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Ubiquitin and ubiquitin-derived peptides as substrates for peptidylglycine α-amidating monooxygenase

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Abstract Ubiquitin (Ub) and the ubiquitin-like proteins (UBLs) mediate an array of cellular functions. These proteins contain a C-terminal glycine residue that is key to their function. Oxidative conversion of C-terminal glycine-extended prohormones to the corresponding α-amidated peptide is one step in the biosynthesis of bioactive peptide hormones. The enzyme catalyzing this reaction is peptidylglycine α-amidating monooxygenase (PAM). We report herein that Ub is a PAM substrate with a $(V/K)_{amidation}$ that is similar to other known peptide substrates. This work is significant because PAM and the UBLs co-localize to the hypothalamus and the adrenal medulla and are both over-expressed in glioblastomas.

Keywords: Peptidylglycine α-amidating monooxygenase; Ubiquitin modification

1. Introduction

Ubiquitin (Ub) is a small, highly conserved 76-residue polypeptide found in all eukaryotes [1]. Ub is the most studied and best understood member of the family of Ub-related proteins, compromised of the Type-1 ubiquitin-like proteins (UBLs) and the Type-2 ubiquitin-domain proteins (UDPs) [2–4]. The post-translational decoration of proteins with Ub or the UBLs mediate an array of cellular functions, including directed protein degradation via the 26S proteasome [5], cell-cycle progression [6], apoptosis [7], regulation of gene expression and DNA repair [7,8], and a sorting signal for vesicular or nuclear transport [2,8]. The UDPs do not function by modifying other proteins, but instead, contain an ubiquitin-like sequence within a larger overall sequence that enables the UDP to bind to the proteasome. The UDP is not degraded by the proteasome in the UDP·proteasome complex, but instead seems to regulate proteasomal function [4].

Critical to the cellular role played by Ub and the UBLs is a conserved glycyl residue at the C-terminus (Table 1). The decoration of proteins with Ub or the UBLs is accomplished by the formation of an isopeptide bond between the C-terminal glycine and the ε-amide of Lys residue within the decorated protein [2] or the formation of a standard peptide bond between the C-terminal glycine and the N-terminus of the ubiquitinated protein [14]. In addition to the attachment of a single Ub or UBL to a protein, polyubiquitin chains can be formed by the attachment of another Ub to Lys-48 of the attached Ub [2]. Polyubiquitination targets the substrate protein for destruction by the proteasome. Elimination of the C-terminal glycine by proteolysis [15,16], site-directed mutagenesis [12,13,17,18], or frame shift mutation [19] prevents Ub/UBL function. These results indicate that any in vivo chemistry that would eliminate the C-terminal glycine would compromise the Ub/UBL system, mostly likely with deleterious health effects [5,12,13,19]. One known reaction that potentially could eliminate the C-terminal glycine of Ub and the UBLs would be C-terminal amidation: $Ub_{1–75}$-CO-NH-CH_2-COOH → $Ub_{1–75}$-CO-NH_2 + CHO-COOH. The non-functional product with an amidated C-terminus, des-Gly-$Ub_{1–75}$-amide in the case of Ub, would be unable to form the critical linkage to either the N-terminus or internal Lys residue of the acceptor protein. In vivo, peptidylglycine α-amidating monooxygenase (PAM, EC. 1.14.17.3) catalyzes the oxidative cleavage of glycyl C_α–N bond in the glycine-extended neuropeptide precursors to the α-amidated peptide and glyoxylate [20,21]. In addition, PAM may also function in the biosynthesis of oleamide and the other fatty acid primary amides via a similar cleavage of long-chain *N*-acylglycine precursors [22,23]. In vitro studies have demonstrated that PAM exhibits a broad substrate specificity not only for glycine-extended peptides [24,25], but also for an extensive set of non-peptide *N*-acylglycines and *N*-arylglycines [22,25–27].

Because PAM and the UBLs co-localize to the hypothalamus [28,29] and the chromaffin granules of the adrenal medulla [30–32] and are both over-expressed in glioblastomas [33,34], we sought to determine if Ub and a number of glycine-extended peptides based on the C-terminus of Ub were amidated by PAM. We report here that Ub and the Ub-derived peptides are PAM substrates with *V*/*K* values that are comparable to glycine-extended precursors of known α-amidated peptide hormones. These data coupled with an earlier report of Ub

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Table 1
Comparison of the C-terminal sequences of Ub and UBLs[a]

Ub or UBL	C-terminal 10 amino acids[b]	Ref.
Ub[c]	LHLVLRLRGG	[8]
SUMO-1	IEVYQEQTGG	[8]
SUMO-2	IDVFQQQTGG	[8]
SUMO-3	IDVFQQQTGG	[8]
NEDD8	LHLVLALRGG	[9]
ISG15	VFMNLRLRGG	[10]
FAT10	LFLASYCIGG	[11]
Ufm1	LRIIPRDRVG	[12]
GATE16	VAYSGENTFG	[13]
GABARAP	VAYSDESVYG	[13]

[a]The sequences are all for the human proteins.
[b]Underlined is the C-terminal glycine residue, a potential site of oxidative cleavage to generate an α-amidated Ub/UBL derivative and glyoxylate.
[c]Human Ub and bovine Ub have the same amino-acid sequence [1].

glutathionylation in HepG2 cells [35] indicate that the post-translational modification of Ub and UBLs may represent an unrecognized process to regulate the function of these proteins. In addition, we find that the *V*/*K* values for the Ub-derived peptides do not correlate well with the value for Ub, bringing into question the in vivo relevance of similar data generated using small, model peptide substrates.

2. Materials and methods

2.1. Materials

Bovine Ub, TPCK-treated trypsin, soybean trypsin inhibitor, Gly–Gly, glycinamide, and *N*-acetylglycine were from Sigma, 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were from Anaspec, *N*-dansyl-Tyr-Val-Gly and 4-(dimethylamino)azobenzene-4′-sulfonyl chloride (dabsyl chloride) were from Fluka BioChemika, bovine catalase was from Worthington, diisopropylethylamine (DIPEA) was from Advanced Chemtech, and Arg–Gly–Gly was from Research Plus, Inc. PAM was gift from Unigene Laboratories, Inc. Pyruvate-extended *N*-acetylphenylalanine (*N*-Ac-Phe-pyruvate, 2,4-diketo-5-acetamido-6-phenylhexanoic acid) was synthesized using a published procedure [36]. All other reagents were of the highest quality available from commercial suppliers.

2.2. PAM-catalyzed consumption of O_2 and the production of glyoxylate from ubiquitin

Reactions at 37 ± 0.1 °C were initiated by the addition of rat PAM (39 μg) into 4.0 mL of 100 mM MES/NaOH, pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0 μM $Cu(NO_3)_2$, 10 μg/mL catalase, 5.0 mM ascorbate, 500 μM ubiquitin (4.25 mg/mL), and either 0 or 45 μM *N*-Ac-Phe-pyruvate. *N*-Ac-Phe-pyruvate inhibits the PAL activity of bifunctional PAM with a $K_i = 0.24$ μM and also weakly inhibits the peptidylglycine α-hydroxylating monooxygenase (PHM) activity with a $K_i \geqslant 25$ μM [36]. In one sample, O_2 consumption was continuously monitored using a Yellow Springs Instrument Model 53 oxygen monitor. In a second matched sample, aliquots were removed at the indicated time and added to a vial containing one-fifth volume of 6% (v/v) TFA to quench the reaction. The concentration of glyoxylate in each quenched sample was determined spectrophotometrically using the method of Christmen et al. [37] as modified by Katopodis and May [26].

2.3. Tryptic digestion of ubiquitin followed by HPLC analysis of the dabsylated reaction products

Ub (4.25 mg in 1.0 mL) was incubated with 43 μg of PAM in 100 mM MES/NaOH, pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0 μM $Cu(NO_3)_2$, 10 μg/mL catalase, and 5.0 mM ascorbate. The control consisted of the same reaction conditions without the addition of PAM. After 1.0 h at 37 ± 0.1 °C, the reactions were terminated by the 0.2 mL of 6% (v/v) TFA and the volumes reduced to ~0.3 mL by ultrafitration. Three changes of 1.0 mL of 25 mM $NaHCO_3$ was sufficient to adjust the pH of the solutions to 8.2–8.5. Trypsin (0.1 mg) was added to the solutions, the solution incubated for 30 min at 37 ± 0.1 °C, and proteolysis terminated by the addition of 0.2 mg soybean trypsin inhibitor. Our procedure is based on the trypsinization procedures of Wilkinson and Audhya [38]. The resulting solution was diafiltered using a Centricon-C3 diafilter and the flowthrough containing the released Gly–Gly dipeptide and glycinamide were dabsylated as described by Stocchi et al. [39]. The dabsylation reaction mixture was analyzed by RP-HLC using a Keystone Scientific Hypersil ODS column (4.6 × 100 mm). Separations were performed at 32 °C using a binary solvent system of 25 mM potassium phosphate, pH 6.8 (solvent A) and acetonitrile (solvent B) as follows: A/B of 80/20 for 0–10 min, A/B of 70/30 for 10–20 min, and A/B of 30/70 for 20–30 min. Analytes were detected at 436 nm using a flow-through UV/visible variable wavelength detector. Under these conditions, dabsyl-Cl had a retention time 8.0 min, glycine of 14.6 min, Gly–Gly of 15.7 min, and glycinamide of 22.7 min.

2.4. Initial rate studies of RGG ($Ub_{74–76}$) amidation

Reactions at 37 ± 0.1 °C were initiated by the addition of PAM (26 μg) into 3.0 mL of 100 mM MES/NaOH, pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0 μM $Cu(NO_3)_2$, 10 μg/mL catalase, 5.0 mM ascorbate, and 0.5–8.0 mM RGG. Initial rates of O_2 consumption were measured using an O_2 electrode. V_{MAX} values were normalized to controls performed at 11.0 mM *N*-acetylglycine. Under these conditions, the initial concentration of O_2 was 217 μM [40].

2.5. Inhibition of N-dansyl-tyr-val-gly amidation

Reactions at 37 ± 0.1 °C were initiated by the addition of PAM (30–40 ng) into 0.25 mL of 100 mM MES/NaOH, pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0 μM $Cu(NO_3)_2$, 10 μg/mL catalase, 6.0 mM ascorbate, 8.0 μM *N*-dansyl-Tyr-Val-Gly, and either Ub or the Ub-derived peptides (concentration range = 1.0–3.0 × K_M). The reaction was terminated at the desired time interval by removing a 40 μL aliquot of the reaction and adding this to a HPLC microvial containing 8 μL of 6% (v/v) TFA. Amidation of *N*-dansyl-Tyr-Val-Gly was then monitored by reverse-phase HPLC [41]. Under these conditions, the K_M for *N*-dansyl-Tyr-Val-Gly is 3.5 μM.

2.6. The V_{MAX} values for the amidation of Ub and the Ub-derived peptides

The quantities of Ub and the Ub-derived peptides required to define the V_{MAX} values at peptide concentration ($\geqslant 10 \times K_M$) were prohibitively expensive. Measurements of the V_{MAX} values were obtained by determining the initial rates of O_2 consumption or glyoxylate production at one peptide concentration and then using the initial rate to calculate the V_{MAX} value from the Michealis–Menton expression.

For Ub, O_2 consumption was monitored using an O_2 electrode following the addition of 100 μg of PAM into 1.6 mL of 100 mM MES/NaOH, pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0 μM $Cu(NO_3)_2$, 10 μg/mL catalase, 5.0 mM ascorbate, and 0.87 mM Ub. The initial rates obtained at 0.87 mM Ub were normalized to values obtained for 11 mM *N*-acetylglycine.

For *N*-Ac-RGG (*N*-Ac-$Ub_{74–76}$), *N*-Ac-LRGG (*N*-Ac-$Ub_{73–76}$), *N*-Ac-LRLRGG (*N*-Ac-$Ub_{71–76}$), *N*-Ac-LHLVLRLRGG (*N*-Ac-$Ub_{67–76}$), and *N*-Ac-QKESTLHLVLRLRGG (*N*-Ac-$Ub_{62P–76}$), glyoxylate production was initiated by the addition of 36 μg of PAM into 0.9 mL of 100 mM MES/NaOH, pH 6.0, 30 mM NaCl, 1.0% (v/v) ethanol, 0.001% (v/v) Triton X-100, 1.0 μM $Cu(NO_3)_2$, 10 μg/mL catalase, 5.0 mM ascorbate, and 1.0 mM peptide. At the desired times, a 75-μL aliquot was removed and added to a vial containing 15 μL of 6% (v/v) TFA to terminate the reaction. The concentration of glyoxylate produced in the acidified samples was determined spectrophotometrically. The initial rates of glyoxylate production from the Ub-derived peptides were normalized to values obtained for 1.0 mM *N*-acetylglycine.

2.7. Data analysis

Kinetic parameters for RGG were obtained by a KaleidaGraph™ fit of the initial velocity (*v*) vs. [RGG] ([S]) data to