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Fourier Transform Ion Cyclotron Resonance Mass Spectrometry for the Analysis of Small Ubiquitin-like Modifier (SUMO) Modification: Identification of Lysines in RanBP2 and SUMO Targeted for Modification during the E3 AutoSUMOylation Reaction

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The attachment of the ubiquitin-like protein SUMO to target proteins is involved in a number of important cellular processes. Typically, SUMO modification occurs on lysine residues within the consensus sequence ψK*x*E/D (ψ is a hydrophobic residue and *x* is any residue), although there are examples of modifications at nonconsensus sites. In most cases, sites of SUMO modification have been inferred from a combination of site-directed mutagenesis and functional analysis; however, these methods have two limitations. They do not directly identify the acceptor lysine, nor are they sufficient to identify acceptor lysine residues in SUMO polymers. Here, we use Fourier transform ion cyclotron resonance (FT-ICR) together with activated-ion electron capture dissociation (AI-ECD) or infrared multiphoton dissociation (IRMPD) mass spectrometry techniques to overcome these restrictions. These approaches were employed to analyze the autoSUMOylation reaction catalyzed by the SUMO E3 ligase RanBP2. Six sites of in vitro SUMOylation in RanBP2 along with four branch-point lysines in SUMO-1 and three in SUMO-2 were identified. In all but one case, SUMOylation occurred within the sequences K*x*E or KψK. These results demonstrate the utility of FT-ICR with AI-ECD or IRMPD mass spectrometry in detecting SUMOylation, and sites of SUMOylation, and their potential roles as complementary tools for proteomic and functional analysis, and provide significant insight into the modification of a SUMO ligase for which conventional techniques have been unsuccessful.

Modification of substrate proteins by the small ubiquitin-like modifier (SUMO) plays an important role in a number of cellular processes,[1−3] and homologues of SUMO are found in all eukaryotes. In higher eukaryotes, three forms of SUMO have been well-characterized (SUMO-1, SUMO-2, SUMO-3), and sequence analysis has suggested that SUMO-2 and SUMO-3 form a subfamily separate from SUMO-1.[4,5] Conjugation of SUMO to substrate proteins occurs via a pathway analogous to that of ubiquitination: SUMO is activated in an ATP-dependent reaction by an E1-like enzyme, the heterodimer SAE1/SAE2 (also known as Uba2/Aos1), and a thioester bond is formed between the C-terminus of the SUMO and a cysteine in SAE2.[6−9] The SUMO protein is then transferred, through a transesterification reaction, to a cysteine residue in the E2 conjugating enzyme Ubc9.[10,11] Finally, an isopeptide bond is formed between the ϵ-amino group of a lysine residue in the substrate protein and the C-terminus of SUMO. Efficient transfer of SUMO from Ubc9 to target proteins can be aided by E3-like SUMO ligases[2], although Ubc9 itself does recognize targets[12,13] and can conjugate SUMO independently of E3 proteins. To date, three types of SUMO E3 ligases have been identified: the PIAS family (protein inhibitor of activated STAT),

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RanBP2 (also known as Nup358), and Pc2 (polycomb protein 2). Unlike ubiquitination, which typically results in targeting of the substrate protein for degradation by the proteasome, SUMOylation results in a number of substrate-specific functions. For example, SUMOylation of RanGAP1 targets the protein to the nuclear pore complex[14,15] whereas PML modification is necessary for formation of mature nuclear bodies.[16,17] SUMOylation of IκBα occurs at the same residue as ubiquitination; thus, activation of the transcription factor NF-κB is inhibited.[18] SUMO attachment activates transcription factors p53[19,20] and HSF2,[21] yet represses the transcriptional activity of LEF[22] and c-Myb.[23] Generally, SUMO proteins are attached to lysine residues within the consensus sequence ψKxE/D, where ψ is a hydrophobic amino acid residue and x any amino acid residue.[24] Exceptions to that rule include sites in E2-25k,[25] Cdc3,[26] human PML,[27] and PCNA.[28] In vitro studies of SUMO conjugation in the absence of E3 proteins has revealed that, unlike SUMO-1, SUMO-2 and SUMO-3 can form polymeric chains. This occurs via a lysine residue within an N-terminal SUMO consensus motif (Lys11) of SUMO-2 and SUMO-3.[29]

As the site of SUMOylation may have important implications for the function of the modified substrate, it is necessary to develop new techniques to specifically identify target lysine residues. The putative sites of SUMOylation are often inferred from site-directed mutagenesis, an indirect approach that detects only lysine residue(s) required for SUMOylation rather than the actual site(s) of modification. Chung et al.[30] recently demonstrated a matrix-assisted laser desorption/ionization (MALDI) time-of-flight time-of-flight (TOF-TOF) approach to identify sites of SUMOylation. These researchers were able to demonstrate in vitro SUMOylation of Ubc9 and of human centromere protein CENP-C fragments at nonconsensus sites.

Proteomics approaches to the identification of novel cellular SUMO substrates have begun to emerge over recent years.[31−37] These methods generally rely upon purification of SUMOylated proteins from cell lysates via affinity tags, followed by protein/peptide analysis by mass spectrometric techniques. Here we investigate the potential of activated ion electron capture dissociation (AI-ECD)[38] to detect SUMOylated proteins. Analysis of the intact SUMO-1−RanGAP1$_{418-587}$ conjugate reveals that the dominant fragmentation channel is loss of the SUMO-1 modification through N−C$_\epsilon$ cleavage within the modified lysine side chain. That cleavage corresponds to the *c*/*z*• -type cleavage[39] typically observed in ECD, in contrast to the cleavages observed in peptides containing small modifications to the lysine side chain.[40,41] The results suggest that AI-ECD may be used as a diagnostic tool in identifying SUMO conjugates.

Recently, we applied a Fourier transform ion cyclotron resonance (FT-ICR)[42] mass spectrometry approach to the identification of sites of ubiquitination in proteins.[40] In this study, we extend that approach to the investigation of SUMOylated proteins. To validate this approach, a number of model substrates were modified by SUMO-1 in vitro and unseparated tryptic digests analyzed by direct-infusion microelectrospray (micro-ESI)[43] FT-ICR mass spectrometry. SUMOylated peptides were identified based on mass, and sites of SUMOylation confirmed by MS/MS; either electron capture dissociation (ECD)[39] or infrared multiphoton dissociation (IRMPD).[44] The SUMO E3 ligase RanBP2 undergoes an autoSUMOylation reaction in vitro generating high molecular weight RanBP2−SUMO conjugates.[45] We therefore used FT-ICR to characterize the SUMO polymers attached to RanBP2. The results confirm that the substrate proteins are modified by polySUMO-1 and polySUMO-2 chains and show that multiple sites of attachment exist within the RanBP2 fragments. With a single exception, SUMO acceptor lysines occurred within either a KxE motif or a KψK motif. The results demonstrate the validity of FT-ICR and AI-ECD mass spectrometric techniques in the proteomic and functional analysis of SUMOylated proteins.

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EXPERIMENTAL METHODS

Protein Expression and Purification. Expression and purification of recombinant IκBα ,[46] GST-PML$_{485-495}$,[24] RanGap$_{418-587}$,[47] SUMO-1/-2,[29] SAE2/SAE1,[8] Ubc9,[11] RanBP2$_{2532-2767}$, and RanBP2$_{2633-2761}$[48] have all been described previously.

Preparation of SUMO-1-Modified RanGAP$_{418-587}$, IκBα, and GST−PML$_{485-495}$. Each reaction contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 0.11 μM SAE2/SAE1, and 4.4 μM Ubc9. Specific conjugation reactions contained either 24 μM RanGap$_{418-587}$ and 35 μM SUMO-1, 7.5 μM IκBα, and 12 μM SUMO-1 or 14 μM GST−PML$_{485-495}$, and 18 μM SUMO-1. Reactions were incubated at 37 °C, and samples were removed throughout for analysis by SDS−PAGE and Coomassie Blue staining to monitor reaction progress. After 4 h, reactions were terminated by addition of EDTA to 10 mM. Samples were dialyzed to 50 mM ammonium bicarbonate (pH 8.0).

Preparation of SUMO-Modified RanBP2$_{2532-2767}$ and RanBP2$_{2633-2761}$. In total, six 1-mL in vitro SUMO conjugation reactions were prepared: Each contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 0.11 μM SAE2/SAE1, and 0.53 μM Ubc9. Concentrations of RanBP2 fragments and SUMOs were varied for each reaction to facilitate the production of RanBP2 modified with either a single copy of SUMO (RanBP2−SUMO$_{x1}$), or multiple copies of SUMO (RanBP2−SUMO$_{xn}$). RanBP2$_{2532-2767}$−SUMO-1$_{x1}$ was produced using 20 μM RanBP2$_{2532-2767}$ and 12 μM SUMO-1. RanBP2$_{2532-2767}$−SUMO-2$_{x1}$ was produced using 20 μM RanBP2$_{2532-2767}$ and 12 μM SUMO-2. RanBP2$_{2532-2767}$−SUMO-1$_{xn}$ was produced using 5 μM RanBP2$_{2532-2767}$ and 24 μM SUMO-1. RanBP2$_{2532-2767}$−SUMO-2$_{xn}$ was produced using 5 μM RanBP2$_{2532-2767}$ and 24 μM SUMO-2. RanBP2$_{2633-2761}$−SUMO-1$_{xn}$ was produced using 5 μM RanBP2$_{2633-2761}$ and 48 μM SUMO-1. RanBP2$_{2633-2761}$−SUMO-2$_{xn}$ was produced using 5 μM RanBP2$_{2633-2761}$ and 12 μM SUMO-2. Reactions were incubated for 5 h at 37 °C before progress was halted by addition of EDTA to 10 mM. Samples were dialyzed against 50 mM ammonium bicarbonate (pH 8.0). Samples of RanBP2$_{2633-2761}$−SUMO$_{x1}$ were not prepared as SUMO modification of this fragment is highly processive, making it difficult to produce large quantities of the singly modified form.

Methylation of SUMO-1 and SUMO-2. To facilitate complete methylation of SUMO-1 and SUMO-2, it was necessary to fully deprotonate all amino groups and denature the SUMO proteins before modification. SUMO protein samples, 10 mg mL^{-1}, were dialyzed against ultrapure water before addition of NaOH to 100 mM to a final volume of 1 mL. The 30-μL sample of 1 M formaldehyde and 60 μL of 1 M sodium borohydride were added in rapid succession while stirring samples in ice, followed 10 min later by addition of 3 μL of 1 M sodium borohydride. This procedure was repeated every 30 min for 3 h. After the last addition, 6 μL of 1 M sodium borohydride was added and the samples were left to stand for 1 h. Methylated SUMO-1 and SUMO-2 (MeSUMO-1 and MeSUMO-2) were refolded by rapid dilution in a 1:10 ratio with 50 mM Tris-HC (pH 7.5), 1 M nondetergent sulfobetane 201,[49] 5 mM DTT, and 1 mM EDTA. MeSUMO-1 and MeSUMO-2 were dialyzed back to 50 mM Tris-HCl (pH 7.5), 1 mM DTT before concentration and storage at −70 °C. Complete methylation was confirmed by LC−MS.

MeSUMO−^{125}I-RanBP2$_{2633-2761}$ Conjugation Reactions. For gel-based analysis, RanBP2$_{2633-2761}$ was labeled with ^{125}iodine as described previously.[29] In vitro conjugation reactions contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 0.11 μM SAE2/SAE1, 0.29 μM Ubc9, 0.65 μM ^{125}I-RanBP2$_{2633-2761}$, and 5 μM MeSUMO-1 or MeSUMO-2. SUMO conjugation in each assay was initiated by addition of the SUMO protein, and samples were removed from reactions at 0, 10, 20, 30 60, 120, and 180 min postinitiation. Reactions were halted by addition to SDS sample buffer containing β-mercaptoethanol, before fractionation by SDS-polyacrylamide gel electrophoresis. Radioactive species could be visualized from dried gels by phosphorimaging. For MS analysis, four 1-mL conjugation reactions were prepared using 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 0.11 μM SAE2/SAE1, 0.5 μM Ubc9, either 5 μM RanBP2$_{2532-2767}$ or 10 μM RanBP2$_{2633-2761}$, and either 40 μM MeSUMO-1 or 10 μM MeSUMO-2. Reactions were incubated for 5 h and dialyzed against 50 mM ammonium bicarbonate.

Preparation of Samples for FT-ICR Mass Spectrometry. *Tryptic digests.* A 100-μL sample of each of the dialyzed reaction mixtures was added to 7 μg/mL sequencing grade modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate, for a substrate/trypsin ratio of 50:1, and incubated at 37 °C overnight. A 10-μL sample of 1:1 water/acetonitrile (or methanol) (J. T. Baker, Philipsburg, NJ) and 2% acetic acid (Aldrich, Milwaukee, WI) was added to 10 μL of the tryptic mixture, and the solution was microelectrosprayed. Calibrant solution (Agilent Technologies, Wilmington, DE) was diluted 1:10 into 1:1 acetonitrile and microelectrosprayed from a second identical microelectrospray emitter located in the dual ESI source.[50]

Intact SUMO-1−RanGAP1$_{418-587}$. A 20-μL sample of the dialyzed reaction mixture was desalted by use of a C4 ZipTip (Millipore, Billerica, MA) and eluted into 5 μL of 1:1 water/acetonitrile, 0.1% formic acid (Aldrich). A further 20 μL of 1:1 water/acetontrile, 0.1% formic acid was added to the eluant, and the solution was microelectrosprayed.

FT-ICR Mass Spectrometry. Peptide and protein samples were analyzed with a home-built, passively shielded, 9.4-T FT-ICR mass spectrometer[51] equipped with an external microelectrospray ionization source.[52] The samples were infused at a flow rate of 300 nL/min through an electrospray emitter consisting of a 50-μm-i.d. fused-silica capillary, which had been mechanically ground to a uniform thin-walled tip.[53] A total of 2.0 kV was applied between the microelectrospray emitter and the capillary entrance. The electrosprayed sample and calibrant ions were delivered alternately into the mass spectrometer through a Chait-style atmosphere-to-vacuum interface and externally accumulated for

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between 2 and 7 s in an rf-only octapole. The ions were transferred through multipole ion guides and trapped in an open[54] cylindrical cell (Malmberg−Penning trap).[55]

Ions were frequency-sweep ("chirp") excited and detected in direct mode (512 kword time-domain data). Ten time-domain data sets were co-added, Hanning apodized, zero-filled once and subjected to fast Fourier transform (FFT) followed by magnitude calculation. The experimental event sequence was controlled by a modular ICR data acquisition system (MIDAS).[56] The FT-ICR mass spectra were internally frequency-to-m/z calibrated[57] with respect to the calibrant ions. The FT-ICR mass spectra were analyzed by use of the MIDAS analysis software package.[58] Ion mass and charge were determined with the THRASH algorithm integrated into MIDAS.[59]

Activated-Ion Electron Capture Dissociation and Infrared Multiphoton Dissociation. For tandem mass spectrometry, the peptide or protein ions were externally accumulated in an rf-only octapole. A front-end resolving quadrupole[60] or a stored-waveform inverse Fourier transform (SWIFT)[61,62] ejection served to isolate the peptide ions of interest.

An indirectly heated dispenser cathode (Heat Wave, Watsonville, CA) mounted on the central axis of the system provided the electrons for ECD.[63] A potential of −5 V was applied to the cathode during the irradiation event. A grid situated in front of the filament was kept at −200 V for most of the experiment and pulsed to +5 or +50 V during the ECD event. An off-axis continuous wave 40-W, 10.6-μm wavelength CO_2 laser (Synrad E48-2-115, Bothell, WA) fitted with a beam expander provided the photons for activated ion ECD. The isolated parent ions were irradiated with electrons (and photons) for 20−50 ms. Between 30 and 100 time-domain data sets were co-added, Hanning apodized, zero-filled once and subjected to FFT followed by magnitude calculation. The ECD mass spectra were internally frequency-to-m/z calibrated with respect to the precursor ion and the charge reduced species or backbone fragments.

The photons for IRMPD were provided by the laser described above. The isolated parent ions were irradiated with photons for 200−300 ms. Between 10 and 50 time-domain data sets were coadded, Hanning apodized, zero-filled once, and subjected to FFT followed by magnitude calculation. The IRMPD mass spectra were internally frequency-to-m/z calibrated with respect to the precursor ion and backbone fragments.

RESULTS AND DISCUSSION

AI-ECD FT-ICR MS of Intact SUMO-1−RanGAP$_{418-587}$. Electrospray ionization[43] produces multiply charged ions. The combination of ESI with FT-ICR MS, with its ultrahigh resolution and mass accuracy, is optimal for the analysis of large species. To test the validity of this technique for detection and characterization of SUMO modifications, SUMO-1-modified RanGAP1$_{418-587}$ was first synthesized in vitro (Figure 1A) for subsequent spectrometric analysis. The intact parent ion is shown in Figure 1B. The calculated mass of the intact conjugate is 29 928.2871 Da. The measured mass was 29 928.2075 Da, i.e., mass accuracy 2.7 ppm. (Note that Cys158 of RanGAP1$_{418-587}$ is modified by mercaptoethanol, i.e., +75.9983 Da.) Figure 1C shows the AI-ECD FT-ICR mass spectrum obtained from the intact SUMO-1−RanGAP1$_{418-587}$ $[M + 23H]^{23+}$ ions. AI-ECD of the intact conjugate results in a number of RanGAP1$_{418-587}$ backbone c and z fragments summarized inset. The modified lysine residue, Lys109, is shown highlighted. That residue corresponds to Lys524 in the WT protein, in agreement with studies published to date.[64,65]

Of particular interest in this spectrum are the peaks at m/z 1254, 1410, and 1612, also shown as insets. These peaks correspond to the SUMO-1 c_{99} fragment ion in the 9+, 8+, and 7+ charge states, respectively. The c_{99} fragment is the only backbone fragment deriving from the SUMO-1 polypeptide and is the result of cleavage of the N−Cϵ bond in the modified lysine residue, i.e., loss of the entire SUMO-1 modification. The results suggest that AI ECD may be used as a diagnostic tool to identify SUMO conjugates. As the fragmentation pattern is characteristic of the modification, rather than the substrate, the method could be applied to unknowns. Following confirmation of the presence of SUMO, further experiments would be required to identify the substrate.

FT-ICR MS and MS/MS of SUMO-1-Modified RanGAP1$_{418-587}$ IκBa and GST−PML$_{485-495}$. To investigate the potential of direct infusion microESI FT-ICR mass spectrometry for identifying sites of SUMOylation, analysis of the tryptic digest of SUMO-1-modified RanGAP1$_{418-587}$ was performed. Trypsin proteolysis of SUMO-1-modified proteins results in peptides that retain the SUMO-1 C-terminus (ELGMEEEDVIEVTQEQTGG). Figure 2A shows the FT-ICR mass spectrum obtained from the unfractionated digest. The sequences of the substrate protein and SUMO-1, together with sequence coverage, are shown as insets. The modified lysine residue is highlighted. The overall sequence coverage for RanGAP1$_{418-587}$ was 90%. The triply protonated SUMO-1 modified peptide[101−113] was observed at m/z 1206.9228 (m/z_{calc} = 1206.9237, Δ 0.7 ppm). To confirm the site of SUMOylation, $[M + 3H]^{3+}$ ions of the modified peptide were isolated and subjected to ECD and IRMPD (see Figure 2B, C). ECD of the modified peptide produced a relatively simple spectrum (Figure 2B). Fragments resulting from cleavage of 9 out of 12 N−Cα bonds in the substrate peptide were observed. Interestingly, only one fragment resulting from cleavage within the SUMO modification was detected. That observation may be due to the acidic nature of the modification; i.e., the lack of basic residues

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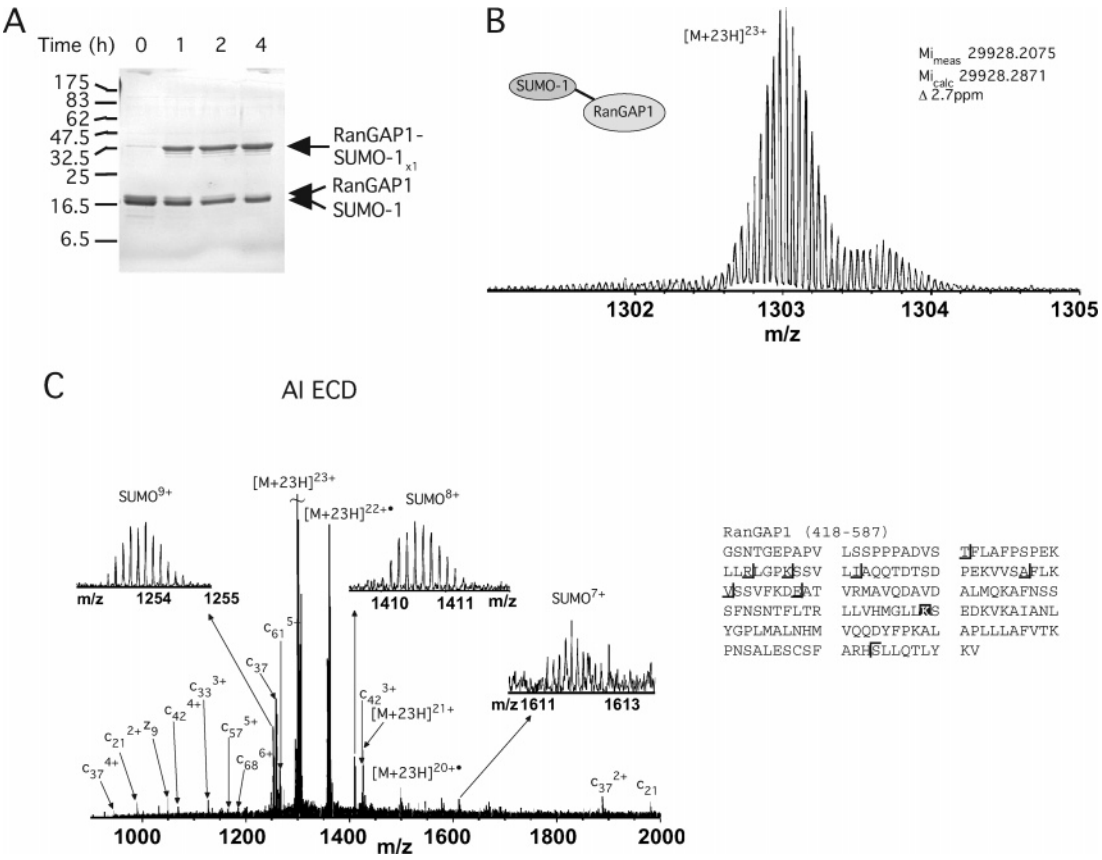

Figure 1. AI-ECD FT-ICR MS analysis of SUMO-1-modified RanGAP1$_{418-587}$. (A). SUMO-modified RanGAP1$_{418-587}$ was synthesized by incubation at 37 °C of recombinant SUMO-1 with RanGAP1$_{418-587}$ in the conjugation assay as described in Experimental Methods. Samples from the conjugation reaction were removed at the times indicated before analysis by Coomassie-stained SDS−polyacrylamide gel electrophoresis. Molecular weight markers, SUMO-1, RanGAP1$_{418-587}$ (RanGAP1), and the RanGAP1$_{418-587}$−SUMO-1 conjugate (RanGAP1−SUMO-1$_{x1}$) are indicated. (B) Precursor [M + 23H]$^{23+}$ ions. (C) Activated ion electron capture dissociation of intact SUMO-1−RanGAP1$_{418-587}$ [M + 23H]$^{23+}$ ions. Expanded *m*/*z* segments showing SUMO-1 c_{99} fragment ions are shown in the inset. The RanGAP1$_{418-587}$ sequence is shown with backbone fragments indicated. The lysine target for SUMOylation is shown in inverse boldface type.

and therefore protonation reduces the likelihood of electron capture in this region. The IRMPD mass spectrum of the modified peptide shows greater complexity (Figure 2C). Fragments resulting from both the substrate and SUMO peptides were observed. All of the b ions, and most of the y ions, arising from cleavage of the peptide bonds[66] in the SUMO modification were accompanied by the loss of water or ammonia. Backbone fragments, in some cases accompanied by water loss, resulting from cleavage of 7 out of 12 peptide bonds in the substrate peptide were observed. Both MS/MS techniques confirm the site of SUMOylation as Lys109, which corresponds to Lys524 in the full protein construct.

To validate the techniques with other SUMO substrate proteins, the procedures were repeated for in vitro SUMOylated GST−PML$_{485-495}$ and IκBα (Supporting Information, Figures 1 and 2). In agreement with the literature, Lys232 from GST−PML$_{485-495}$ (corresponding to Lys490 from WT PML) and Lys23 from IκBα (Lys21 in WT) were confirmed as the sites of SUMO modification.

FT-ICR MS and MS/MS of Mono- and PolySUMOylated RanBP2$_{2532-2767}$. RanBP2 is conjugated to SUMO[67] although the precise site(s) of modification has yet to be established, because RanBP2 is a SUMO E3 ligase and undergoes an autoSUMOylation reaction in which it is modified by multiple copies of SUMO-1.[45] Therefore, as a more unorthodox and complex SUMO substrate, RanBP2 is an ideal candidate for analysis by FT-ICR MS and MS/MS methodology to identify the lysine target(s). Although it has been previously noted that a single lysine in RanBP2 is the "primary acceptor"[68] (the first or major site of modification in the protein), our biochemical experiments suggested that additional sites may be modified. To accurately determine the acceptor lysine(s) in RanBP2$_{2352-2767}$, it was necessary to synthesize a sample of monoSUMOylated RanBP2 in vitro by incubating 20 μM RanBP2$_{2352-2767}$ with 12 μM SUMO-1 in the SUMO conjugation assay as described in Experimental Methods. The almost 2-fold molar excess of RanBP2 over SUMO results in accumulation of predominantly singly modified forms of RanBP2 (see Figure 3A). The reaction products were digested with trypsin, and the unseparated digests were subjected to direct-infusion ESI FT-ICR analysis. Five sites of SUMO-1 modification were identified in RanBP2$_{2532-2767}$ (Figure 3B). See Table 1 for summary. Peaks corresponding to SUMO-1-modified triply charged [24−44] peptide ions (m/z_{meas} 1429.9649; m/z_{calc} 1429.9787, Δ 9.8 ppm), triply charged [51−63] peptide ions (m/z_{meas} 1194.5431; m/z_{calc} 1191.5408, Δ 1.9 ppm), triply charged [62−81] peptide ions (m/z_{meas} 1486.6994;

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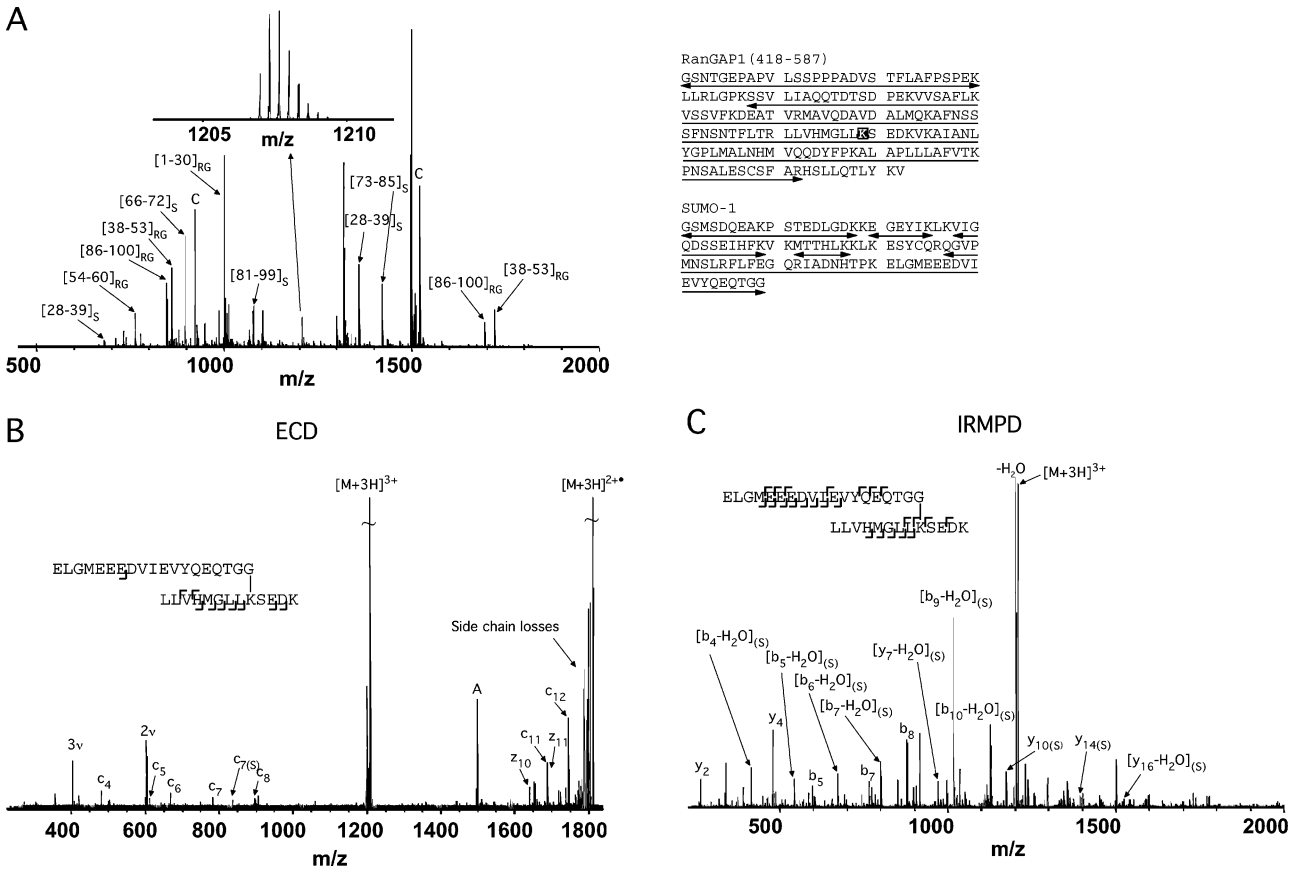

Figure 2. Electrospray FT-ICR mass spectrum of a tryptic digest of SUMO-1−RanGAP$_{418-587}$ and ECD and IRMPD mass spectra of the triply protonated modified tryptic [103−113] fragment. (A) FT-ICR mass spectrum of SUMO-1−RanGAP$_{418-587}$ (S is SUMO-1, RG is RanGAP$_{418-587}$). Sequence coverage (underlined) and expanded *m*/*z* ranges showing modified peptides are shown in the inset. The modified lysine residue is shown in inverse boldface type. c denotes peaks corresponding to calibrant ions. (B) Electron capture dissociation FT-ICR mass spectrum of triply protonated SUMO-1-modified RanGAP1$_{418-587}$ [101−113] tryptic peptide ions. (C) Infrared multiphoton dissociation FT-ICR mass spectrum of triply protonated SUMO-1-modified RanGAP1$_{418-587}$ [101−113] tryptic peptide ions. (S) refers to cleavage within the SUMO modification. Backbone cleavages are shown in the inset. ν = harmonic and A= artifact not removed in quadrupole/SWIFT isolation.

Table 1

RanBP2 fragment	SUMO modification	identified target lysine(s)		sequence coverage	
		RanBP2	SUMO	RanBP2	SUMO
2532−2767	monoSUMO-1	2571, 2592, 2594, 2723, 2725	-	2555−2575, 2582−2612, 2622−2645, 2662−2682, 2688−2695, 2717−2730, 2760−2765	1−16, 18−23, 26−37, 40−45, 64−70, 79−97
	polySUMO-1	2571, 2592	16	2555−2575, 2582−2612, 2662−2682, 2688−2695,	1−23, 26−37, 40−45, 55−97
	monoSUMO-2	2592	-	2555−2571, 2582−2612, 2622−2645, 2688−2695, 2717−2723, 2760−2765	8−32, 36−44, 59−92
	polySUMO-2	2592	11	2582−2612, 2622−2645, 2662−2682,	8−32, 36−44, 59−92
2633−2761	polySUMO-1	2650, 2723	16, 37, 39, 46	2646−2652, 2663−2682, 2688−2695, 2731−2759	1−23, 26−48, 55−70
	polySUMO-2	2725	5/7, 11, 41	2633−2695, 2724−2759	1−32, 36−44, 59−92

m/z_{calc} 1486.7005, Δ 0.7 ppm), doubly charged [186−194] peptide ions (m/z_{meas} 1583.2607; m/z_{calc} 1583.2614, Δ 0.4 ppm), and doubly charged [193−199] peptide ions (m/z_{meas} 1441.6856; m/z_{calc} 1441.6848, Δ 0.6 ppm) were observed (Figure 3B) revealing that SUMOylation occurred at Lys40, Lys61, Lys63, Lys192, and Lys194 (corresponding to lysines 2571, 2592, 2594, 2723, and 2725 in the whole protein). Figure 3E shows the AI-ECD mass spectrum obtained from triply protonated modified [51−63] peptide ions. Backbone fragments deriving from cleavage of 9 out of 12 N−Cα bonds within the substrate peptide were observed, in addition to

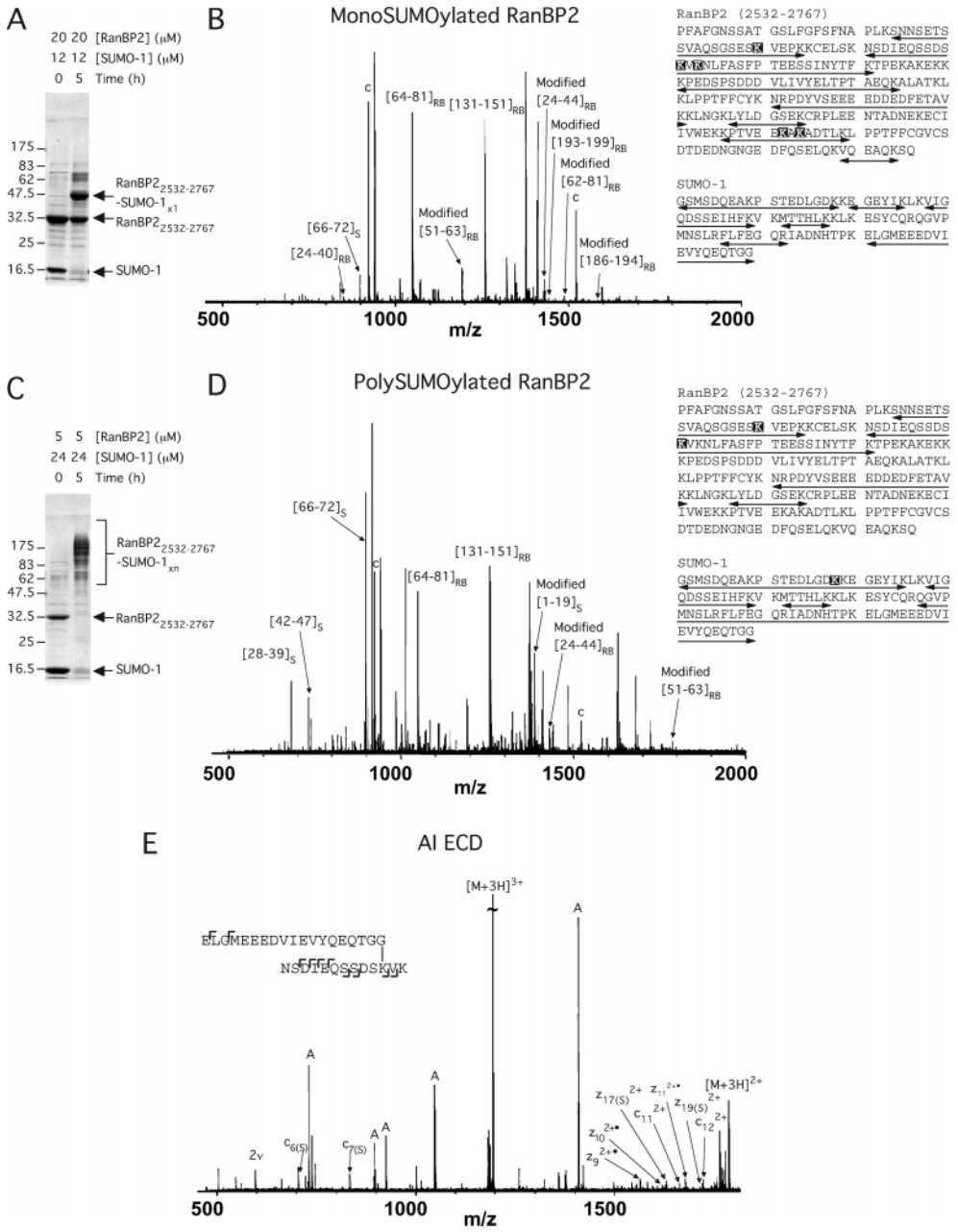

Figure 3. Identification of lysine residues involved in conjugation and polySUMOylation in SUMO-1−RanBP2$_{2532-2767}$ conjugates. (A) A 1-mL sample of RanBP2$_{2532-2767}$ conjugated to a single copy of SUMO-1 was generated in vitro by incubation for 5 h at 37 °C of 20 μM RanBP2$_{2532-2767}$ with 12 μM SUMO-1 in the conjugation assay as described under Experimental Methods. A 10-μL sample was removed before and after incubation and was fractionated by electrophoresis on a 10% polyacrylamide gel containing SDS. Proteins were visualized in the gel by Coomassie staining. Molecular weight markers and the positions of SUMO-1, RanBP2$_{2532-2767}$, and RanBP2$_{2532-2767}$−SUMO-1$_{x1}$ are shown. (B) Electrospray FT-ICR mass spectrum of a tryptic digest of RanBP2$_{2532-2767}$−SUMO-1$_{x1}$. S is SUMO-1, and RB is RanBP2$_{2532-2767}$. Sequence coverages (underlined) are shown in the inset. Modified lysine residues are shown in inverse boldtype face. c denotes peaks corresponding to calibrant ions. (C) A 1-mL sample of RanBP2$_{2532-2767}$ attached to multiple copies of SUMO-1 was produced as described in (A) except the RanBP2$_{2532-2767}$ concentration was reduced to 5 μM and the SUMO-1 concentration increased to 24 μM. The ∼5-fold molar excess of SUMO over RanBP2$_{2532-2767}$ potentiates the formation of the multiply modified form of RanBP2. Samples were analyzed as described in (A). The multiply modified forms of RanBP2$_{2532-2767}$ are indicated (RanBP2$_{2532-2767}$−SUMO-1$_{xn}$). (D) Electrospray FT-ICR mass spectrum of a tryptic digest of RanBP2$_{2532-2767}$−SUMO-1$_{xn}$. Symbols and sequence presentation are as in (B). (E) Activated ion electron capture dissociation FT-ICR mass spectrum of triply protonated RanBP2$_{2532-2767}$−SUMO-1$_{x1}$ [51−63] tryptic peptide ions (see (A)). Backbone cleavages are shown in the inset. ν = harmonic and A = artifact not removed by quadrupole/SWIFT isolation.

some fragments from the SUMO-1 peptide. RanBP2 is therefore modified by SUMO at multiple sites including Lys61 (Lys2592) of RanBP2$_{2532-2767}$.

As it has been shown that RanBP2 can be conjugated to SUMO-1 polymeric chains,[45] we therefore decided to identify the lysine residues in SUMO-1 used for the chain formation. A sample of polySUMOylated RanBP2$_{2532-2767}$ was generated by the same assay described above although an ∼5-fold molar excess of SUMO-1 over RanBP2$_{2532-2767}$ was used to encourage polySUMOylation of RanBP2 (Figure 3C). The ESI FT-ICR mass spectrum

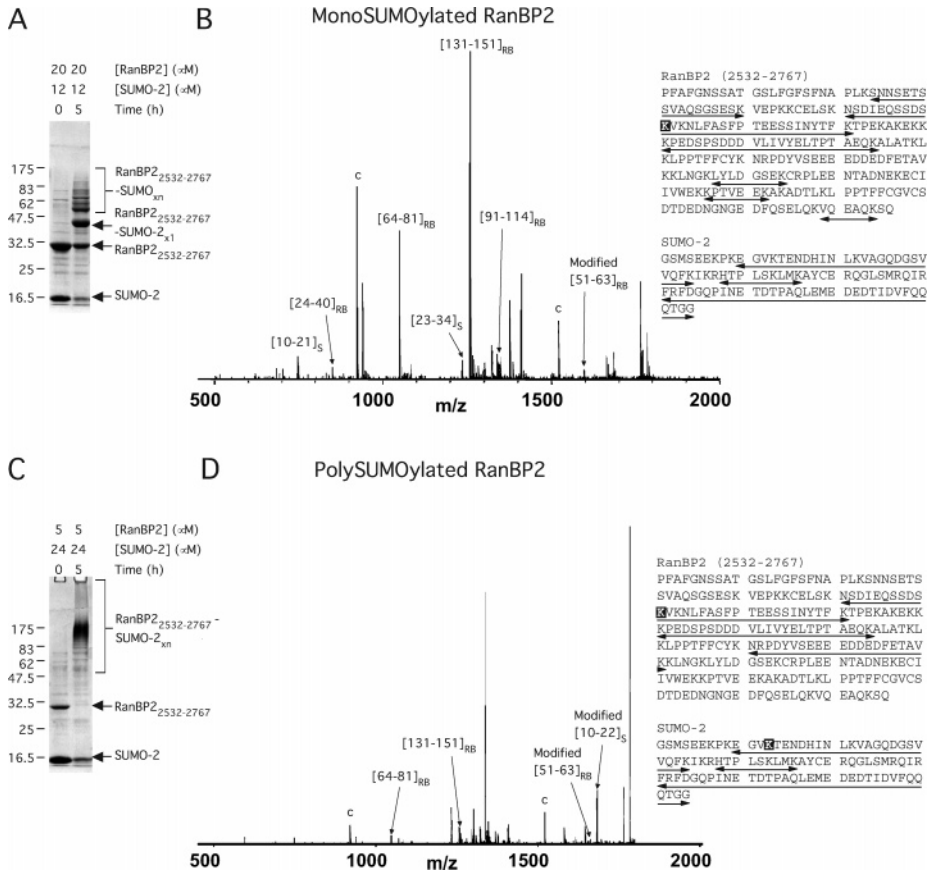

Figure 4. Identification of lysine residues targeted for modification in $RanBP2_{2532-2767}$−SUMO-2 conjugates. (A−D) $RanBP2_{2532-2767}$−SUMO-2_{x1} and $RanBP2_{2532-2767}$−SUMO-2_{xn} were synthesized in vitro, and tryptic digests were analyzed by electrospray FT-ICR MS. Preparation and analysis are as described for Figure 3A−D with SUMO-1 replaced by SUMO-2 in the conjugation reactions.

of polySUMOylated RanBP2 reveals a single site of conjugation in SUMO-1. The peak at m/z 1386.9542 corresponds to triply protonated modified[1−19] SUMO-1 peptide ions (m/z_{calc} 1386.9549, Δ 0.4 ppm). The site of SUMO-1 polymerization is therefore identified as Lys18 (Lys16 in the WT protein). Peaks corresponding to the modified $RanBP2_{2532-2767}$ peptides,[62−81] [186−194] and [193−199], were not observed in the mass spectrum shown in Figure 3D. Because that experiment had a 2-fold greater concentration of SUMO-1 than the previous experiment, the absence of the modified peptides is more likely the result of ion suppression by the SUMO-1 peptides rather than a lack of modifications at Lys63 (Lys2594), Lys192 (Lys2723), and Lys194 (Lys2725).

As mentioned earlier, two subfamilies of SUMO have been identified with significant differences in their cellular function[4] and metabolism by RanBP2.[48] Therefore it was important to compare the findings for SUMO-1 modified RanBP2 with that of another member of the SUMO family, i.e., SUMO-2. Figure 4 shows the direct infusion ESI FT-ICR mass spectra of tryptic digests of RanBP2−SUMO-2 conjugates in which the predominant species were either monoSUMOylated (B) or polySUMOylated (D). Trypsin proteolysis of SUMO-2-modified proteins results in peptides that retain the SUMO-2 C-terminus (FDGQPINETDTPAQLEMEDEDTIDVFQQQTGG), which is much larger than for SUMO-1. Nevertheless, one site of SUMOylation at Lys61 (Lys2592) was identified in the substrate $RanBP2_{2532-2767}$. A peak corresponding to $[M + 3H]^{3+}$ ions of modified peptide$^{51-63}$ (m/z_{meas} 1662.7380; m/z_{calc} 1662.7462, Δ 4.9 ppm) was observed (Figure 4B). This residue also acted as a lysine acceptor for SUMO-1. A single site of SUMOylation at Lys13 (Lys11) was identified in SUMO-2. A peak corresponding to $[M + 3H]^{3+}$ ions of modified peptide$^{10-22}$ (m/z_{meas} 1682.7760; m/z_{calc} 1682.7742, Δ 1.0 ppm) was observed (Figure 4D). That is the site previously identified as the target for E3-independent polySUMO-2 formation.[29]

WT RanBP2 is a 358-kDa nucleoporin, and sequence and functional analyses have revealed a defined domain structure. In particular, the SUMO E3 ligase section of the protein has been shown to contain two internal sequence repeats (IR1 and IR2) in the region 2633−2761.[69] Interestingly, this fragment undergoes a far more extensive self-modification with SUMO-1 and SUMO-2 than the 2532−2767 fragment (M.H.T. unpublished results). To compare these two RanBP2 fragments for their sites of self-modification and polymerization on SUMO-1 and SUMO-2, samples of polySUMOylated $RanBP2_{2633-2761}$ were prepared and analyzed by ESI FT-ICR mass spectrometry (for summary, see Table 1; for details see Supporting Information, Figure 3). For SUMO-1, two sites of RanBP2 SUMOylation were identified at Lys45 (Lys2650) and Lys118 (Lys2723), and four sites of SUMOylation were identified in SUMO-1: Lys18 (Lys16), Lys39 (Lys37), Lys41 (Lys39), and Lys48 (Lys46). Identical analysis of polySUMO-2 conjugated $RanBP2_{2633-2761}$ revealed two sites of SUMO-2 modification at Lys47 (Lys2652) and Lys120 (Lys2725). Within SUMO-2 itself, Lys13 (Lys11) and Lys43 (Lys42) were found to be targeted

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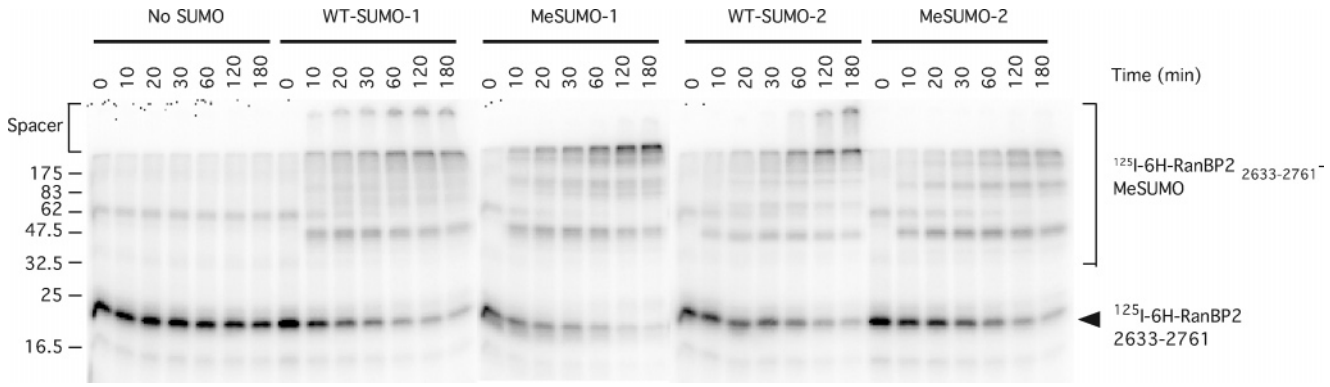

Figure 5. Multiple copies of methylated SUMO conjugate to RanBP2. ^{125}I-Labeled 6H−RanBP2$_{2633-2761}$ was conjugated to WT and methylated SUMO-1 and SUMO-2 by incubation at 37 °C in the in vitro conjugation assay as described in Experimental Methods. Samples were removed at the times indicated and fractionated on 10% polyacrylamide gels containing SDS, before drying and phosphorimaging analysis. Unconjugated ^{125}I-6H−RanBP2$_{2633-2761}$ and the MeSUMO conjugates are indicated. The position of the spacer gel and molecular weight markers are also shown.

for SUMOylation. A low-magnitude peak corresponding a further site of SUMOylation, localized to either Lys7 (Lys5) or Lys9 (Lys7), was also observed.

Methylated SUMO-1 and SUMO-2 Form High Molecular Weight Conjugates with RanBP2. The mass spectrometric experiments described above provide two unexpected findings with respect to RanBP2 self-modification and SUMO polymer formation. First, they show that SUMO chains can be generated from more than one lysine residue in each SUMO protein and, second, that more than one lysine in RanBP2 is targeted for modification. To confirm independently that multiple sites of SUMOylation exist within RanBP2, the conjugation of WT-SUMOs was compared to that for methylated WT-SUMOs (MeSUMO-1/MeSUMO-2). Methylation of SUMO blocks all branched chain formation, and hence, any modified forms of RanBP2 observed in assays containing these proteins will be a result of attachments of only one SUMO per lysine. As can be seen from Figure 5, conjugation reactions containing MeSUMO-1 and MeSUMO-2 still accumulated high molecular weight RanBP2 conjugates (>200 kDa) although no RanBP2 was detected in the "spacer" region of the gel as seen for WT proteins. These results confirm that under these conditions multiple (possibly as many as 15) lysine targets in RanBP2 are used for SUMOylation. These results suggest that, as RanBP2 appeared to be modified by more than six single MeSUMOs under these conditions, either we were unable to detect all target lysines by mass spectrometry or blockage of chain formation caused more lysines in RanBP2 to be utilized. To address this question, tryptic digests of MeSUMOylated samples of RanBP2$_{2532-2767}$ and RanBP2$_{2633-2762}$ were analyzed by direct-infusion ESI FT-ICR mass spectrometry. For the Me-SUMO-1 modified species, 10 acceptor lysines were identified: WT Lys2571, Lys2576, Lys2592, Lys2618, Lys2650, Lys2682, Lys2683, Lys2723, Lys2725, and Lys2765 (see Figure 6). For the Me-SUMO-2-modified species, two acceptor lysines were identified: WT Lys2723 and Lys2725. These findings are consistent with an in vitro study of the branching activity of the yeast SUMO homologue Smt3,[70] which showed that an Smt3 mutant with all lysines mutated to arginine could still form high molecular weight species with the yeast RING-type E3 ligase Siz1.

A

		KxE	
RanBP2	2566	SGSESKVEPKKCE	2578
SUMO-1	11	EDLGDKKEGEYIK	22
SUMO-2	6	PKEGVKTENDHIN	18

B

		KψK	
RanBP2	2587	QSSDSKVKNLFAS	2599
RanBP2	2587	QSSDSKVKNLFAS	2599
RanBP2	2645	KALATKLKLPPTF	2657
RanBP2	2718	PTVEEKAKADTLK	2730
RanBP2	2718	PTVEEKAKADTLK	2730
SUMO-1	32	SEIHFKVKMTTHL	44
SUMO-1	32	SEIHFKVKMTTHL	44
SUMO-1	41	TTHLKKLKESYCQ	53
SUMO-2	1	MSEEKPKEGVKT	12
SUMO-2	36	HTPLSKLMKAYCE	48

Figure 6. Sequence alignments of regions surrounding lysine resides identified in RanBP2, SUMO-1, and SUMO-2 as acceptors of SUMO modifications. (A) Sequences from RanBP2, SUMO-1, and SUMO-2 that contain a SUMO-modified lysine that conforms to a K*x*E sequence motif. (B) Sequences from RanBP2, SUMO-1, and SUMO-2 that contain a SUMO-modified lysine that conforms to a KψK sequence motif, where ψ corresponds to a hydrophobic residue. Modified lysines are shown in boldface type.

CONCLUSION

FT-ICR offers two major advantages for the study of SUMOylation in proteins. The resolution and mass accuracy of the technique allows accurate mass determination of large species, e.g., SUMO−substrate conjugates. Second, the resolution and mass accuracy mean that FT-ICR is ideally suited for complex mixture analysis. The technique may be applied to the investigation of unseparated (no liquid chromatography) protein digests thereby reducing analysis time. The mass accuracy allows confident identification of peptides. An arsenal of MS/MS techniques (ECD, IRMPD, SORI-CID) may be used, if necessary, for confirmation of assignment.

We have applied an FT-ICR mass spectrometry approach for the characterization of sites of SUMOylation in proteins. The results obtained from intact SUMO-1-RanGAP1$_{418-587}$ suggest that AI ECD may be used as a diagnostic tool to identify SUMO conjugates. In vitro SUMOylated proteins RanGAP1$_{418-587}$, IκBα, and GST−PML$_{485-495}$ were digested with trypsin, and the unfrac-

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tionated digests were analyzed by direct infusion (i.e., no on-line separation) ESI FT-ICR mass spectrometry. Unlike trypsin digestion of ubiquitinated proteins which results in modified peptides retaining the ubiquitin C-terminal Gly-Gly motif, trypsin digestion of SUMOylated proteins results in peptides that retain a much longer modification (ELGMEEEDVIEVYQEQTGG for SUMO-1; FDGQPINETDTPAQLEMEDEDTIDVFQQQTGG for SUMO-2). Nonetheless, comparison with in silico digests allows identification of modified peptides. Assignments were confirmed by MS/MS, either ECD, IRMPD, or both.

The SUMO E3 ligase RanBP2 undergoes an autoSUMOylation reaction in vitro generating high molecular weight RanBP2−SUMO conjugates.[45] The results revealed six sites of in vitro SUMOylation in RanBP2 along with four branch-point lysines in SUMO-1, and three in SUMO-2 were identified. In all but one case, SUMOylation occurred within the sequences K*x*E or KψK. Ten sites of in vitro MeSUMOylation in RanBP2 were revealed. Modifications occurred within the sequences K*x*E, KψK, or KK. These results demonstrate the utility of FT-ICR with AI-ECD or IRMPD mass spectrometry in detecting SUMOylation, and sites of SUMOylation, and their potential roles as complementary tools for proteomic and functional analysis, and provide significant insight into the modification of a SUMO ligase for which conventional techniques have been unsuccessful.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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