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HCF-1 is cleaved in the active site of O-GlcNAc transferase

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Abstract

Host Cell Factor-1 (HCF-1), a transcriptional co-regulator of human cell-cycle progression, undergoes proteolytic maturation in which any of six repeated sequences is cleaved by the nutrient-responsive glycosyltransferase, O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT). We report that the tetratricopeptide-repeat domain of O-GlcNAc transferase binds the C-terminal portion of an HCF-1 proteolytic repeat such that the cleavage region lies in the glycosyltransferase active site above UDP-GlcNAc. The conformation is similar to that of a glycosylation-competent peptide substrate. Cleavage occurs between cysteine and glutamate residues and results in a pyroglutamate product. Conversion of the cleavage site glutamate into serine converts an HCF-1 proteolytic repeat into a glycosylation substrate. Thus, protein glycosylation and HCF-1 cleavage occur in the same active site.

O-GlcNAc transferase (OGT) is a Ser/Thr (S/T) glycosyltransferase that O-GlcNAcylates nuclear and cytoplasmic proteins, thus influencing their activity, localization, and overall function(1–3). Because OGT activity is sensitive to UDP-GlcNAc concentrations, OGT is proposed to regulate cellular responses to nutrient status(4–6). Human HCF-1 is a transcriptional co-regulator involved in regulating cell-cycle progression(7, 8). In an unusual proteolytic maturation process(9–11), any of six centrally located 20–26 amino acid sequence repeats called HCF-1PRO repeats (Fig. 1A) are cleaved by OGT in the presence of UDP-GlcNAc(12), providing a link between cell-cycle progression and nutrient levels. The HCF-1PRO repeats contain two essential regions for proteolysis: a threonine-rich region proposed to be an OGT-binding site and the cleavage site, which contains a conserved Cys-Glu-Thr (CET) sequence (10, 11).

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OGT can cleave a fragment of HCF-1, called HCF-1rep1, which contains the first HCF-1\textsubscript{PRO} repeat plus N-terminal HCF-1 sequences containing several O-GlcNAc sites\textsuperscript{(12)} (Fig. 1A). To elucidate the cleavage process, we first analyzed the impact of amino acid substitutions in OGT (Fig. 1B and fig. S1) and the HCF-1\textsubscript{PRO} repeat (Fig. 1C) on cleavage and glycosylation. Three OGT active site residues implicated in S/T glycosylation were evaluated: K842, which is involved in binding and activation of UDP-GlcNAc for glycosyltransfer; H498, which contacts the C2-N-acetyl group of UDP-GlcNAc; and H558, which contacts a backbone carbonyl of glycosylation substrates\textsuperscript{(13–17)}. Substitution of K842 with methionine prevented S/T glycosylation upstream of the proteolytic repeat as well as cleavage within the repeat region. Substitution of H498 or H558 with alanine decreased S/T glycosylation but had a negligible effect on the extent of cleavage after 16 hours. K842 is an essential residue for glycosylation\textsuperscript{(14, 15)} and its importance in cleavage suggests that UDP-GlcNAc is involved in the cleavage mechanism.

Next, we tested substitutions in the proteolytic repeat of HCF-1rep1. We previously showed that alanine substitution of glutamate E10 leads to loss of cleavage. To probe the role of E10 in more detail, we substituted it with glutamine (E10Q), aspartate (E10D) and serine (E10S). All three substitutions blocked cleavage (Fig. 1C), indicating that the chemical nature of the glutamate residue is critical for OGT-mediated HCF-1\textsubscript{PRO}-repeat cleavage. In contrast, the C9 position can tolerate alanine and serine substitution (ref. 12 and fig. S2).

Because S/T glycosylation upstream of the cleavage site in HCF-1rep1 complicates study of the cleavage requirements, we identified a cleavage substrate consisting of the first three proteolytic repeats (HCF3R, Figure 1A), which did not undergo substantial glycosylation (see below). No cleavage products were observed when HCF3R was incubated with OGT alone or in the presence of UDP, but several products were observed in reactions containing both OGT and UDP-GlcNAc (Fig. 2A). These products did not form if HCF3R was incubated with a K842A OGT mutant incapable of catalyzing glycosylation\textsuperscript{(13, 16)}, nor when wild-type OGT was pretreated with 1.5 equivalents of a previously described inhibitor that covalently inactivates the enzyme by crosslinking the active site\textsuperscript{(18)}. Cleavage was also inhibited if UDP was added to reactions containing OGT and UDP-GlcNAc (fig. S3A), but accelerated by adding alkaline phosphatase, which destroys UDP. Since intact UDP-GlcNAc, but not UDP, promoted HCF3R cleavage, we tested cleavage in the presence of UDP-5SGlcNAc, an isostere of UDP-GlcNAc that adopts the same conformation within the active site, yet is resistant to glycosylation and hydrolysis\textsuperscript{(14, 19)}. Almost no peptide cleavage was observed (Fig. 2A and fig. S3B), implying that the UDP-GlcNAc does not simply fulfill a structural function, but must react for HCF3R cleavage to occur. Consistent with this proposal, UDP-5SGlcNAc was found to inhibit cleavage of HCF-1rep1 when added to reactions containing UDP-GlcNAc (fig. S3C).

We next examined the cleavage products using LC-MS. Previous analysis of products isolated from cells concluded that cleavage occurs at the E-T peptide bond of the CET sequence\textsuperscript{(10, 11)} but none of the four products we observed had masses consistent with E-T cleavage (Fig. 2B and fig. S4). Instead, they were consistent with cleavage at the preceding C-E bond except that all the C-terminal fragments were 18 Da lower in mass than expected. We hypothesized that the 18 Da difference resulted from generation of an N-terminal pyroglutamate. Indeed, cleavage of a simplified HCF3R substrate possessing a single active HCF-1\textsubscript{PRO} repeat (HCF3R-EAA) led to the production of one dehydrated C-terminal product (Fig. 2C and fig. S5). Pyroglutamate aminopeptidase (PGAP) treatment reduced the mass of this product by 111 Da, corresponding to loss of pyroglutamate (Fig. 2C and fig. S6). The earlier proposed cleavage site was probably misidentified because N-terminal pyroglutamates are resistant to the Edman sequencing method used in those studies, and cellular PGAPs likely processed some cleavage products\textsuperscript{(10, 11)}. 
To gain additional insight into how OGT cleaves HCF-1, we solved crystal structures of OGT-HCF-1PRO-repeat complexes (Supplementary Tables 1 and 2). We solved a 1.8 Å structure of OGT containing UDP and a 16 amino acid peptide comprising the threonine-rich region of the HCF-1PRO repeat (Fig.s 3A and 3B). We also solved a 1.9 Å structure with UDP and a full HCF-1PRO repeat containing an E10A mutation, but density was only observed for the threonine-rich portion of the repeat (Fig. 3C and fig. S7). These two structures show the threonine-rich peptide bound in an extended conformation along the channel formed by the tetratricopeptide-repepase (TPR) domain(13, 20, 21) of OGT (Fig. 3B and fig. S7). Five conserved asparagine residues within the TPR domain form a series of interactions, four being bidentate, with the amides of alternating residues along the peptide backbone (Fig. 3C). A lysine side chain also contacts the peptide backbone. Four aspartates of OGT form hydrogen bonds to the threonine side chains of the HCF-1 repeat. The binding mode in the crystal structures is consistent with mutational data demonstrating the importance of the OGT asparagines (Fig. 3D and fig. S8) and the conserved HCF-1PRO-repeat threonines (ref. 12 and Fig. 3E, see also Fig. 1C) for both cleavage and binding.

Because intact UDP-GlcNAc is required for OGT-catalyzed cleavage of HCF-1PRO-repeats, we thought the UDP-5SGlcNAc analogue might stabilize density for a full repeat. Indeed, we obtained a structure of OGT with UDP-5SGlcNAc and a 26 amino acid peptide corresponding to HCF-1PRO rep2, but with an E10Q substitution. In this structure the C-terminal threonine-rich region binds to the TPR domain as described above, and the N-terminal cleavage region is now visible (Fig. 4A) and forms an extensive binding interface with UDP-5SGlcNAc (Fig. 4B). A structure containing UDP-GlcNAc and a wild-type repeat confirms the binding mode of the E10Q peptide (fig. S9). Remarkably, the cleavage region binds in a mode almost identical to that of a glycosylation-competent peptide substrate(14), and residue 10 aligns perfectly with the glycosyl acceptor amino acid (Fig. 4C). The structures suggested an E10S mutation, which prevents cleavage (Fig. 1D), would be glycosylated at residue 10. Indeed, unlike HCF3R-EAA, the E10S analog (HCF3R-SAA) was glycosylated effectively (Fig. 4D and fig. S10). Thus, the identity of the amino acid at position 10 of an HCF-1PRO repeat — glutamate or serine — can dictate whether OGT cleaves or glycosylates the substrate.

Previous hypotheses suggested that OGT contains a dedicated protease active site or acts as a co-protease to template HCF-1 autocatalysis(12). Instead, OGT promotes cleavage of the HCF-1PRO repeat using the same catalytic region as for glycosylation. The threonine-rich region of the HCF-1PRO repeat binds in the channel formed by the TPR domain of OGT, stabilized by the contacts described above. The cleavage region threads into the active site and binds over UDP-GlcNAc in the same conformation that a glycosylation substrate would, with the glutamate side chain positioned near the anomeric carbon of the sugar. Since a pyroglutamate product is formed, and spontaneous cyclization of N-terminal glutamates is kinetically very slow(22), the glutamate side chain is likely activated by formation of an ester species as part of the cleavage mechanism. We speculate that the glutamate side chain traps a transient oxocarbenium ion formed within the active site, producing a glutamyl ester that can undergo intramolecular attack, leading ultimately to formation of the N-terminal pyroglutamate. We note that pyroglutamates are proposed species in other biological phenomena(23). Possible mechanisms for cleavage proceeding from a glutamyl ester are suggested (fig. S11). Although direct physical evidence for a glutamyl-sugar intermediate has yet to be obtained, glycosylation of the glutamate side chain prior to cleavage is consistent with the structural data, the strict requirement for glutamate at the cleavage site, the formation of a pyroglutamate product, and the observation that cleavage of HCF-1PRO repeats requires UDP-GlcNAc and depends on an OGT residue, K842, which is essential for catalyzing glycosylation.
These studies provide insight into two important aspects of OGT function. First, they provide a view of a peptide bound to OGT’s TPR domain, which is thought to play a central role in substrate selection (20, 21, 24, 25). The structures reported suggest that some glycosylation substrates may bind in a manner similar to the HCF-1PRO repeats, with the glycosylation site separated by several residues from a C-terminal recognition motif that binds in the channel formed by the TPR domain. Adaptor proteins that recruit glycosylation substrates to OGT might also contain threonine-rich recognition motifs. Second, they suggest an unprecedented mechanism of proteolysis in which OGT uses UDP-GlcNAc as a co-substrate in a cleavage reaction that takes place in the active site for glycosylation. Indeed, we show that two very different post-translational protein modifications — proteolysis and addition of a sugar residue — can occur in the same active site with the outcome determined by the identity of a single amino acid in the substrate.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments
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References and Notes


Fig. 1. Effect of mutations in OGT and HCF-1 constructs on cleavage and glycosylation

(A) Top. Schematic of HCF-1 showing the six proteolytic repeats (rep1-rep6) with the amino acid identities of a representative pro repeat shown. The conserved residues are shown in yellow, with the E10 glutamate essential for cleavage shown in red. The repeats are subdivided into cleavage and threonine-rich regions(12). Site-specific proteolysis by OGT leads to the formation of HCF-1_N and HCF-1_C subunits. Bottom. Schematic of HCF-1 constructs used in this study. GST–HCF-1rep1 contains the first HCF-1_PRO repeat and surrounding sequences fused to GST. Several S/T glycosylation sites are found in the HCF-1rep1 construct as schematized. The HCF3R construct contains only the first three
HCF-1\textsubscript{PRO} repeats fused to an N-terminal His-tag. (B) Comparative cleavage and glycosylation activities of WT OGT and several catalytic domain mutants. GST–HCF-1rep1 was incubated in the absence (lane 1) or presence of WT OGT (lane 2) or the indicated mutants (lanes 3–5). HCF-1rep1 cleavage was detected by western blot analysis with anti-GST antibody and HCF-1rep1 glycosylation was detected with anti-O-GlcNAc antibody (RL2). (C) Cleavage activities of WT and mutant HCF-1rep1 constructs. WT GST–HCF1rep1 (lanes 1 and 2) or a threonine-rich region mutant (T17-22A; lanes 3–4) or the indicated E10 cleavage site mutants (E10A, E10Q, E10D, E10S; lanes 5–12) were incubated in the absence (–) or presence (+) of WT OGT as described in Materials and Methods. Cleavage was detected as in (B).
Fig. 2. HCF-1PRO-repeat cleavage results in formation of a pyroglutamate product

(A) Cleavage of HCF3R requires UDP-GlcNAc. HCF3R was incubated with WT OGT (lanes 2–6), with K842A OGT (lane 7) or with OGT treated with a previously reported (18) covalent inhibitor BZX2 (lane 8), in the presence of UDP (lane 3), UDP-GlcNAc (lanes 4, 5, 7, 8), or UDP-5S-GlcNAc (5S, lane 6). Alkaline phosphatase (AP) was added to some reactions, as indicated. Cleavage products were separated by SDS-PAGE and stained with Coomassie Blue. (B) LC-MS analysis of untreated HCF3R (black) and HCF3R cleavage products (red) following incubation with OGT and UDP-GlcNAc shows unexpected mass peaks. Detected and predicted MS peaks for different cleavage products are tabulated. (C) Mutation of E10 to alanine in the cleavage region of the second and third HCF-1PRO repeats produces a construct, HCF3R-EAA, containing only a single cleavable repeat. Pyroglutamate (pyroGlu) aminopeptidase removed a 111 Da fragment from the HCF3R-EAA C-terminal cleavage product, confirming the formation of pyroglutamate in the cleavage reaction. 
**Fig. 3.** The threonine-rich region of the HCF-1\textsubscript{PRO} repeat binds in the channel formed by the TPR domain of OGT

(A) Overall structure of the OGT:UDP:HCF-1\textsubscript{11–26} peptide complex. A 16-residue peptide comprising the threonine-rich region of HCF-1\textsubscript{PRO} repeat 2 (THETGTTNTATTATSN) was co-crystallized with UDP and a previously described\textsuperscript{13} N-terminally truncated OGT construct (hOGT\textsubscript{4.5}) and refined to 1.8 Å. The OGT catalytic domain (red) and TPR domain (grey) along with the HCF-1\textsubscript{11–26} peptide (cyan) and UDP (yellow) are shown. (B) Close-up view of OGT-peptide interactions. The electron density around the visible portion of HCF-1\textsubscript{11–26} is shown as an F\textsubscript{O}–F\textsubscript{C} difference map contoured at 3σ. The peptide is shown in cyan. OGT sidechains that contact the peptide backbone are shown in yellow and OGT.

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sidechains that contact HCF-1 peptide sidechains are shown in magenta. (C) Schematic of contacts between OGT sidechains and the threonine-rich region of the HCF-1PRO repeat 2 from the complex of OGT:UDP:HCF-1-E10A1–26. OGT sidechains are numbered and colored as in panel (B). (D) Mutations in the TPR domain of OGT (5N-5A) inhibit cleavage. Cleavage and glycosylation of GST–HCF-1rep1 were assayed, as in Figure 1C, in the absence (lane 1) or presence (lane 2) of WT OGT or the 5N-5A TPR-domain mutant in which Asn residues 322, 356, 390, 424 and 458 are mutated to alanine (lane 3). (E) OGT does not bind effectively in vitro to an HCF-1PRO repeat mutant containing mutations in the threonine-rich region (T17–22A). WT (lane 2) and mutant (lanes 1, 3 and 4) GST–HCF-1rep1 substrates were tested for OGT binding in the presence of UDP-GlcNAc using an OGT-directed pull-down assay. Anti-GST and anti-T7 antibodies were used to detect GST–HCF-1rep1 (upper panel) and OGT (lower panel), respectively, by western blotting.
Fig. 4. HCF-1 cleavage takes place in the glycosyltransferase active site of OGT
(A) Overall structure of the OGT:UDP-5SGlcNAc:HCF-1-E10Q_1-26 complex. The HCF-1 peptide is shown as spheres in cyan with the UDP-5SGlcNAc in yellow. (B) Close-up view of the two substrate analogs shown in yellow in the OGT active site. The entire cleavage region can be seen and the C-E10Q-T residues are annotated. The anomeric carbon of UDP-5SGlcNAc is indicated (C1). (C) Overlay of the substrate analogs from the OGT:UDP-5SGlcNAc:HCF-1 peptide complex (yellow) and the previously reported OGT:UDP-5SGlcNAc:CKIIA complex (cyan). CKIIA is a well-characterized OGT glycosylation substrate. The E10Q sidechain of the HCF-1 peptide is shown as transparent just after the β-carbon. (D) Mutating E10 to S in an HCF-1PRO repeat converts a cleavage substrate (HCF3R-EAA) into a glycosylation substrate (HCF-SAA), which is defective in cleavage. (Left panel) Cleavage products of HCF3R-EAA and HCF3R-SAA were separated by SDS-PAGE and stained with Coomassie Blue. (Right panel) Glycosylation of wild-type and mutant HCF3R substrates was carried out with $^{14}$C-UDP-GlcNAc and analyzed by PAGE. Full gels are shown fig. S10.