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The p66 and p12 subunits of DNA polymerase δ are modified by ubiquitin and ubiquitin-like proteins

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Abstract

Modification by ubiquitin-like proteins is now known to be important for the functions of many proteins involved in DNA replication and repair. We have investigated the modification of human DNA polymerase δ by ubiquitin and SUMO proteins. We find that while the p125 and p50 subunits were not modified, the p12 subunit is ubiquitinated and the p66 subunit can be modified by ubiquitin and SUMO3. We show that levels of p12 are regulated by the proteasome, either directly or indirectly, through a mechanism that is not dependent upon p12 ubiquitination. We have mapped two sites of SUMO3-specific modification on the p66 subunit. SUMOylation by SUMO3 but not SUMO2 is unusual: their level of homology is so high that they are normally classified as variants of the same protein. However, our findings show that these two proteins can be distinguished *in vivo* and may have specific functions.

Keywords: DNA polymerase delta; Ubiquitin; SUMO; DNA replication; PCNA

The highly complex and interrelated processes of DNA replication and repair are regulated by the assembly and disassembly of large protein complexes. Many of the protein molecules involved are stable and in some cases very abundant; it is becoming clear that post-translational modification plays an essential role in the regulation of their functions and interactions. Recently, a range of proteins involved in DNA replication and repair have been identified as targets for modification by ubiquitin and ubiquitin-like proteins. These include translesion polymerases, PCNA, the WRN helicase, topoisomerases I and II, the thymine DNA glycosylase TDG and the nucleotide excision protein XPC (reviewed by [1,2]). The results of modification of these proteins do not follow a single theme, but can result in diverse effects ranging from regulating protein–protein interactions in the case of PCNA, to regulating substrate turnover as is seen with TDG.

One of the most striking examples of how ubiquitination and SUMOylation can affect protein function is that of PCNA in budding yeast *Saccharomyces cerevisiae*. Following DNA damage, PCNA can be mono-ubiquitinated, poly-ubiquitinated or SUMOylated on the K164 residue and each modification results in a different outcome in DNA synthesis and repair (reviewed by Watts [3]). Mono-ubiquitinated PCNA directs translesion synthesis via error-prone DNA polymerases, while poly-ubiquitinated PCNA is associated with an error-free DNA repair pathway [4,5]. However, the mechanism by which PCNA is directed to these differing functions is still not clear. Mono-ubiquitination of PCNA *in vitro* does not affect its functional interaction with DNA polymerase δ and does not stimulate the PCNA-dependent activities of the translesion polymerases η, ζ or Rev1 [6]. SUMO modification has recently been shown to recruit the helicase Srs2 to replication forks, where it functions to inhibit recombination during S phase [7,8]. However, although K164 is highly conserved within the PCNA sequence, no SUMOylated

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forms have been detected in human PCNA, suggesting that a different regulatory mechanism exists in higher eukaryotes [4].

Since protein modification by ubiquitin and ubiquitin-like proteins clearly has a role in regulating the function of proteins involved in DNA replication and repair, we decided to investigate whether the subunits of DNA polymerase δ were subject to this form of post-translational modification. The DNA polymerase δ holoenzyme consists of PCNA and its clamp loader (replication factor C) and the four subunit polymerase complex [9]. The catalytic subunit is the largest, at 125 kDa in human cells, and this forms a stable heterodimer with the p50 regulatory subunit. For some time this was thought to be the complete polymerase complex, until the discovery of a third, non-catalytic subunit. This was first identified as POL32 in budding yeast and Cdc27 in fission yeast, followed shortly afterwards by the identification of the p66 subunit in human cells [10–14]. p66 interacts with both PCNA and with the p50 subunit forming an essential bridge between these two proteins [10,12,14,15]. It has an elongated shape, causing it to run aberrantly in gel filtration analysis [16]. More recently, the p12 subunit of DNA polymerase δ was identified [17,18]. The role of this protein is still unclear, though it has been shown to interact with the p125 and p50 subunits and data from *in vitro* DNA replication assays indicate that it is involved in the stability of the polymerase complex [19,20].

Materials and methods

Plasmid constructs. The p12 coding region was amplified by PCR as a *Bam*HI–*Hin*dIII fragment with a sequence encoding a 10 amino acid HA epitope at the N-terminus. This was cloned into pcDNA3.1/myc-His(−)C (Invitrogen) to give pcDNA3.1-HAp12. A stop codon immediately before the *Hin*dIII site meant that neither myc nor His tags were expressed. The p66 coding region was cloned as an *Xho*I–*Hin*dIII fragment into pcDNA3.1/myc-His(−)C to give pcDNA3.1-p66myc-His which expressed full length human p66 with C-terminal myc-His tags. To create p66 constructs which expressed only the myc tag, site-directed mutagenesis was used to insert a stop codon between the myc and His coding regions. K to R mutants of both p12 and p66 were made by site-directed mutagenesis. All site-directed mutagenesis was carried out using a method similar to that described for the QuikChange site-directed mutagenesis kit (Stratagene). Expression constructs for His-ubiquitin, His-SUMO1, His-SUMO2, and His-SUMO3 were as described by Li et al. [21], Rodriguez [22].

Cell culture and transfection. U2OS osteosarcoma cells were grown at 37 °C and 5% carbon dioxide, in a humidified atmosphere. Cells were grown in DMEM (PAA Laboratories, GmbH), supplemented with 10% v/v foetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and harvested 24 h post-transfection. Treatment with MG132 (Calbiochem) was carried out at a final concentration of 10 μM for 4 h prior to harvesting cells.

Preparation of cell lysates and His-tag affinity purification. Transfected cells in a 10 cm dish were washed twice with ice-cold PBS and then harvested into 1 ml of 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris–HCl, and pH 8.0. DNA was sheared by taking through 23 gauge needle. Protein concentration was measured with a BCA kit (Pierce) and normalised. DTT was added to 10 mM final concentration. Eight hundred microlitres of protein extract was incubated overnight with 75 μl of a 50% slurry of nickel (Ni–NTA)–agarose beads (Qiagen) and incubated with mixing overnight at 4 °C. The beads were washed with 750 μl of the following buffers with 5 min mixing at room temperature for each washing step: (1) 6 M Guan–HCl, 0.1 M $NaHPO_4$, 0.01 M Tris–HCl, pH 8.0, and 10 mM DTT; (2) 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris–HCl, pH 8.0, and 10 mM DTT; (3) 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris–HCl, pH 6.3, 10 mM DTT, and 0.2% Triton X-100; (4) 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris–HCl, pH 6.3, and 10 mM DTT; (5) 8 M urea, 0.1 M $NaHPO_4$, 0.01 M Tris–HCl, pH 6.3, 10 mM DTT, and 0.1% Triton X-100. Following these washes, bound proteins were eluted with 100 μl of 2× SDS sample loading buffer (Invitrogen) containing 200 mM imidazole and 10 mM DTT. Samples were heated to 95 °C for 5 min, cooled, and a typical volume of 20 μl loaded on each lane for Western blot analysis [21].

SDS–PAGE and Western blotting. Electrophoresis was performed using the NuPage system (Invitrogen). Either 10% or 4–12% Bis–Tris gradient gels were run with MES buffer as supplied by the manufacturer. Proteins were transferred to Immobilon-P membranes (Millipore). Blots were blocked with 5% (w/v) milk in PBS for 1 h at room temperature and then rinsed twice with PBS. Primary antibodies were diluted in 2% milk in PBS and added to the blot at a concentration of 2 μg/ml of purified antibody or 1:1000 ascites/serum. This was incubated for 1 h at room temperature followed by extensive washing with PBS 0.2% Tween 20. HRP-conjugated secondary antibodies were added, incubated, and washed as for primaries. Rat monoclonal antibodies (5G1 and 7B4) were used to analyse the ubiquitination and SUMOylation of the p125 and p50 subunits of DNA polymerase δ. Commercially available mouse monoclonal antibodies to the His tag, the HA epitope (12CA5), and the myc epitope (9E10) were used. Peroxidase-coupled donkey anti-mouse IgG (Jackson Immunoresearch) was used as secondary antibody. Immunoblots were visualised by enhanced chemiluminescence (ECL, Amersham Pharmacia or DURA, Pierce), according to the manufacturers' instructions.

Results and discussion

Recent results have underlined the importance of the ubiquitin and SUMO modification of proteins involved in DNA replication and repair. We therefore decided to investigate whether the subunits of DNA polymerase δ were subject to this form of post-translational modification. In vertebrates, four SUMO family proteins have been reported, though SUMO4, if expressed, would not be able to form conjugates *in vivo* [23]. We have examined the conjugation of ubiquitin, SUMO1, SUMO2, and SUMO3. It was not possible to detect forms of the p125 and p50 subunits modified with ubiquitin or SUMO1, 2 or 3 (data not shown); however, modified forms of the p12 and p66 subunits could be detected and their analysis is described.

The p12 subunit of DNA polymerase δ is ubiquitinated

To analyse the modification of the smallest DNA polymerase δ subunit, p12, an HA epitope-tagged form of p12 was co-expressed with His-tagged forms of ubiquitin, SUMO1, SUMO2, and SUMO3 in human U2OS osteosarcoma cells. His-tagged proteins were purified from cell lysates using Ni-bead chromatography and the results analysed by SDS–PAGE and Western blotting. Fig. 1 shows that p12 can be ubiquitinated, predominantly as a mono-ubiquitinated form, and that SUMOylated species could not be detected. The addition of the proteasome inhibitor MG132 substantially increases the levels of p12 in this assay and there is a concomitant increase in the levels, though not the proportion, of ubiquitinated p12.

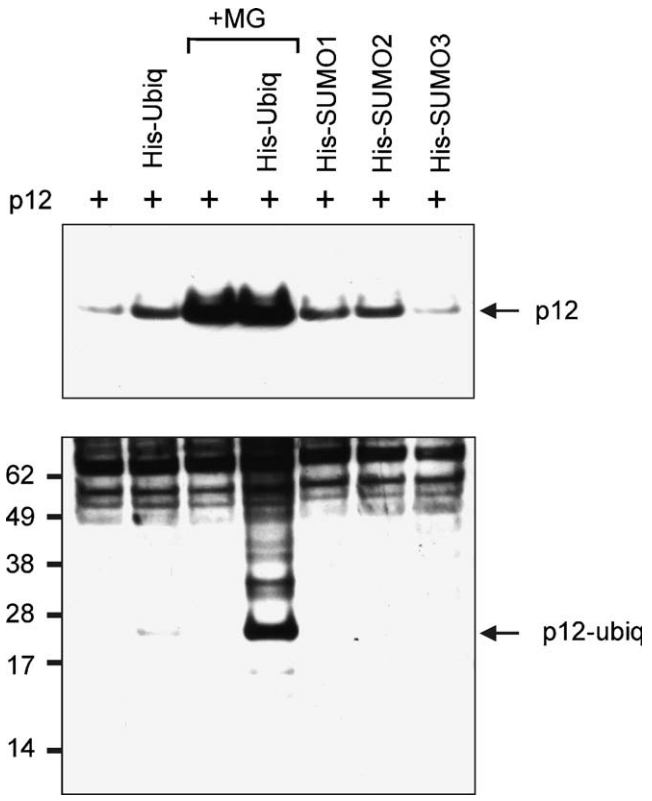

Fig. 1. p12 is ubiquitinated, but not SUMOylated. U2OS cells were transiently transfected with constructs expressing proteins as shown. In lanes 3 and 4 the extracts were made from cells treated with the proteasome inhibitor MG132 (MG). The upper panel shows the input levels of HA-tagged p12 by Western blot analysis. The lower panel shows Western blot analysis of proteins isolated by Ni-bead purification and probed with anti-HA antibody to reveal p12 proteins covalently linked to His-tagged ubiquitin or SUMO proteins. Bands corresponding to p12 and mono-ubiquitinated p12 are indicated.

Following treatment with MG132, di- and tri-ubiquitinated forms can also be seen. The observation that these forms are represented by a doublet may either be due to structural factors resulting from modification, or may be due to modification by both His-tagged and endogenous, un-tagged forms of ubiquitin. To investigate further which residues were being ubiquitinated within p12, a series of mutant proteins were tested for ubiquitination. In these proteins, one of each of the 5 lysine (K) residues was mutated to arginine (R) and a 5KR mutant form was also tested in which all five residues were mutated. As shown in Fig. 2B, each K to R mutant protein can still be ubiquitinated, suggesting that ubiquitination does not take place on a single lysine residue. However, ubiquitination of the 5KR mutant could not be detected, indicating that ubiquitination is taking place upon some combination of lysine residues and not at the free amino terminus, as has been shown for p21(WAF1/Cip1) [24]. The treatment of cells with the proteasome inhibitor MG132 resulted in highly increased levels of the wild type as well as each of the mutant proteins, including the 5KR mutant (Fig. 2A), suggesting that the mechanism of stabilisation is somehow dependent upon the proteasome, but is not directly dependent upon p12 ubiquitination.

One possible explanation for these observations is that p12 is degraded by the proteasome through a non-ubiquitin-dependent pathway. A few examples of such a process exist, the best studied of which is ornithine decarboxylase (ODC) where it has been shown that the interaction of ODC with the proteasome is mediated by the small Antizyme protein [25]. A model could be proposed whereby p12 stability is regulated by a second protein which is itself degraded by the proteasome in a ubiquitin-dependent manner. Following the addition of MG132, increased levels of this protein could stabilise p12 by a direct protein–protein interaction. Although levels of p12 are clearly regulated in a proteasome-dependent manner, ubiquitination of p12 does not appear to be linked to its stability. The role played by the p12 subunit in the DNA polymerase δ complex is still not completely clear, but it has been shown to contribute to the stability of the complex [19,20]. It is possible that p12 ubiquitination plays a role in regulating protein–protein interactions, either within the polymerase complex, or with other proteins involved in DNA replication.

The p66 subunit of DNA polymerase δ is ubiquitinated

The p66 subunit of DNA polymerase δ is essential for the interaction of the p125/p50 dimer with PCNA and it contains two separable interaction domains for the p50 subunit and PCNA. To investigate the ubiquitination of p66, similar assays were performed to those described above using myc-tagged p66 constructs and the results are shown in Fig. 3. p66 is clearly ubiquitinated and the majority of the modified species are mono-ubiquitinated. It is also possible to see low levels of a high molecular weight "smear" of multiply ubiquitinated species, though it is not possible to distinguish whether these represent multiply mono-ubiquitinated forms, or poly-ubiquitin chains. Within p66, 59 out of its 466 amino acids are lysine, making it the most abundant amino acid, so the analysis of modified residues by arginine substitution is not feasible, given that more than one residue is likely to be ubiquitinated. However, in contrast to the results seen with p12, the treatment of cells with MG132 does not significantly increase the levels of p66 and does not affect either the levels or the proportions of mono- or poly-ubiquitinated species, suggesting that p66 is not targeted to the proteasome by a ubiquitin-dependent mechanism.

p66 is specifically SUMOylated by SUMO3

Similar assays were carried out to determine if p66 is modified by the SUMO family using His-tagged SUMO1, SUMO2, and SUMO3 and myc-tagged p66. As can be seen in Fig. 4A, very low levels of SUMO1-p66 and SUMO2-p66 are seen, but p66 is significantly modified by SUMO3. A doublet of mono-SUMOylated forms is visible, which could represent two or more SUMOylated forms with

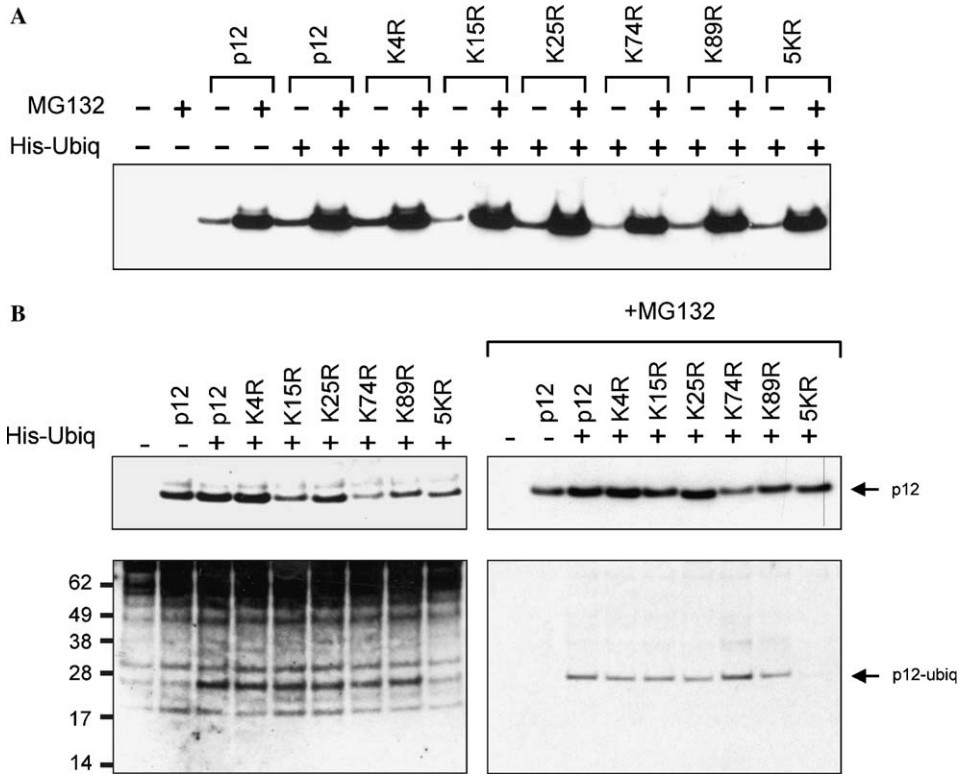

Fig. 2. p12 is significantly stabilised by the proteasome inhibitor MG132, but this effect is not dependent upon ubiquitination. Western blot analysis is shown of HA-tagged p12 and p12 K to R mutant proteins. (A) The input levels of p12 and p12 K to R mutant proteins isolated from MG132 treated and untreated cells. (B) The input levels (above) and ubiquitinated forms of p12 isolated by Ni-bead purification (below). Bands corresponding to p12 and mono-ubiquitinated p12 are indicated.

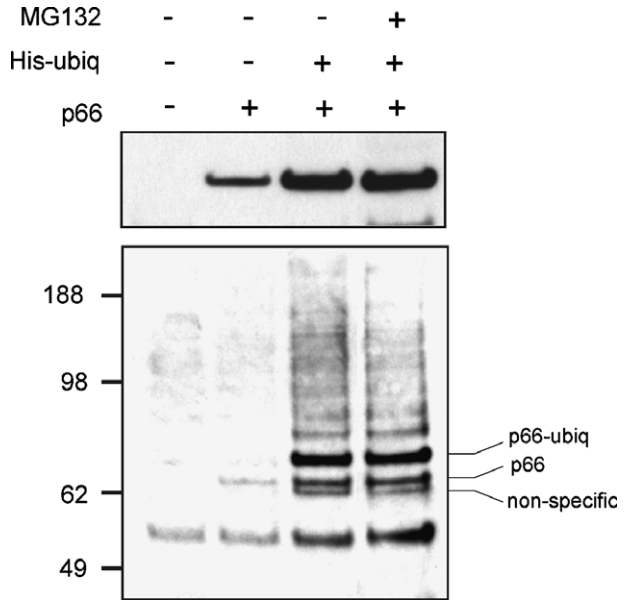

Fig. 3. Modification of p66 by ubiquitin. Western blot analysis is shown of myc-tagged p66 constructs which were co-transfected with a His-tagged ubiquitin construct and either untreated or treated with MG132 as shown. The upper panel shows the level of p66 as detected by an anti-myc antibody and the lower panel shows proteins isolated by Ni-bead purification. A very low level of p66 can be seen binding non-specifically to the Ni beads. A non-specific band represents an unrelated, ubiquitinated protein.

slightly different structural properties (see below). A low abundance species is also present with a molecular weight consistent with that of p66 conjugated to two SUMO molecules. These results are not due to differing levels of expression of the His-tagged SUMO constructs which are very similar (Fig. 4C). This is a very unusual finding, as SUMO2 and SUMO3 are very similar. At the N-terminus, these proteins only differ by three amino acids and are identical until the double glycine motif near the C-terminus. Although there is some divergence following the GG motif, these sequences are cleaved during pre-conjugation processing by SUMO-specific proteases. These C-terminal sequences are not present in the His-tagged constructs used in these experiments, so they are not contributing towards the SUMO3 specificity seen here.

The differential SUMOylation of p66 by SUMO2 and SUMO3 is intriguing and several mechanisms for this can be proposed. Different SUMO family members are known to be preferentially conjugated to specific substrates. For example, RanGAP1 is predominantly modified by SUMO1 and topoisomerase II is predominantly modified by SUMO2 and SUMO3 [26–28]. SUMOylation of target proteins occurs through a pathway analogous to the ubiquitination reaction. The process requires a heterodimeric E1-activating enzyme (SAE1/SAE2), an E2-conjugating enzyme (Ubc9), and an E3 ligase which facilitates SUMOylation of specific substrates [29–31]. One model to explain

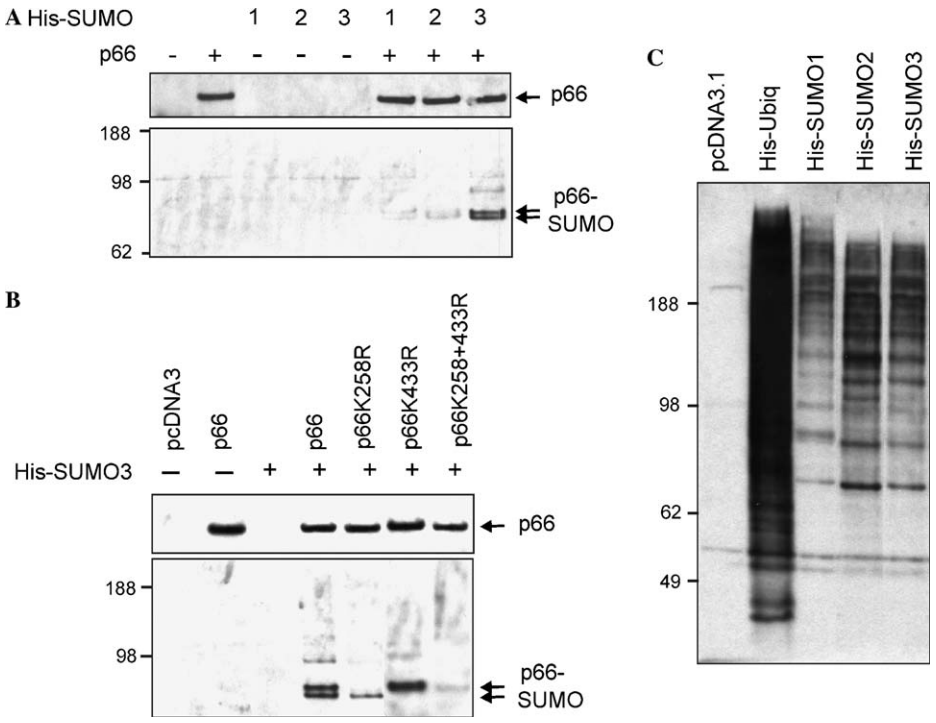

Fig. 4. p66 is modified specifically by SUMO3 on residues K258 and K433. (A) Western blot analysis of cells transiently transfected with constructs expressing myc-tagged p66 and one of SUMO1, SUMO2, or SUMO3 as shown. The upper panel shows the levels of p66 in the input lysates. The lower panel shows proteins isolated by Ni-bead purification. A doublet representing p66 conjugated to SUMO3 can be seen. (B) A similar analysis using constructs expressing His-tagged SUMO3 and mutant p66 proteins which have specific lysine (K) residues mutated to arginine (R) as shown. (C) Western blot analysis of cells transfected with His-tagged ubiquitin, SUMO1, SUMO2, and SUMO3 as shown probed with an anti-His antibody.

the SUMO3-specific modification observed here is the existence of a SUMO3-specific E3 ligase with an affinity for p66. One example of an E3 ligase contributing towards SUMO paralog selection has been shown in the case of RanBP2. This E3-ligase can promote the modification of target proteins with both SUMO1 and SUMO2 via a mechanism which involves a direct interaction with Ubc9. However, a second SUMO1-specific mechanism also occurs whereby a Ubc9-SUMO1 complex interacts with a SUMO1-binding site on RanBP2. The consequence of SUMO1 binding is that RanBP2 is preferentially autoSUMOylated with SUMO1 and target proteins are SUMOylated with SUMO2 [32]. It has also been proposed that regulatory components, such as co-factors for the E3 ligases, contribute towards substrate specificity, but there is no concrete data to support this idea as yet [33].

Another possible model to explain our results is that p66 is modified by all or some of SUMO1, SUMO2, and SUMO3 and that SUMO-specific proteases act to remove SUMO1 and SUMO2. SUMO proteases have two essential functions: processing of nascent SUMO to its mature form (C-terminal hydrolyases activity) and the cleavage of isopeptide bonds between SUMO and its targets (isopeptidase activity). All known SUMO proteases fall into the Ulp1 cysteine protease family and seven human genes encoding proteins with homology to Ulp1-like proteases have been identified. Five of these SENP genes have been confirmed to encode SUMO proteases [33]. The isopeptidase activity of SENP5 is preferentially targeted to removing SUMO2 and SUMO3 from substrates compared to SUMO1 [34]. In contrast, gene knockout experiments suggest that SENP1 preferentially removes SUMO1 from a range of substrates [35]. However, there is no evidence to date of a SUMO-specific protease which distinguishes between SUMO2 and SUMO3.

Mapping of sites of SUMO modification within p66

In order to predict which residues within p66 were likely to be SUMOylated, the p66 sequence was analysed using the SUMOplot™ program available from Abgent. Most SUMO-modified proteins contain the tetrapeptide motif B-K-x-D/E where B is a hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid, D or E is an acidic residue [36]. The SUMOplot™ program predicts the probability for the SUMO consensus sequence (SUMO-CS) to be engaged in SUMO attachment. For p66, three motifs scored highly as potential sites of SUMOylation: those containing lysines 258 and 433 scored equal top with a score of 0.93. The third motif containing lysine 325 was third with a score of 0.84.

To investigate the SUMOylation of these residues, myc-tagged constructs were used in Ni-bead affinity assays as described above and the results are shown in Fig. 4B. Mutation of K258 to R resulted in the disappearance of the upper band of the SUMOylated p66 doublet. Converse-

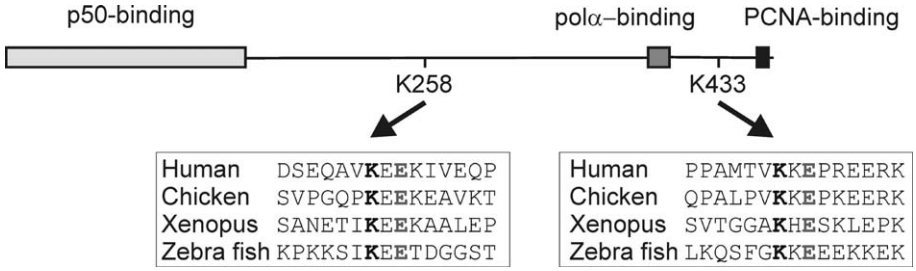

Fig. 5. SUMO3 modification of p66 does not occur within known p66 interaction motifs. This schematic diagram drawn approximately to scale shows the location of known interaction motifs within the primary structure of the p66 protein. The locations of the K258 and K433 residues are shown with their surrounding sequences aligned with regions of homologous sequence from chicken (*Gallus gallus*), *Xenopus laevis*, and zebrafish (*Danio rerio*). Within the conserved B-K-x-D/E SUMOylation consensus, the lysine residue is shown in bold and the conserved acidic residues in grey. The aligned sequences shown here are taken from a global alignment of the full length sequences of these p66 homologues created using CLUSTALW. All the motifs shown were rated with a high probability of modification by SUMO by the Abgent SUMOPLOT™ program, with the exception of the motif in the chicken sequence corresponding to human K258, which scored as a potential site, but with a low probability.

ly, mutation of K433 to R resulted in the disappearance of the lower band. No significant levels of SUMOylation could be detected in the K258R K433R double mutant. Mutation of the third site, K325R, did not affect the pattern or level of p66 SUMOylation (data not shown). These results are strongly suggestive that only K258 and K433 are SUMOylated in p66 and that the doublet of SUMOylated proteins is due to structural differences between these two SUMOylated forms in SDS–PAGE analysis.

Several functional interaction domains have been determined within p66 (Fig. 5). A large region at the N-terminus is involved in an interaction with the p50 subunit of DNA polymerase δ and a small region at the C-terminus containing a conserved PCNA-interacting motif (PIP box) is required for the p66 interaction with PCNA [10,12,15,37,38]. A region capable of interacting with the catalytic subunit of DNA polymerase α has also been delineated as lying between the p50- and PCNA-interacting domains. This domain does not seem to be essential for the function of Cdc27, which is the *Schizosaccharomyces pombe* homologue of p66, though this domain is highly conserved throughout evolution, indicating that it must have an important role to play in optimal DNA replication [39,40]. As can be seen from Fig. 5, the two SUMOylation domains identified here do not lie within or immediately adjacent to any of the known p66 interaction domains, so it is difficult from these data to predict their function. However, it is clear that the canonical SUMOylation sequence B-K-x-D/E is conserved as far as the bony fishes, suggesting that SUMOylation at these sites is a conserved process. The primary role of p66 is that of a molecular scaffold which acts to bring together elements of the replication machinery. An attractive model is one in which ubiquitination and/or SUMOylation of p66 alters its conformation to affect the overall structure and function of the polymerase complex.

Summary

In this study, we have investigated the modification of the subunits of human DNA polymerase δ by ubiquitin and SUMO proteins. We have shown that both the p12 and p66 subunits can be modified by ubiquitin in predominantly mono-ubiquitinated forms. Our results indicate that levels of p12 are regulated by the proteasome, either directly or indirectly, but through a mechanism that is not dependent upon p12 ubiquitination. It is possible that levels of p12 are affected by its interaction with another interacting protein which is subject to proteasome-dependent degradation. The p66 subunit is specifically SUMOylated by SUMO3 and we have identified the two sites of SUMO3 modification; these are evolutionarily conserved, but lie outside previously identified interaction domains within p66. Our observation that p66 is specifically modified by SUMO3 is highly unusual: SUMO2 and SUMO3 are very homologous and are normally classified as variants of the same protein. However, our findings show that these two proteins can be distinguished *in vivo* and may have specific functions.

Acknowledgments

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