Proteolytic Cleavage Driven by Glycosylation

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Proteolytic processing of human host cell factor 1 (HCF-1) to its mature form was recently shown, unexpectedly, to occur in a UDP-GlcNAc-dependent fashion within the transferase active site of O-GlcNAc-transferase (OGT) (Lazarus, M. B., Jiang, J., Kapuria, V., Bhuian, T., Janetzko, J., Zandberg, W. F., Vocadlo, D. J., Herr, W., and Walker, S. (2013) Science 342, 1235–1239). An interesting mechanism involving formation and then intramolecular rearrangement of a covalent glycosyl ester adduct of the HCF-1 polypeptide was proposed to account for this unprecedented proteolytic activity. However, the key intermediate remained hypothetical. Here, using a model enzyme system for which the formation of a glycosyl ester within the enzyme active site has been shown unequivocally, we show that ester formation can indeed lead to proteolysis of the adjacent peptide bond, thereby providing substantive support for the mechanism of HCF-1 processing proposed.

O-GlcNAc-transferase (OGT) is an essential glycosyltransferase that transfers GlcNAc to the side chain hydroxyl groups of serine and threonine in nuclear and cytoplasmic proteins in response to triggers such as increased glucose. Higher glucose levels lead to higher concentrations of UDP-GlcNAc, which in turn result in higher levels of O-GlcNAcylation. These levels are believed to reflect the nutrient status of the cell and to correlate with a plethora of changes in gene expression (1, 2). However, the molecular mechanisms underlying this regulatory process remain poorly understood. Intriguingly OGT was recently shown to catalyze another, completely unexpected, reaction: proteolytic cleavage of the cell cycle regulator host cell factor 1 (HCF-1) (1, 3). The cleavage requires UDP-GlcNAc as a co-substrate, implying that maturation of the cell cycle regulator and levels of cellular glucose are linked through this proteolytic activity of OGT (3).

It was initially assumed that OGT must possess a separate protease site to carry out this reaction. However, recent structural and mechanistic studies of HCF fragments bound to an inactive form of OGT showed that cleavage of the HCF peptide occurs when it is bound adjacent to UDP-GlcNAc within the OGT active site, and further that the inert analogue UDP 5-thioGlcNAc does not support proteolysis (3). Cleavage occurs between the cysteine and glutamate residues of a conserved CET sequence, releasing the C-terminal fragment with a pyroglutamate residue as its N terminus. The conformation of the bound peptide substrate is similar to that of a glycosylation-competent peptide substrate. Indeed, replacement of the cleavage site glutamate with serine converts the HCF-1 proteolytic site into a glycosylation substrate when UDP-GlcNAc is present (3). These findings show that protein glycosylation and HCF-1 cleavage occur in the same active site and imply that proteolysis relies on some kind of glycosylation event. The authors suggest that the proteolytic reaction proceeds via the OGT-catalyzed transfer of GlcNAc onto the substrate glutamate, forming a transient glycosyl ester as a key intermediate. This then undergoes reaction of the type shown in Fig. 1a, driven by the presence of the sugar leaving group.

Such dual glycosyltransferase/protease activity is unprecedented and highly important as it may well have broader implications. Although the mechanism proposed for this process is reasonable, it rests entirely upon the notion of the formation of this key glycosyl ester intermediate and its ability to set up cleavage of the peptide backbone. The intermediate, however, remains hypothetical. Further it seems unlikely that the intermediate will ever be isolated given the failures to isolate equivalent intermediates on other glycosyltransferases and the inherent reactivity of the intermediate in this case.

We therefore set out to test this hypothesis using a model enzyme system in which a glycosyl ester is known to form at a glutamic acid side chain. Retaining glycosidases use a two-step double-displacement mechanism in which a covalent glycosyl–enzyme intermediate is formed and hydrolyzed. Typically, the catalytic nucleophile is a glutamic or aspartic acid residue; thus a glycosyl ester intermediate is formed. With their natural substrates, however, these glycosyl esters are very short lived, as the first displacement reaction is usually rate-limiting and turnover numbers around 100 s⁻¹ are common. In contrast, reaction of such enzymes with activated 2-deoxy-2-fluoro glycoside substrates generates stable 2-deoxy-2-fluoroglycosyl esters (4, 5), which have proved extremely informative in elucidating the structures and mechanisms of glycoside hydrolases by protein crystallography, NMR, and mass spectrometry. These stable covalent intermediates therefore provide ideal systems with which to evaluate the proposed mechanism of proteolytic cleavage of HCF-1, because the 2-substituent (NAc) is not thought to play any chemical role. If the mechanism proposed is correct, we might expect cleavage of the peptide bond adjacent to the catalytic nucleophile to occur in the glycosyl enzyme species but not the apo-enzyme. As our model system, we chose the ~50-kDa Agrobacterium sp. β-glucosidase (Abg), (6) a retaining glycoside hydrolase from CAZy (Carbohydrate-active Enzymes Database) family GH1 that has been studied.
extensively in our laboratory (7, 8). Abg has been shown to form a stable, covalent intermediate with Glu-359 (underlined residue in the sequence YITENGA using numbering that includes the N-terminal Met) upon treatment with an appropriate 2-fluoro sugar such as 2-deoxy-2-fluoro β-glycosyl fluoride (2FGlcF, see Fig. 1b for the active site structure of a closely homologous enzyme trapped as its 2-fluoroglycosyl intermediate (9–11) and supplemental Figs. 1 and 2 for high resolution ESI-MS spectra of apo-Abg and 2FGlc-Abg).

Experimental Procedures

2FGlcF was synthesized as described previously (12–14). All materials were commercially available if not indicated otherwise. For all experiments, ultrapure water (from Millipore) and HPLC grade organic solvents were used.

Bacterial Expression and Purification of Abg.—Protein expression was conducted in *Escherichia coli* (BL21(DE3) transformed with pET29b (+) Abg: Ndel, Xhol). Cultures were grown at 37 °C in 1 liter of lysogeny broth (10 g/liter Tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) medium containing 50 μg/ml kanamycin. Isopropyl-1-thio-β-d-galactopyranoside was added to a final concentration of 0.05 mM at mid-log phase, and incubation continued at 30 °C for 5 h. Cells were collected by centrifugation and resuspended in 40 ml of binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 5 mM imidazole). To the suspension, 10 units of Benzonase nuclease (Novagen) were added, and it was passed through a pressure homogenizer three times. The cell extract was clarified by centrifugation at 16,000 × g for 20 min at 4 °C. The supernatant was passed through a 0.22-μm Millipore Millex® syringe filter and purified by binding to a nickel-nitrilotriacetic acid column (1 ml), washed with 10 ml of binding buffer and 5 ml of the same buffer containing 10 mM imidazole. The protein was eluted with an imidazole gradient (20 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 20–250 mM imidazole, 20 ml) at 1 ml/min over 5 ml using an ÄKTA purifier. 1-ml fractions were collected. Protein elution was monitored by absorption at 280 nm, and the purity of the fractions was checked by SDS-PAGE. Fractions were combined and concentrated using a 30,000 molecular weight cut-off spin filter (Millipore), and the buffer was exchanged to 10 mM sodium phosphate (pH 6.5) using the same spin filter.

Preparation of 2-Deoxy-2-fluoro-glucosyl Abg and Cleavage Reaction—To 100 μl of a solution of 3.3 mg/ml Abg in 10 mM sodium phosphate buffer (pH 6.5), 5 μl of 2FGlcF (20 mM in water) was added (final concentration of 2FGlcF was 1 mM). The solution was incubated at 37 °C for 40 min. The sample of Abg-2FGlc was then heated to 95 °C for 10 min to induce cleavage. Cleavage samples prepared for MS analysis were maintained in a reduced state by adding 5 mM DTT prior to heating (DTT is, however, not required for the cleavage process); 10 mM iodoacetamide was added after heating to obtain chemically homogenous fragments. After incubating 30 min at room temperature, 20 mM DTT was added to quench excess iodoacetamide. The sample was then diluted with 25 μl of acetone/1 HCl and acidified by adding 0.5 μl of TFA, and insoluble protein was removed by centrifugation. The sample was filtered through a 0.22-μm centrifugal filter device (Millipore) and subjected to HPLC purification. For the cleavage reaction induced by denaturation by urea, Abg-2FGlc was concentrated in a 30,000 molecular weight cut-off spin filter to ~10 mg/ml, and 25 μl was diluted into 500 μl of buffer containing 8 M urea, 10 mM phosphate, pH 7.0, and 5 mM DTT. The reaction mixture was incubated at room temperature for 1 h, and 15-μl aliquots were taken at the indicated time points, mixed with 5 μl of 4X SDS loading buffer, and subjected to SDS-PAGE analysis without heating.

HPLC Purification of the Abg Fragments—Purification of the Abg fragments was carried out using an Agilent 1200 LC system equipped with an analytical Vydac C18 column (4.6 × 250 mm, 5-μm particle size, 300 Å pore size) at 1.0 ml/min flow rate. The mobile phase was a mixture of solvent A (water with 0.1% TFA)
and solvent B (acetonitrile with 0.1% TFA). A gradient from 20 to 95% solvent B was applied over 30 min. The chromatograms were monitored with a diode array UV detector at 214 and 280 nm. Fractions absorbing at 214 nm (threshold 5 mAU; slope 5 mAU/s) were collected. To concentrate the samples for further analysis, the fractions were freeze-dried and redissolved in 100 μl of 1:1 acetonitrile/water, 0.1% TFA. After analyzing 5 μl of each fraction by SDS-PAGE, 5 μl of the fractions containing the small and the large protein fragments was subjected to high resolution ESI-QTOF MS.

High Resolution ESI-QTOF MS Analysis of Intact Abg and Its Fragments—Samples were analyzed with a Waters nanoAC-QUITY UPLC equipped with a C4 cartridge and coupled to a high resolution ESI-QTOF MS.

**FIGURE 2.** The glycosyl enzyme intermediate undergoes autolysis at the site of the glycosylation upon denaturation. a, SDS-PAGE showing that denaturation of the glycosyl enzyme at room temperature in 8 M urea and 10 mM sodium phosphate, pH 6.5, is sufficient to induce autolysis. Lane 1, 5 min; lane 2, 10 min; lane 3, 20 min; lane 4, 40 min; lane 5, 60 min. Heating to 70 °C for 10 min has the same effect in 8 M urea (lane 6) or 10 mM phosphate buffer alone (lane 7). Without chemical or thermal denaturation, the glycosyl enzyme remains intact (lane 8). Treatment of the apo-enzyme under the same conditions does not induce cleavage (lanes 10 – 13: denatured by 8 M urea and/or heat; lane 14: not denatured). The arrow indicates the faint band of the smaller fragment. Note that denaturation results in a small shift of mobility (lanes 8 and 14, which have not been denatured, versus the rest). b, purification of the small fragment (13.9 min) from the larger fragment and the intact protein (15.4 min) by reversed phase HPLC as verified by SDS-PAGE (supplemental Fig. 3), c, high resolution ESI MS spectrum of the fraction collected at 13.9 min showing the C-terminal fragment corresponding to a cleavage at Glu-359 and formation of a pyroglutamate (m/z monoisotopic [M+H]+ calcld. 2234.9; found 2235.1). Abs. intensity, absolute intensity. b, The MALDI-TOF MS spectrum of the sample after digestion with pyroglutamyl aminopeptidase indeed shows a mass difference of −111 m/z for the C-terminal peptide (see inset, m/z monoisotopic [M+H]+ calcld. 2123.9; found: 2124.0). The two smaller peaks (m/z +16) correspond to partial methionine oxidation of the same peptides. See supplemental Table 1 for a complete listing of tryptic peptides and their masses.

**FIGURE 3.** Digest with pyroglutamyl aminopeptidase shows the presence of N-terminal pyroglutamate within the small C-terminal fragment. a, MALDI-TOF MS spectrum of the tryptic digest of the small fragment. The inset focuses on the signal of the N-terminal tryptic peptide, which potentially carries a pyroglutamate (m/z monoisotopic [M+H]+ calcld. 2234.9; found 2235.1). Abs. intensity, absolute intensity. b, The MALDI-TOF MS spectrum of the sample after digestion with pyroglutamyl aminopeptidase indeed shows a mass difference of −111 m/z for the C-terminal peptide (see inset, m/z monoisotopic [M+H]+ calcld. 2123.9; found: 2124.0). The two smaller peaks (m/z +16) correspond to partial methionine oxidation of the same peptides. See supplemental Table 1 for a complete listing of tryptic peptides and their masses.

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Waters Xevo G2 QTOF. Raw data were deconvoluted using the maximum entropy algorithm.

MALDI-TOF MS Analysis of Tryptic Peptides—The sample was desalted by binding the peptides to a C18 ZipTip (Millipore) and washing four times with 0.1% TFA in water. The peptides were eluted with 1.4 µl of 60% acetonitrile/water containing 0.1% TFA. The eluted sample was mixed with 1.0 µl of 7 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile/water, 0.1% TFA and applied to the MALDI target. Analysis was carried out on a Bruker AutoFlex III MALDI-TOF mass spectrometer in the positive ion mode using a reflector.

Tryptic Digest of the C-terminal Fragment—From the redisolved HPLC fraction containing the small fragment (concentration ~1 mg/ml), 20 µl was neutralized by adding 1 µl of saturated sodium bicarbonate solution. The protein solution was diluted with 20 µl of 200 mM ammonium bicarbonate buffer, 1 µl of trypsin (1 µg/µl, MS grade, Sigma) was added, and the solution was incubated at 37 °C for 1.5 h. Trypsin was then heat-inactivated by incubating at 95 °C for 10 min. For MALDI-TOF MS analysis, 5 µl of the trypsic digest was diluted with 5 µl of double-distilled H2O containing 0.1% TFA and desalted using the ZipTip protocol described above.

Pyroglutamyl Amino Peptidase Digestion of Tryptic Peptides—A sample (5 µl) of the trypsic digest was freeze-dried and reconstituted in 10 µl of pyroglutamyl aminopeptidase buffer (Takara Clontech). Pyroglutamate aminopeptidase (0.5 µl = 0.1 milliunits from Pyrococcus furiosus, Takara Clontech) was added, and the sample was incubated at 50 °C for 2 h. The sample was then freeze-dried, reconstituted in 13 µl of water with 0.1% TFA, and desalted using the ZipTip protocol described above.

Results

To monitor possible cleavage, we first incubated Abg with 2FGlcF to form the ester (see Fig. 4) and then subjected samples to either chemical or thermal denaturation and analyzed the samples by SDS-PAGE. As can be seen in Fig. 2a, time-dependent cleavage of Abg into two fragments occurs upon incubation in 8 M urea. The size of these fragments (~10 and 40 kDa) is consistent with cleavage approximately at the site of the catalytic nucleophile. The autolysis reaction occurs around neutral pH (6.0 – 8.0 have been tested) and is independent of additives to the buffer or the mechanism of denaturation. The covalent intermediate cleaves at room temperature in high concentrations of denaturants as well as in 10 mM phosphate buffer at temperatures >70 °C. In contrast, no autolysis is seen with unmodified Abg under otherwise identical conditions. To identify the fragments, the cleaved Abg sample was purified by reversed phase HPLC (Fig. 2b) and subjected to mass analysis. It proved to be critical to reduce/alkylate the cysteine residues after cleavage to obtain high resolution mass spectra, revealing the masses of the two fragments as 11,498.8 and 40,044.0 Da (Fig. 2, c and d). These values correspond, with 2 and 8 ppm accuracy, respectively, to the expected masses for cleavage at Glu-359 according to the mechanism shown in Fig. 1a. To confirm that the mass of 11,498.8 for the C-terminal fragment is indeed consistent with N-terminal pyroglutamate formation and not due to some other dehydration reaction, we digested the sample with trypsin and analyzed the peptides by MALDI-TOF MS. Six of the seven expected peptide ions were found, including the N-terminal peptide of this small C-terminal fragment, with a monoisotopic mass of 2235.1 corresponding to the expected masses for cleavage at Glu-359 according to the mechanism shown in Fig. 1c.

FIGURE 4. Proposed cleavage reaction mechanism for 2-deoxy-fluoro glucopyranosyl-Abg. The covalent inhibitor 2FGlcF (6) forms a stable glycosyl ester (8) with the nucleophilic catalyst Glu-359 of Abg (7). Upon denaturation, the amide nitrogen of Glu-359 attacks the activated carbonyl to form the 1-acylpyrrolidin-2-one (9). This diacylamide is preferentially hydrolyzed at the backbone carbonyl function, yielding the N-terminal fragment (10) and the C-terminal fragment (11), which features an N-terminal pyroglutamyl residue. The chemical steps are analogous to the OGT-induced cleavage mechanism shown in Fig. 1a.
that of 2235.1. This −111 Da difference is indeed that expected
from the loss of a pyroglutamate residue.

**Discussion**

Our data demonstrate that the γ-glycosyl ester of glutamic acid is indeed able to activate the side chain sufficiently to induce hydrolysis of the peptide bond between the glutamic acid and the preceding residue according to the mechanism shown in Fig. 4, which is analogous to the proposed cleavage mechanism for HCF-1 in Fig. 1a. These findings provide solid support for the hypothesis that such a glycosyl ester is the key intermediate formed during the cleavage of HCF-1 in the active site of OGT. Alternative proposed mechanisms for OGT-cata
yzed HCF-1 lysis involving participation of the cysteine residue preceding Glu in HCF-1 (3) appear to be unlikely in light of our results. However, it is possible that co-evolution of OGT and HCF-1 has delivered an environment that allows more facile cleavage through hydrogen bonding and acid/base interactions.

Although isolated examples are found in the literature in which proteolytic cleavage has been induced by formation of active site esters, primarily in acyltransferases, so far the ester
tified intermediates have been elusive (15). Early observations from peptide chemistry show that activated glutamyl side
chains can undergo backbone autolysis. Model peptides that have been transiently converted into the respective side chain acyl chloride, or even benzyl ester, were shown to form the internal 1-acetylpyrrolidin-2-one by the mechanism shown in Figs. 1a and 4 (16). In the absence of steric or conformational hindrance, this 1-acetylpyrrolidin-2-one, which is a diaacilamide, undergoes specific cleavage at the backbone amide rather than the lactam amide because nucleophilic attack by water is favored at the more electrophilic carbonyl (15, 17). In our model system, about half of the protein underwent cleavage, with the other half likely undergoing competing direct hydrolysis of the glycosyl ester (18). However, for enzymes that potentially activate glutamyl side chains for autolysis by means of esterification, formation of the 1-acetylpyrrolidin-2-one intermediate could be accelerated by general base catalysis in the active site, thereby ensuring a higher conversion rate. Strikingly, the crystal structure of the ternary product complex of OGT (Protein Data Bank code 4GYW) indeed shows an interaction that could promote formation of the 1-acetylpyrrolidin-2-one in HCF-1: the α-phosphate of UDP forms a hydrogen bond with the amide NH of the glycosylated residue of the peptide, thereby potentially enhancing its nucleophilicity (19). Furthermore, very recent results from the Herr group (20) indicate that the scissile glutamic acid in HCF-1 exhibits unfavorable interactions with the OGT/UDP-GlcNAc complex, thereby potentially applying strain to the backbone, reducing the planarity of the amide bond and positioning the nitrogen for a nucleophilic attack. This mechanism could further add to the catalytic effi
ciency of OGT-promoted proteolysis of HCF-1 as opposed to our model system, where denaturation allows the reaction to occur by widening the conformational space.

Taken together, our data on cleavage of a characterized glycosyl ester within a protein provide the missing experimental link between glycosyltransferase activity and proteolysis, albeit in a glycosidase hydrolase rather than a glycosyltransferase. The
general chemical mechanism underlying this process could, at least in principle, also be exploited by other glycosyltransferases and transglycosylases acting on protein side chains to effect specific chain cleavages. This intriguing process therefore gives a new perspective on proteolytic maturation mechanisms in vivo.

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