Identification of a Substrate Recognition Site on Ubc9*

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Human Ubc9 is homologous to ubiquitin-conjugating enzymes. However, instead of conjugating ubiquitin, it conjugates a ubiquitin homologue, small ubiquitin-like modifier 1 (SUMO-1), also known as UBL1, GMP1, SMTP3, PIC1, and sentrin. The SUMO-1 conjugation pathway is very similar to that of ubiquitin with regard to the primary sequences of the ubiquitin-activating enzymes (E1), the three-dimensional structures of the ubiquitin-conjugating enzymes (E2), and the chemistry of the overall conjugation pathway. The interaction of substrates with Ubc9 has been studied using NMR spectroscopy. Peptides with sequences that correspond to those of the SUMO-1 conjugation sites from p53 and c-Jun both bind to a surface adjacent to the active site Cys39 of human Ubc9, which has been previously shown to include residues that demonstrate the most significant dynamics on the microsecond to millisecond time scale. Mutations in this region, Q126A, Q130A, A131D, E132A, Y134A, and T135A, were constructed to evaluate the role of these residues in SUMO-1 conjugation. These alterations have significant effects on the conjugation of SUMO-1 with the target proteins p53, E1B, and promyelocytic leukemia protein and define a substrate binding site on Ubc9. Furthermore, the SUMO-1 conjugation site of p53 does not form any defined secondary structure when either free or bound to Ubc9. This suggests that a defined secondary structure at SUMO-1 conjugation sites in target proteins is not necessary for recognition and conjugation by the SUMO-1 pathway.

SUMO-11 (also known as PIC1, sentrin, UBL1, SMTP3, and GMP1) is a ubiquitin homologue, and it has been shown to play an important role in cellular functions such as DNA repair and p53-dependent processes (for a review, see Ref. 1). The SUMO-1 and ubiquitin conjugation pathways share many similarities (for a review on ubiquitination, see Ref. 2) in the primary structures of the activating enzymes (E1), the three-dimensional structures of the conjugating enzymes (E2), and the mechanism of substrate modifications. In the SUMO-1 pathway, SUMO-1 is first activated by a heterodimeric SUMO-activating enzyme (SAE1/SAE2) (3–6) through hydrolysis of ATP to form a high energy thioester bond between the C-terminal Gly residue of SUMO-1 and a Cys residue in SAE2. Then, SUMO-1 is transferred to the SUMO-conjugating enzyme, Ubc9, in a transesterification reaction whereby the C-terminal Gly of SUMO-1 is conjugated to the SH group of the active site Cys39 of Ubc9. In the final step, SUMO-1 is transferred from the SUMO-1-Ubc9 conjugate to the target protein. Similar to the ubiquitination pathway, the C-terminal Gly residue of the SUMO-1 molecule is involved in covalent linkage to the e-amino group on a Lys residue of the target protein. At least in vitro, the SUMO-1 pathway does not appear to require the participation of activities equivalent to ubiquitin-protein isopeptide ligases (E3).

The SUMO-1 pathway has diverse substrate proteins that include transcription factors (p53, c-Jun, and tramtrack), topoisomerases, GTPase-activating protein RanGAP1, oncogene product MDM-2, cell cycle-related protein CDC3, the nuclear dot protein sp100, the promyelocytic leukemia gene product (PML), the bovine papillomavirus E1 protein, homeodomaining protein kinase 2, and IκBα, which is an inhibitor of the transcription factor nuclear factor-κB (1, 7, 8). Many other proteins have been shown to interact with Ubc9, including the DNA repair proteins RAD51 and RAD52, glucocorticoid receptor, the negative regulatory domain of the Wilms’ tumor gene product, CLB2 (an M-phase cyclin), and CLB5 (an S-phase cyclin). Many of these proteins may well be SUMO-1 target proteins or potential regulators of the pathway. Unlike ubiquitination, SUMO-1 modification does not target proteins for degradation. SUMO-1 conjugation to RanGAP1 and PML appears to target these proteins to the nucleus or to subnuclear structures (9–12). However, SUMO-1 modification of IκBα antagonizes ubiquitination and stabilizes the proteins in the cell (13, 14). SUMO-1 modification activates p53 (15, 16), although the mechanism is unclear.

It appears that the SUMO-1 modification machinery recognizes diverse target proteins that contain the sequence KXE (consensus SUMO modification site, where K represents a large hydrophobic residue, and X represents any residue) (17, 18). Short peptides that contain the KXE consensus sequence were shown to be sufficient for SUMO-1 conjugation. This suggests that the consensus sequence is important for the recognition of diverse target proteins by the SUMO-1 modification system.

The three-dimensional structures of human Ubc9 and several other E2 proteins have been determined (19–25), and they reveal that the E2 enzymes have a highly conserved three-dimensional structure. Furthermore, the conformational flexi¬
bility of Ubc9 has been characterized using NMR methods (26), which show that Ubc9 has an overall rigid conformation but that several regions have higher than average flexibility. In particular, a few residues near the active site have high mobility on the microsecond to millisecond and picosecond to nanosecond time scales. These residues have been proposed to play a role in substrate recognition or catalytic activity.

We have examined the interactions of Ubc9 with two peptide corresponding to the sequences surrounding the conjugation sites in p53 and c-Jun. Some residues surrounding the active site, Cys93, of Ubc9 showed significant chemical shift perturbation by the binding of either peptide. These residues have been altered by mutation to examine their functional significance. SUMO-1 conjugation assays using Ubc9 containing these mutations show that these amino acid residues play important roles in target protein conjugation with SUMO-1.

**MATERIALS AND METHODS**

**cDNA Cloning—Mutants of Ubc9 (Q126A, Q130A, E132A, A131D, and E132A) were created using a three stage PCR process as described previously (27), using external primers 5′-CCAGGCGGCCG-CAAGGGATACCATCTAGTAC-3′ and 5′-GAAGGAGATATATCATGGCCTAATCCACAGGG-3′; the primer 5′-CCAGGCGGCCGCCC-3′, and the mutant internal primers 5′-GGACCTTCTAAAGTTATATGGGAAATCGGCAGAACTGAAAGCTGCTGACGATCT-3′ (Q126A), 5′-CCAAATTTCCAAGGAGGAGCGCTGACAGGACCATCTGACGATCT-3′ (Q130A), 5′-CTATGAAACATCAGCTCAAGAACAGCAG-3′ (E132A), 5′-GACCGACCGATCATCAGCTCAAGAACAGCAGG-3′ (A131D), and 5′-GCCAAGGTGTCTTCTGTTGATG-3′ (E132A). Wild-type (wt) human Ubc9 and the mutants were subcloned into vector PET28 as described previously (28).

To generate a recombinant p53 substrate, Ubc9 was fused to an 11-amino acid sequence (29) "KKLVGRPRKTVS" representing amino acids 381–391 of p53 (17). This was constructed by annealing the primers 5′-GATCCAAAAATTGATGTTCAAGACGGAAGGCCCTGACTA-3′ and 5′-AATTCTCATTCTGTTCTTACATCATTACCACCAT-3′ into a pGEX2T expression plasmid cleaved with BamHI and EcoRI restriction enzymes. GST and the 11-amino acid peptide were separately expressed in E. coli BL21(DE3) and purified by nickel-nitrilotriacetic acid resin (Qiagen) as follows. The supernatant was loaded onto the column equilibrated with buffer A. The column was washed with 10 volumes of buffer A followed by 10 volumes of buffer B (20 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9), and then eluted with 5 column volumes of buffer C (600 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9). The eluted proteins were concentrated and then exchanged to the buffer for the NMR studies (100 mM sodium phosphate, pH 6.0, 0.02% NaN3, 5 mM dithiothreitol, in 90% H2O/10% D2O). The purity of the protein was confirmed by SDS-PAGE. The concentration of the protein was estimated using Bradford's method (32) and one-dimensional proton NMR spectra. The structural integrity of the mutant proteins was confirmed by one-dimensional proton NMR spectra. Other recombinant protein concentrations were determined or verified using Bradford's method or calculated extinction coefficients for absorbance measurements at 280 nm.

The p53 and c-Jun peptides were synthesized by solid phase synthesis in the Peptide and Nucleic Acid Synthesis Facility of the City of Hope National Medical Center and verified by mass spectroscopy. The p53 peptide contains the last 30 amino acid residues of p53: AHSSHLKPRLQALKEEKPVQH. The c-Jun peptide contains 25 amino acid residues corresponding to the sequence at the SUMO-1 modification site of c-Jun: QMPVQH.

**NMR Measurements—** All NMR spectra were acquired on a Varian UNITY-plus 500 MHz NMR spectrometer equipped with four channels, pulsed field gradient, z-axis field gradient, and 2D NOE methods. Chemical shifts for the p53 peptide or 25 mM c-Jun peptide to molar ratios of 1:3.9 or 1:11, respectively. Titration with the p53 and c-Jun peptides took 8 and 11 steps, respectively. At each titration point, two-dimensional 1H-15N HSQC spectra (34) were recorded for bound 15N-labeled Ubc9. In addition, two-dimensional ω1-15N-half-filtered TOCSY and ω2-15N-half-filtered NOESY spectra (35, 36) were recorded for bound p53 at molar ratios of 1:1, 1:2.4, and 1:3.9. The spectral widths in the HSQC spectroscopy experiments were 1300 Hz in F1 and 6000 Hz in F2 dimensions, with 128 and 512 complex points in the F1 and F2 dimensions, respectively. The ω1- and ω2-half-filtered TOCSY and NOESY spectra, quadrature detections in the F1 dimension were achieved using the TIPPI approach. In these experiments, spectral widths of 5995 Hz were used in both dimensions, and 512 and 1024 complex points were used in the F1 and F2 dimensions, respectively. Linear prediction in the indirect dimension was performed. Linear dimensions in both were used before Fourier transformation.

**Determination of the Dissociation Constant Kd—** The peptides and Ubc9 have a charge difference with respect to the antibodies in chemical shifts between the free and bound forms. Therefore, a single resonance is observed, which is the population-weighted average of the chemical shifts of the free and bound forms. Under this condition, the dissociation constant can be estimated based on chemical shift changes. The resonances of residues Ala129, which have the largest chemical shift changes upon conformation of the complex, were used to estimate Kd.

**Calculations of Electrostatic Potentials—** The surface electrostatic potentials for Ubc9 were calculated using the DelPhi module of INSIGHTII (MSI, Inc.) and the crystal structure of Ubc9 (21, 24). The solvent dielectric constant was set to 80. The radius of the probe water molecule was 1.4. The grids in the calculation of the electrostatic potentials were with a spacing of 1.5.

**Secondary Structure Prediction—** Secondary structure prediction was done using the PHD program on the entire sequences of RanGAP1, c-Jun, and PML and the first 100 amino acids of IκBα (10, 11, 14, 37). Sequences were deemed to be helical only when the program returned a predicted secondary structure content of >50% or more adjacent amino acid residues.

**Kinetic Analysis of Ubc9 Mutants—** Conjugation assays were set up using 6.44 μM 125I-labeled SUMO-1-C52A and various concentrations of defined GST tag substrates (see above). SUMO-1-C52A was used instead of the wt to avoid experimental interference from the formation of SUMO-1-SUMO-1 disulfide-linked dimers. Assays were performed in 10-μl reaction volumes and also contained an ATP regeneration system.
described above (50 mM Tris, pH 7.5, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 unit/ml creatine kinase), 0.6 unit/ml inorganic pyrophosphatase, 120 ng of SAE1/SAE2, and 2.76 μM of each Ubc9 protein. Under these conditions wt-Ubc9 was seen to be rate-limiting, and the increase in concentration of the GST substrate-SUMO-1 conjugate was proportional to time over the 60-min incubation time (data not shown). Each Ubc9 mutant was assayed in triplicate using concentrations ranging from 1 to 30 μM of three different GST substrates. Reactions were incubated at 37 °C for 60 min before the addition of SDS sample buffer containing β-mercaptoethanol. Assay samples were subsequently fractionated by electrophoresis in 12% polyacrylamide gels containing SDS, stained, destained, and dried before phosphorimaging analysis. Initial reaction velocity rates (V₀) could then be calculated and used to extrapolate kinetic information for each Ubc9 mutant for three different recombinant substrates.

RESULTS

Mapping the Binding Site of the p53 and c-Jun Peptide on Ubc9

It has been shown recently that short peptides containing the SUMO-1 modification consensus sequence, ψKXE, are sufficient for in vitro SUMO-1 modification by Ubc9 (17). We used chemical shift perturbation to determine whether peptides corresponding to the sequences surrounding the SUMO-1 conjugation sites of p53 and c-Jun form specific interactions with Ubc9 and to determine which residues from Ubc9 are involved in the interaction. The sequences of the two peptides are given in Fig. 1A.

Chemical shift perturbation is extremely sensitive to molecular interactions and has been widely used to map binding surfaces. Specific chemical shift changes usually correlate to specific interactions. When two molecules form a specific complex, changes in the environments of the nuclei at the interface will inevitably cause changes in chemical shifts. Conformational changes due to the interaction will result in additional chemical shift perturbation beyond the direct contacting surface. Because chemical shift perturbation is sensitive to specific interactions over a wide range of affinities, it has been successfully used in drug screening to identify molecules that bind to a protein target. This approach is known as “SAR (structure activity relationship) by NMR” (38).

<15N-1H HSQC spectra were used to identify the interaction between Ubc9 and the p53 or c-Jun peptide and to map the binding site of these peptides on Ubc9. 15N-enriched Ubc9 and unlabeled p53 or c-Jun peptides were used for this study. 15N-1H HSQC spectroscopy selectively observes signals from Ubc9 in the complex with the p53 and c-Jun peptides. Specific chemical shift perturbation was observed in 1H-15N HSQC spectra of Ubc9 upon complex formation with the p53 or c-Jun peptide. These changes were consistent from the beginning of the titration, when the concentration of Ubc9 was ~0.7 mM and that of the p53 (or c-Jun) peptide was 0.3 mM (or 0.5 mM for the c-Jun peptide), until the final concentrations of Ubc9 and the p53 (or c-Jun) peptide reached ~0.6 and 2.3 mM respectively (or 0.5 mM for Ubc9 and 6.0 mM for the c-Jun peptide). The affinity of the interaction between Ubc9 and the p53 or c-Jun peptide was estimated as described under “Materials and Methods” from the chemical shift changes of Ala129, and the dissociation constant was ~3–6 mM (data not shown). Superpositions of the HSQC spectra of free Ubc9 and that in complex with the p53

![Image](http://www.jbc.org/)

FIG. 1. A, sequences of the p53 and c-Jun peptides used for NMR chemical shift perturbation studies. The SUMO-1 conjugation sites are boxed, and the consensus sequence is highlighted. Positively and negatively charged amino acid residues are shown in blue and red, respectively. B and C show the superposition of a region of the 1H-15N HSQC spectra of 15N-labeled Ubc9, free (red) and in the complex (green) with (B) the unlabeled p53 peptide and (C) the c-Jun peptide. The molar ratios of p53 to Ubc9 and c-Jun to Ubc9 are 3.9:1 and 11:1, respectively. Only peaks affected significantly upon complex formation are indicated with their assignments.
and c-Jun peptides are shown in Fig. 1, B and C, respectively. Plots of chemical shift changes in Ubc9 upon binding to the p53 and c-Jun peptides versus residue number are shown in Fig. 2A. The low affinity is partly responsible for the small chemical shift changes observed. Residues that show significant chemical shift changes induced by the p53 and c-Jun peptides are indicated in red in the three-dimensional structure of Ubc9 as shown in Fig. 2B. Most resonances of Ubc9 were not affected, indicating that complex formation does not cause overall conformational changes in Ubc9.

Both peptides, which have no apparent sequence similarity outside the consensus sequence, produced similar chemical
shift changes in residues surrounding the active site Cys^{93} of Ubc9. Ala^{129} displays the largest chemical shift change that accompanies binding to either peptide. The side chain of Ala^{129} approaches that of the conjugation active site Cys^{93}, and the two side chains are less than 5 Å apart. In addition, residues Leu^{94}, Gln^{99}, Gln^{126}, and most of the observable residues between residues 129 and 135 show significant chemical shift changes. This surface is adjacent to the conjugation active site. Residue Val^{148} also displayed chemical shift changes induced by complex formation with both peptides. This residue is next to the segment composed of residues 129–135 in the structure. Because both peptides produced similar chemical shift changes in residues near Cys^{93}, it is likely that these residues are important for target protein recognition.

A number of residues that are not adjacent to the active site also show some chemical shift perturbation specific to either the c-Jun or p53 peptide. It is most likely that these correspond to nonspecific interactions. For example, some residues that only show chemical shift changes upon binding of the p53 peptide are scattered on a surface that has a strong negative electrostatic potential. The p53 peptide has a net overall positive charge; therefore, the p53 peptide and this surface on Ubc9 have opposing electrostatic potentials, and nonspecific interactions between them may occur. Electrostatic interactions are generally long range and not highly specific and may generate the pattern of chemical shift changes observed in these residues.

**Substrate Binding Site Mutants of Ubc9 Affect the Transfer of SUMO-1 to Protein Substrates**—In the SUMO-1 pathway, the Ubc9-SUMO-1 conjugate targets interact with target proteins, to which it attaches SUMO-1. To investigate whether the residues near the active site of Ubc9 identified by NMR chemical shift perturbation studies are relevant to target protein recognition in the SUMO-1 pathway, the mutants Q126A, Q130A, A131D, E132A, Y134A, and T135A were made and tested in *in vitro* conjugation assays. The side chains of these amino acid residues are located on the surface of the protein, and their alteration did not cause overall structural disruption, as determined by one-dimensional NMR analysis of the mutant proteins (Fig. 3). The region of the spectra displayed (Fig. 3) corresponds to signals of methyl and methylene groups, which include those from amino acid residues in the hydrophobic core of the protein, such as Ile, Leu, and Val. The one-dimensional spectra indicate that the mutants have three-dimensional structures very similar to that of the wt protein. The residue showing the largest chemical shift perturbation in the NMR binding studies, Ala^{129}, was not altered due to its apparent involvement in important hydrophobic contacts in the Ubc9 structure.

To probe the roles of these residues in substrate modification by the SUMO-1 pathway, steady-state kinetics studies were conducted for the wild-type and mutant versions of Ubc9. In both ubiquitin and SUMO modification pathways, the E1 enzyme catalyzes three distinct reactions: (a) adenylation of SUMO, (b) formation of E1-SUMO thiolester, and (c) transfer of SUMO from E1 to form an E2-SUMO thiolester (39). Like the previously described reactions in the ubiquitin systems, the reactions mediated by the SUMO-activating enzyme occur very quickly, and the overall rate of the conjugation reaction is limited by the rate at which Ubc9 transfers SUMO to substrates, (in the absence of an E3 enzyme).^2^ There are two reactions involving Ubc9: (a) formation of SUMO-Ubc9 thiolester, and (b) transfer of SUMO from Ubc9 to substrates. This reaction therefore fits the ping-pong Bi Bi mechanism (40), where SUMO-E1 conjugate is the first substrate (S1), Ubc9 is the unmodified enzyme, SUMO-Ubc9 thiolester is the modified enzyme (E'), release of E1 is the first product (P1), substrate protein is the second substrate (S2), and SUMO-modified substrate is the second product (P2). Although the SUMO-E1 conjugate is the first substrate, its concentration depends on the concentration of SUMO-1 because the formation of SUMO-E1 conjugate is fast and not rate-limiting for the overall reaction. This reaction mechanism and related rate constants are summarized as shown below.

\[
E + S1 \rightarrow ES1 \rightarrow P1 + E'
\]

\[
E' + S2 \rightarrow E'S2 \rightarrow P2 + E
\]

**REACTION SCHEME 1**

The Dalziel approach (40) is more straightforward than the Michaelis-Menten approach (41) for the description of steady-state kinetics of enzymes that involve more than one substrate. The ping-pong Bi Bi mechanism can be described by the Dalziel approach as:

\[
e V_c = \frac{e}{V_0} \phi_0 + \frac{e}{V_0} \phi_1 + \frac{e}{V_0} \phi_2 = \frac{k_2 + k_5}{k_3 k_6} \phi_2 = k_5 + k_6
\]

(Eq. 1)

where \( e \) is the total Ubc9 concentration, and \( \Phi_0, \Phi_1, \) and \( \Phi_2 \) are given by Eq. 2.

\[
\phi_0 = \frac{1}{k_3} + \frac{1}{k_6} \phi_1 = \frac{k_2 + k_5}{k_3 k_6} \phi_2 = \frac{k_5 + k_6}{k_3 k_6}
\]

(Eq. 2)
The double reciprocal plot of $1/V_o$ versus the inverse of the substrate concentration or SUMO concentration should be linear, and the slope of the plot is proportional to $K_2$ or $\Phi_2$, respectively. $1/\Phi_1$ or $1/\Phi_2$ corresponds to the rate of $S_1$ or $S_2$ going to $E_S1$ or $E'S2$ multiplied by the probability of $E_S1$ or $E'S2$ going to $P_1$ or $P_2$. Therefore, $1/\Phi_1$ and $1/\Phi_2$ are the effective transfer rate constants or net transfer rate constants for SUMO-1 from E1 to E2 and from E2 to substrate proteins, respectively.

The initial rates ($V_o$) of the overall conjugation reaction were measured under the conditions where Ubc9 was rate-limiting at various substrate concentrations of three purified substrates, the GST fusion peptides of p53, PML, and E1B (Fig. 4A). For each concentration of a substrate and with either the wild-type or mutant Ubc9, $V_o$ was measured three times to estimate the uncertainties in the measurements and to extract uncertainties in the calculated slopes of the double reciprocal plot. Assays were halted by the addition of SDS sample buffer containing $\beta$-mercaptoethanol, and then samples were fractionated on polyacrylamide gels containing SDS, which were subsequently stained, destained, and dried. Transfer of $^{15}$N-labeled SUMO-1 to each substrate was imaged (Fig. 4B), and the amount of product was quantitated by phosphorimaging analysis (Fig. 4, C and D). Double reciprocal plots for the three substrates using wild-type and mutant Ubc9 proteins are linear, which verifies that Eq. 1 is appropriate for the description of the reaction mechanism. Slopes ($\Phi_{fe}$) of these double reciprocal plots are proportional to $\Phi_2$ (Eq. 2), which is the inverse of the effective rate constant of SUMO-1 transferring from Ubc9 to substrates. $\Phi_2$ is independent of the transfer of SUMO from E1 to E2 (Eq. 2). The slopes of the double reciprocal plots are well determined, and the inversedes of the slopes are plotted in Fig. 4E.

Comparison of the values in Fig. 4E shows that nearly all amino acid substitutions had a significant effect on transferring SUMO-1 from mutant versions of Ubc9 to substrate and reduced the net transfer rate constant. Q130A and A131D appear to have the most significant effects and reduce the net transfer rate constants of all three substrates. Other mutants appear to have substrate-specific effects. Q126A reduced the effective rate constants of transferring SUMO-1 from Ubc9 to the p53 and PML peptides by 50% but reduced the effective rate constant by approximately a factor of 4 for the E1B peptide. Other than Q130A and A131D, the Y134A and T135A mutants had the largest reduction on the net transfer rate constants of SUMO-1 from Ubc9 to the p53 and PML peptides. However, Q126A and T135A reduced the net transfer rate constant for the E1B peptide more significantly. This suggests that residues outside of the consensus modification motif in p53 may also play a role in Ubc9 interactions.

**Structural Characterization of the p53 Peptide**—The sequence of the p53 peptide corresponds to the unstructured C-terminal domain in the unbound state. TOCSY and ROESY experiments were performed on the free p53 peptide to characterize its structural property. Sequence-specific resonance assignments were obtained using a combination of two-dimensional ROESY and TOCSY spectra. The free p53 peptide has a random structure in solution without any nonsequential NOEs. This result is consistent with a recent study (42).

It is possible that the SUMO-1 conjugation sites of target proteins are generally unstructured, as is observed in p53. The c-Jun peptide corresponds to a region of the protein that is highly rich in Pro residues (Fig. 1A) and is therefore likely also to be unstructured. Significant information is now available to correlate primary sequences to secondary structures. Secondary structure prediction based on primary sequences is successful in most cases, although the boundary of the secondary structures cannot be reliably predicted. Secondary structure prediction using the program PHD (43) indicates that the conjugation sites of known SUMO-1 target proteins RanGAP1, IxBo, AdE1B, c-Jun, and PML (10, 11, 14, 37) are not in the predicted regular secondary structures such as $\alpha$-helices or $\beta$-sheets, but in predicted loops (data not shown).

We have investigated the bound conformation of p53 in the complex with Ubc9. The structure of the p53 peptide in the complex can be characterized by transferred NOEs because transferred NOEs are dominated by NOEs of the bound conformation of the peptide (44). $^{15}$N-filtered NOESY spectra were acquired at two titration points, where the molar ratios of the p53 peptide to Ubc9 were ~1:1 and 2:1:2. The $^{15}$N-filtered NOESY spectrum acquired when the concentration of the peptide was ~1.5 mM and the ratio of p53 to Ubc9 was ~2:1:1 is shown in Fig. 5A. The ROESY spectrum of the same region of the free p53 peptide is shown in Fig. 5B for comparison. The $^{15}$N-filtered NOESY spectrum has a small number of cross-peaks. Only some of the intraresidue NOEs and NOEs between sequentially connected residues of the peptide are observed in the $^{15}$N-filtered NOESY spectra. The p53 peptide does not appear to form a regular secondary structure, such as $\alpha$-helix or $\beta$-sheet, when bound to Ubc9. In these heteronuclear filtered spectra, chemical shift changes of the p53 peptide are small because of a low percentage of p53 in the complex.

**DISCUSSION**

**Interaction between Ubc9 and the Target Proteins**—The role of Ubc9 in the SUMO-1 conjugation pathway is to interact with the SUMO-1/SAE1/SAE2 thioester complex to accept SUMO-1 and then to bind target protein substrates, to which the SUMO-1 molecule is finally attached. Two ubiquitin-E2 complexes have been characterized by NMR studies (45, 46). Both studies show that ubiquitin and E2 have considerably independent motion in solution and that the thioester conjugate does not form a compact structure. This is evident by comparisons between NMR resonance linewidths of ubiquitin or E2 in both the free states and in the conjugates, which were shown to be similar. Additionally, the ubiquitin and E2 NMR resonances have different linewidths in the conjugates, suggesting that they do not form a single tight unit in solution. Because of the similarities in protein structures and in the chemistry of the conjugation between the ubiquitin and SUMO-1 pathways, the SUMO-1-Ubc9 conjugate is likely to have a similar structural property as the ubiquitin-E2 conjugates. SUMO-1 and Ubc9 are likely to have considerably independent motions connected by a flexible linker when forming the covalent complex. Therefore, specific side chain interactions between the target proteins and Ubc9 in the conjugate may not be significantly affected by the covalently bound SUMO-1.

The relatively low affinity between Ubc9 and substrate peptides is consistent with the results of kinetics studies. In the steady-state kinetic studies described in Fig. 4, initial rates ($V_o$) increase almost linearly with the increase of substrate concentrations (Fig. 4C). This correlates to small intercepts of the double reciprocal plots of $1/V_o$ versus 1/$S$ (Fig. 4D). This observation is consistent with the mechanism that enzyme-substrate intermediate dissociates rapidly, and in this situation, $\Phi_2$ (Eq. 1) approaches 0 (40). It has also been shown recently that in fully defined in vitro assays using recombinant SUMO-1, SAE1/SAE2, and substrate, Ubc9 has a relatively low substrate turnover rate (~3/h) (30). These data are consistent with our findings that Ubc9 has a relatively low affinity for peptide substrates. Two types of E3 ligase enzymes for SUMO modification have been discovered recently (47–52). Whereas E3 ligase enzymes are not absolutely required for the SUMO...
FIG. 4. Steady-state kinetic analysis of substrate-binding site mutants of Ubc9. Assays for the conjugation of 125I-SUMO-1 to GST tag substrates using rate-limiting concentrations of Ubc9 were developed such that the increase in the concentration of the product (GST-substrate-SUMO-1 conjugate) was linear with respect to time over a 60-min period (see "Materials and Methods" for details). The 10-μl assays contained one of three different GST tag substrates containing residues 381–391 from human p53 (left column), 485–495 from human PML (center column), or 99–109 from adenovirus E1B (right column). Schematic representations of the substrates are shown in A, with the target lysine shown in bold. Reactions containing substrate concentrations varying from 1 to 30 μM were performed in triplicate for each substrate and each Ubc9 mutant over a 60-min period at 37 °C. Samples were then fractionated by electrophoresis on 12% polyacrylamide gels, and radioactive species were detected by phosphorimaging analysis of dried gels. B shows a sample of the raw data for assays containing either no Ubc9 (None) or 2.76 μM of each indicated.
conjugation system in vitro, they increase the efficiency of SUMO-1 conjugation by Ubc9. Unlike the ubiquitin system, where no general consensus modification sequence exists, the requirement of such a sequence for SUMO modification suggests that any E3 ligase enzymes are unlikely to alter the substrate specificity but may make additional stabilizing contacts between Ubc9 and substrates. If the SUMO-1 moiety of the Ubc9-SUMO-1 thiolester also interacts with target proteins, the affinity between the peptides and Ubc9 observed in this study may not represent the affinity of target proteins to the SUMO-1-Ubc9 conjugate.

Dynamics and Substrate Recognition—The surface of Ubc9 that is likely to be important in target protein recognition has been identified from chemical shift perturbation and site-directed mutagenesis. This site is adjacent to the active site of Ubc9 and is located in the region of the highest conformational flexibility on the microsecond to millisecond time scale and significant dynamics on the picosecond to nanosecond time scale in Ubc9 (26). Gln126, Asp127, Ala129, Gln130, and Glu132 are near the active site Cys91 and have higher conformational flexibility than average residues in the picosecond to nanosecond and microsecond to millisecond time scale. Among these residues, Ala129 and Glu132 have the highest flexibility on the microsecond to millisecond time scale (Rex > 4 s⁻¹) in Ubc9. In particular, Glu132 has the largest Rex term of the entire molecule (14.7 s⁻¹). Thus, the region on Ubc9 that is involved in binding both target peptides has significant conformational flexibility.

Although three-dimensional structures of proteins and their complexes provide important insights into the determinants of binding affinity and specificity, dynamics clearly play important roles in molecular recognition and enzyme activities (53, 54). For example, in a recent study of the bacterial response regulator protein Spo0F, dynamics on the microsecond to millisecond time scale correlate with residues and surfaces that are known to be critical for protein-protein interactions (55). The flexible regions at the interface usually become more rigid upon complex formation. This induced structural formation is likely to be important for binding specificity because nonspecific interactions are unable to generate such “induced fits.” In addition, changes in flexibility should modulate the affinity of the interaction through changes in entropy and its contribution to free energy changes. The correlation between chemical shift changes and dynamics of residues near the active site further suggests the importance of these residues in substrate recognition.

Conformational Flexibility of the Substrates—It is likely that the SUMO-1 conjugation site on target proteins is located in flexible surface loops or termini, as shown in p53 and predicted for other SUMO-1 target proteins where the conjugation sites have been identified. The KXE sequence is not always a signal for SUMO-1 conjugation. For example, IsxBc contains two of these sequences around lysines 21 and 38, but only Lys21 is the SUMO-1 conjugation site. It is possible that some of the consensus sequences form a well-defined structure and that the side chains are buried, and therefore these sites cannot be recognized and modified by SUMO-1. Because no specific secondary structure has been found for the p53 peptide in the complex with Ubc9, it is postulated that the SUMO-1 conjugation of the p53 peptide is maintained by Ubc9 protein in the presence of 15 μM GST-substrate. The positions of both free and GST-substrate-conjugated ¹⁵N-SUMO-1 are indicated. Initial velocities (V₀) were calculated for each assay condition, and the mean values for each Ubc9 protein at each substrate concentration were presented graphically both as V₀ against [GST-substrate] (C) and double reciprocal plots (D) (error bars represent 1 S.D. from the mean). Double reciprocal data were fit using nonlinear least squares regression analysis. The gradients of best-fit lines were used to calculate the slope values for each Ubc9 protein, the inverses of which are represented as bar graphs in E (error bars reflect uncertainties in the measurement and deviation of the data from the linear equation).
SUMO-1 target proteins showed no evidence of induced flexible termini. The interaction between Ubc9 and the flexible or unstructured, such as in exposed surface loops or in target proteins are likely to be located in regions that are structural variation among the three-dimensional structures of second to millisecond time scales and has a larger than average has high flexibility in the picosecond to nanosecond and micro-second to millisecond time scales. Because the E2 proteins may in general be involved in homologous pathways.

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