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Glycosylation promotes random coil to helix transition in a region of a protist Skp1 associated with F-box binding

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Abstract

Cullin-Ring-Ligases mediate protein polyubiquitination, a signal for degradation in the 26S-proteasome. The CRL1 class consists of Skp1/cullin-1/F-box protein/Rbx1 (SCF) complexes that cyclically associate with ubiquitin-E2 to build the polyubiquitin chain. Within the SCF complex, the 162-amino acid DdSkp1 from *Dictyostelium* bridges cullin-1 with an F-box protein (FBP), the specificity factor for substrate selection. The hydroxylation-dependent glycosylation of Pro143 of DdSkp1 by a pentasaccharide forms the basis of a novel O₂-sensing mechanism in the social amoeba *Dictyostelium* and other protists. Previous evidence indicated that glycosylation promotes increased α -helical content correlating with enhanced interaction with three F-box proteins. To localize these differences, we used NMR methods to compare non-glycosylated DdSkp1 and a glycoform with a single GlcNAc sugar (Gn-DdSkp1). We report NMR resonance assignments of backbone ¹H^N, ¹⁵N, ¹³C ^{α} and ¹³CO nuclei, as well as side-chain ¹³C ^{β} and methyl ¹³C/¹H nuclei of Ile(δ 1), Leu, and Val in both unmodified DdSkp1 and Gn-DdSkp1. Random Coil Index (RCI) and ¹⁵N{¹H}-HNOE indicate that the C-terminal region, which forms a helix-loop-helix centered on Pro143 at the crystallographically-defined binding interface with F-box domains, remains dynamic in both DdSkp1 and Gn-DdSkp1. Chemical shifts indicate that conformation variation in Gn-DdSkp1, relative to DdSkp1, is limited to this region, and characterized by increased helical fold. Extension of the glycan chain results in further changes, also limited to this region. Thus,

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Author Contributions

XX conducted the experiments. AE, MOS, JHP and CMW advised on design and interpretation. XX wrote the initial manuscript draft, which was edited by all authors and finalized by AE and CMW. All authors approved the final version.

Notes

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ASSOCIATED CONTENT

Supporting Information

The Supplement PDF contains methods, references, Tables S1-S2, Figures S1-S4, and is available free of charge on the ACS Publications website.

glycosylation may control F-box protein interactions via a local effect on DdSkp1 conformation, by a mechanism that may be general to many unicellular eukaryotes.

Graphical Abstract

A major pathway for protein degradation involves tagging of proteins with ubiquitin chains that are recognized by the 26S-proteasome. Skp1/cullin-1/F-box protein/Rbx1 (SCF) complexes are major effectors of protein polyubiquitination.¹ SCF complexes dock to an E2 ubiquitin-donor, which is tethered via Rbx1, cullin-1 and Skp1 to an F-box protein, which serves as a receptor that presents a target substrate for multiple rounds of ubiquitin conjugation. Eukaryotes express dozens to hundreds of F-box proteins (FBPs), and FBPs can in turn recognize up to dozens of substrates, so SCF complexes potentially regulate a broad subset of the cellular proteome. SCF-mediated polyubiquitination is regulated at multiple levels. Target proteins are often primed by a posttranslational modification that promotes recognition by their respective FBP(s), whose selective expression represents another level of control. The SCF complex itself is regulated by neddylation of cullin-1, which is under the control of Nedd8 E3's and the Cop9 signalosome. Neddylation inhibits binding of Cdn1, which dissociates Skp1 from its Cul1 docking site. We have identified another level of regulation that involves, in many unicellular eukaryotes, posttranslational hydroxylation and glycosylation of Skp1, which in the social amoeba *Dictyostelium* promotes association with select FBPs *in vitro* and in cells.² Here we investigate structural alterations of *Dictyostelium* Skp1 (DdSkp1) that may mediate this regulatory signal.

In the presence of sufficient O₂, Pro143 of DdSkp1, and Skp1 in other protists including *Toxoplasma gondii*,⁴ is oxidized to 4*R*,2*S*-hydroxyproline (Hyp) by the non-heme dioxygenase PhyA.^{2,5} The resulting Hyp143 is susceptible to sequential modification by 5 glycosyltransferase activities, resulting in the formation, in *Dictyostelium*, of the pentasaccharide Gal α 1,3Gal α 1,3Fuca1,2Gal β 1,3GlcNAc α 1-, or GGFGGn-.²⁶ Similar glycosylation occurs on Skp1 from another protist, the parasite *Toxoplasma gondii*.⁷ Based on analysis of mutants that are unable to assemble the glycan, this posttranslational modification serves as an O₂-sensor that controls *Dictyostelium* development, and contributes to proliferation of *Toxoplasma* in host cells. Interactome studies have shown enhanced interaction between DdSkp1 and three FBPs as DdSkp1 glycans are extended, whereas association with Cul1 was not affected (unpublished data).⁸ Glycosylation also promoted interaction with a heterologous soluble mammalian FBP.⁹ The enhanced

interaction might be due to the glycan affecting DdSkp1 rather than directly interacting with FBP, because the glycan moieties are evolutionarily confined to protists. Increased interactions of FBPs with DdSkp1 may contribute to enhanced turnover of FBP substrates, or FBPs themselves, when enough O₂ is available to permit DdSkp1 glycosylation that promotes assembly of FBP/Skp1 complexes.

A characteristic of FBPs is a 40-amino acid F-box domain that docks to the C-terminal region of Skp1. In crystal structures of FBP/Skp1 complexes from yeast, plants and animals, the F-box domain forms a compact structure comprised of three α -helices that associate with a region of Skp1 usually comprised of 3–4 α -helices.^{310–12} In human Skp1 (HsSkp1) helices H6 and H7, the H6-H7 linker loop, and the proximal part of the H7-H8 linker loop, form the primary interaction site with the human FBP Skp2,¹⁰ and the distal part of the H7-H8 loop, along with H8, contribute to the so-called secondary F-box interface (Fig. 1A).

DdSkp1, with 88% sequence identity to HsSkp1 over the C-terminal 72 amino acids that contribute to the binding interface with FBPs (Fig. 2), likely folds in a similar manner. FBPs are typically insoluble without Skp1 making the interaction difficult to analyze biochemically. Phosphorylation of Skp1 H8, and the AAA+ ATPase Cdc48/p97 and chaperones, have been implicated in influencing the FBP/Skp1 interaction,^{13–16} and modification of Pro143, at the start of H8, is posited to regulate this interaction in protists.²

To determine whether glycosylation affects DdSkp1 polypeptide conformation, various glycoforms were previously compared using circular dichroism and small angle X-ray scattering (SAXS).⁹ DdSkp1 is, like its human counterpart HsSkp1,¹⁷ a homodimer in the absence of an FBP. Glycosylation was shown to decrease structural disorder and increase α -helical content in Gn-DdSkp1 and GGFGGn-DdSkp1 vs. unmodified DdSkp1.⁹ According to SAXS data the dimer envelope became more slender and extended, but which amino acids were affected remained unknown. Thus far Skp1 has not been crystallized without an FBP binding partner, but a recent NMR study of free HsSkp1 in solution indicated a lack of helical structure in the H8 region.¹⁸ A molecular dynamics simulation also indicated a higher degree of motion for the entire H6-H7-H8 segment in free HsSkp1 as compared to its complex with FBP.¹⁹ Since vertebrate Skp1's lack the necessary Pro143 residue, and thus cannot be glycosylated, we initiated an amino acid level analysis of DdSkp1 to understand whether glycosylation affects the conformation of the FBP binding interface.

For NMR studies, DdSkp1 was expressed in *E. coli* as its native sequence in the presence of precursors isotopically enriched in ¹³C, ¹⁵N and ²H, and purified to >90% homogeneity under non-denaturing conditions. DdSkp1 glycoforms were obtained by a combination of *in vivo* and *ex vivo* enzymatic reactions (see Supporting Information). Owing to its dimeric status, NMR spectra of DdSkp1 and its glycoforms were acquired at 35 °C using a 900 MHz spectrometer equipped with a cryogenic probe.

We obtained well-dispersed NMR spectra for DdSkp1 and Gn-DdSkp1 (Table S1, Fig. 3), which allowed nearly complete sequence specific resonance assignments (Table S2). We were unable to obtain any resonance assignments only for residues 1, 2, 82, 123, 124, and the segment 128–138. Chemical shift differences between backbone ¹H^N, ¹⁵N, ¹³C ^{α} and

^{13}C spins in DdSkp1 and Gn-DdSkp1 demonstrate that glycosylation of Pro143 impacts primarily the segment 139–153 (Fig. 4A, S2), which corresponds to H8 and H7-H8 linker regions in the HsSkp1 complex with FBP. Comparison of 2D [^{15}N , ^1H] TROSY spectra of Gn-DdSkp1 and GGFGGn-DdSkp1 indicated further peak shifts for the same residue range 139–153, as well as Glu17, with no significant changes for the rest of the polypeptide. Differences between the Gn-DdSkp1 and GGFGGn-DdSkp1 spectra indicate further conformational changes with the extension of the glycan, consistent with previously observed further changes in the CD profiles.⁹

An important finding from comparison of chemical shifts of DdSkp1 and Gn-DdSkp1 is that glycosylation has an impact on ^{13}C spins as well as $^1\text{H}^{\text{N}}$ and ^{15}N spins (Fig. S2). While the chemical shifts of $^1\text{H}^{\text{N}}$ and ^{15}N spins can be sensitive to small changes in sample conditions and hydrogen bond geometry, ^{13}C shifts are better indicators of regular secondary structure elements.²⁰ CSI 3.0 software²¹ can accurately predict such elements in proteins based on amino acid composition and backbone chemical shifts. For most of the polypeptide chain the regular secondary structure elements are identical for both DdSkp1 and Gn-DdSkp1 (Fig. 2). Their positions are also consistent with secondary structures observed in the HsSkp1/FBP complex, though CSI 3.0 was unable to detect a short gap between H1 and H2, or identify the two-residue β 3-strand. For Gn-DdSkp1, CSI prediction indicates the presence of H8 in the same position, but slightly shorter than in the HsSkp1/FBP complex. In contrast, this region in unmodified DdSkp1 is predicted to be in random coil. This finding is further supported by the ^{15}N -edited NOESY spectra (Fig. S3), which exhibit sequential $^1\text{H}^{\text{N}}$ - $^1\text{H}^{\text{N}}$ NOE cross-peaks characteristic of helical structure in the 145–150 residue segment of Gn-DdSkp1, but not in unmodified DdSkp1.

With regard to side-chain assignments, only $^{13}\text{C}^{\delta 1}/^1\text{H}^{\delta 1}$ chemical shifts of Ile149, out of all 44 assigned Leu, Val and Ile ($\delta 1$) methyl groups moieties, appear shifted in Gn-DdSkp1 relative to DdSkp1 (Fig. S4). All other assigned Leu, Val and Ile ($\delta 1$) methyl groups are found only in the residue range 4–114, and lack of chemical shift perturbation is consistent with the DdSkp1 hydrophobic protein core being unaffected by glycosylation. The effect on Ile149 indicates a change in its molecular environment or a change in its conformation, consistent with the CSI and NOESY results.

To assess the effect of glycosylation on backbone dynamics, we collected $^{15}\text{N}\{^1\text{H}\}$ -HNOE data (Fig. 4A). Backbone amides in the ordered core regions of DdSkp1 and Gn-DdSkp1, which include strands β 1 and β 2 as well as helices H1-H6, have HNOE values around 0.6 or higher, while lower values are characteristic of increased motions in disordered segments. The latter include residues in the extended loops linking H2 with H3, and H3 with H4, as well as the short segment 89–91 between H4 and H5. The entire C-terminal region 140–162 also exhibits low overall HNOE values in both DdSkp1 and Gn-DdSkp1. Random Coil Index (RCI)²² values also remain elevated in this region. Since RCI values are directly proportional to expected atomic coordinate RMSD in MD simulations or NMR structure ensembles, together with HNOE values they indicate a high degree of structural flexibility in the H8 region. This experimental observation is consistent with MD simulations of HsSkp1¹⁹ and NMR relaxation data measured for the first GlcNAc sugar in GGFGGn-DdSkp1.⁶ Correspondingly, RCI values for residues 141–154 in Gn-DdSkp1 are lower than

those in unmodified DdSkp1, indicating a reduction in structural disorder upon glycosylation. HNOE values for residues Glu146 and Arg150 in Gn-DdSkp1 are significantly higher than in unmodified DdSkp1. These represent i+3 and i+7 positions relative to Pro143, suggesting a decrease in molecular motion on one side of H8 in Gn-DdSkp1.

We have generated a homology model of DdSkp1 based on the X-ray structure (PDB ID 3WOS) of HsSkp1 in complex with an FBP protein as a template (Fig. 1B). Short (<5.5 Å) $^1\text{H} - ^1\text{H}$ distances were computed and, along with assigned DdSkp1 and Gn-DdSkp1 chemical shifts, were used to predict the expected NOESY peak lists. These were checked for consistency with ^{15}N - and ^{13}C -edited NOESY spectra by searching for unexplained NOE cross-peaks. The presence of such peaks would either indicate a deviation of DdSkp1 subunit structure from the homology model, or identify the residues producing intermolecular NOEs across the dimer interface. No such NOE peaks were identified, suggesting that individual DdSkp1 subunits within the homodimer assembly adopt a similar structure to that in HsSkp1/FBP heterodimer. However, this also meant that the available NMR data were insufficient to accurately predict the dimer interface. The dimer interface likely involves residues not directly affected by glycosylation, not only because inter-chain contacts might be expected to restrict molecular motions, but also because a C-terminally truncated fragment of DdSkp1 (residues 1–122) forms a stable homodimer on its own based on analytical ultracentrifugation (unpublished data).

Since our data indicate a higher degree of motion for the C-terminal tail in both DdSkp1 and Gn-DdSkp1, it is unlikely that the H8 region in solution forms a well-defined α -helix as in the protein core. We think that this segment samples a range of conformations, but glycosylation with the first GlcNAc shifts the equilibrium towards a helical conformation. Extension to the complete pentasaccharide further impacts the distribution of possible conformations, consistent with MD simulations that correlated with measurements of glycan motions.⁶ Thus, glycosylation of DdSkp1 may accelerate the association rate with FBPs, by increasing the tendency of the C-terminal region to adopt a conformation typically found in the complex with FBPs. This may explain why glycosylation of DdSkp1 enhances interactions with the mammalian FBP Fbs1 *in vitro*,⁹ and with three native FBPs in cells (*op. cit.*). Interestingly, in a crystal structure of HsSkp1 in complex with Fbs1,¹⁰ the H8 region was observed adopting an extended conformation. Therefore, glycosylation may also affect selectivity of DdSkp1 docking to FBPs, whose different F-box sequences might be predisposed to favor interactions with a helical or extended conformation of the H8 region. There is limited precedent for glycosylation effects on peptide conformation,^{e.g.,23–25} and even less for full proteins.²⁶ Further studies are warranted to investigate the mechanism by which glycosylation modulates the secondary interface of DdSkp1 with FBPs.

Supplementary Material

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Acknowledgments

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ABBREVIATIONS

CSI	Chemical Shift Index
DdSkp1	<i>Dictyostelium discoideum</i> Skp1
FBP	F-box protein
GGFGGn-	full-length glycan
H8	α -helix-8
HNOE	heteronuclear NOE
HSQC	heteronuclear single quantum coherence spectroscopy
HsSkp1	human Skp1
NOESY	nuclear Overhauser enhancement spectroscopy
RCI	Random Coil Index
SAXS	small angle X-ray scattering
SCF	Skp1/cullin-1/FBP/Rbx1 complex
TROSY	transverse relaxation optimized spectroscopy

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Figure 1.

Skp1 fold and structural context of Skp1/FBP interaction. (A) Representation of HsSkp1 (tan) in complex with the FBP Fbg3 (blue and green) based on PDB entry 3WSO,³ illustrating the interface between HsSkp1 and the F-box domain (helices 1–3, green) of Fbg3. (B) Homology model of DdSkp1. Pro143, the site of glycosylation, is in red. Residues 138–153, whose chemical shifts are significantly perturbed in Gn-DdSkp1 relative to DdSkp1, are shown in yellow and orange, with orange indicating the predicted helical segment 145–150 in Gn-DdSkp1. Residues without chemical shift assignments are in gray.

Figure 2.

Sequence alignment of DdSkp1 and HsSkp1 generated with Clustal-Omega. Regular secondary structure elements identified by CSI 3.0 in DdSkp1 and Gn-DdSkp1 are indicated above the alignment. Regular secondary structure elements in the crystal structure of HsSkp1 in complex with Fbs3 (PDB ID 3WSO) are indicated underneath. Blue square = Pro143 glycosylation site in DdSkp1.

Figure 4.

Residue-specific effects of glycosylation in DdSkp1. (A) Weighted root mean square (RMS) of chemical shift differences for $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}^{\alpha}$ and ^{13}CO spins between DdSkp1 and Gn-DdSkp1 according to residue number. See Supporting Information for weighted RMS formula. Chemical shift differences for each spin type are in Fig. S2. Residues whose $^1\text{H}^{\text{N}}$ and ^{15}N chemical shifts are significantly perturbed in GGFGGn-DdSkp1 relative to Gn-DdSkp1 are in magenta. Secondary structure elements identified by CSI 3.0 for Gn-DdSkp1 are at the top. (B) $^{15}\text{N}\{^1\text{H}\}$ -HNOE and random coil index (RCI) of DdSkp1 and Gn-DdSkp1. HNOE error bars represent two standard deviations.