p14 Arf Promotes Small Ubiquitin-like Modifier Conjugation of Werners Helicase*

Received for publication, May 14, 2004, and in revised form, August 17, 2004
Published, JBC Papers in Press, September 7, 2004, DOI 10.1074/jbc.M405414200

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Here we demonstrate a novel p53-independent interaction between the nucleolar tumor suppressors, p14 Arf and Werners helicase (WRN). Binding of p14 Arf to WRN is multivalent and resembles the binding of p14 Arf to Mdm2. Residues 2-14 and 82-101 of p14 Arf and residues in the central region and C terminus of WRN have particular importance for binding. p14 Arf promotes small ubiquitin-like modifier (SUMO) modification of WRN in a synergistic manner with the SUMO-conjugating enzyme, UBCH9. p14 Arf causes redistribution of WRN within the nucleus, and this effect is reversed by expression of a SUMO-specific protease, thus implicating the SUMO conjugation pathway in WRN re-localization. We establish that the ability to promote SUMO conjugation is a general property of the p14 Arf tumor suppressor.

The INK4a-ARF locus encodes two different cell cycle inhibitors p16 INK4a and p14 Arf by alternative splicing and the use of alternative reading frames (reviewed in Ref. 1). p14 Arf (p19 Arf in mice) is an arginine-rich protein and is localized to the nucleolus. p19 Arf is a tumor suppressor, and p19 Arf null mice are highly tumor prone. p19 Arf acts upstream of p53 to cause growth arrest at the G1 stage of the cell cycle (2).

Early observations indicated that Arf mediates a p53-dependent checkpoint that responds to oncogenic, hyperproliferative signals. In mice, p19 Arf expression is induced by oncogenic signals such as Myc, E2F1, and oncogenic Ras, and human p14 Arf expression is also induced by high levels of E2F (3). Recent work has argued against a critical role for p19 Arf in regulating Mdm2 function under physiological conditions and has suggested that the situations in which Arf regulates the p53/Mdm2 pathway might be restricted to those in which cellular stresses activate p53 during the tumorigenic process (4).

At the molecular level, p14 Arf overcomes the ability of the ubiquitin E3 ligase, Mdm2, to repress p53 (5, 6). It has been proposed that the interaction of p14 Arf with Mdm2 inhibits its E3 ligase activity toward p53, resulting in p53 accumulation and induction of the p53 response (7-9). It has also been suggested that p14 Arf sequesters Mdm2 in the nucleolus causing physical segregation of Mdm2 from p53 (10). However, under some circumstances, Arf can stabilize endogenous Mdm2 and p53 without quantitative relocation of either protein (11). Another model proposes that p53 is targeted for degradation via a nucleolar route of export to the cytoplasm (12) and that p14 Arf and many other stimuli induce p53 activation by causing nucleolar disruption (13).

Additional p53-independent functions of Arf have been reported. Knockout mice lacking p19 Arf, p53, and Mdm2 develop a wider spectrum of tumors, with higher frequency, than those observed in animals lacking both p53 and Mdm2. In addition, p19 Arf halts proliferation of mouse embryo fibroblasts lacking both Mdm2 and p53 at the G1/S-phase boundary of the cell cycle, implying that at least one p19 Arf target, when regulated, is multivalent and resembles the binding of p14 Arf and Mdm2 (1). Recent work has argued against a critical role for p19 Arf in regulating Mdm2 function under physiological conditions and has suggested that the situations in which Arf regulates the p53/Mdm2 pathway might be restricted to those in which cellular stresses activate p53 during the tumorigenic process (4).

The human Werners helicase (WRN) gene encodes a 160-kDa protein that is a member of the RecQ family of DNA helicases, also including BLM and RecQ4. WRN is unique within this family, in that it also has an N-terminal 3'-5'-exonuclease activity. In man, germ line mutations in WRN give rise to a rare autosomal recessive disorder, termed Werners syndrome. Werners syndrome is a disease of premature aging that is associated with an elevated risk of cancer (reviewed in Ref. 19). Fibroblast cells derived from Werners syndrome patients exhibit a prolonged G1 phase and display genomic instability (20, 21). Numerous studies have suggested potential functions of WRN based upon its other binding partners, including roles in DNA replication and restoration of replication at sites of fork blockage, rRNA transcription, homologous recombination, repair of breaks in double-stranded DNA, and telomere maintenance (reviewed in Ref. 22).

Here we report a novel interaction between p14 Arf and WRN. p14 Arf causes redistribution of WRN within the nucleus and promotes SUMOylation of WRN, in a synergistic manner with the SUMO-conjugating enzyme UBCH9.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—[35S]Methionine and Amplify reagent were purchased from Amersham Biosciences. Ni2+-NTA-agarose was
from Qiagen. Streptavidin-agarose was from Sigma. NuPAGE pre-cast gradient gels were from Invitrogen.

**Antibodies—** WRN protein was detected using a monoclonal antibody to the WRN C terminus (Transduction Laboratories). FLAG-tagged WRN was detected using the monoclonal anti-FLAG antibody clone M2 (Sigma). p14 Arf was detected using a polyclonal rabbit antiserum, a kind gift from Dr. Karen Vousden (Beatson Institute for Cancer Research, Glasgow, Scotland, UK), or by a mouse monoclonal antibody to the p14 Arf protein, Clone 14P03 (NeoMarkers). Mdm2 was detected using the 4B2 monoclonal antibody (23). UBC9 was detected by a sheep polyclonal antibody, generously provided by Professor Ronald T. Hay (University of St. Andrews, Scotland, UK). SUMO-1 (GMP-1) was detected by a mouse monoclonal antibody, clone 21C7 (Zymed Laboratories Inc.), and ubiquitin was detected by a mouse monoclonal antibody, ab2754 (abcam). Immunofluorescence staining was performed with fluorescein isothiocyanate-conjugated anti-mouse IgG (The Jackson Laboratory). Mouse and rabbit immunoglobulins used in control experiments were from Sigma.

**Expression Constructs—** FLAG-WRN expression constructs were kindly provided by Dr. Moshe Oren (Weizmann Institute, Israel) and are described in Ref. 24. HA-WRN constructs were prepared by removing the sequence encoding the FLAG tag by restriction digestion and replacing it with a sequence encoding the HA tag. WRN point mutants were constructed using QuickChange XL kit, according to the manufacturer’s instructions (Stratagene). UBC9 and SSP3 expression constructs were generously provided by Professor Ronald T. Hay (University of St. Andrews, Scotland, UK). p14 Arf, His₆-SUMO-1, and His₆-ubiquitin expression constructs have been described previously (9, 17). pSUPER vectors were kindly provided by Dr. Sonia Lain (Ninewells Hospital, Dundee, Scotland, UK).

**Cell Culture—** H1299 and U2-OS cell lines were obtained from the ATCC. H1299 cells were cultured in RPMI medium and U2-OS cells in Dulbecco’s modified Eagle’s medium, each supplemented with 10% FCS and containing 50 μg/ml gentamycin. Cells were grown at 37 °C, 5% CO₂ in a humidified atmosphere.

**Transient Transfections—** H1299 cells were seeded onto 10-cm tissue culture plates and transfected 24 h later using the calcium phosphate method. Typically, 5 μg of expression plasmids for WRN, p14 Arf, UBC9, SSP3, and His₆-SUMO-1 were transfected per 10-cm plate, unless otherwise stated. DNA amount was normalized with pcDNA3 vector.

**Immunoprecipitations—** For immunoprecipitation (IP), or peptide pull-down, cells were lysed in IP buffer: 50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.1% Nonidet P-40, 150 mM NaCl, supplemented with complete protease inhibitor mixture (Roche Applied Science) and passed several times through a narrow gauge needle. Immunoprecipitation was carried out overnight co-immunoprecipitated with anti-WRN antibody but not with control mouse IgG (Fig. 1A), thus indicating that p14 Arf can interact with WRN independently of Mdm2.

**RESULTS**

*p14 Arf Physically Associates with WRN—* Residues 1–14 are the most highly conserved in Arf proteins from different mammalian species. This region also contributes strongly to the high affinity interaction between p14 Arf and Mdm2, although other regions of p14 Arf are also involved (8, 25). A green fluorescent protein fusion peptide encompassing the first 20 residues of p14 Arf can activate p53 when introduced into cells by transfection (8). p19 Arf mutants lacking residues 2–14 are defective in arresting the proliferation of cultured primary mouse embryo fibroblasts or NIH-3T3 cells (14), suggesting that this region is critically important in Arf function.

We therefore attempted to identify novel p14 Arf-interacting proteins in a peptide binding approach using a bacterially produced peptide encompassing the first 20 residues of p14 Arf. As controls, we employed a high affinity, biotinylated, Mdm2-binding peptide (12/1-WT), which was isolated during a screen of phage display libraries for ligands that would interfere with the Mdm2-p53 interaction, and a non-Mdm2-binding mutant peptide in which the key contact residues were mutated to alanine (12/1-Ala) (26). The peptides were coupled to streptavidin-agarose beads and incubated with H1299 (p53 null) cell extract. We observed a high molecular weight protein band, which bound specifically to the p14 Arf-derived peptide and not to 12/1-WT or to 12/1-Ala (data not shown). The band was excised and digested with trypsin, and mass spectrometry analysis revealed peptide masses matching those derived from WRN with 14% protein coverage. The presence of WRN protein in the p14 Arf peptide binding experiment was confirmed by immunoblotting with a monoclonal antibody specific for the WRN C terminus (Fig. 1A). Both p14 Arf (1–20) and 12/1-WT bound endogenous Mdm2 from the H1299 cell extract, whereas only the p14 Arf-derived peptide captured endogenous WRN (Fig. 1A), thus indicating that p14 Arf can interact with WRN independently of Mdm2.

To establish the interaction of endogenous p14 Arf and WRN proteins, reciprocal immunoprecipitation (IP) reactions were carried out in H1299 cells. Endogenous WRN co-immunoprecipitated with anti-p14 Arf antibody but not with control mouse IgG (Fig. 1B). In the reciprocal experiment, endogenous p14 Arf co-immunoprecipitated with anti-WRN antibody but not with control mouse IgG (Fig. 1B). Therefore, the p14 Arf-WRN complex occurs in vivo.

**Physical Mapping of the p14 Arf/WRN Interaction—** In order to define the regions of WRN involved in p14 Arf binding in vivo, FLAG-tagged WRN truncation mutants were expressed

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*p14 Arf Promotes SUMO Conjugation of WRN*
Western blot.

anti-WRN antibody or anti-p14 Arf polyclonal rabbit serum.

IP eluates were then electrophoresed and immunoblotted with mouse IgG, mouse anti-WRN, and anti-p14 Arf monoclonal antibody.

described under “Experimental Procedures,” employing control Mdm2 (lower panel) or 4B2 antibody, which detects Mdm2 (lower panel). B, reciprocal IP reactions were performed as described under “Experimental Procedures,” employing control mouse IgG, mouse anti-WRN, and anti-p14 Arf monoclonal antibody. IP eluates were then electrophoresed and immunoblotted with mouse anti-WRN antibody or anti-p14 Arf polyclonal rabbit serum. WB, Western blot.

with p14 Arf in H1299 cells. The FLAG-tagged WRN truncation mutants have been described previously and encode N-terminal, C-terminal, and central regions of the WRN polypeptide (24). The full-length, central region, and C terminus of WRN were co-immunoprecipitated with p14 Arf rabbit serum but not with control rabbit serum (Fig. 2A). In addition, the level of WRN present in the IPs with anti-p14 Arf antibody was increased by co-transfection of p14 Arf (Fig. 2B). Therefore, the co-immunoprecipitation was specific and not because of direct recognition of WRN by the anti-p14 Arf antibody. In each case, the complex of WRN with endogenous p14 Arf was also detected when longer immunoblot exposures were examined (data not shown). Co-transfection of p14 Arf resulted in the increased expression of each of the truncation mutants of WRN but not of the full-length protein (Fig. 2C). p14 Arf also increases levels of full-length Mdm2 (17). The reason for this striking effect of p14 Arf is currently unknown, and further work to determine its mechanism is in progress.

To delineate the regions of p14 Arf involved in interaction with WRN, we employed a series of 20-mer overlapping biotinylated peptides encompassing the entire polypeptide sequence of p14 Arf (8). Peptides were coupled to streptavidin-agarose and incubated with H1299 extract. Bound proteins were eluted in sample buffer, resolved by SDS-PAGE, and visualized by immunoblotting. Endogenous WRN was predominantly captured by p14 Arf-derived peptides encompassing regions 1–60 and 81–100 of p14 Arf (Fig. 3, upper panels). This pattern was similar to that observed with endogenous Mdm2 (Fig. 3, lower panels), and the same results were obtained with U2-OS osteosarcoma (p14 Arf null) cells (data not shown).

To characterize further the multivalent nature of the p14 Arf/WRN interaction, each of the four major WRN-binding peptides from p14 Arf were incubated with IVT 35S-labeled full-length WRN or the N-terminal, central, or C-terminal truncated proteins. Residues 1–20, 11–30, and 1–60 of p14 Arf interacted most strongly in vitro with the central region of WRN and the N terminus (Fig. 4A). These constructs express overlapping regions of WRN (residues 326–365), possibly implicating these WRN residues in the p14 Arf interaction. The central and N-terminal regions of WRN did not interact as strongly with p14 Arf in the IP experiments from cell extracts (Fig. 2). However, these WRN regions completely lack the C-terminal nuclear localization sequence (NLS) (27), and so the observed differences between the in vitro and IP experiments probably reflect a lack of nuclear localization of the N-terminal and central fragments in vivo. Strikingly, residues 81–100 of p14 Arf preferentially bound to the C-terminal WRN (Fig. 4A), suggesting that a distinct p14 Arf-binding site, which interacts with p14 Arf residues 82–101, lies in the WRN C terminus. These data indicate that there are multiple p14 Arf-binding sites in WRN and multiple WRN-binding sites in p14 Arf. This situation resembles the multivalent nature of the p14 Arf/Mdm2 interaction (25).

To confirm the regions of p14 Arf required to interact with WRN in vivo, previously described p14 Arf truncation mutants were employed (9). The p14 Arf Δ2–14, Δ82–101, and Δ2–14/
82–101 truncation mutants were expressed in H1299 cells together with WRN. Full-length p14 Arf and the 2–14 and 82–101 mutants co-immunoprecipitated with WRN; however, the 2–14/82–101 mutant was greatly impaired in its ability to co-immunoprecipitate with WRN (Fig. 4B), indicating that removal of both the 2–14 and 82–101 regions of p14 Arf are required to ablate interaction with WRN in vivo.

p14 Arf Promotes SUMO Conjugation of WRN—Recently, it was demonstrated that p14 Arf could promote modification of Mdm2 and p53 with the ubiquitin-like protein SUMO-1 (17, 18). Murine WRN has been reported to interact with the SUMO-conjugating enzyme UBCH9 by yeast two-hybrid analysis (28). We therefore compared the abilities of p14 Arf and UBCH9 to promote SUMO-1 conjugation of WRN. H1299 cells were transfected with expression constructs for FLAG-WRN, p14 Arf or UBCH9, and His6-tagged SUMO-1. Cells were lysed and SUMO assays performed as described under “Experimental Procedures.” Aliquots of the inputs and Ni2+-NTA column eluates were electrophoresed on 3–8% or 4–12% gradient gels and immunoblotted with anti-WRN antibody, or anti-UBCH9 antibody (lower panel), respectively. HA-tagged UBCH9 migrates above the endogenous protein upon SDS-PAGE, due to the additional mass contributed by the tag. B, H1299 cells were transfected with the indicated combination of plasmids expressing the C terminus of WRN (C), p14 Arf, His6-SUMO-1 (S), or UBCH9. Lysates were prepared and analyzed as for A. Ni2+-Ag, Ni2+-agarose.

Higher molecular weight species of WRN were enriched in the Ni2+-NTA-agarose purified material compared with the input, and this enrichment was entirely dependent upon co-transfection with His6-SUMO-1 (Fig. 5A). These high molecular weight species therefore represent SUMO-modified WRN. It is possible that the major band observed contains multiple modified species of WRN that are difficult to fully resolve due to the large molecular weight of SUMO-modified forms of WRN (~180 kDa). Following co-transfection of p14 Arf and His6-SUMO-1, the single major band of modified WRN was significantly enhanced, and a ladder of multiple higher molecular
weight bands was also observed (Fig. 5A). SUMO-1 cannot multimerize; therefore, the laddering pattern might represent SUMO modification of WRN at multiple single sites. Overexpression of HA-tagged UBCH9 also promoted His<sub>6</sub>-SUMO-1 conjugation of WRN (Fig. 5A). However, in contrast to p14 Arf, UBCH9 only stimulated the accumulation of the single major band of SUMO-modified WRN. Co-transfection of p14 Arf with UBCH9 induced a robust synergistic SUMO-1 conjugation of WRN, resulting in a ladder of high molecular weight-modified WRN species (Fig. 5A). This was not due to an effect of p14 Arf on the level of UBCH9 protein (Fig. 5A, lower panel).

**p14 Arf Promotes SUMO-1 Conjugation of the WRN C Terminus**—To investigate WRN SUMO-1 conjugation in more detail, we examined the ability of UBCH9 and p14 Arf to promote modification of fragments of the WRN protein. Unlike p14 Arf, UBCH9 was unable to promote significantly SUMO-1 conjugation or to increase expression of the isolated WRN C terminus (Fig. 5B), presumably because this WRN region lacks the UBCH9 binding region, previously identified in murine WRN (28). Strikingly, co-transfection of p14 Arf and UBCH9 promoted a robust synergistic modification of the WRN C terminus (Fig. 5B). p14 Arf did not induce SUMO-1 conjugation of the N terminus or central WRN regions in vivo (data not shown). The N-terminal and central WRN regions completely lack the C-terminal NLS (27). Because nuclear localization is important for SUMOylation of substrates in general (29), this lack of nuclear localization probably prevented the SUMO-1 conjugation of these regions in vivo.

We also attempted to reconstitute this effect of p14 Arf in an *in vitro* reaction, employing a recombinant SUMO-activating enzyme (E1-activating enzyme), UBCH9 (E2-conjugating enzyme), [<sup>35</sup>S]methionine-labeled WRN fragments, and an ATP-regenerating system (described in Ref. 30). Robust SUMO-1 conjugation of the central WRN region was observed in an *in vitro* reaction in the presence of recombinant UBCH9 (data not shown). However, in this system p14 Arf did not induce SUMO-1 conjugation of WRN or act synergistically with UBCH9 to promote WRN SUMO-1 conjugation, even at limiting concentrations of UBCH9 (data not shown). Similarly, other investigators have experienced difficulty in establishing a cell-free system that reconstitutes the p14 Arf effect on SUMO conjugation of these regions *in vivo*.

**Endogenous p14 Arf Is Required for WRN SUMO Conjugation**—To determine whether endogenous p14 Arf is required for SUMO conjugation of WRN, we examined WRN SUMO-1 modification in the p14 Arf null human osteosarcoma U2-OS cell line. H1299 or U2-OS cells were transfected with expression constructs for FLAG-WRN, p14 Arf, or UBCH9 and His<sub>6</sub>-tagged SUMO-1 and analyzed as before. In direct comparison with H1299 cells, basal SUMO modification of WRN was reduced in U2-OS cells, and UBCH9 was severely impaired in its ability to promote SUMO modification of WRN (Fig. 6A). Moreover, reintroduction of p14 Arf into U2-OS cells caused a massive stimulation of WRN SUMO conjugation that was much greater than the effect observed in H1299 cells. This could not be further increased by co-expression of UBCH9 (data not shown). Thus, WRN SUMO conjugation in U2-OS cells appears to be highly sensitive to the reintroduction of p14 Arf.

We also examined the effect of siRNA-mediated knockdown of p14 Arf upon SUMO conjugation of endogenous WRN in the presence or absence of His<sub>6</sub>-tagged SUMO-1. p14 Arf siRNA expression reduced endogenous p14 Arf levels by more than 70% in the whole cell population, although levels of SUMO-1 and UBCH9 were unaffected (Fig. 6B). A band representing modified endogenous WRN was observed after long exposure (Fig. 6B, indicated with an asterisk), which was increased by overexpression of His<sub>6</sub>-tagged SUMO-1 in cells transfected with empty pSUPER vector. However, this modification of WRN was impaired in p14 Arf-depleted cells expressing p14 Arf siRNA (Fig. 6B). These data indicate that endogenous p14 Arf is required for SUMO modification of WRN.

**The Modification Induced by p14 Arf Is Reversed by a SUMO-specific Protease and Dominant Negative UBCH9 and Consists Predominantly of SUMO**—To confirm further that the higher molecular weight forms of WRN induced by p14 Arf co-expression contained SUMO-1-modified forms of WRN, a SUMO-specific protease (SSP3) was employed. Exogenous expression of SSP3 results in the highly specific deconjugation of SUMO-modified substrates *in vivo* (31). SSP3 is unable to remove ubiquitin and other related molecules. Co-transfection of SSP3 ablated the ability of p14 Arf to promote modification of both full-length and C-terminal WRN, without affecting SUMO-1 levels (Fig. 7, A and B). This demonstrates that the ladder of modified WRN induced by p14 Arf is completely dependent upon SUMO. Most interestingly, co-expression of SSP3 increased expression of full-length WRN protein but not the isolated C terminus (Fig. 7, A and B, middle panel).

In addition, we employed an active site cysteine mutant of UBCH9 (C93S), which acts as a dominant negative inhibitor (31). Co-expression of increasing amounts of C93S UBCH9 also blocked the ability of p14 Arf to promote SUMO-1 conjugation of both full-length and C-terminal WRN in a dose-dependent manner (Fig. 7, C and D, upper panels) but did not affect levels of WRN or SUMO-1 (Fig. 7, C and D, lower panels). These
experiments confirm that p14 Arf induces formation of higher molecular weight species of WRN that are entirely dependent upon SUMO-1 modification. p14 Arf has also recently been reported to induce ubiquitinization of B23 (32). The ladder of modified WRN species induced by p14 Arf must contain SUMO, but the higher molecular weight species could represent mixed His$_{12}$SUMO-1/ubiquitin species containing endogenous ubiquitin that have been enriched during Ni$^{2+}$-NTA-agarose purification. To investigate this possibility, cells were transfected with p14 Arf and His$_{12}$-SUMO-1 or His$_{12}$-ubiquitin. Immunoprecipitation experiments with anti-WRN antibody were performed on the Ni$^{2+}$-NTA-agarose eluates, which were then analyzed by immunoblotting with anti-SUMO or anti-ubiquitin antibodies. The major SUMO-modified WRN band is indicated with an arrow. Ni$^{2+}$-NTA eluates were diluted into IP buffer, and IP reactions were carried out as described under “Experimental Procedures,” employing anti-WRN monoclonal antibody. Aliquots of IP eluates were electrophoresed on 3–8% gradient gels and immunoblotted with either anti-SUMO or anti-ubiquitin antibodies. The major SUMO modified WRN band is indicated with an arrow. Ni$^{2+}$-Ag, Ni$^{2+}$-agarose;

p14 Arf required to promote SUMO-1 conjugation of WRN, p14 Arf truncation mutants were expressed in H1299 cells, together with either full-length or C-terminal WRN and His$_{12}$-SUMO-1. As observed previously, wild-type p14 Arf promoted SUMO-1 conjugation of both full-length and C-terminal WRN while increasing expression of C-terminal WRN (Fig. 9). In contrast, the p14 Arf Δ2–14 and Δ2–14/82–101 mutants were unable to support SUMO-1 modification of WRN or increase the level of C-terminal WRN protein (Fig. 9). The Δ82–101 mutant was also greatly impaired in promoting SUMO-1 conjugation when compared with WT p14 Arf (Fig. 9). p14 Arf Δ82–101 was still able to increase expression of C-terminal WRN, although it would be predicted to have impaired binding to this region of WRN (Fig. 4A). This situation is similar to the p14 Arf-induced SUMO-1 conjugation of Mdm2, in which deletion of the 82–101 region also dramatically impaired p14 Arf-induced SUMO-1 conjugation, although p14 Arf Δ82–101 can still increase expression of Mdm2 (17, 18). Thus the effect of p14 Arf on protein levels is independent of its ability to promote SUMOylation. These results indicate that regions of p14 Arf involved in the binding to WRN are also required for p14 Arf-dependent SUMO conjugation; however, binding of p14 Arf per se is not sufficient to promote WRN SUMO modification.

p14 Arf Deletion Mutants Are Deficient in Promoting SUMO-1 Conjugation to WRN—To determine the regions of

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FIG. 7. The modification induced by p14 Arf is reversed by a SUMO-specific protease and dominant negative UBCH9. A, H1299 cells were transfected with full-length (FL) WRN and the indicated combination of p14 Arf, His$_{12}$-SUMO-1 (S), and SSP3-expressing plasmids. Cells were lysed, and SUMO assays were performed as described under “Experimental Procedures.” Lysates and Ni$^{2+}$-NTA eluates were electrophoresed on 3–8% gradient gels and immunoblotted with anti-WRN antibody. B, H1299 cells were transfected with C-terminal WRN (C) and the indicated combination of plasmids as for A. Cells were lysed, and SUMO assays were performed as described under “Experimental Procedures.” Aliquots of the inputs and Ni$^{2+}$-NTA column eluates were electrophoresed on 4–12% gradient gels and immunoblotted with anti-WRN antibody. C, H1299 cells were transfected with full-length WRN, p14 Arf, His$_{12}$-SUMO-1, and increasing amounts of a UBCH9 C93S dominant negative mutant. Samples were analyzed as for A. D, H1299 cells were transfected with full-length WRN, p14 Arf, His$_{12}$-SUMO-1, and increasing amounts of a UBCH9 C93S dominant negative mutant. Samples were analyzed as for A. Ni$^{2+}$-Ag, Ni$^{2+}$-agarose.

p14 Arf Expression Causes Nucleolar Exclusion of WRN—WRN is a predominantly nucleolar protein in a variety of exponentially growing cell lines (33). Residues 949–1092 are important for nucleolar targeting, although this polypeptide also requires a contiguous intact NLS region to mediate nuclear localization (34). Upon serum starvation or treatment with cellular stresses, WRN migrates from nucleoli to discrete nuclear foci (33, 35). DNA damage also induces the formation of nuclear foci containing the homologous recombination and
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Here we characterize a novel interaction between the p14 Arf and WRN, and we demonstrate that p14 Arf can promote SUMO modification of WRN in a synergistic manner with the SUMO-conjugating enzyme UBCH9. The ability to promote SUMO conjugation is likely to be a general property of p14 Arf, because in addition to p53 and Mdm2, WRN represents the third reported substrate for p14 Arf-stimulated SUMO modification. This work establishes a novel, biochemical property of p14 Arf.

Multivalent Nature of the p14 Arf/WRN Interaction—The binding of p14 Arf and WRN involves multiple interacting regions in both p14 Arf and WRN, with residues 2–14 and 82–101 of p14 Arf, and residues in the central region and C terminus of WRN having particular importance for binding (Figs. 2–4). Similar results have been reported for the p14 Arf-Mdm2 interaction. The first 35 amino acids of p14 Arf make multiple contacts with Mdm2 (8, 25, 38), and analysis of p14 Arf also indicates a second Mdm2-binding site within residues 65–132 (39). We have further defined a second Mdm2-binding region to within residues 82–101, and we demonstrated that a similar interaction pattern was observed for p14 Arf and WRN (Figs. 3 and 4). Like WRN, Mdm2 also has at least two binding sites for p14 Arf (38).

p14 Arf greatly stimulated formation of a high molecular weight species of WRN (Fig. 5A). These species must consist of SUMOylated forms of WRN, as they were dependent upon co-transfection with His$_6$-SUMO-1 and enriched in the Ni$^{2+}$-NTA-agarose purified material (Fig. 5). This modification of WRN was impaired in both p14 Arf null U2-OS cells and in H1299 cells depleted of p14 Arf by siRNA expression (Fig. 6). In addition the modified species were removed by co-expression of a SUMO-specific protease, which cannot remove related ubiquitin-like molecules, and conjugation was inhibited by a dominant negative UBCH9 mutant (Fig. 7). Deletion of the 2–14 or 82–101 regions dramatically impairs p14 Arf-induced SUMO-1 conjugation of WRN (Fig. 9), as for Mdm2 (17). The same p14 Arf regions are involved in binding to WRN, and both must be deleted to ablate binding to WRN (Fig. 4B). These observations are consistent with a requirement for direct binding of p14 Arf to promote SUMO modification of WRN, but they indicate that binding alone is not sufficient for p14 Arf to promote SUMO conjugation. Perhaps binding of both p14 Arf domains to WRN is required to allow a conformational change necessary for SUMO modification. Alternatively, these two domains in p14 Arf might be required to recruit additional proteins of the SUMO conjugation pathway. Further work to fully establish the requirements for p14 Arf-stimulated SUMO conjugation is required.

Potential Mechanisms of p14 Arf-promoted SUMO Conjugation—p14 Arf may actively promote SUMO modification. Alternatively, p14 Arf may prevent removal of SUMO-1 rather than directly stimulating its conjugation. Such a scenario could explain why the stimulatory effect of p14 Arf on SUMO-1 conjugation is difficult to reconstitute in vitro. The data presented here indicate an active role for p14 Arf in the SUMO conjugation process. Overexpression of UBCH9 alone cannot stimulate SUMO-1 conjugation of the C terminus of WRN (Fig. 5B). This is consistent with the fact that this region lacks the residues required for UBCH9 binding identified in murine WRN (28). However, co-expression of p14 Arf allows a synergistic effect of UBCH9 and p14 Arf on the isolated C terminus of WRN (Fig. 5B), strongly suggesting that p14 Arf has a positive stimulatory role in SUMO modification. In addition, p14 Arf is unable to stimulate SUMO-1 conjugation of another unrelated nuclear DNA repair proteins, Rad 51 and Rad 52, which partially co-localize with WRN (35, 36).

SUMO modification also has an established role as a regulator of protein localization (reviewed in Ref. 37). To determine whether p14 Arf co-expression influenced WRN localization, as well as SUMO modification, immunofluorescence experiments were performed in H1299 cells. As expected, transfected WRN showed a predominantly nuclear localization, which was confirmed by phase contrast microscopy (data not shown). Nucleoli are indicated by the arrows in Fig. 10. However, p14 Arf co-expression induced a clear redistribution of WRN within the nucleus (Fig. 10). Co-expression of SSP3, which ablates p14 Arf-mediated SUMO conjugation of WRN (Fig. 7), restored the nucleolar localization of WRN in ~30% of the p14 Arf-transfected cells (Fig. 10). This implicates the SUMO conjugation pathway in p14 Arf-induced redistribution of WRN. Similar results were obtained with the C terminus of WRN (data not shown).

Mapping of the SUMO Conjugation Sites—UBCH9 can interact directly with its substrates via a conserved motif $\Phi$KXE (where $\Phi$ is typically a large hydrophobic residue and K is the target residue for SUMO modification). WRN has 117 lysine residues, four of which constitute good UBCH9 consensus motives. To identify the sites of SUMO-1 conjugation in vivo, putative WRN UBCH9 consensus site mutants were expressed in H1299 cells either in the presence of p14 Arf or UBCH9, and the cell lysates were analyzed for total WRN levels and His$_6$-SUMO-1-WRN isolated on Ni$^{2+}$-NTA-agarose beads, as before. The intensity and thickness of the major UBCH9-induced SUMOylated forms of WRN, as they were dependent upon co-expression of p14 Arf and WRN (Figs. 3 and 4). Like WRN, Mdm2 also has at least two binding sites for p14 Arf (38).

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UBCH9 substrate.\textsuperscript{2} These data indicate that p14 Arf is not merely a general inhibitor of SUMO proteases. It is possible that p14 Arf recruits UBCH9 to WRN or relocates WRN within the nucleus to be proximal to UBCH9 or other components of the SUMO conjugation pathway.

The pattern of SUMO modification induced by p14 Arf is qualitatively different from that induced by UBCH9. UBCH9-mediated SUMO conjugation generally produced one major band of SUMO-modified WRN (Fig. 5A), whereas p14 Arf induces a ladder of bands including higher molecular weight species (Fig. 5A). Perhaps p14 Arf binding changes the protein conformation of WRN, thereby allowing additional lysine residues to become amenable to SUMO modification, resulting in multiple forms of WRN that are mono-SUMO-modified on different combinations of individual lysines. Alternatively, p14 Arf may preferentially induce SUMO conjugation of WRN species that have already been otherwise modified, for example by ubiquitin or other related molecules, and the laddering pattern therefore represents mixed species of modified forms of WRN.

The mammalian PIAS proteins act as SUMO E3 enzymes for LEF1, p53, and c-Jun (43–45). The nucleoporin RanBP2 also functions as a SUMO E3 ligase for substrates such as Sp100, HDAC4, and Mdm2 (46–48). A more recent report (49) demonstrated that the polycomb protein Pc2 enhances the SUMO modification of the transcriptional co-repressor CtBP1 by recruiting UBCH9 and Pc2 into polycomb group (PcG) nuclear bodies, and can hence be considered a distinct type of non-enzymatic SUMO E3. p14 Arf represents another important addition to the group of proteins capable of promoting SUMO modification.

Despite the fact that a substrate may carry all the information necessary to specify its SUMO modification by virtue of its KXE motif(s), the existence of SUMO E3-like activities provides a potential mechanism for fine-tuning substrate specificity. Moreover, potential SUMO substrates, which lack the KXE-targeting motif, may be SUMO-conjugated \textit{in vivo} provided the correct E3 is present. For example, Pc2 can enhance UBCH9-mediated SUMO modification of CtBP2, which has no consensus motif (49). WRN is a large protein of 160 kDa and has 117 lysine residues, four of which constitute good consensus motifs. We mutated these SUMO consensus sites in WRN either alone or in combination, and although we were able to reduce SUMOylation of WRN, we were unable to ablate it completely (Fig. 11). p14 Arf could recruit UBCH9 to allow modification of nonconsensus sites, because co-expression of p14 Arf allows a synergistic effect of UBCH9 and p14 Arf on the C terminus of WRN, although there are no clear KXE consensus...
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**Functional Consequences of WRN SUMO Conjugation—p14 Arf causes a clear redistribution of WRN within the nucleus (Fig. 10). The vast majority of p14 Arf-interacting proteins appear to be sequestered in the nucleolus. To our knowledge a molecular switch that in its SUMO-modified form may play a role in the WRN nuclear re-localization induced by p14 Arf displays an intriguing ability to promote SUMO conjugation of WRN and cause WRN redistribution within the nucleus. This effect of p14 Arf is reversed by expression of a SUMO-specific protease, thus implicating the SUMO conjugation pathway in WRN re-localization. The biochemical analysis of the p14 Arf/WRN interaction performed here places p14 Arf in a novel signaling pathway with WRN, and future genetic dissection of the pathway is now possible.**

**REFERENCES**

1. Sherr, C. J. (2001) *Nat. Rev. Mol. Cell. Biol.* 2, 731–737
2. Kamiya, T., Zandy, P., Roussel, M. F., Donawig, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997) *Cell* 91, 649–659
3. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998) *Nature* 395, 124–125
4. O’Leary, K. A., Mendez, S. M., Vacara, A., and Perry, M. E. (2004) *Mol. Cell. Biol.* 24, 186–191
5. Pomerantz, J., Schreiber-Agus, N., Lipes, N. J., Silverman, A., Alland, L., Chen, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Cordon-Cardo, C., and DePinho, R. A. (1998) *Cell* 92, 713–723
6. Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998) *Cell* 92, 725–734
7. Honda, R., and Yasuda, H. (1999) *EMBO J.* 18, 22–27
8. Midgley, C. A., Desterro, J. M., Saville, M. K., Howard, S., Sparks, A., Hay, R. T., and Lane, D. P. (2000) *Oncogene* 19, 2312–2323
9. Xiromidas, D., Saville, M. K., Edging, C., Lane, D. P., and Lain, S. (2001) *Oncogene* 20, 4972–4983
10. Weber, J. D., Jeffers, J. E., Randle, D. H., Lozano, G., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. (1999) *Nat. Cell Biol.* 1, 20–26
11. Llanos, S., Clark, P. A., Rowe, J., and Peters, G. (2001) *Nat. Cell Biol.* 3, 445–452
12. Tao, W., and Levine, A. J. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 6937–6941
13. Rubin, C. P., and Mindor, J. (2003) *EMBO J.* 22, 6068–6077
14. Weber, J. D., Jeffers, J. R., Beh, J. E., Randle, D. H., Lozano, G., Roussel, M. F., Sherr, C. J., and Zambetti, G. P. (2000) *Genes Dev.* 14, 2358–2365
15. Yarbrough, W. G., Beesho, M., Zanaton, A., Bisi, E. J., and Xiong, Y. (2002) *Cancer Res.* 62, 1171–1177
16. Kuo, M. L., Duncavage, E. J., Mathew, R., den Besten, W., Pei, D., Naeve, D., Yamamoto, T., Cheng, C., Sherr, C. J., and Roussel, M. F. (2003) *Cancer Res.* 63, 1046–1053
17. Xiromidas, D. P., Chisholm, J., Desterro, J. M., Lane, D. P., and Hay, R. T. (2002) *FEBS Lett.* 528, 207–211
18. Chen, L., and Chen, J. (2003) *Oncogene* 22, 5438–5437
19. Chen, L., and Oshima, J. (2002) *J. Biomed. Biotechnol.* 2, 46–54
20. Poot, M., Hoehn, H., Runger, T. M., and Martin, G. M. (1992) *Exp. Cell Res.* 202, 267–273
21. Fukushima, K., Martin, G. M., and Monnat, R. J., Jr. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 5893–5897
22. Bachrati, C. Z., and Hickson, I. D. (2003) *Biochem. J.* 374, 577–606
23. Chen, J., Marechal, V., and Levine, A. J. (1995) *Mol. Cell. Biol.* 15, 4107–4114
24. Blander, G., Kipnis, J., Leal, J. F., Yu, C. E., Schellenberg, G. D., and Oren, M. (1999) *J. Biol. Chem.* 274, 29463–29469
25. Clark, P. A., Llanos, S., and Peters, G. (2002) *Oncogene* 21, 4498–4507
26. Bottger, A., Biettger, V., Stojoez, A., Liu, W. L., Howard, S. P., and Lane, D. P. (1997) *Curr. Biol.* 7, 860–869
27. Matsumoto, T., Imamura, O., Goto, M., and Furuchi, Y. (1998) *Int. J. Mol. Med.* 1, 71–76
28. Kawase, Y., Seki, M., Seki, T., Wang, W. S., Imamura, O., Furuchi, Y., Saitoh, H., and Enomoto, T. (2000) *J. Biol. Chem.* 275, 20963–20966
29. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001) *J. Biol. Chem.* 276, 26545–26559
30. Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999) *EMBO J.* 18, 6455–6461
31. Girdwood, D., Rumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., Garcia-Wilson, E., Perkins, N. D., and Hay, R. T. (2003) *Mol. Cell* 11, 1043–1054
32. Iihana, K., Bhat, K. P., Jin, A., Iihana, Y., Hawke, D., Kobayashi, R., and Zhang, Y. (2003) *Mol. Cell* 12, 1151–1164
33. Gray, M. D., Wang, L., Youssefian, H., Martin, G. M., and Oshima, J. (1998) Exp. Cell Res. 242, 487–494
34. Von Kobbe, C., and Bohr, V. A. (2002) J. Cell Sci. 115, 3901–3907
35. Sakamoto, S., Nishikawa, K., Heo, S. J., Goto, M., Furuichi, Y., and Shimamoto, A. (2001) Genes Cells 6, 421–430
36. Baynton, K., Otterlei, M., Bjoras, M., Von Kobbe, C., Bohr, V. A., and Seeberg, E. (2003) J. Biol. Chem. 278, 36476–36486
37. Pichler, A., and Melchior, F. (2002) Traffic 3, 381–387
38. Weber, J. D., Kuo, M. L., Bothner, B., DiGiammarino, E. L., Kriwacki, R. W., Roussel, M. F., and Sherr, C. J. (2000) Mol. Cell. Biol. 20, 2517–2528
39. Lohrum, M. A., Ashcroft, M., Kubbutat, M. H., and Vousden, K. H. (2000) Curr. Biol. 10, 539–542
40. Schwartz, D. C., and Hochstrasser, M. (2003) Trends Biochem. Sci. 28, 321–328
41. Johnson, E. S., and Gupta, A. A. (2001) Cell 106, 735–744
42. Takahashi, Y., Toh-e, A., and Kikuchi, Y. (2001) Gene (Amst.) 275, 223–231
43. Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001) Genes Dev. 15, 3088–3103
44. Kahyo, T., Nishida, T., and Yasuda, H. (2001) Mol. Cell 8, 713–718
45. Schmidt, D., and Muller, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2872–2877
46. Pichler, A., Gast, A., Seeler, J. S., Dejean, A., and Melchior, F. (2002) Cell 108, 109–120
47. Kirsh, O., Seeler, J. S., Pichler, A., Gast, A., Muller, S., Miska, E., Mathieu, M., Harel-Bellan, A., Kouzarides, T., Melchior, F., and Dejean, A. (2002) EMBO J. 21, 2882–2891
48. Miyashita, Y., Yosogawa, S., Honda, R., Nishida, T., and Yasuda, H. (2002) J. Biol. Chem. 277, 50131–50136
49. Kagey, M. H., Melhuish, T. A., and Wertten, D. (2003) Cell 113, 127–137
50. Duprez, E., Saurin, A. J., Destervo, J. M., Lallemant-Breitenbach, V., How, K., Boddy, M. N., Solomon, E., de The, H., Hay, R. T., and Freemont, P. S. (1999) J. Cell Sci. 112, 381–393
51. Blander, G., Zalle, N., Daniely, Y., Taglick, J., Gray, M. D., and Oren, M. (2002) J. Biol. Chem. 277, 59934–59940
52. Cheng, W. H., Von Kobbe, C., Opresko, P. L., Fields, K. M., Ren, J., Kufe, D., and Bohr, V. A. (2003) Mol. Cell. Biol. 23, 6385–6395
53. Stelter, P., and Ulrich, H. D. (2003) Nature 425, 188–191
54. Menendez, S., Khan, Z., Coomber, D. W., Lane, D. P., Higgins, M., Koufali, M. M., and Lain, S. (2003) J. Biol. Chem. 278, 18720–18729