skp1 and GGFGGn-Skp1 revealed glycosylation-dependent changes in the chemical environment of residues 132–156, based on chemical shift differences in an \(^{15}\text{N},\text{H}\) HSQC experiment, \(^{15}\text{N}\) NOESY-HSQC, and constant-time \(^{13}\text{C},\text{H}\) HSQC. This interval is most affected by the presence of GGFGGn in the MD simulations (Fig. 10).

To assist in evaluating the all-atom MD simulations of the glycoprotein motions, an algorithm was developed to extract \(R_1\) and \(R_2\) values from the trajectories (see supplemental Methods). The \(^{13}\text{C}\)Gal relaxation kinetics of three of the six trajectories examined exhibited a good match with the experimental NMR values (supplemental Results and Tables S5 and S6). These simulations exclusively showed the glycan to fold back onto the loop connecting helix-7 (H7) with helix-8 (H8) and contact H7. Three hydrogen bonds appear to stabilize the association. Coincidentally, these three trajectories exhibit an increased average distance between the protein core and the end of helix-8 (Fig. 10). This was not observed in the three trajectories of the glycoprotein that did not match the NMR data or in the 18 trajectories of the other Skp1 isoforms. The extension of helix-8 away from the protein core was highly dynamic and may enhance the accessibility or selectivity of the F-box–binding site (Fig. 9C). This may explain the altered SAXS envelope observed upon glycosylation, whose terminal expansions might reflect excursions of helix-8, rather than excursions of the glycan as originally hypothesized (12). Amino acids involved in these hydrogen bonds are in similar positions within the crystal structure of unglycosylated Skp1 bound to an F-box (Fig. 11), suggesting that the position of the glycan does not interfere with further motions of helix-8 as it assumes its final position in the bound conformation with the F-box.

**Evolutionary conservation of the FBP-combining region**

The 68-amino acid FBP-combining region of human Skp1 has been described as having primary and secondary subsites, which mediate 14 and 9 direct crystallographic contacts,
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respectively, with the FBP Skp2 (18, 19). These subsites consist approximately of H5-loop-H6 (36 amino acids) and H7-loop-H8 (32 amino acids), respectively (see supplemental Fig. S11). The described hydrogen-bonded residues at the equivalent positions of Glu-129, Asn-146, and Asn-139, which reside in the secondary subsite, are perfectly conserved among Dictostelium discoideum, Toxoplasma gondii (whose Skp1 also is modified by a pentasaccharide) (42), yeasts, and humans (supplemental Fig. S11). Because yeast does not evidently modify the Pro, and humans (and all chordates) lack the equivalent of Pro-143 in their Skp1, it is likely that these residues are subject to additional selective pressure to conserve their identity, which implies that they have functions beyond hydrogen-bonding to the glycan as proposed here. This interpretation is supported by the perfect conservation of the entire FBP-combining region among cellular slime molds that diverged over 300 million years ago.

Many species have amplified their Skp1 genes, allowing for varied functions associated with differences in their sequences. Several cellular slime molds have two Skp1 genes, the duplicate copy of which experienced conserved substitutions in their FBP-combining region (supplemental Fig. S11). Of the 15–21 changes in three species, the majority (70–75%) occur in the secondary interaction region, and only one or two of these involve residues that directly interact with the F-box of Skp2. Glu-129, Asn-146, and Asn-139, which do not contact the F-box directly, are each (with one exception) semi-conservatively substituted in the duplicate Skp1. Changes of these residues might modulate the strength of the hydrogen bonds with the glycan. In four species of stramenopiles, which conserve duplicates might modulate the strength of the hydrogen bonds with respectively substituted in the duplicate Skp1. Changes of these residues that directly interact with the F-box of Skp2. Glu-129, Asn-146, and Asn-139, which do not contact the F-box directly, are each (with one exception) semi-conservatively substituted in the duplicate Skp1. Changes of these residues might modulate the strength of the hydrogen bonds with the glycan. In four species of stramenopiles, which conserve the glycan.

**Experimental procedures**

**Native Skp1 from D. discoideum**

Skp1, consisting of a mixture of Skp1A and Skp1B, which differ by a single amino acid at codon 39, was purified from D. discoideum strain HW302 grown in standard HL-5 growth medium, as described previously (49). Briefly, a S100 cytosolic fraction was prepared from a 30-liter culture by filter lysis and ultracentrifugation, and Skp1 was enriched by DEAE-Sepharose chromatography and frozen at −80 °C. DEAE pools were combined from eight batches (2.4 × 10^12 total cells), and Skp1 was purified by phenyl-Sepharose, Q-Sepharose, and mAb 3F9 affinity chromatographies and finally C<sub>6</sub>-reversed phase HPLC, yielding 1.6 mg (80 nmol) of highly purified Skp1. After treatment with endo-Lys-C, the glycopeptide was purified by HPLC on a Phenomenex PolymerX RP-1 column (3-μm particle diameter, 100-Å pore diameter, 150 × 4.1 mm), using an ascending gradient of ACN in 10 mM NH<sub>4</sub>OH, followed by further purification on a Phenomenex Jupiter C<sub>18</sub> Proteo column (4 μm, 90 Å, 250 × 2 mm), using an ascending gradient of ACN in 0.1% TFA on a Pharmacia SmartSystem. The glycopeptide was recovered at <10% yield (~6 nmol), based on the <br>absorbance, and was contaminated by another Skp1 peptide (ENEWCEDK) at about 0.12 (mol/mol) abundance, based on its <br>value and intrinsic Trp absorbance (Fig. 1B). Instability in the presence of TFA, formic acid, or acetic acid was also observed during work-up of a synthetic glycopeptide corresponding approximately to the trisaccharide tryptic peptide (27).

**Expression and purification of recombinant glycosylated Skp1**

Glycoforms of untagged, full-length Skp1A, referred to simply as Skp1, were prepared as described previously (12). Briefly, Escherichia coli BL21-Gold cells harboring pET19b-Skp1A (Amp<sup>8</sup>), pET19b-Skp1A-PhyA (Amp<sup>8</sup>), or pET19b-Skp1A-PhyA and pACYCDuet-DdDpGnt1 (Cm<sup>8</sup>) were used as sources of Skp1, HO-Skp1, or Gn-Skp1, respectively. A typical preparation consisted of 4 × 1 liter of LB medium containing 100 μg/ml ampicillin (34 μg/ml chloramphenicol as appropriate) in baffled Erlenmeyer flasks incubated at 37 °C. At an A<sub>600</sub> (1 cm) of 0.6–0.7, E. coli were harvested by centrifugation at 5000 × g for 5 min at 4 °C; resuspended to 1 liter of M9 minimal salts medium supplemented with 18.7 mM NH<sub>4</sub>Cl (Cambridge Isotope Laboratories, Inc.), 2 mM MgCl<sub>2</sub>, 0.4% (w/v) d-glucose, 0.1 mM CaCl<sub>2</sub>, 10 μg/ml thiamine, 10 μg/ml biotin, 100 μg/ml ampicillin, and 34 μg/ml chloramphenicol in 100% D<sub>2</sub>O (Cambridge Isotope Laboratories, Inc.); and shaken at 37 °C for 1 h.
Protein expression was induced by the addition of 0.5 mM isopropyl-1-thio-β-d-galactopyranoside in D₂O at 22 °C. After 16 h, cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C. Cell pellets were resuspended in 20 mM Tris-HCl (pH 8.0), pelleted again as before, and stored at −80 °C until lysis and purification was performed as described previously (12). Reaction progress was monitored by MALDI-TOF-MS analysis on a Bruker Ultraflex II instrument. Expected m/z for unmodified Skp1 with natural isotope abundance is 18,587, and for 15N/2H-Skp1 it is 20,101. Actual m/z observed for 15N/2H-Skp1 was 19,635, indicating 97% isotopic labeling.

Purified 15N/2H-Gn-Skp1 was reacted with the dual-function (β3-galactosyltransferase/α2-fucosyltransferase) glycosyltransferase PgtA (49) to yield 15N/2H-Fucα1,2Galβ1,3GlcNAcα1-Skp1 (15N/2H-FGGn-Skp1), as described previously (12). To 13C-label, the terminal Gal residues of the Skp1 pentasaccharide, 15N/2H-FGGn-Skp1 was reacted with the dual-function α-galactosyltransferase, AgtA (26), as described previously (12) in the presence of UDP-α-d-[UL-13C6]galactose (Omicron Biochemicals, Inc.) to yield 15N/2H-G-G*FGGn-Skp1. The asterisks indicate positions of 13C-sugars. Expected m/z for 15N/2H-G*G*FGGn-Skp1 is 21,022. Actual m/z was measured at 20,415, indicating 97% isotopic labeling of nitrogen and exchangeable hydrogen sites. We confirmed that the outer Gal residues are α-linked, as expected, based on sensitivity to release of 3H from a reaction employing UDP-[3H]Gal by green coffee bean α-galactosidase but not β4-galactosidase (data not shown). As also observed for the native in vivo structure (24), X. manihotis α1,3/6-galactosidase preferentially removed only a single Gal.

To 13C-label the internal GlcNAc residue, 15N/2H-HO-Skp1 was reacted with Gnt1 (50) as described previously (12) to yield 15N/2H-Gn*-Skp1, except that UDP-α-d-[UL-13C6]N-acetyl-d-glucosamine (Omicron Biochemicals, Inc.) served as the sugar-nucleotide donor. Glycosylation was completed to yield 15N/2H-GGFGGn*-Skp1. Expected m/z for 15N/2H-GFGGn*-Skp1 is 21,016. An actual m/z of 20,615 was observed, indicating 98% isotopic labeling.

**Skp1 glycopeptide purification**

FGGn-Skp1 was prepared as described above without 15N/2H-labeling, and the final two αGal residues were incorporated in a reaction consisting of 10 μM FGGn-Skp1, 25 μM UDP-α-d-[1-13C]galactose (Omicron), 50 mM HEPES-NaOH (pH 7.2), 50 mM NaCl, 2 mM MnCl₂, 5 mM DTT and incubated at 22 °C for 40 h in the presence of 8 nM purified His₆-AgtA. A separate aliquot of 17 μg (1%) was instead labeled with UDP-α-d-[6-3H]galactose (denoted as #) to allow tracking of the glycopeptide during development of the purification protocol. 5–15 μM GGF-GGn-Skp1 (1.7 mg) was incubated with 0.01 mg/ml Pronase in 50 mM HEPES-NaOH (pH 7.2), 50 mM NaCl, 2 mM MnCl₂, 3 mM CaCl₂, 1 mM DTT. The samples were digested for 6 h at 50 °C, at which time additional Pronase was added to 0.02 mg/ml, followed by a further 14-h incubation at 50 °C. The radioactive sample was processed identically in parallel in a scaled down reaction.

Previous work with the synthetic Skp1 glycopeptide indicated poor recovery of native or synthetic versions of the glycopeptide under acidic conditions, so a neutral pH method was developed guided by recovery of the 3H-glycopeptide. Radioactive fractions were mixed with 6 ml of Biosafe II scintillation mixture and counted in a Beckman LS 6500 scintillation counter. In the optimized protocol, Pronase digests were applied to a 100-μg C₁₈ cartridge (Waters) that had been pre-equilibrated with H₂O using a vacuum manifold. The C₁₈ flow-through was pooled with four additional washes each of 1 ml of H₂O, yielding 84% 3H-glycopeptide recovery. This pool was applied to a 150-μg Carbograph cartridge (Grace-Alltech) pre-equilibrated with H₂O. The Carbograph was sequentially washed four times with 1.2 ml of ml H₂O, one time with 1.2 ml of 50 mM NH₄CO₃, four times with 1.2 ml of H₂O, and one time with 1.2 ml of 50% ACN. The Carbograph was then eluted with 7 × 1.2 ml of 25 mM NH₄CO₃ in 50% ACN. The pooled fractions were dried down and resuspended in 8 ml of H₂O, yielding 90% 3H-glycopeptide recovery. The pool was reapplied to a fresh Carbograph cartridge, and the cartridge was washed with 4 × 1 ml of H₂O, 1.2 ml of 25 mM NH₄CO₃ in 10% ACN, 2 × 1.2 ml of 25 mM NH₄CO₃ in 20% ACN, 3 × 1.2 ml of H₂O, 2 × 5 mM NH₄CO₃ in 1.2 ml of 40% ACN, 6 × 1.2 ml of 25 mM NH₄CO₃ in 50% ACN. The 3H-glycopeptide was found in the 25 mM NH₄CO₃ in the 10% ACN fraction at 71% overall recovery. Corresponding fractions from the 13C-glycopeptide purification were spotted on a MALDI target plate with dihydroxybenzoic acid, dried down, and analyzed on a Bruker Ultraflex II in positive-ion mode. Fractions that were most enriched in glycopeptide ions, which confirmed predictions from the 3H-glycopeptide analysis, were lyophilized.

**Tandem mass spectrometry of the permethylated glycopeptide**

The glycopeptide was prepared as described above from Skp1 that had been glycosylated using unlabeled UDP-Gal. The sample was first N-acetylated to modify the N terminus and other amines on the peptide portion of the glycopeptide to eliminate positive charges. This was achieved by cooling the sample on ice; treatment with ice-cold 1 mM sodium bicarbonate; the slow, dropwise addition of ice-cold acetic anhydride; mixing; incubation on ice for 10 min; the further addition of acetic anhydride; and incubation at room temperature for 30 min with occasional shaking. The sample was then desalted using Dowex-50 (H⁺ form), permethylated (51), and desalted on a C₁₈ cartridge. To monitor N-acetylation and permethylation, an aliquot was profiled by MALDI/TOF-MS on a AB SCIEX TOF/TOF™ 5800 (AB SCIEX), in reflector positive-ion mode using 20 mg/ml α-dihydroxybenzoic acid in 50% MeOH/water (v/v) as the matrix. The verified sample was analyzed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher) equipped with a nanospray ion source. The N-acetylated, permethylated glycopeptide-rich fraction was dissolved in 1 mM NaOH in 50% MeOH and infused directly into the instrument at a constant flow rate of 0.5 μl/min. A full Fourier transform MS spectrum was collected at 30,000 resolution. The capillary temperature was set at 210 °C, and MS analysis was performed in the positive-ion mode.
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NMR spectroscopy of the Skp1 glycopeptide

NMR spectroscopy of the Skp1 native glycopeptide was performed at 900 MHz on a Varian VNMR spectrometer using a triple-resonance cold probe equipped for application of z-gradients at the Rocky Mountain regional NMR facility at the University of Colorado Denver, Anschutz Medical Campus. Spectra were recorded in 5-mm Shigemi tubes, susceptibility-matched to water, in D$_2$O using a trace of acetone as an internal standard at δ = 2.225 ppm at 25 °C. One-dimensional experiments were carried out by averaging 1024 or 2048 transients, spectral width of 8000 Hz, 16 K data points, and preparatory delay of 1 s. gCOSY experiments (52) were carried out with or without the WALTZ-16 decoupling scheme (53) applied to appropriate carbon frequencies during acquisition. gTOCSY experiments employed the DIPSI-3 mixing sequence (54), followed by a zero-quantum dephasing period using a simultaneous 180° BIP pulse and gradient z-filter (55) to generate purely absorptive in-phase multiplets, using WALTZ-16 for decoupling carbon during acquisition when desired. gHMBC experiments were performed with a set of delays to optimize different $J_{C,H}$ values (56). A value near 3.5 Hz appeared close to optimal for observing intensities of interresidue $J_{C,H}$ couplings, at least, in these [1-13C]Gal-labeled glycopeptides.

NMR spectroscopy of Skp1

NMR spectroscopy was performed on instruments operating at 21.1 or 18.8 teslas and equipped with a Varian VNMR console and a 5-mm cryogenically cooled triple-resonance and z-gradient–equipped probe at the University of Georgia. NMR experiments were performed at 35 °C after stabilization and shimming, based on calibration with an ethylene glycol standard under conditions approximating data acquisition. The unmodified 15N/1H-Skp1 sample consisted of 5 mg of protein in 300 µl. The 15N/13C-G+GGG-Skp1 sample consisted of 1.4 mg of protein in 300 µl. The 15N/13C-GGGGn-Skp1 NMR sample consisted of 2.2 mg of protein in 300 µl. Samples were analyzed in 100% D$_2$O NMR buffer containing 20 mM sodium phosphate, pH 7.0, 50 mM NaCl, 1 mM MgCl$_2$, 0.1 mM EDTA, 1 mM tris(2-carboxyethyl)phosphine, and 0.05% NaN$_3$, except for 1H–15N experiments, which contained 10% D$_2$O. Samples were filtered before analysis. NMR pulse sequences were obtained from the Varian BioPack software package. Spectra were processed using NMRPipe (57) and analyzed using Sparky (58) or NMR-View (59). Peaks of the 1H–13C HSQC spectra were provisionally assigned by comparison with predicted chemical shifts calculated by the CASPER program (28). To quantitate the similarities, the absolute value of the difference between each experimental and CASPER calculated chemical shift was determined. Due to the different sensitivities of 1H and 13C shifts to structure, 13C chemical shifts were scaled by a factor that was calculated from the ratio of the difference between the minimum and maximum chemical shift values in each dimension (scaling factor = $\frac{\text{Max}^{1}H\text{ppm}-\text{Min}^{1}H\text{ppm}}{\text{Max}^{13}C\text{ppm}-\text{Min}^{13}C\text{ppm}}$ = 0.03891). The 13C or 1H chemical shift difference (experimental – calculated) for each 13C–1H assignment within each glycosidic linkage configuration was summed (denoted as $\Sigma^{13}C\Delta\text{ppm}$ and $\Sigma^{1}H\Delta\text{ppm}$), and the $\Delta\text{ppm}$ values presented in supplemental Fig. S3 were calculated by the equation, $\Delta\text{ppm} = \sqrt{(\Sigma^{13}C\Delta\text{ppm})^2 + (\Sigma^{1}H\Delta\text{ppm})^2}$.

The 13C-filtered band-selective NOESY-HSQC was conducted as a selective NOESY experiment rather than a full 2D NOESY. Only the anomeric protons (as filtered by C-13 range) are inverted for the NOE part of the sequence, as for a selective proton NOE experiment.

Relaxation measurements

$T_1$ or $T_2$ values were extracted by measuring the intensities of cross-peaks in 1H–15N or 1H–13C NMR spectra using different relaxation delay times and fitting the intensities to the equation, $I(t) = I_0 e^{-\frac{t}{T_1}}$ where $I$ is the intensity of a peak at relaxation delay time = $t$, and $T_1$ or $T_2$. The region defined by 8.2–9.5 ppm (1H) and 115–130 ppm (15N), was chosen for 15N polypeptide relaxation measurements. Relaxation rates $R_1$ and $R_2$ are related to $T_1$ or $T_2$ by the relationship, $R_1 = 1/T_1$, and $R_2 = 1/T_2$. Rotational correlation time ($\tau_r$) estimates using the ratio of $R_2/R_1$ and Lipari–Szabo model-free analysis (37, 38) were performed using the ModelFree program (Palmer Lab) (60, 61).

Molecular dynamics conditions

MD simulations were performed with the pmemd.cuda version of AMBER14 (62). The amino acid and carbohydrate residues were parameterized with the FF12SB and GLYCAM06 (I-1) force fields, respectively (63, 64). The systems were neutralized with Na$^+$ ions and solvated using the TIP3P water model (65) in a truncated octahedral box with 15-Å distance from the solute to the end of the unit cell. Electrostatic interactions were treated with the particle mesh–Ewald algorithm, and a cut-off for non-bonded interactions was set to 8 Å (66). SHAKE was employed to constrain hydrogen-containing bonds, enabling an integration time step of 2 fs. Restraints were imposed in specific situations and were enforced with a 10-kcal/mol Å$^2$ energy barrier in each case. Each minimization step consisted of 1000 cycles of the steepest descent method (1000 cycles), followed by 24,000 cycles using the conjugate gradient approach. The systems were heated to 300 K under NVT conditions over 60 ps, employing the Berendsen thermostat with a coupling time constant of 1 ps. The subsequent simulations were performed under NPT conditions. A torsion term that corrects 4(trans)-hydroxyproline residue (Hyp) ring puckering was included in simulations of the O-linked residue type (OLP) based on previous studies that indicate that the ring is primarily exo when glycosylated (67, 68). This torsion term has been adopted in GLYCAM06 (version K).

Model construction

A model of the glycan was created with the GLYCAM web builder (www.glycam.org)5 per the NMR data described in this study: GalNAc1,3Galα1,3Fucα1,2Galβ1,3GlCNacα1-(trans)Hyp. The pentasaccharide was linked to hydroxyproline (residue ID OLP) and capped by N-acetyl and N-methylamido groups.

5 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
The Dictyostelium Skp1A sequence (UniProtKB P52285) was submitted to the SWISS-MODEL web server (69, 70). The top-ranked model was constructed based upon PDB entry 2ASS, which has a 68.2% sequence identity to the Dictyostelium sequence. The GMQE and QMEAN4 scores were 0.75 and −4.04, respectively. Chimera was employed to complete the model by adding two residues on the N terminus and six on the C terminus (71). These new amino acids were relaxed by first minimizing, heating, and equilibrating while maintaining Ca restraints on every residue for 10 ns, followed by removing the restraints from the eight Chimera-built residues for an additional 40 ns. The final frame from this sequence represented the starting structure for each of the four Skp1 isoforms.

Three additional isoforms of Skp1 were created by 1) converting Pro-143 to Hyp-143, 2) linking GlcNAc to the Hyp residue, and 3) linking the full pentasaccharide to residue 143. The glycosidic linkages of the pentasaccharide were explicitly defined based on the low-energy structure generated by the GLYCAM web builder. Six independent simulations of each of the four Skp1 isoforms were performed, three of which followed the same procedure as described for the glycan and ended after 250 ns. The remaining three trajectories consisted of minimization, heating, and an initial 50 ns in which the Ca atoms of the protein backbone were restrained before the 250-ns production run. This second procedure was performed to allow the glycan to equilibrate to the environment without affecting the protein structure before continuing the simulation for an additional 250 ns without restraints. This procedure was repeated for the isoforms lacking the glycan to match the amount of time that was simulated for each model. A single simulation was performed with the fully glycosylated Skp1 model in complex with the human FBP Skp2 from PDB entry 2ASS (chain B, residues 8–578). This trajectory also involved 50 ns of restrained Ca simulation before the 250-ns production run.

Analysis of MD trajectories

Analysis of the Skp1 isoforms was performed on the latter 200 ns of the simulations, and the latter 450 ns of the free glycan. Structural metrics, such as distances, solvent-accessible surface area, root mean square deviations, hydrogen bond properties, and coordinate averaging were generated using the cpptraj module of AmberTools14 (72). The default hydrogen bond cut-off of 3.3 Å was employed, and only those cumulatively attaining >20% occupancies were reported. The $\phi$ and $\psi$ glycosidic torsion angles were defined by the H1-C1-Ox-Cx and C1-Ox-Cx-Hx atomic sequences, respectively. Solvent-accessible surface area was calculated on residues 93–162, which correspond to H5–H8 of the Skp1 protein. Significance was determined with a $t$ test. All images and videos were created using the VMD (Visual Molecular Dynamics) program (73). Schematic representations of glycans follow the SNFG format (74), and the 3D shapes were created with the 3D-SNFG plugin (75).

Calculations of $T_1$ and $T_2$ values from MD simulations

Spin-lattice and spin-spin relaxation rates (denoted by $R_1$ and $R_2$) (76) were calculated from MD trajectories, using correlation functions calculated directly from vector fluctuations in the trajectories and Fourier transformation of those correlation functions rather than assuming a $1/t^6$ distance dependence and curve-fitting exponents to the correlation functions to extract correlation times (77). A description of the theory and calculations, as extended for the present application, is provided in the supplemental material. Two programs were developed as a C++ package. The first program is designed to calculate relaxation rates of carbohydrates and small molecules. The second program is for large molecules, such as proteins, and applies a “sphere approximation” to add artificial tumbling to an aligned trajectory. The latter program is suitable if there is a lack of isotropic sampling of molecular orientations. The programs and documentation are available at the Glycam website (www.glycam.org).5

Author contributions—M. O. S. and C. M. S. prepared the samples, B. B. conducted the glycopeptide NMR studies, M. O. S. and J. N. G. conducted the glycoprotein NMR studies, M. I. and P. A. performed the MS+ studies, D. T. performed the theoretical studies, and G. C. wrote the software for extracting relaxation rates from MD simulations. All authors interpreted their results and provided draft sections and figures. J. H. P., B. B., R. J. W., and C. M. W. conceived the experimental strategies for the project and reviewed the interpretations. C. M. W. assembled and edited the manuscript, which was approved by all authors.

Acknowledgments—We are grateful to Jennifer Johnson and Catherine Xu (OUHSC) for assistance in preparing the native glycoprotein from Dictyostelium.

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