The Small Ubiquitin-like Modifier-1 (SUMO-1) Consensus Sequence Mediates Ubc9 Binding and Is Essential for SUMO-1 Modification*

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SUMO-1 is a ubiquitin-related protein that is covalently conjugated to a diverse assortment of proteins. The consequences of SUMO-1 modification include the regulation of protein-protein interactions, protein-DNA interactions, and protein subcellular localization. At present, very little is understood about the specific mechanisms that govern the recognition of proteins as substrates for SUMO-1 modification. However, many of the proteins that are modified by SUMO-1 interact directly with the SUMO-1 conjugating enzyme, Ubc9. These interactions suggest that Ubc9 binding may play an important role in substrate recognition as well as in substrate modification. The SUMO-1 consensus sequence (SUMO-1-CS) is a motif of conserved residues surrounding the modified lysine residue of most SUMO-1 substrates. This motif conforms to the sequence "ΨKXE," where Ψ is a large hydrophobic residue, K is the lysine to which SUMO-1 is conjugated, X is any amino acid, and E is glutamic acid. In this study, we demonstrate that the SUMO-1-CS is a major determinant of Ubc9 binding and SUMO-1 modification. Mutating residues in the SUMO-1-CS abolishes both Ubc9 binding and substrate modification. These findings have important implications for how SUMO-1 substrates are recognized and for how SUMO-1 is ultimately transferred to specific lysine residues on these substrates.

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SUMO-1 is a member of a family of ubiquitin-like proteins that are post-translationally conjugated to other proteins (1). The specific effects of SUMO-1 modification appear to be substrate dependent, but they are clearly distinct from the effects of ubiquitination in mediating protein degradation. In a number of cases, SUMO-1 modification regulates the subcellular localization of specific substrates. For example, SUMO-1 modification targets RanGAP1 from the cytoplasm to the nuclear pore complex (2, 3) and PML from the nucleoplasm to PML nuclear bodies (4). SUMO-1 modification of certain other substrates may play a role in antagonizing ubiquitin-mediated proteolysis. Idβ and MDM2, for example, are both modified by SUMO-1 on lysine residues that also function as sites for ubiquitination (5, 6). SUMO-1 modification of these lysines has been proposed to stabilize the substrates by blocking ubiquitin modification. For a growing list of other substrates, the exact effects of SUMO-1 modification remain to be determined. A majority of these substrates, including p53 (7–9), c-Jun (7), and topoisomerase I and II (10, 11) are nuclear proteins that function in regulating transcription or chromatin structure.

Immunofluorescence analysis and cell fractionation studies further indicate that the majority of proteins modified by SUMO-1 are nuclear and that they correspond to only a small subfraction of all cellular proteins (12). The specific subfraction of proteins modified by SUMO-1 also varies throughout the cell cycle (13), and possibly in response to cellular growth conditions, indicating that SUMO-1 modification and de-modification are dynamic processes. However, the precise mechanisms involved in substrate selection and in regulating the timing of modification or demodification are poorly defined.

Many steps involved in SUMO-1 modification parallel those involved in ubiquitination. Like ubiquitin, SUMO-1 is synthesized as a precursor that is proteolytically processed to generate the mature, active polypeptide (13, 14). Once processed, SUMO-1 is activated in an ATP-dependent reaction that creates a thioester intermediate between the active-site cysteine of the SUMO-1 activating enzyme (E1) and the carboxyl terminus of SUMO-1. The SUMO-1 E1 enzyme, a heterodimer consisting of Aos1 and Uba2, is structurally and functionally related to the ubiquitin E1 enzyme (14–16). Following activation, SUMO-1 is transferred from the E1 enzyme to Ubc9, a protein similar in structure and function to ubiquitin E2 enzymes (17–21). How SUMO-1 is subsequently transferred from Ubc9 to specific protein substrates is the most poorly defined step in the SUMO-1 conjugation pathway. Although SUMO-1 modification must be quite specific by virtue of the limited number of cellular proteins that are modified, there is very little, if any, homology among the currently known substrates. Probably all ubiquitination reactions involve E3 ligases, factors that mediate the transfer of ubiquitin from E2 enzymes to specific protein substrates (22, 23). In general, E3s function in substrate recognition and are responsible for the high degree of specificity that is characteristic of most ubiquitination reactions. Currently no E3-like factors have been identified for SUMO-1 conjugation, so how specific proteins are recognized as substrates for SUMO-1 conjugation remains unknown.

Relative to ubiquitin-specific E2 enzymes, an unusual feature of Ubc9 is that it interacts directly with many SUMO-1 substrates (1). These interactions suggest that Ubc9 may play a direct role in recognizing SUMO-1 substrates, as well as in modifying them. In this study, we demonstrate that Ubc9 binds to the SUMO-1 consensus sequence (SUMO-1-CS), a motif of conserved residues surrounding the modified lysine of many SUMO-1 substrates. We further demonstrate that the binding of Ubc9 to the SUMO-1-CS is essential for SUMO-1 modification.

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Ubc9 Binding Is Required for SUMO-1 Modification—The carboxyl-terminal domain of RanGAP1 is modified by SUMO-1 at lysine residue 526 (3, 24). We have previously shown that modification at this site is dependent on a ~120-amino acid domain of RanGAP1 extending from residue 470 to the carboxyl terminus (3) (summarized in Fig. 1). The ability of this domain to specify SUMO-1 modification was demonstrated by in vitro translation in rabbit reticulocyte lysate, where SUMO-1 modification is mediated by endogenous Aos1/Uba2 and Ubc9 activities (3) (Fig. 2, lanes 1 and 7). A significant fraction of the proteins modified by SUMO-1 are known to interact directly with the SUMO-1-conjugating enzyme, Ubc9 (1). Although RanGAP1 is one of the best characterized SUMO-1 substrates, its interactions with Ubc9 have not been analyzed previously. To investigate the functional relevance of Ubc9-substrate interactions, we assayed for the ability of Ubc9 to bind to RanGAP1 and also to the various mutant and heterologous proteins summarized in Fig. 1. GST-Ubc9 (or GST alone as a control) was immobilized on glutathione-Sepharose beads and incubated with [35S]labeled proteins in the presence of [35S]methionine as described by the manufacturer (Promega Corp., Madison, WI). The carboxyl-terminal domain of mouse RanGAP1 (N419: amino acids 420–589) was similarly amplified from a cDNA clone by polymerase chain reaction and subcloned into pGEX-5X-1 (Promega Corp., Madison, WI). GST-N419 and GST-Ubc9 fusion proteins were expressed in bacteria, purified by affinity chromatography on glutathione-Sepharose beads, and cleaved from the beads by Factor Xa as outlined by the manufacturer (Amersham Pharmacia Biotech).

Gel filtration analysis of the carboxyl-terminal domain of RanGAP1 and Ubc9 was performed on a Amersham Pharmacia Biotech Superdex 75 chromatography column. The column was equilibrated and proteins were fractionated with buffer containing 110 mM potassium acetate, 2 mM magnesium acetate, 20 mM HEPES (pH 7.3), and 1 mM dithiothreitol. 20 μg of each protein was loaded either individually or together following mixing and incubation for 30 min at room temperature. 0.5-ml fractions were collected, trichloroacetic acid precipitated, and analyzed by SDS-PAGE. Full-length RanGAP1 interacted specifically with GST-Ubc9, or GST alone, was bound to 20 μl of glutathione-Sepharose beads (1 mg of protein/ml beads) in phosphate-buffered saline containing 1 mM dithiothreitol. Nonspecific protein-binding sites were blocked by incubation with 2% bovine serum albumin for 60 min at 4 °C. An equivalent amount (radioactive counts) of each in vitro translated protein was incubated with the beads in 100 μl of binding buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20) for 30 min at room temperature. Beads were washed three times with binding buffer followed by elution of the bound proteins with SDS-PAGE sample buffer. Binding was analyzed by SDS-PAGE, or by quantifying counts in a liquid scintillation counter. The Ubc9 binding observed for each mutant protein relative to that of wild-type mouse RanGAP1, C232, N3419/PK, and NΔ502/PK were constructed as previously described (3). Site-directed mutagenesis was performed using the GeneEditor Mutagenesis System (Promega Corp., Madison, WI) and mutations were verified by DNA sequencing. A cDNA coding for human Ubc9 was obtained from a fetal liver cDNA library using the GeneEditor Mutatgenesis System (Promega Corp., Madison, WI). The carboxyl-terminal domain of mouse RanGAP1 (N419: amino acids 420–589) was similarly amplified from a cDNA clone by polymerase chain reaction and subcloned into pGEX-5X-1.

**Experimental Procedures**

**Plasmid Constructions**—Expression vectors coding for wild-type mouse RanGAP1, C232, N3419/PK, and NΔ502/PK were constructed as previously described (3). Site-directed mutagenesis was performed using the GeneEditor Mutagenesis System (Promega Corp., Madison, WI) and mutations were verified by DNA sequencing. A cDNA coding for human Ubc9 was obtained from a fetal liver cDNA library using the GeneEditor Mutatgenesis System (Promega Corp., Madison, WI). The carboxyl-terminal domain of mouse RanGAP1 (N419: amino acids 420–589) was similarly amplified from a cDNA clone by polymerase chain reaction and subcloned into pGEX-5X-1 (Amersham Pharmacia Biotech, Piscataway, NJ). The carboxyl-terminal domain of mouse RanGAP1 was expressed in bacteria, purified by affinity chromatography on glutathione-Sepharose beads, and cleaved from the beads by Factor Xa as outlined by the manufacturer (Amersham Pharmacia Biotech).

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**Results**

Ubc9 Binding Is Required for SUMO-1 Modification—The carboxyl-terminal domain of RanGAP1 is modified by SUMO-1 at lysine residue 526 (3, 24). We have previously shown that modification at this site is dependent on a ~120-amino acid domain of RanGAP1 extending from residue 470 to the carboxyl terminus (3) (summarized in Fig. 1). The ability of this domain to specify SUMO-1 modification was demonstrated by in vitro translation in rabbit reticulocyte lysate, where SUMO-1 modification is mediated by endogenous Aos1/Uba2 and Ubc9 activities (3) (Fig. 2, lanes 1 and 7). A significant fraction of the proteins modified by SUMO-1 are known to interact directly with the SUMO-1-conjugating enzyme, Ubc9 (1). Although RanGAP1 is one of the best characterized SUMO-1 substrates, its interactions with Ubc9 have not been analyzed previously. To investigate the functional relevance of Ubc9-substrate interactions, we assayed for the ability of Ubc9 to bind to RanGAP1 and also to the various mutant and heterologous proteins summarized in Fig. 1. GST-Ubc9 (or GST alone as a control) was immobilized on glutathione-Sepharose beads and incubated with [35S]-labeled proteins produced by translation in rabbit reticulocyte lysate. Bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE. Full-length RanGAP1 interacted specifically with Ubc9 (Fig. 2, lanes 1–3), as did N3419/PK (Fig. 2, lanes 7–9). Significantly, both of these proteins are also modified by SUMO-1. Notably, both unmodified and SUMO-1-modified RanGAP1 and N3419/PK interacted with Ubc9. In contrast, C232 (Fig. 2, lanes 4–6) and NΔ502/PK (Fig. 2, lanes 10–12) did not interact with Ubc9. As apparent in lanes 4 and 10, these same two proteins also failed to be modified by SUMO-1. These results indicate a correlation between the ability of these proteins to interact with Ubc9 and their ability to be modified by SUMO-1.

The binding reactions described above were done in the presence of rabbit reticulocyte lysate, making it possible that the observed interaction between Ubc9 and RanGAP1 was indirect. To investigate whether Ubc9 and the carboxyl-terminal domain of RanGAP1 could form a complex in the absence of other factors, we analyzed the proteins by gel filtration chromatography either alone or together after mixing and incubating at room temperature. Bacterially expressed Ubc9 and N3419 were purified and individually fractionated by gel filtration chromatography on a Superdex 75 column (Amersham Pharmacia Biotech). Under these conditions, both proteins eluted as apparent monomers with calculated molecular masses of 20 kDa (Fig. 3). When Ubc9 and N3419 were incubated together and subsequently fractionated on the same column they co-eluted as an apparent heterodimer with a calculated molecular mass of 40 kDa (Fig. 3). This result demonstrates that Ubc9 and the carboxyl-terminal domain of
FIG. 2. A 120-amino acid domain near the carboxyl terminus of RanGAP1 mediates Ubc9 binding. Wild-type RanGAP1 (lanes 1–3), the carboxyl-terminal deletion mutant C23 (lanes 4–6), and the pyruvate kinase fusion proteins N419/PK (lanes 7–9), and ND502/PK (lanes 10–12) were transcribed and translated in rabbit reticulocyte lysates in the presence of [35S]methionine and incubated with immobilized GST-Ubc9 or GST. Bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. The amount of protein loaded in “input” is equivalent to 40% of the amount of protein assayed in each binding reaction. Molecular mass standards are indicated on the left and asterisks indicate SUMO-1-modified proteins. Unmodified RanGAP1 and pyruvate kinase fusion protein translation products all appear as triplets following separation by SDS-PAGE, possibly due to initiation of translation at internal start sites or to phosphorylation.

FIG. 3. The carboxyl-terminal domain of RanGAP1 and Ubc9 interact directly to form a 40-kDa heterodimer. N419 and Ubc9 were expressed in bacteria and purified. The purified proteins were analyzed individually on a Superdex 75 gel filtration column (top two panels) or analyzed together following preincubation for 30 min at room temperature (bottom panel). Column fraction numbers are indicated at the top. The elution positions of standard protein molecular mass markers are also indicated.

RanGAP1 interact directly to form a complex and that the complex is sufficiently stable to allow purification by gel filtration chromatography. This result also demonstrates that Ubc9 can bind to RanGAP1 prior to forming a thiol ester with SUMO-1.

Residues Surrounding the SUMO-1 Modification Site Are Essential for Modification—The precise lysine residues modified by SUMO-1 have been identified in approximately a dozen known substrates (1). The majority of these modification sites conform to a consensus sequence that we refer to as the SUMO-1 consensus sequence, or SUMO-1-CS. The SUMO-1-CS is defined by four amino acids with the sequence “ΨKXE” (where Ψ is a large hydrophobic amino acid, K is the lysine residue modified by SUMO-1, X is any amino acid, and E is glutamic acid). Positive interactions with Ubc9 are indicated. References: RanGAP1 (2, 3), PML (4, 26, 37), Sp100 (28), p53 (7–9, 34), IκBα (5, 42), c-Jun (7, 35), IΕ2 (43), HSF2 (45), and androgen receptor (AR) (44).

| Substrate | Target Site | Ubc9 Interaction |
|-----------|-------------|------------------|
| RanGAP1   | H M G L K S D E K V | + |
| PML(1)    | H Q W F L K H E A R P | + |
| PML(2)    | F R K V I K M S E E E | + |
| Sp100     | R L V D I K K E K F F | ? |
| p53       | K L M F K T E G F D | + |
| IκBα      | F R D G L K K E R L L | + |
| c-Jun     | K L Q A L K K E F P Q T | + |
| IΕ2 (1)   | M D P I K Q E G K E | + |
| IΕ2 (2)   | K Q E D I K K E F P D F | + |
| HSF2      | D S G I K K Q E R D G | + |
| AR        | F H A R I K L E N P L | + |

CONSENSUS (Ψ) Κ Ε

FIG. 4. The SUMO-1-CS defines the SUMO-1 modification site of many SUMO-1 substrates. Defined SUMO-1 modification sites in the indicated proteins were aligned, revealing the SUMO-1-CS: ΨKXE (where Ψ is a large hydrophobic amino acid, K is the SUMO-1 modified lysine residue, X is any amino acid, and E is glutamic acid). Positive interactions with Ubc9 are indicated. References: RanGAP1 (2, 3), PML (4, 26, 37), Sp100 (28), p53 (7–9, 34), IκBα (5, 42), c-Jun (7, 35), IΕ2 (43), HSF2 (45), and androgen receptor (AR) (44).
the wild-type protein. Mutations of histidine 521 (Fig. 6, lanes 4–6) and lysine 530 (Fig. 6, lanes 22–24) to alanine had only modest effects on Ubc9 binding, reducing the interaction by 50 and 30%, respectively. Mutating the actual modification site, lysine 526, to arginine (Fig. 6, lanes 16–18) had no noticeable effect on Ubc9 binding, possibly due to the conservative nature of this amino acid substitution. These results again demonstrate a correlation between Ubc9 binding and SUMO-1 modification: proteins that retained their ability to interact with Ubc9 were modified by SUMO-1 (with the exception of K526R), whereas those that did not bind Ubc9 were not modified by SUMO-1. These results further demonstrate that conserved residues surrounding the SUMO-1 modification site are essential for Ubc9 binding.

SUMO-1 Contributes to Interactions between Ubc9 and SUMO-1-modified RanGAP1—Despite the apparent role of the SUMO-1-CS (including the targeted lysine) in mediating Ubc9 binding, we observed that SUMO-1 modified RanGAP1 bound to Ubc9 as well as, and possibly better than, unmodified RanGAP1. To investigate whether SUMO-1 itself, after being conjugated to RanGAP1, could contribute to Ubc9 binding, we assayed for the binding of Ubc9 to SUMO-1-modified RanGAP1 and unmodified RanGAP1 in the presence of increasing concentrations of free SUMO-1. Surprisingly, we found that free SUMO-1 had a significant effect on the binding of SUMO-1-modified RanGAP1 to Ubc9, but had only a modest effect on the binding of unmodified RanGAP1 (Fig. 7). We observed an ~60% reduction in the binding of SUMO-1-modified RanGAP1 when binding assays were performed in the presence of 3.25 mg/ml SUMO-1 (corresponding to an ~15-fold excess over immobilized Ubc9) (Fig. 7, lanes 1 and 5). In the same assay, the binding of unmodified RanGAP1 was reduced by less than 20% (Fig. 7, lanes 1 and 5). These findings indicate that Ubc9 interacts with SUMO-1 modified RanGAP1 through SUMO-1, possibly independent of direct interactions with RanGAP1.

DISCUSSION

SUMO-1 modification modulates protein functions by altering protein-protein interactions, protein-DNA interactions, protein subcellular localization, and possibly by directly altering protein activity. It is anticipated that SUMO-1 modification is highly regulated, with substrates being selectively recognized and modified in response to specific cellular signals. Several studies, for example, have demonstrated that SUMO-1-modified proteins vary with changes in the cell cycle (13) and in response to cellular growth conditions (10, 25). However, the mechanisms by which proteins are selectively recognized as substrates for SUMO-1 modification or de-modification and
how such mechanisms might be regulated are not yet understood.

We have begun to investigate how proteins are recognized as substrates for SUMO-1 modification and present evidence that direct interaction with the E2 enzyme, Ubc9, is an important part of this process. Using a domain derived from a well-characterized SUMO-1 substrate, RanGAP1, we have demonstrated that SUMO-1 modification correlates with the ability to directly interact with Ubc9. This domain of RanGAP1 contains a consensus sequence, the SUMO-1-CS, which is found in nearly all known SUMO-1 substrates. The SUMO-1-CS contains the lysine residue to which SUMO-1 is covalently attached, and several highly conserved residues that flank this lysine. Although recognized by several other groups (26–28), the functional significance of the SUMO-1-CS was not previously characterized. To investigate its role in SUMO-1 modification, we made alanine substitutions of the conserved residues in the SUMO-1-CS. These mutations were found to inhibit both Ubc9 binding and SUMO-1 modification. These findings indicate that the SUMO-1-CS plays a direct role in mediating the binding of Ubc9 to SUMO-1 substrates and that this binding is essential for substrate modification.

The actual lysine acceptor in the SUMO-1-CS of RanGAP1 is important for the interaction with Ubc9, as an alanine substitution at this position inhibits Ubc9 binding. In apparent contradiction to this finding, it was also observed that SUMO-1-modified RanGAP1 and free RanGAP1 interact equally well with Ubc9. Competition experiments using excess free SUMO-1, however, indicated that modified RanGAP1 likely interacts with Ubc9 through the SUMO-1 moiety. It remains to be determined whether the binding of modified RanGAP1 to Ubc9 is mediated solely through interactions with SUMO-1 or through a combination of interactions with RanGAP1 and SUMO-1. Previous studies have indicated that Ubc9 and free SUMO-1 can form direct, noncovalent interactions (29). It will be interesting to determine whether other SUMO-1-modified substrates interact similarly with Ubc9, or whether this interaction is specific for modified RanGAP1. In vivo, Ubc9 is centered at the nuclear envelope at sites that overlap with the localization of SUMO-1-modified RanGAP1 (19). Although Ubc9 has been shown to interact with the nucleoporin Nup358 (18), it remains to be determined whether this interaction is direct or mediated by SUMO-1-modified RanGAP1.

In addition to demonstrating a role for the SUMO-1-CS in Ubc9 binding, we also found that ~50 amino acids on either side of the consensus sequence were required for Ubc9 binding and SUMO-1 modification. Comparison of the amino acid sequence of this larger domain with other SUMO-1 substrates revealed no obvious homologies outside of the SUMO-1-CS. It therefore remains to be determined whether additional residues outside of the SUMO-1-CS are directly involved in Ubc9 binding, or whether they indirectly affect binding by influencing the proper folding and exposure of a smaller domain containing the SUMO-1-CS. Evidence that the precise position of the SUMO-1-CS within a protein can be an important determinant of its ability to function in SUMO-1 modification is provided by analysis of the heat shock transcription factor, HSF2. HSF2 contains three putative SUMO-1-CS motifs, but is modified at a lysine residue in only one of these motifs (45). The precise position of the SUMO-1-CS within a protein is likely to determine its accessibility and, therefore, its potential to interact with Ubc9. The presence of a SUMO-1-CS in a protein is therefore not an absolute indicator of whether protein will be a substrate for SUMO-1 modification. Further analysis of the amino acid residues surrounding the SUMO-1-CS and their contributions to Ubc9 binding will be needed to refine the definition of a SUMO-1 substrate.

In this particular study, we examined the SUMO-1-CS of RanGAP1, but we propose that the SUMO-1-CS of other SUMO-1 substrates will function similarly to mediate Ubc9 binding and facilitate SUMO-1 modification. Ubc9-binding domains have been partially mapped in a large number of known and putative SUMO-1 substrates. Many of these domains contain SUMO-1-CS motifs, including the Ubc9-binding domains found in WT1 (30), TEL (31), Dorsal (33), c-Jun (35), glucocorticoid receptor (35), and poly(ADP-ribose) polymerase (36). Other motifs in addition to the SUMO-1-CS may also mediate Ubc9 binding, however. Most notably, Ubc9 also interacts with the RING domain of PML (26). The functional significance of this interaction remains unclear as mutations that disrupt interactions between Ubc9 and the PML RING domain have very little effect on the SUMO-1 modification of PML (26, 37). It has also recently been proposed that PEST sequences may play a role in regulating the interaction of Ubc9 with at least some SUMO-1 substrates (1, 38). Although PEST sequences are best known for their involvement in ubiquitin-mediated proteolysis (39), they are found in more than half of the known SUMO-1 substrates.

The binding of Ubc9 to the SUMO-1-CS argues against a direct requirement for E3 ligases in SUMO-1 modification. However, it is possible that the modification of substrates with a SUMO-1-CS may be regulated or facilitated by the action of E3-like factors in vivo. Regardless of this consideration, a more detailed analysis of the direct interactions between Ubc9 and the SUMO-1-CS will provide important insights into the mechanisms involved in the ultimate transfer of SUMO-1 to specific substrates. Despite crystallographic studies of ubiquitin E2-E3 complexes (40, 41), the exact mechanism that underlies the transfer of ubiquitin from upstream factors to protein substrates remains poorly defined. Given the high degree of homology between Ubc9 and ubiquitin E2 enzymes, it is likely that the transfer of SUMO-1 to protein substrates will be very similar to the process of ubiquitination. Detailed characterization of the interactions between Ubc9 and the SUMO-1-CS...
should, therefore, provide important insights into the mechanisms of both ubiquitination and SUMO-1 modification. It will also be important to further characterize the role of amino acids surrounding the SUMO-1-CS, to determine whether they also be important to further characterize the role of amino
nisms of both ubiquitination and SUMO-1 modification. It will should, therefore, provide important insights into the mecha-

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