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Covalent modification of stathmin by CCNU determined by FTMS analysis of modified proteins and tryptic peptides

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Abstract

Chemical modification of proteins is often carried out to generate protein-small molecule conjugates for various applications. The high resolution and mass accuracy of a Fourier transform mass spectrometer is particularly useful for assessing the extent or sites of covalent modifications. As protein-small molecule reactions often produce products with variable numbers of the compound incorporated at different sites, a direct mass analysis of the reaction products at times yields mass spectra hard to interpret. Chromatographic separation at protein level could reduce the complexity of a sample, thus allowing more accurate mass spectrometric analysis. In this report, we demonstrate the utility of reversed-phase protein chromatography and FT-ICR mass spectrometry in analyzing CCNU (lomustine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-urea, MW: 233.7 Da) modification of stathmin. With this combined approach, we determined the stoichiometry as well as sites of CCNU incorporation into the protein, demonstrating differential reactivity of several lysyl residues to CCNU alkylation.
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Keywords: RP-HPLC; Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS or FTMS); 1-(2-Chloroethyl)-3-cyclohexyl-1-nitroso-urea (CCNU); Stathmin; Stoichiometry

Mass spectrometry has been used as a tool for characterizing protein-small molecule interactions in solution [1–6]. One of the critical aspects of such studies is the ability to assess the extent and/or sites of small molecule incorporation. Since chemical modification of a protein is usually amino acid-specific, the formation of a mixture of products with variable numbers of bound molecules at different sites is not uncommon. A direct mass spectrometric analysis of the reaction products often leads to mass spectra difficult to interpret due, in part, to the presence of heterogeneous products formed from, for example, protein isoforms, proteolytic products, or different post-translationally modified proteins in the sample. Chromatographic separation by size or hydrophobicity following chemical modification could be carried out to resolve heterogeneous products into distinctive fractions prior to mass spectrometric analysis. A study using hydrophilic interaction chromatography (HILIC) to pre-fractionate various mono- to tri-acetylated histone H3 conjugates prior to mass spectrometric analysis has been recently reported [7]. Histones, being DNA binding proteins, are highly basic and polar in aqueous solution, thus ideal for HILIC separation. Reversed-phase separation on a C4, C8, or C18 column [8,9] is however more suitable for most other proteins.

Stathmin is a relatively small protein (mw ~18.5 kDa) present ubiquitously in cells which exhibits pleotropic roles in signaling pathways important in the control of cell proliferation, differentiation, and motility (for a review, see Ref. [10]). We have previously conducted proteomic profiling on two types of malignant glioma cell lines, one with normal chromosome 1p ($1p^{+/+}$) and resistant to nitrosourea chemotherapy and the other missing a region of chromosome 1p ($1p^{+/-}$) and sensitive to nitrosourea chemotherapy. We showed that stathmin was significantly down-regulated in the $1p^{+/-}$ cells [11]. Since stathmin gene

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is localized at chromosome 1p36.11, we suspected that a decrease in stathmin protein might account for sensitivity of $1p^{+/-}$ gliomas to chemotherapy with nitrosourea derivatives such as CCNU (lomustine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-urea) or BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea). CCNU is a DNA alkylating agent but also known to carbamoylate proteins on the N-terminal amino ($-NH_2$) group or the ε-amino groups of lysine [12]. Because stathmin is relatively rich in lysine (~15%), we were wondering whether stathmin would be a target for CCNU, and if so, what lysyl groups are susceptible to modification. We therefore set out to investigate the stoichiometry of CCNU–stathmin interaction by using RP-HPLC fractionation of the modified proteins, followed by mass analysis using a Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR MS). Herein we provide evidences that multiple molecules of CCNU could be incorporated into stathmin and that various lysyl residues exhibit differential reactivity to the modification.

Materials and methods

Expression and purification of His-tagged human stathmin. His-tagged human stathmin protein was prepared as previously described [11]. Briefly, the nucleotide sequence 5′-CACCACCATCACCATCATTAA-3′, which encodes for six histidines, was inserted at the 3′ end to replace the stop codon of a full-length human stathmin cDNA (Origene Technologies; Rockville, MD). This modified cDNA was subcloned into the pIX 2.0 bacterial expression vector (Qiagen; Valencia, CA) to yield the pIX 2.0-STMN-6× His plasmid. BL21(DE3) *Escherichia coli* (Novagen; San Diego, CA) transformed with pIX 2.0-STMN-6× His were grown and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Novagen, San Diego, CA) to synthesize 6× His-tagged human stathmin. The recombinant protein was purified from bacterial lysates using TALON CellThru resin (Clontech, Mountain View, CA), an immobilized metal affinity chromatography (IMAC) resin.

Reaction of CCNU with stathmin. TALON CellThru purified 6× His-tagged stathmin in 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM sodium chloride was chemically modified by incubation with 10 μM CCNU in DMSO (final DMSO conc. 0.005%) at 37 °C for 4 h. The control protein was treated with the same concentration of the solvent.

Reversed phase HPLC fractionation. Reversed phase protein purification was conducted using Beckman ProteomeLab PF 2D system (Beckman/Coulter, Fullerton, CA). Proteins were fractionated using a non-porous reversed-phase C18 column (4.6 × 33 mm) and a gradient consisting of solvent A (0.1% trifluoroacetic acid (TFA) in water) and solvent B (0.08% TFA in CH_3CN). The separation was performed at 50 °C with a flow rate of 0.75 ml/min and the effluent monitored by absorbance at 214 nm. The column was first equilibrated with 10 column volumes of 100% solvent A prior to each injection. Two minutes after sample injection, bound proteins were eluted with a gradient of 0–100% solvent B in solvent A over 30 min, followed by 100% solvent B for 4 min. The column was then re-equilibrated with 100% solvent A. The fractions were collected in 96 well plates every 0.25 min.

LTQ/FT-ICR mass spectrometry for intact proteins. The solvent in collected HPLC fractions was evaporated in a SpeedVac to near dryness. To each fraction, 50 μl of a H_2O:CH_3CN mixture (50:50) containing 1% formic acid (FA) was then added. The acquisition of spectra for intact proteins was carried out using an LTQ/FT-ICR MS (7 T, Thermo Electron, San Jose, CA) equipped with an electrospray ionization probe (ESI). Each reconstituted protein solution was infused at 5 μl/min into the mass spectrometer. Charge states of samples were determined from the spacing of isotopic peaks (resolution 100,000 at $m/z = 400$), while molecular weights were calculated from charge-state deconvoluted spectra, as described by others [13].

LTQ/FT-ICR mass spectrometry for tryptic peptides. Proteins in reconstituted fractions in 50:50 H_2O:ACN with 1% FA, as described above, were diluted with 4 volumes of 50 mM NH_4HCO_3 to reduce the CH_3CN content to 10%. The pH of the solution was adjusted to around 8.0 with NH_4OH prior to the addition of trypsin (1:50 ratio) for digestion at 37 °C overnight. Tandem mass spectra of peptides were obtained following the same instrumental settings as reported previously [14]. SEQUEST/Bioworks 3.2 was used to confirm the identity of peptides and sites of CCNU modification. This was done by matching the acquired MS/MS spectra to those of peptides generated in silico in the Swiss-Prot human database, in which stathmin sequence had been replaced with that of the His-tagged stathmin. Differential modifications of peptides with CCNU at lysyl residues were included in the database search. Criteria of spectra/peptide matches were set at the same level as reported previously [15]; namely, matches were considered significant if they had a normalized difference in cross-correlation scores (ΔC_n) of at least 0.1 and minimum cross-correlation scores (XCorr) of 2.0 for +1, 2.5 for +2, and 3.5 for +3 charged ions.

Results and discussion

Chromatography of control and CCNU-modified stathmin

TALON CellThru-purified 6× His-tagged stathmin was first analyzed by RP-HPLC to check for purity. As shown in the inset of Fig. 1B, the majority of protein was eluted in a symmetric peak, suggesting that it was relatively pure. A few minor peaks were present, two of which (eluted at about 11 min) were identified by MS analysis to be proteolytic products of stathmin, which retained the C-terminal 6× His but lacked the N-terminal region (data not shown). Only the protein eluted at about 14.3 min was used for CCNU modification study. The reaction between the ε-NH_2 group of lysine and CCNU is depicted in Fig. 1A. Following CCNU modification, both the hydrophobicity and the mass of stathmin are expected to increase due to incorporation of a hydrophobic *N*-cyclohexyl group that adds 125.0841-Da to protein mass. HPLC elution profiles (Fig. 1B) indicate that control stathmin was eluted as a large symmetric peak (solid line), while CCNU-treated stathmin (dash line) was eluted in peaks with delayed retention time and reduced intensity, consistent with increased hydrophobicity of modified proteins with varied numbers of CCNU molecules. Eluted proteins were collected in fractions at 0.25 min intervals.

FT-MS analysis of intact proteins

To determine the stoichiometry of CCNU incorporation per protein in different fractions, mass determination of intact stathmin was first established using FT-ICR MS. The charge-state envelope of stathmin and the isotopic envelope of the +22-charged protein are shown in Fig. 2A and B, respectively. An apparent mass of 18434.70 Da was derived from deconvoluted spectra (theoretical average mass = 18434.62 Da, based on the amino acid sequence composition shown in Fig. 4A), suggesting an intact stathmin unmodified by the solvent. The spectra of the successive

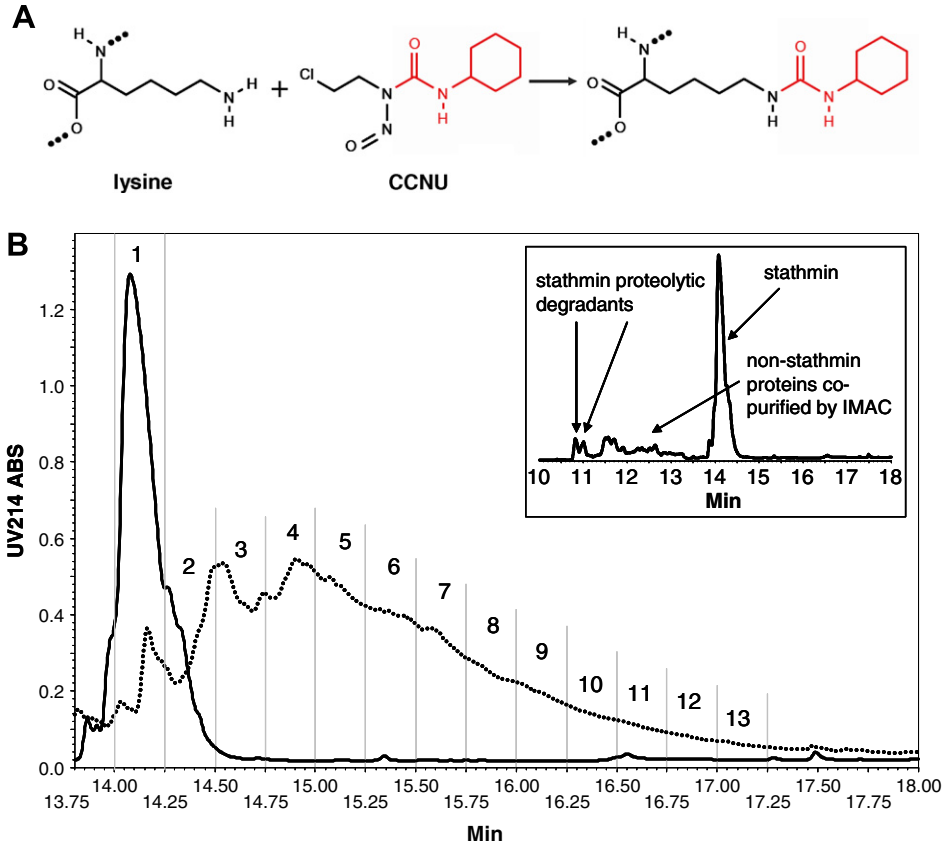

Fig. 1. (A) A proposed chemical reaction between ε-amino group of lysine and CCNU. The cyclohexylcarbamoyl group is shown in red. (B) HPLC elution profiles of control (solid) and CCNU (dash) treated stathmin proteins. The fractions were collected every 0.25 min. Numbers 1–13 are fraction numbers. The inset indicates the elution profile of control His tagged stathmin, showing minor proteolytic degradants and non-stathmin contaminants present in the sample purified from bacterial lysates through TALON CellThru resin.

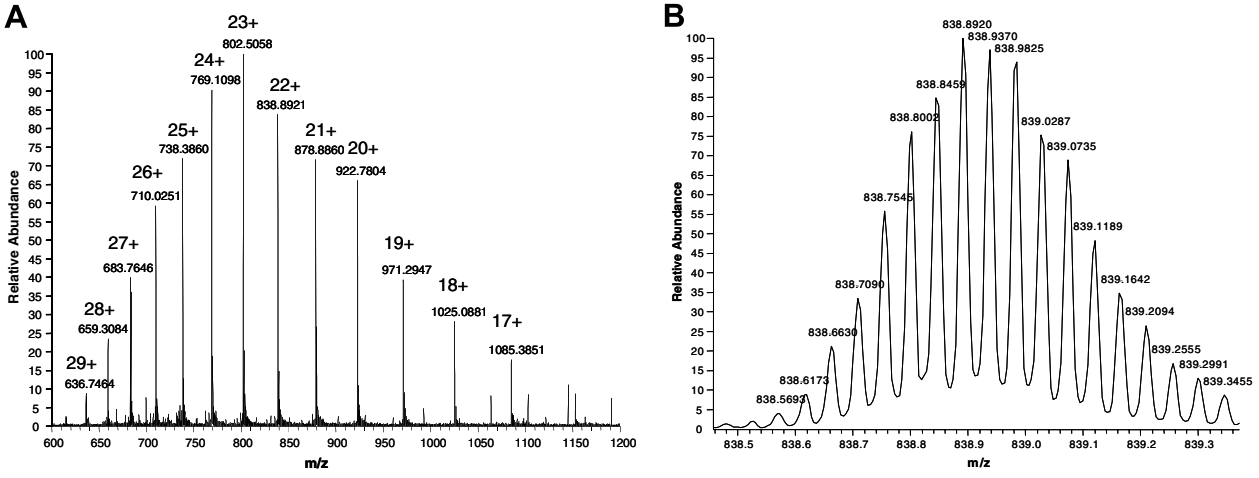

Fig. 2. FT-ICR mass spectra of (A) the charge-state envelope of control stathmin; and (B) the isotopic envelope of control stathmin at charge state of +22.

HPLC fractions of CCNU-treated sample (fractions 1–13 in Fig. 1B) were similarly analyzed. A series of mass spectra at +22 charge state representing the control and modified stathmin molecules is presented in Fig. 3. The molecular weights of the 6 most dominant CCNU-modified stathmin species in fractions 1–10 exhibited an increment of 125 Da, suggesting incorporation of 1–6 copies of cyclohexylcarbamoyl group (mw 125 Da) into the protein. The relatively broader peak area around fractions 3–5 (Fig. 1B) suggests that, under the reaction conditions used, a stoichiometry of 2–3 CCNU/protein is most prevalent, although products with CCNU/protein ratio as high as 5–8 could also be seen.

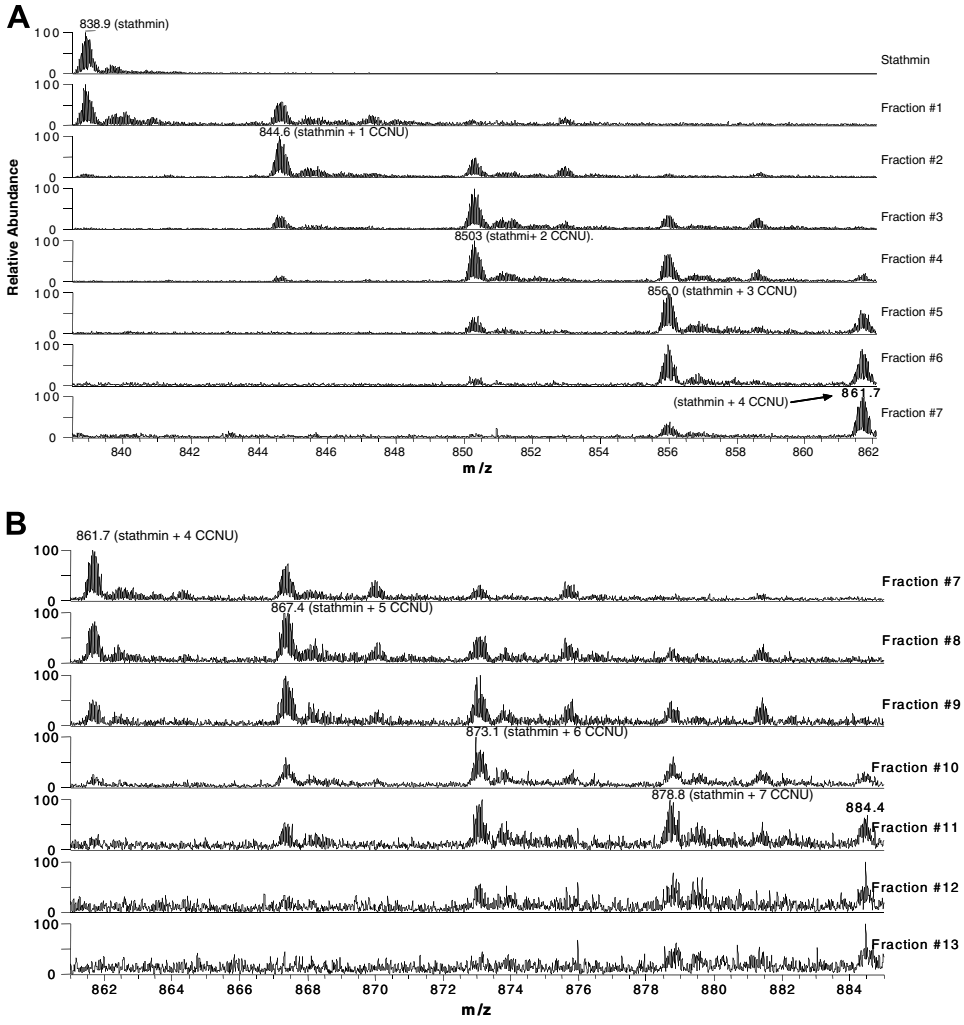

Fig. 3. FT-ICR mass spectra of charge state of +22 proteins infused from: (A) control stathmin and fractions 1–7 of CCNU modified stathmin; (B) fractions 7–13 of CCNU modified stathmin. Note the difference in m/z at the X-axis.

CCNU modifications of lysyl residues determined by mass analysis of tryptic peptides

To see if modification of stathmin by CCNU proceeds with a preferred pathway, i.e., modification of one lysyl group at a specific site precedes the next modification at other sites, proteins from control and fractions 1–10 were digested with trypsin and the products analyzed by LTQ–FTMS. By PeptideMass (online software from Expasy) analysis, 16 theoretical tryptic peptides having $[M + H]^+$ masses greater than 500 Da, including 12 lysine-ended peptides, 3 arginine-ended peptides, and 1 C-terminal peptide, are expected. Of those 12 lysine-ended peptides (highlighted in alternate color in Fig. 4A), mass spectra of the 4 smaller peptides containing 4–5 amino acids (ELEK, position 9–12; QLAEK, position 70–74; EHEK, position 76–79; MEANK, position 104–108) could not be consistently acquired, presumably due to poor retention by the trap/PicoFrit column (Thermo Electron, Keystone, PA) used or poor ionization due to high content (>40%) of acidic amino acids (E and D). Thus, the analysis of CCNU incorporation focuses only on 8 of the 12 lysine-ended peptides.

The extent of CCNU modification on the lysyl residues of these eight peptides could be analyzed by either direct or indirect estimation. A direct estimation relies on the peak intensities of CCNU-modified peptides, while an indirect estimation infers CCNU-modification from quantity reduction of the intact peptide. This is because modification of lysine by CCNU prohibits trypsinization at that site. Hence the survey MS intensity of a peptide ending with a lysine becomes less in the digest from a modified sample than that from an unmodified sample. The decrease in intensity could be inferred to as reflecting the degree of modification of that lysine. Since the amounts of stathmin varied among different fractions (Fig. 1B), the extent of CCNU modification on a specific lysine-ended peptide needs to be normalized. For this purpose, peak intensities of peptides of interest in any given fraction were divided by that of ASGQAFELILSPR (aa 14–26, Fig. 4A), a peptide not bracketed by lysine on either end, in the same fraction.

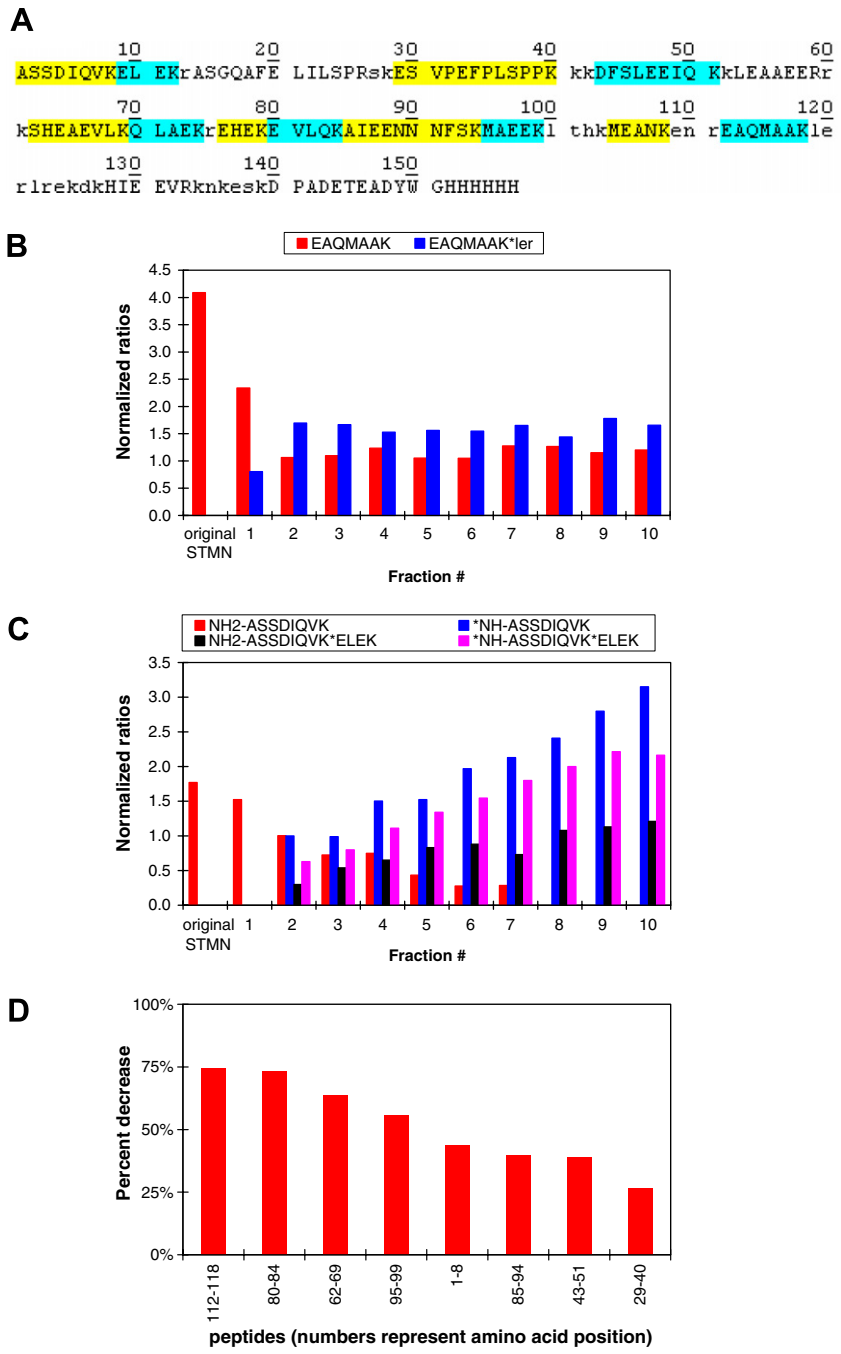

Fig. 4. (A) Amino acid sequence of the expressed His-tagged stathmin. The sequences in capital letters represent tryptic peptides having a mass greater than 500 Da. Lysine-ended peptides are highlighted in alternate colors to denote distinctive peptides. (B) Relative amounts of the unmodified (red) and CCNU modified (blue) peptides of ($_{112}$EAQMAAK$_{118}$) in different fractions. (C) Relative amounts of N-terminal peptides and CCNU-modified peptides in the N-terminal region. Colored bars indicate N-terminal peptide (NH_2-ASSDIQVK) and those resulted from modification. (D) Percent decrease of eight lysine-ended peptides due to CCNU incorporation in fraction #2 (predominantly one CCNU incorporation). Note: Due to the relatively large denominator (the intensity of peptide 14–26), 10× the normalized ratios are shown in (B) and (C) for clarity. The star ∗, denotes CCNU incorporation.

For the peptide EAQMAAK (aa 112–118), CCNU incorporation was confirmed by detecting EAQMAAK∗ler (∗ denotes CCNU) in tandem MS. In direct estimation, the amount of EAQMAAK∗ler in fraction #2 was similar to those in fractions #3–10 (blue bars, Fig. 4B), indicating that K118 was relatively reactive or readily accessible to modification. Consistent with this observation, a steep decrease of the EAQMAAK peptide to 60% and 28% (the red bars in Fig. 4B) in fraction #1 and #2, respectively, was observed by indirect estimation. The level remained rather constant in the subsequent fractions, where more CCNU was incorporated per protein, suggesting that addition of CCNU to K118 might be affected by modifications occur at other sites.

Analysis of the N-terminal peptide (aa 1–8, NH_2-ASSDIQVK) revealed a different pattern in that disappearance of the peptide was accompanied by the formation of three modified ions: *NH-ASSDIQVK, NH2-ASSDIQVK*ELEK, and *NH-ASSDIQVK*ELEK (* denotes CCNU). By indirect estimation (red bars, Fig. 4C), it was found that the amounts of N-terminal peptide became less and less in latter fractions, indicating that more K8 became modified as more CCNU was incorporated into the protein (latter fractions are more hydrophobic due to more CCNU in the protein). This is supported by the direct determination in that more and more *NH-ASSDIQVK, NH_2-ASSDIQVK*ELEK, and *NH-ASSDIQVK*ELEK (denoted by the blue, black, and pink bars, respectively, Fig. 4C) were seen in higher number fractions. This trend of a gradual loss of the unmodified peptide, concomitant with a gradual increase in modified peptides, suggests that the N-terminal NH2 group or the ε-NH2 group of lysine was less reactive to CCNU modification, compared to K118, and also not significantly affected by incorporation of CCNU at other sites.

Among the other six lysine-ended peptides (29–40; 43–51; 62–69; 80–84; 85–94; 95–99), two are flanked by more than one lysine residue (e.g. 29–40; 43–51) on the C-terminal end, three are adjacent to each other (e.g. 80–84; 85–94; 95–99), and one is preceded by RK at the N-terminal end (e.g. 62–69). A direct estimation of CCNU incorporation is rather challenging because the large number of possible combination of modifications that could occur on the flanking or adjacent lysine residues, which is further complicated by potential enzyme missed cleavages. Indirect estimation by the disappearance of target peptides, as shown previously, appears to be a more feasible method. Using the indirect strategy, the percent decrease of all 8 lysine-ended peptides in fraction 2, where the majority of protein is mono-CCNU modified stathmin (Fig. 3A) was determined to see if any lysyl group was more susceptible to modification. Fig. 4D shows that there indeed are significant differences in terms of percent decrease of peptides due to CCNU modification. The range of percent peptide decrease varied from 74% (K118 and K84) to 27% (K40), suggesting that K118 and K84 are most reactive or accessible to initial CCNU modification. The result is consistent with that of Fig. 4B, showing a significant decrease of the K118-containing peptide when stathmin is predominantly singly modified by CCNU (see Fig. 3A, fraction #2).

Summary

Haploinsufficiency of stathmin was thought to be a contributing factor to chemosensitivity of $1p^{-/+}$ gliomas to CCNU. The contention was further supported by the demonstration that stathmin knockdown increased the sensitivity of glioma cells i*n vitro* and *in vivo* [11]. Whether a single modification at the most sensitive lysine or modification at multiple lysine residues in the proteins is required to confer chemosensitivity of $1p^{-/+}$ gliomas remains to be elucidated. The identification of a couple of highly susceptible and several reactive lysyl residues to CCNU modification should prove useful for future delineation of the mechanism by which stathmin may play in chemotherapy sensitivity to nitrosourea derivatives. Our studies illustrate the power of FT-ICR mass spectrometry in providing accurate mass information for intact proteins and peptides, thus facilitating analysis of the interaction between a small therapeutic agent and its target protein.

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