Peptide toxin glacontryphan-M is present in the wings of the butterfly *Hebomoia glaucippe* (Linnaeus, 1758) (Lepidoptera: Pieridae)

Narkhyun Bae[a,1], Lin Li[a,1], Martin Lödl[b], and Gert Lubec[a,2]

[a]Department of Pediatrics, Medical University of Vienna, 1090 Vienna, Austria; and [b]2nd Department of Zoology, Naturhistorisches Museum Wien, 1010 Vienna, Austria

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Protein profiling has revealed the presence of glacontryphan-M, a peptide toxin identified only in the sea snail genus *Conus*, in the wings of *Hebomoia glaucippe* (HG). The wings and body of HG were homogenized and the proteins were extracted and analyzed by 2D gel electrophoresis with subsequent in-gel digestion. Post-translational protein modifications were detected and analyzed by nano–LC-MS/MS. An antibody was generated against glacontryphan-M, and protein extracts from the wings of HG samples from Malaysia, Indonesia, and the Philippines were tested by immunoblotting. Glacontryphan-M was unambiguously identified in the wings of HG containing the following posttranslational protein modifications: monoglutamylation at E55, methylation at E53, quinone modification at W61, cyanylation at C56, and amidation of the C terminus at G63. Immunoblotting revealed the presence of the toxin in the wings of HG from all origins, showing a single band for glacontryphan-M in HG samples from Malaysia and Philippines and a double band in HG samples from Indonesia. Intriguingly, sequence analysis indicated that the *Conus* glacontryphan is identical to that of HG. The toxin may function as a defense against diverse predators, including ants, mantes, spiders, lizards, green frogs, and birds.

mass spectrometry | venom

Although a large number of low molecular weight deterrents and toxins have been demonstrated to exist in Lepidoptera (1), information on the peptide or protein sequences is limited to a few of these toxins.

The cloning and purification of pierisin-2, an apoptosis-inducing protein from *Pieris brassicae*, was reported in 2000 (2). The protein shows homology with other ADP ribosylating toxins, including the A subunit of cholera toxin and pierisin-1. Pierisin-1–induced apoptosis is mediated by mono-ADP ribosylation of DNA (3). This toxin, isolated from the cabbage butterfly *Pieris rapae*, was found to induce mutations of the hypoxanthine-phosphoribosyltransferase (4). The toxin is highly expressed during the larval stage and rapidly decreases before pupation to the very low levels maintained in adults (5). Another protein, papiliocin, is a 37-residue cecropin-like peptide isolated from the larvae of the swallowtail butterfly *Papilio xuthus* (6). This protein shows low toxicity toward mammalian cells but demonstrates antimicrobial activity (7).

In conducting a proteomic profile of the wings of *Hebomoia glaucippe* (HG) (8, 9), we observed a protein that was unambiguously identified as glacontryphan-M (GT). Study of this protein toxin was motivated by the result that GT, intriguingly, so far only been reported in the sea snail *Conus marmoreus*, was observed by 2D gel electrophoresis (2DE) in butterfly wing protein samples at relatively high abundance but was not detected in samples from the body. Furthermore, we were interested in studying Lepidoptera toxins because of the potential medical relevance of these toxins in causing human disease (10, 11). The aim of the present study was to characterize GT using a combination of full-length peptide sequencing and the determination of the protein posttranslational modifications (PTMs) of the toxin. Additional goals included determining whether GT was found in the butterfly wing and demonstrating that the protein toxin was present on HG samples originating from various southeastern Asia regions.

Results

Characterization and Peptide Identification of GT by 2DE and MS, Respectively. Protein analysis of dry season HG wings as well as the HG caterpillar by 2DE gave rise to a single spot with an apparent molecular weight of ∼8 kDa (Fig. 1 and Fig. S1). GT from dry season HG wings was fully sequenced, with complete sequence coverage achieved by multienzyme digestions using trypsin, chymotrypsin, subtilisin, and Asp-N. A list of the resulting peptides is given in Table 1. The peptide sequences and parameters for identification are also listed, as well as the enzymes used to generate the individual peptides.

As shown in Fig. S1, GT from the caterpillar was detected with high sequence coverage (34.92%), unambiguously identifying the proteotoxin in caterpillar skin.

Determination of PTMs. A series of dry season wing GT protein modifications and PTMs were observed in the peptides, including quinone modification at W61, C-terminal amidation at G63, methylation at E53, monoglutamylation at E55, and cyanylation at C56. The corresponding mass spectra are shown in Figs. S2–S4.

Methylation at E53 was verified by the observation of a methyl modification in samples in which ethanol had been substituted for methanol in all analytical steps (Fig. S3).

Verification of monoglutamylation at E55 was obtained by in-gel treatment with carboxypeptidase G, which revealed a shift in the observed mass consistent with cleavage of this residue from the GT peptide (Fig. S4).

As shown in Fig. 2, a 2D Western blot combined with a Coomassie-stained 2DE gel indicated the presence of a single spot for GT at ∼8 kDa. This figure also presents evidence for the observation of GT in the wings, but not in the body, of HG.

Evidence for the presence of GT in wings of HG samples from different origins is given in Fig. 3. Fig. 3*A* shows a comparison of control GT (control) and GT isolated from HG wings. The HG sample has minor immunoreactive bands for GT that may indicate protein modifications or splice variants. Fig. 3*B* shows that the immunoreactive GT in the wings of HG was similar for

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[1]N.B. and L.L. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: gert.lubec@meduniwien.ac.at.

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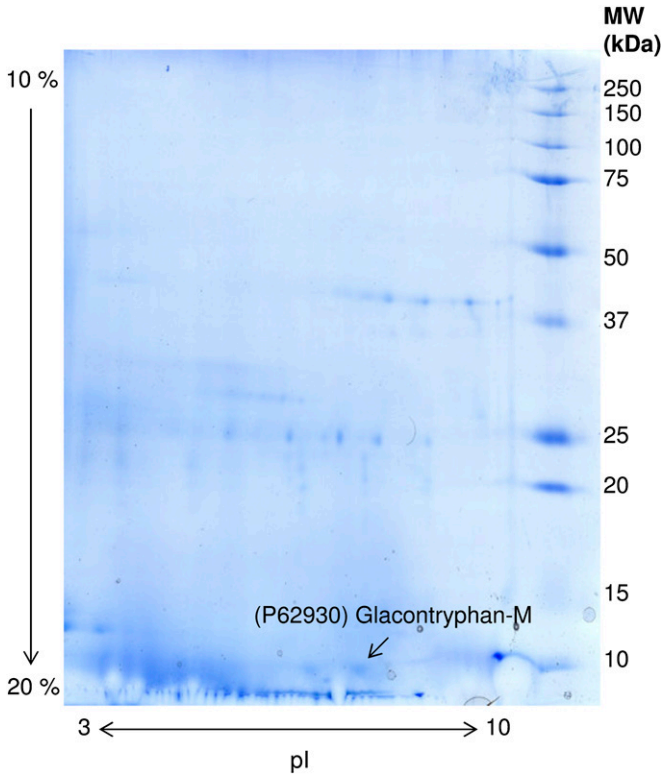

Fig. 1. Two-dimensional gel electrophoresis of HG wing samples. A representative Coomassie blue-stained gel is shown, indicating the presence of GT. Other proteins identified by gel staining are reported elsewhere (8).

the Malaysian and the Philippine samples, and the Indonesian sample showed a double band with an approximate apparent molecular weight of 20 kDa. This finding may be interpreted as indicative of the presence of multiple GT isoforms or of protein modifications.

Clear evidence for the presence of GT in dry and wet season HG wings, as well as in caterpillar skin, was revealed, presenting at the identical apparent molecular weight (Figs. S5 and S6).

Discussion

GT was unambiguously identified in the wings of HG, and its sequence was determined to share 100% identity with the GT from the sea snail *C. marmoreus* [UniProtKB: P62903.1; National Center for Biotechnology Information (NCBI): GI:51701274]. Moreover, the presence of GT was observed in the HG caterpillar skin. This protein, a member of the contryphan family of conotoxins, was found in the wings of HG, both dry and wet season forms, but not in the body of the butterfly. Determination of the complete sequence was achieved using a gel-based mass spectrophotometry method following multienzyme digestion of the protein spot (12) and the subsequent use of two different fragmentation methods on an ion trap mass spectrometer. Using these methods, protein changes and PTMs were identified.

GT is a protein of 63 amino acids with a known conformation and a series of PTMs (13), including a specific γ-carboxyglutamic acid modification (14). Characteristic features of the GT from *C. marmoreus* are a conserved disulfide-bonded loop, the presence of a D-tryptophan, a histidine (H) within the intercystine loop, and two γ-carboxyglutamic acid residues (13).

An analogous γ-carboxyglutamic acid residue was not detected in the GT from butterfly wings; however, tandem MS/MS suggested that the glutamic acid was modified by monoglutamylation. This finding was verified by treatment with glutamyl hydrolase and analysis of the resulting mass shift changes. This modification has already been shown to be present in several proteins and to modify protein function (15). GT from *C. marmoreus* contains proline hydroxylations, similar to those found at prolines P57 and P60 in the GT from HG, and a C-terminal amidation, confirmed to occur at G63 in the HG sample. In the present study, a series of additional modifications were detected; for example, methylation at E53 was confirmed by running gels in which methanol was replaced with ethanol (16). This PTM increases the hydrophobicity of the protein, thereby modifying the binding to the calcium channel target of GTs. Oxidative changes that would not be expected from ambient oxygen exposure or artifacts from the analytical procedure were observed on tryptophans, including dioxidation at W58 and quinone modification at W61. This finding is distinct from the oxidation of methionine (M48), which is known to occur during analytical steps. The functional relevance of tryptophan oxidations remains elusive.

Formation of 2-oxo-histidine is likely catalyzed by heavy metals, such as copper (17), but the source of copper or heavy metals for histidine oxidation (H59) of GT remains unknown. The butterflies do, however, experience oxidative stress as a result of feeding on some plants. Some plants use peroxidases as defensive mechanisms, possibly explaining why oxidative modifications are observed on the Lepidoptera feeding on them (18). Furthermore, Lepidoptera and other arthropods are known to contain phenol oxidases that may be responsible for quinone modification of GT of HG (19).

The underlying cause of cysteine cyanylation, observed at C56 in GT from HG, is unclear; although, it is known that some Lepi-

Table 1. Peptides identified in glacontryphan-M (P62903)

Peptide sequence	Observed	M_r (expt)	M_r (calc)	Δ	Score	Enzyme*
1 -MGKLTIL.V 7	508.830	1015.645	1015.556	0.090	37	C
4 K.LTILVLVAAVLLSTQVMVQGDR.D 25	785.795	2354.363	2354.361	0.003	44	A
15 L.LSTQVMVQGDRDQPADRN.A 32	682.660	2044.958	2044.960	−0.002	42	C
32 R.NAVPRDDNPGR.A 42	488.340	1465.157	1465.020	0.138	44	T
33 N.AVPRDDNPGRARRKRMKVL.N 51	745.820	2234.438	2234.265	0.172	38	S
37 R.DDNPGRAR.R 44	450.760	899.505	899.421	0.085	34	T
48 R.MKVLNESECPWHPWCG 63	682.685	2045.033	2044.859	0.174	54	T
49 M.KVLNESECPW.H 58	631.360	1260.705	1260.580	0.124	47	C
50 K.VLNESECPWHPW.C 61	777.430	1552.845	1552.677	0.169	66	T
50 K.VLNESECPWHPWCG.- 63	885.920	1769.825	1769.729	0.096	63	T
52 L.NESECPWHPW.C 61	671.310	1340.605	1340.524	0.081	41	S
52 L.NESECPWHPWCG-63	779.846	1557.820	1557.576	0.106	64	S
53 N.ESECPWHPWCG.- 63	780.270	1558.525	1558.600	−0.075	37	A

*A, Asp-N; C, Chymotrypsin; S, Subtilisin; T, Trypsin; M_r, molecular weight.

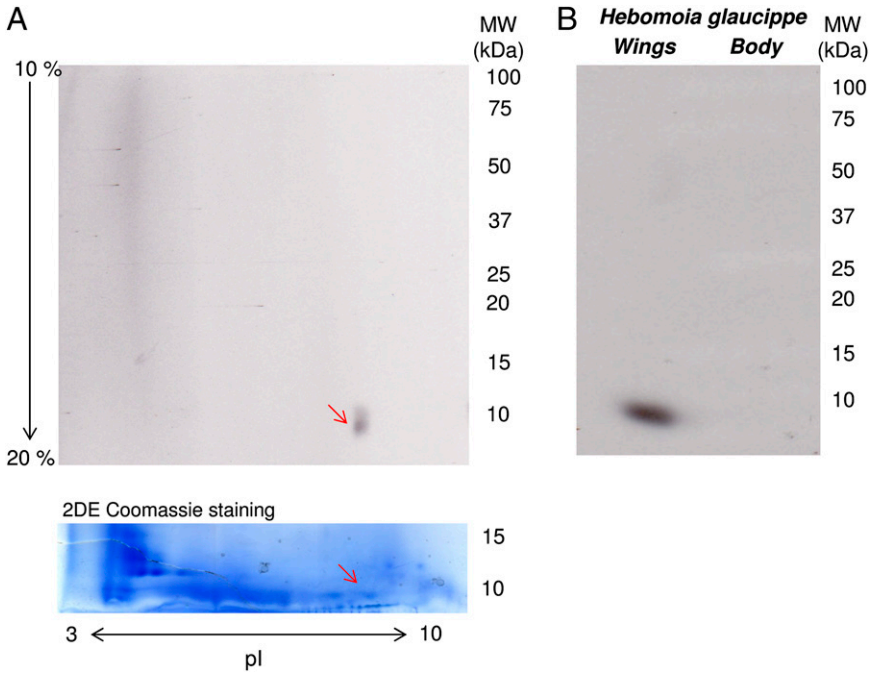

Fig. 2. Western blotting of GT using 2DE and 1D immunoblotting. (*A*) The 2D position of GT is demonstrated by immunoblotting (*Upper*). The Coomassie-stained gel showing the GT, indicated by the arrowhead, is shown below (*Lower*). (*B*) Immunoblotting demonstrates that GT is located in the wings of HG, but not in the body.

doptera feed on cyanogenic plants and use cyanogens as a defense against predators (20, 21), possessing the machinery for the de novo synthesis of these toxins (22, 23). HG both feed and deposit their eggs on plants of the family Capparaceae (24), a species that are capable of cyanogenesis (25).

Using a specific antibody against GT, we identified the protein in the wings but not the bodies of HG samples originating from the Cameron highlands, Malaysia, Mindoro, Philippines, and Buton, Indonesia. Although the electrophoretic mobility between samples from the first two locations was comparable, migrating with

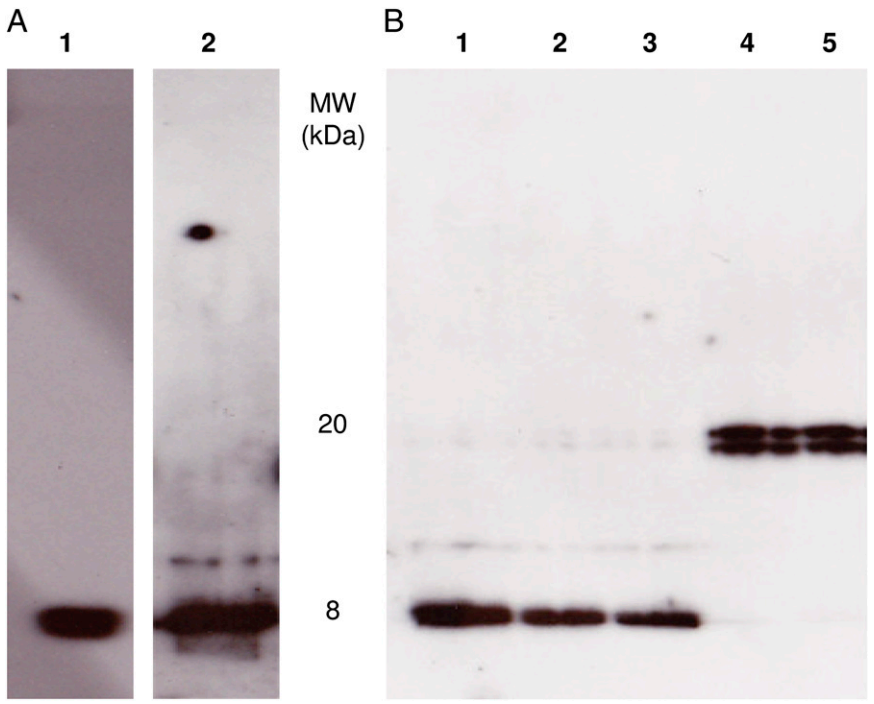

Fig. 3. Western blotting showing GT immunoreactivity. (*A*) A control sample of the recombinant protein GT (lane 1) compared with the pattern observed in the wings of HG (lane 2). (*B*) The immunoblot pattern observed from HG samples of the following origins: Malaysia (lane 1), Philippines (lanes 2 and 3), and Indonesia (lanes 4 and 5). The last sample shows a double band at a higher apparent molecular weight of ~20 kDa.

an apparent molecular weight of 8 kDa, the Indonesian sample showed a double band with an apparent molecular weight of ~20 kDa. This finding points to the presence of a homolog or the presence of heavy protein modifications (e.g., glycosylations) that lead to decreased mobility on the gel. A single band at ~8 kDa was observed when using the recombinant GT; thus, additional bands observed in the wing GT samples likely indicate isomers or the presence of PTMs. Taking these data together, we have uniquely demonstrated the presence of a specifically modified GT in the wings of HG samples from several geographical areas. The protein modifications and PTMs may result from exogeneous sources, such as food plants, or may be genetically encoded.

These findings raise a series of questions, including the probable role of GT as a defensin, as is suggested from the observation that HG predators usually avoid the wings, including birds, ants, and orchid mantis (Movie S1), although another predator, *Nephila pilipes*, would kill and suck out the body of HG (Movie S2) and *Gekko smithii* would devour the entire animal (Movie S3). It remains open if this gecko is resistant to the toxin or if the toxin is not powerful enough to generate toxic effects in this species. As to the toxin levels on wings of HG, these are in the order of magnitude of levels present in *C. marmoreus* (14), which may well exert toxic effects on small predators. Other questions involve GT's function as an L-type voltage-gated Ca-channel blocker, and the homology of protein sequences and modifications in GTs from different geographical areas. Our laboratory is not only initiating investigation of GT protein chemistry in HG samples from across the world but also in samples from a series of other toxic animals to possibly bridge the large gap between the evolutionarily totally open question of why a butterfly and a sea snail share the identical toxin GT.

Methods

Sample Preparation for 2DE. Samples of wet season and dry season HG wings (Figs. S5 and S7) and HG caterpillars (Fig. S6), 0.5 g (dry weight), were ground to a fine powder in liquid nitrogen and directly incubated with 5 mL lysis buffer [10% (wt/vol)] containing 2.6 M thiourea, 5 M urea, 4% (wt/vol) CHAPS, 1% dodecyl maltoside, 20% (vol/vol) glycerol, 100 mM dibasic sodium phosphate (pH 7.6), and 1 mM PMSF at 37 °C for 2 h. Samples were mixed by repeated inversion of the centrifuge tube, then filtered and centrifuged at 15,000 × *g* for 10 min at 4 °C. The sample supernatants were pooled and centrifuged again at 15,000 × *g* for 60 min at 4 °C. Protein was precipitated from the final supernatant using the 2D Cleanup Kit (BioRad), following the manufacturer's protocol. The resulting proteins were resuspended in fresh urea buffer: 20 mM Tris, 7 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, 10 mM 1,4-dithioerythritol, 1 mM EDTA, 1 mM PMSF, and one tablet Complete (Roche Diagnostics). The resuspension was centrifuged at 10,000 × *g*. Samples were homogenized and suspended in 1.5 mL of the urea buffer described above. The suspension was sonicated on ice for 30 s and centrifuged 15,000 × *g* for 120 min at 10 °C. Samples were desalted using an Ultrafree-4 centrifugal filter unit with a 3-kDa molecular weight cutoff (Millipore) at 3,000 × *g* at 10 °C until the filtrate volume was ~4 mL and the retentate was 200 μL. Protein sample concentrations were determined using the BCA protein assay kit (Pierce) with BSA as the standard (26).

Two-Dimensional Gel Electrophoresis. The precipitated protein (300 μg) from HG samples was immobilized on 18-cm pH 3–10 nonlinear gradient strips (GE Healthcare). The focusing voltage was gradually increased from 200 to 8,000 V at 4 V/min, then held constant at the maximum voltage for an additional 3 h (~150,000 V applied in total). Before separation in the second dimension, strips were equilibrated twice in 10 mL of SDS equilibration buffer [50 mM Tris•HCl, pH 8.8, 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS and trace bromophenol blue] for 15 min per incubation with gentle shaking. DTT [1% (wt/vol)] was added to the first incubation mixture, and iodoacetamide [4% (wt/vol)] was added to the second mixture. The second-dimensional separation was performed on 10–18% gradient SDS/PAGE gels. After fixing proteins for 12 h in 50% methanol and 10% acetic acid, the gels were stained with colloidal Coomassie blue (Novex) for 8 h, and the excess dye was washed from the gels with distilled water. Molecular masses were determined by comparison with precision protein standard markers (BioRad) spanning the 10- to 250-kDa molecular weight range. Isoelectric point values were determined as instructed by the supplier of the immobilized pH gradient strips.

In-Gel Digestion. Gel fragments of interest were excised, placed in a 1.5-mL tube, and repeatedly washed with solutions of 10 mM ammonium bicarbonate and 50% acetonetrile (ACN) in 10 mM ammonium bicarbonate. Addition of ACN caused the gel to shrink, and the shrunken gel plugs were dried in a Speedvac Concentrator 5301 (Eppendorf). Dried gel pieces were reswollen and the proteins were digested in the gel matrix by incubating overnight at 37 °C with 40 ng/mL trypsin (Promega) in digestion buffer [5 mM octyl-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate, pH 7.8]. Digestion with the endoprotease Asp-N (AspN; Roche Diagnostics) was performed in 25 mM NH_4HCO_3 maintained at 37 °C for 16 h, whereas digestion with 25 ng/mL chymotrypsin (Roche Diagnostics) was performed in 25 mM NH_4HCO_3 with 5 mM OGP (pH 7.8) at 30 °C for 4 h. Finally, digestion with 20 ng/μL subtilisin (a protease from *Bacillus subtilis* var. *biotecus* A.; Sigma) was conducted in a buffer of 6 M urea and 1 M Tris, pH 8.5 at 37 °C for 1 h. Peptide extraction was performed with 15 μL of 1% formic acid (FA) in 5 mM OGP for 30 min, 15 μL of 0.1% FA for 30 min, and 15 μL of 0.1% FA in 20% ACN for 30 min. The extracted peptides were pooled for nano-LC-electrospray ionization (ESI)–collision-induced dissociation (CID)/electron transfer dissociation (ETD) MS/MS analysis (27).

Nano–LC-ESI-CID/ETD-MS/MS. Samples were analyzed by HPLC on an Ultimate 3000 system (Dionex) equipped with a PepMap100 C-18 trap column (300 mm × 5 mm) and a PepMap100 C-18 analytic column (75 mm × 150 mm). Solvents A and B consisted of 0.1% FA in water and 0.08% FA in ACN, respectively. The elution program was as follows: 4–30% solvent B from 0 to 105 min, 80% solvent B from 105 to 110 min, and 4% solvent B from 110 to 125 min. The flow rate was 300 nL/min from 0 to 12 min, 75 nL/min from 12 to 105 min, and 300 nL/min from 105 to 125 min. An HCT ultra-ETD II (Bruker Daltonics) was used to record peptide spectra over the mass range of *m/z* 350–1,500, and MS/MS spectra using information-dependent data acquisition over the mass range of *m/z* 100–2,800. MS spectra were recorded first, followed by three data-dependent CID MS/MS spectra and three ETD MS/MS spectra generated from three highest-intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect low abundant peptides. The applied voltage between the ion-spray tip and spray shield was set to 1,500 V. The drying nitrogen gas was heated to 150 °C and maintained at a flow rate of 10 L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Several charged peptides were chosen for MS/MS experiments because of their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated using DataAnalysis 4.0 (Bruker Daltonics). Peptide searches were performed using MASCOT 2.2.06 (Matrix Science) software and were compared with the latest NCBI database and UniProtKB database. The search parameters were as follows: the protease was selected as trypsin (or corresponding enzymes) with a maximum of two missed cleavage sites, the species taxonomy was limited to other Metazoa, a mass tolerance of 0.2 Da was set for peptide tolerance and MS/MS tolerance, and the fixed modification of carbamidomethyl (C) formation and variable modifications of methionine oxidation or phosphorylation (Y, T, S) were specified. Positive protein identifications were based on a significant MOWSE score. After protein identification, an error-tolerant search was performed to detect unspecific cleavage and unassigned modifications. The computed protein identification and modification data were manually inspected and filtered to confirm protein identification and modifications. PTM searches were also performed using the Modiro software with following parameters: the appropriate protease was selected with a maximum of two missing cleavage sites, a mass tolerance of 0.2 Da was set for peptide mass tolerance and the fragment mass tolerance, and the modifications of carbamidomethyl (C) modification and methionine oxidation were specified. Searches for unknown mass shifts, for amino acid substitution, and for calculation of significance were selected from the advanced PTM explorer search algorithms. Protein identification was initially performed by inspection of spectra; subsequent peptide identification was based on the ion-charge status of the peptide, b- and y-ion fragmentation quality, an ion score >200, and a significance score >80, as suggested by the manufacturer's manual (http://www.protagen.com/customers_downloads/MAN_Modiro_v1.1.zip). A list of 172 common modifications was selected and added to the theoretical peptide fragments, which were compared with experimentally obtained MS/MS spectra (12).

Verification of Methylation and Monoglutamylation. To verify the methylation of E53, gels were processed in either methanol or ethanol, and the resulting proteins analyzed individually by MS. Methylation was considered when

a mass shift corresponding to this modification was maintained in the ethanol-processed gel.

To verify monoglutamylation, in-gel digestion was performed with 0.5 mL of a solution of carboxypeptidase G (γ-glutamyl hydrolase, E.C. 3.4.17.11; Sigma) in enzyme buffer (50 mM Tris•HCl, pH 7.3, 0.1 mM $ZnCl_2$) at 30 °C for 2 h. Observation of the appropriate mass shift following incubation of the GT peptide was considered verification of monoglutamylation.

Western Blotting. Protein extracts (40 μg) from HG samples were loaded on a 1D SDS/PAGE gel. Proteins were transferred to PVDF membranes (Millipore) using a semidry BioRad transfer system. Membranes were blocked by incubation for 1 h in PBS containing 5% nonfat dried milk powder (BioRad) and 0.1% Tween 20. After washing, membranes were incubated with dilute primary anti-GT antibody (1:3,000; Genscript) at 4 °C overnight. The membranes were washed three times by gentle agitation in PBS with 0.1% Tween 20. Proteins were detected using horseradish peroxidase-coupled secondary anti-rabbit IgG antibodies (1:5,000; Abcam). Membranes were developed with the Amersham ECL plus Western blotting detection system (GE Healthcare).

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