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Cellular dynamics and modulation of WRN protein is DNA damage specific

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Abstract

The human premature aging protein Werner (WRN), deficient in Werner syndrome (WS), is localized mainly to the nucleolus in many cell types. DNA damage or replication arrest causes WRN to redistribute from the nucleolus to the nucleoplasm into discrete foci. In this study, we have investigated DNA damage specific cellular redistribution of WRN. In response to agents causing DNA double strand breaks or DNA base damage, WRN is re-distributed from the nucleolus to the nucleoplasm in a reversible manner. However, after ultraviolet (UV) irradiation such redistribution of WRN is largely absent. We also show that WRN is associated with the insoluble protein fraction of cells after exposure to various kinds of DNA damage but not after UV irradiation. Further, we have studied the DNA damage specific post-translational modulation of WRN. Our results show that WRN is acetylated after mytomycin C or methyl methane-sulfonate treatment, but not after UV irradiation. Also, DNA damage specific phosphorylation of WRN is absent in UV irradiated cells. Inhibition of phosphorylation fails to restore WRN localization. Thus, our results suggest that the dynamics of WRN protein trafficking is DNA damage specific and is related to its post-translational modulation. The results also indicate a preferred role of WRN in recombination and base excision repair rather than nucleotide excision repair.
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Keywords: DNA damage; DNA repair; WRN distribution; Phosphorylation of WRN; Acetylation of WRN

1. Introduction

Werner syndrome (WS) is an autosomal recessive disorder with distinct premature aging features and with a high incidence of age associated diseases including cancer (soft tissue) and diabetics meliitus, etc. (Hickson, 2003). The Werner syndrome protein (WRN) plays an important role in the maintenance of genomic integrity. WS cells display extensive genomic instability, such as chromosomal translations, and extensive deletions (Opresko et al., 2003). Cells carrying a mutation in the WRN gene show hypersensitivity to certain DNA damaging agents including some that arrest cells in S-phase. WRN interacts with a number of cellular proteins and is likely to participate in more than one DNA repair pathway (Opresko et al., 2003). Accelerated aging and predisposition to cancer are hallmarks of WS patients and lack of DNA repair with accumulation of DNA damage may be responsible for phenotypical changes seen in WS. WRN is a member of the highly conserved family of RecQ helicases, extending from bacteria to humans. It is the only family member that contains an exo-nuclease function in addition to the helicase activity (Opresko et al., 2003). While there have been many studies on the biochemical properties of WRN, there is a relative lack of knowledge about the cellular distribution, localization and function of WRN under various forms of cellular stress.

DNA damage can be removed by a number of distinct DNA repair pathways (Wood et al., 2001). Bulky lesions are removed by nucleotide excision repair (NER), while monofunctional lesions and oxidative base modifications are removed by base excision repair (BER). Double strand

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DNA breaks (DSB) and interstrand crosslinks are removed by recombinational DNA repair. These pathways contain subpathways; for example, there are two subpathways of NER, transcription coupled repair (TCR) and global genome repair (GGR). BER can be mediated through long patch or short patch subpathways, and non-homologous end joining (NHEJ) and homologous recombination (HR) are two pathways for DSB repair. While induction of the different DNA repair pathways to some extent is DNA damage specific, there is a small, but increasing number of proteins that participate in more than one DNA repair pathway and may function as modulators of DNA repair (Mitchell et al., 2003). Most types of cellular stress give rise to a spectrum of different types of DNA damage that are then removed simultaneously by the different repair pathways. WRN appears to participate in more than one DNA repair pathway as we and others have demonstrated roles in BER and recombination (Opresko et al., 2004).

Here, we have examined DNA repair pathways, which involve the WRN protein. An important feature of WRN is that it is localized to the nucleoli (Marciniak et al., 1998; Von kobbe and Bohr, 2002). It was also shown that after cellular stress, WRN leaves the nucleoli to function in the general nucleus. In previous studies, we and others have emphasized the importance of this cellular relocation of WRN, the basis of which is not yet understood (Cheng et al., 2003; Joaquin et al., 2003). WRN has been shown to localize to the nucleolus of many cells, but after certain types of DNA damages it leaves the nucleolus and forms distinct foci in the nucleoplasm (Sakamoto et al., 2001). The formation of these foci is likely to reflect an important stage in the DNA damage response. Several cellular DNA repair proteins have been shown to change their nuclear locations and concentrate at discrete sites to form foci after DNA damage. These proteins are important markers for DNA repair. After irradiation, the Mre11/RAD50/Nbs1 (M/R/N) complex rapidly migrates to sites of DSBs and forms foci, which persist until DSB repair is complete. Mre11 and RAD50 play direct roles in DSB repair, while Nbs1 appears to be involved in DNA damage signaling (Uziel et al., 2003; Petrini and Theunissen, 2004).

In response to ionizing radiation and other DNA damaging agents, the RAD51 protein, which is essential for homologous recombination, relocalizes within the nucleus to form distinct foci that can be visualized by microscopy and are thought to represent sites where DNA repair reactions take place. The formation of RAD51 foci in response to DNA damage is dependent upon BRCA2 and upon a series of proteins known as the RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3), indicating that the components present within foci assemble in a carefully orchestrated and ordered manner (Miyazaki et al., 2004; Tarsounas et al., 2003).

After replication arrest or transcription arrest the WRN protein relocalizes to the nucleoplasm (Constantinou et al., 2000b; Suzuki et al., 2001). WRN has been proposed to resolve abnormal DNA structures that arise after replication fork arrest. This is also consistent with the observation that WS cells show prolonged S-phase (Poot et al., 1992). Thus, RecQ helicases play a significant role in replication arrest recovery (Blank et al., 2004; Chen et al., 2003). The cellular WRN protein has also been shown to form distinct nuclear foci after DNA damage in cells that are not in S-phase (Sakamoto et al., 2001).

The interaction between WRN and other DNA repair proteins including PCNA, replication protein A (RPA), DNA polymerase β, flap-endonuclease (Fen)-1, Ku, RAD51/52 and others prompted us to investigate the DNA repair pathways in which WRN might be involved. Also, recently it was observed that post-translational modulation of WRN protein can regulate its catalytic activity and/or nuclear localization (Karmakar et al., 2002; Blander et al., 2002; Cheng et al., 2003).

Because WRN interacts with so many proteins, it of interest to investigate the dynamics of WRN trafficking after different kinds of DNA damage and also how post-translational modulation of WRN is involved. Here, we show that the nuclear relocalization and modulation of WRN takes place after many kinds of DNA damage, but not after UV. Thus, the results indicate a preferred role of WRN in recombination and base excision repair rather than in nucleotide excision repair.

2. Materials and methods

2.1. Cell lines

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. MRC 5 and WRN −/− primary fibroblast (AG03141C) were grown in minimum essential medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1% vitamins, and 1% amino acids (Life Technologies, Inc.).

2.2. Chemicals

Bleomycin, mytomycin C (MMC), methyl methanesulfonate (MMS) were purchased from Sigma (Sigma, St. Louis, MO), Wortmannin was from Alexis Biochemicals; 4NQO was from Janssen Chemical.

2.3. Antibodies

We used WRN antibodies (ab200; Novus, Littleton, CO) for immunolabeling or immunoblotting (clone 30; BD Biosciences, Lexington, KY), respectively. Goat polyclonal anti p89 and mouse monoclonal anti-nucleolin (c23) was from Santa Cruz. Mouse monoclonal anti-acetylated lysine and rabbit polyclonal anti-phospho ser/thr antibody were purchased from Cell Signaling. Mouse monoclonal anti-

phospho tyrosine was from Sigma. All secondary antibodies were purchased from Jackson laboratories.

2.4. Treatment

Cells were grown to 90% confluence and incubated in serum free medium for 12 h (at least 65% of cells are in G1 phase as analyzed by FACS). The cells are then treated with the DNA damaging agents for the indicated time. After the incubation, cells were washed twice with PBS and incubated further with complete medium as necessary.

2.5. Immunolabeling

Cells were washed with PBS, fixed with freshly prepared 4% paraformaldehyde for 10 min at room temperature. They were then washed 3× with PBS containing 100 mM glycine. The cells were permeabilized with 0.25% Triton X-100 in ice for 10 min and washed with cold PBS for 3×. They were then incubated with 1% BSA in PBS for 30 min at room temperature. Proteins were detected immunologically by incubating the coverslips with the appropriate antibodies for 16 h at 4 °C. After washing 3× (10 min each) with PBS containing 0.05% Tween and 0.1% BSA, the coverslips were incubated simultaneously with secondary antibody conjugated with fluorescence dye for 1 h at RT. After washing 3× (10 min each), the coverslips were mounted on Vectashield (Vector Laboratories) and viewed with a fluorescence microscope (Zeiss Axiovert 200 M), and images were processed by using adobe photoshop software.

2.6. Cell fractionation

Cells treated with either DNA damaging agent or mock treated were collected and washed with PBS. The cells were resuspended in cold PB buffer (100 mM KH_2PO_4, 130 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.4) containing 0.5% Triton X-100 and incubated for 30 min in ice. The cells were then centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was collected in a separate tube (soluble fraction). The pellet was then dissolved in PB buffer containing Triton X-100 with 450 mM NaCl. It was briefly sonicated and 2× volume of PB was added. Finally, the suspension was centrifuged and the supernatant collected (insoluble fraction). The protein content of each fraction was measured and equal amount of protein was loaded for Western blot analysis. Prior to loading, samples were resuspended in Laemmli buffer and boiled for 5 min.

2.7. Immunoprecipitation

Approximately 5×10^8 cells were used for each point. The cells were washed with PBS, lysed with RIPA buffer (150 mM NaCl, 1% Triton, 0.1% SDS, 10 mM Tris (pH 8), 0.5% sodium deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 units/ml DNAse) supplemented with phosphatase inhibitors (1:1000, Sigma). After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was precleared with protein G-agarose beads (Calbiochem) and incubated with rabbit polyclonal WRN antibody for 16 h at 4 °C. The immune complexes were then collected by adding G protein-agarose beads and washed 3× with RIPA buffer. The precipitated complex was separated in a 4–15% Tris–glycine polycrylamide gel and transferred to a PVDF membrane. The membrane was immunoblotted with suitable primary antibody followed by a 1 h incubation with horseradish peroxidase-conjugated secondary antibody. The resulting signal was visualized with ECL plus a Western blotting detection system (Amersham Biosciences) and analyzed by ImageQuant software (Molecular Dynamics)

3. Results

3.1. DNA damage specific relocation of WRN from the nucleolus to the nucleoplasm

We examined cellular localization of WRN after DNA damage. G1 synchronized Hela cells were treated with different DNA damaging agents. Indirect immunolabeling was performed on paraformadehyde fixed cells. As seen in Fig. 1(A), endogenous WRN is mainly localized in the nucleolus in undamaged cells. Such localization of WRN was also found in many other cell types including primary fibroblasts (data not shown). When cells were treated with agents that cause DNA double strand breaks (X-rays, Bleomycin), interstrand crosslinks (mytomycin C (MMC)), or DNA base damage (H_2O_2, 4-nitro quinoline (4NQO), MMS), WRN leaves the nucleolus and relocates to the nucleoplasm to form distinct punctate foci (Fig. 1). However, when cells were irradiated with UV, this redistribution of WRN was absent or much less prominent (Fig. 1). The DNA damage induced by the agents that we have used is either repaired by DNA DSB repair or by BER. The exception is UV, which is removed by NER. Thus, WRN does not relocate significantly during NER. Table 1

Table 1
Percentage of cells showing WRN localization in the nucleoplasm after the treatment of HeLa cells with different DNA damaging agents

Damaging agents	Time	% of cells with WRN in the nucleoplasm
Undamaged		16 ± 6
UV (20 J/m^2)	30 min	30 ± 8
X-rays (10 Gy)	30 min	75 ± 5.5
MMC (10 μg/ml)	1 h	68 ± 6.5
4NQO (0.1 mg/ml)	8 h	60 ± 7
H_2O_2 (250 μM)	30 min	71 ± 9
Bleomycin (10 μg/ml)	6 h	73 ± 12
MMS (1 mM)	1 h	69 ± 11

Cells were incubated in the presence of damaging agents for the indicated time. For X-rays and UV, cells were incubated 30 min after irradiation before processing for immunolabeling.

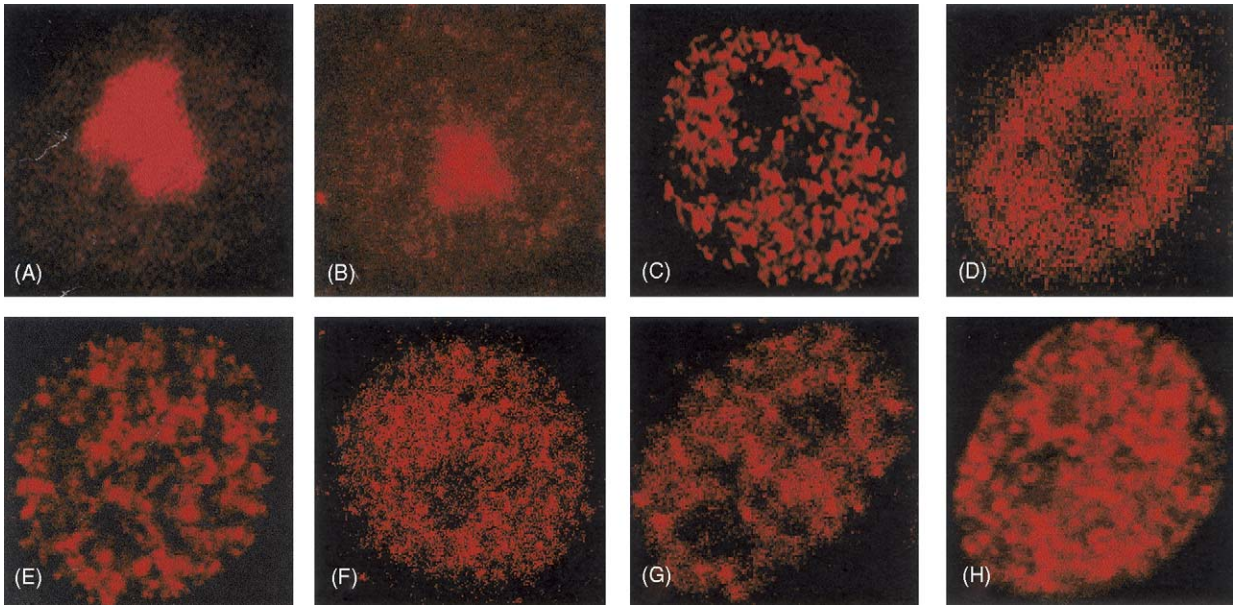


Fig. 1. Distribution of WRN after DNA damage. After treatment with different DNA damaging agents. (A) No damage; (B) UV(20 J/m^2); (C) X-irradiation (10 Gy); (D) 4NQO (0.1 mg/ml) for 8 h; (E) methane methyl-sulfonate (MMS), 1 mM for 1 h; (F) H_2O_2, 250 μM for 30 min; (G), bleomycin, 10 μg/ml for 6 h; (H) mitomycin C (MMC) (10 μg/ml) for 1 h.

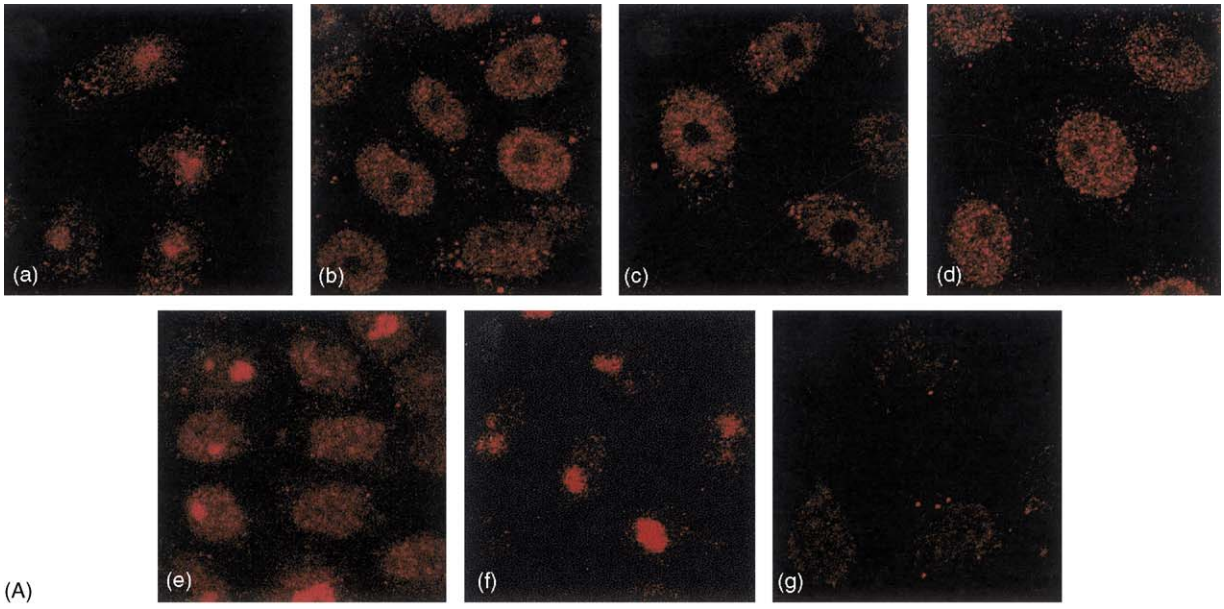
shows the percent of the cells in which WRN was localized in the nucleoplasm. The cells were immunolabeled for WRN and viewed under a fluorescence microscope. The field was selected randomly and the number of cells showing WRN localization in the nucleoplasm was counted along with the total number of cells. At least 100 cells were scored for each point. From Table 1 it is also evident that UV produces much less of an effect on WRN distribution than the other DNA damaging agents. It is important to emphasize that the dose of the different DNA damaging agents was taken from other studies representing physiological conditions (Gray et al., 1998; Pichierri et al., 2001; Poot et al., 1992). Moreover, we have tested some damaging agents (like H_2O_2, MMC, X-rays, MMS) at various concentrations by the MTT assay and optimum concentrations were chosen. UV was tested in the physiological range of 10–20 J/m^2.

3.2. WRN relocates to the nucleolus after removal of the DNA damaging agent

We then determined whether the redistribution of WRN was reversible. Cells were allowed to incubate after the DNA damaging agent was removed. At different time points the cells were collected and immunolabeled to visualize WRN distribution. The recovery of WRN distribution is shown for H_2O_2 treated cells (Fig. 2A). H_2O_2 (250 μM, 30 min) treated cells typically recover WRN localization 8 h after removal of H_2O_2 (∼71% cells showed WRN in the nucleolus). Such recovery of WRN distribution was studied in cells treated with different DNA damaging agents. At each time point the cells were immunolabeled for WRN and at least 100 cells were scored under a fluorescence microscope for the nucleolar localization of WRN in randomly selected fields. A graph was plotted for the percent of cells showing WRN localization in the nucleolus at different times. As seen in Fig. 2B, in undamaged cells WRN localization does not change within 24 h. H_2O_2 and 4NQO treated cells recovered WRN nucleolar localization within 8 h of the post-incubation period. However, in cells treated with MMC, MMS and bleomycin, this recovery was much slower and was not complete even after 24 h. The extent of damage induced by these agents may partly regulate this kinetics.

3.3. WRN may not be significantly involved in nucleotide excision repair

An important observation listed above is that among all the cellular stresses we had imposed on the cells, UV was the exception where WRN left the nucleolus to a much smaller extent than for the other damages. This would suggest a stronger association for WRN in other DNA repair pathways than NER. UV induced nucleotide excision repair is a distinct pathway involving many protein complexes. TFIIH, the basal transcription factor II, plays an important role in NER. We immunolabeled WRN along with p89, one of the components of TFIIH. For these experiments we used human primary fibroblasts, MRC5. In undamaged cells, TFIIH is localized as discrete foci in the nucleus, but after UV irradiation TFIIH redistributed further to small foci throughout the nucleus, as also previously observed (Volker et al., 2001; Karmakar et al., 1998). These small foci are sites of active excision repair. After UV irradiation, cells were incubated for 30 min before they were labeled simultaneously for WRN and p89. As seen in Fig. 3a, TFIIH redistributed to smaller nuclear foci after UV while WRN



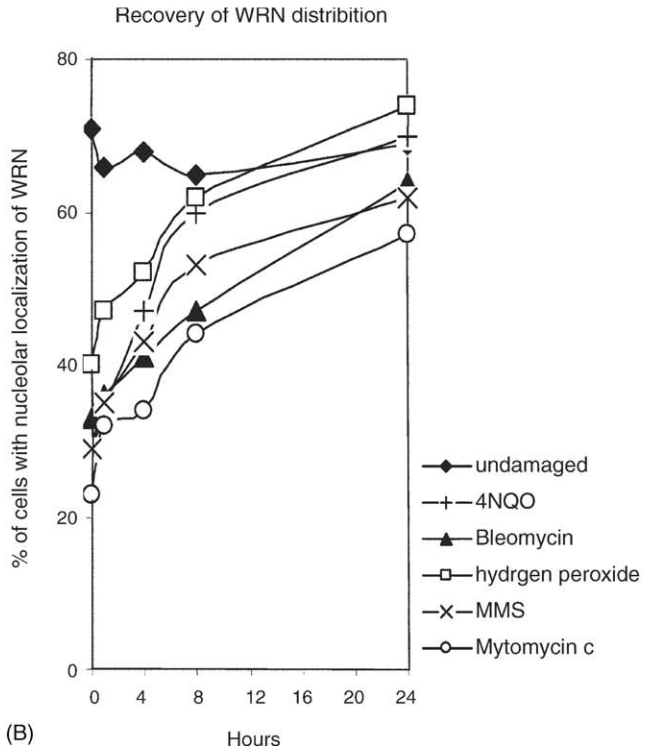


Fig. 2. (A) WRN distribution after recovery from H_2O_2 treatment. HeLa cells were treated with 250 μM $H_2O_2$for 30 min. Cells were then washed and incubated in fresh medium. At different time points cells were processed for indirect immunolabeling as described in Section 2 (a) untreated cells and (b) H_2O_2 treated cells (250 μM $H_2O_2$for 30 min). After H_2O_2 treatment, cells were incubated further for 30 min (c), 2 h (d), 4 h (e) and 8 h (f). WRN −/− cells were used as a control (g). (B) Percentage of cells with nucleolar localization of WRN was plotted as a time course. Cells were allowed to incubate in fresh medium after the removal of the damaging agent. At different time points cells were processed for indirect immunolabeling to visualize WRN distribution. At each point the field was selected randomly and the number of cells showing WRN localization in the nucleolus was scored. At least 100 cells were scored at each point. (◆) Undamaged cells, (+) 4NQO (0.1 μg/ml for 10 h), (△) bleomycin (10 mg/ml for 6 h), (□) hydrogen peroxide (250 μM, 30 min), (×) MMS (1 mM, 1 h), (○) mytomycin C (10 μg/ml, 10 h).

localization did not change significantly. We used up to a maximum of 40 J/m^2 UV and the TFIIH distribution increased with the increasing dose of UV. TFIIH distribution was restored after 4 h. There was no significant change in WRN localization although TFIIH localization was restored (Fig. 3b). In the bottom panel, WRN deficient primary fibroblasts were used for analysis of the distribution of TFIIH after UV irradiation. As seen in Fig. 3b, the TFIIH

distribution profile after UV irradiation in WRN deficient cells followed the same pattern as in normal cells. To see the localization of WRN after UV irradiation, we colabelled cellular WRN with nucleolin (C23). Nucleolin is the most abundant protein in the nucleoli and it is highly concentrated in the nucleolus compared with other parts of the cell, and thus it is an excellent marker of the nucleolus.

As seen in Fig. 3c, cellular WRN and nucleolin are colocalized in control cells. After irradiation with UV at different doses the distribution of WRN changes to a lesser extent compared to what we observed for the other DNA damaging agents that we used. The nucleolin localization did not change at all after UV. As a positive control we labeled WRN and nucleolin simultaneously in mytomycin treated cells. Here, WRN distribution changes while nucleolin remains in the nucleolus. These results suggest that WRN and nucleolin might have a common function, and they also support the conclusion that WRN leaves the nucleolus after damage.

3.4. DNA damage specific association of WRN with DNA

We then explored whether the WRN foci were associated with DNA after the cellular stress. Permeabilized cells were treated with DNAse for 15 min and washed extensively before fixation. Cellular localization of WRN was visualized by indirect immunolabeling. As seen in Fig. 4a, the distinct WRN foci induced by MMC or MMS were absent after DNAse treatment. The overall intensity of WRN foci in the DNAse treated cells was reduced. All images were captured by a Zeiss microscope with equal time exposure. In the undamaged or UV treated cells WRN distribution and the intensity of the protein was not reduced significantly. Thus,

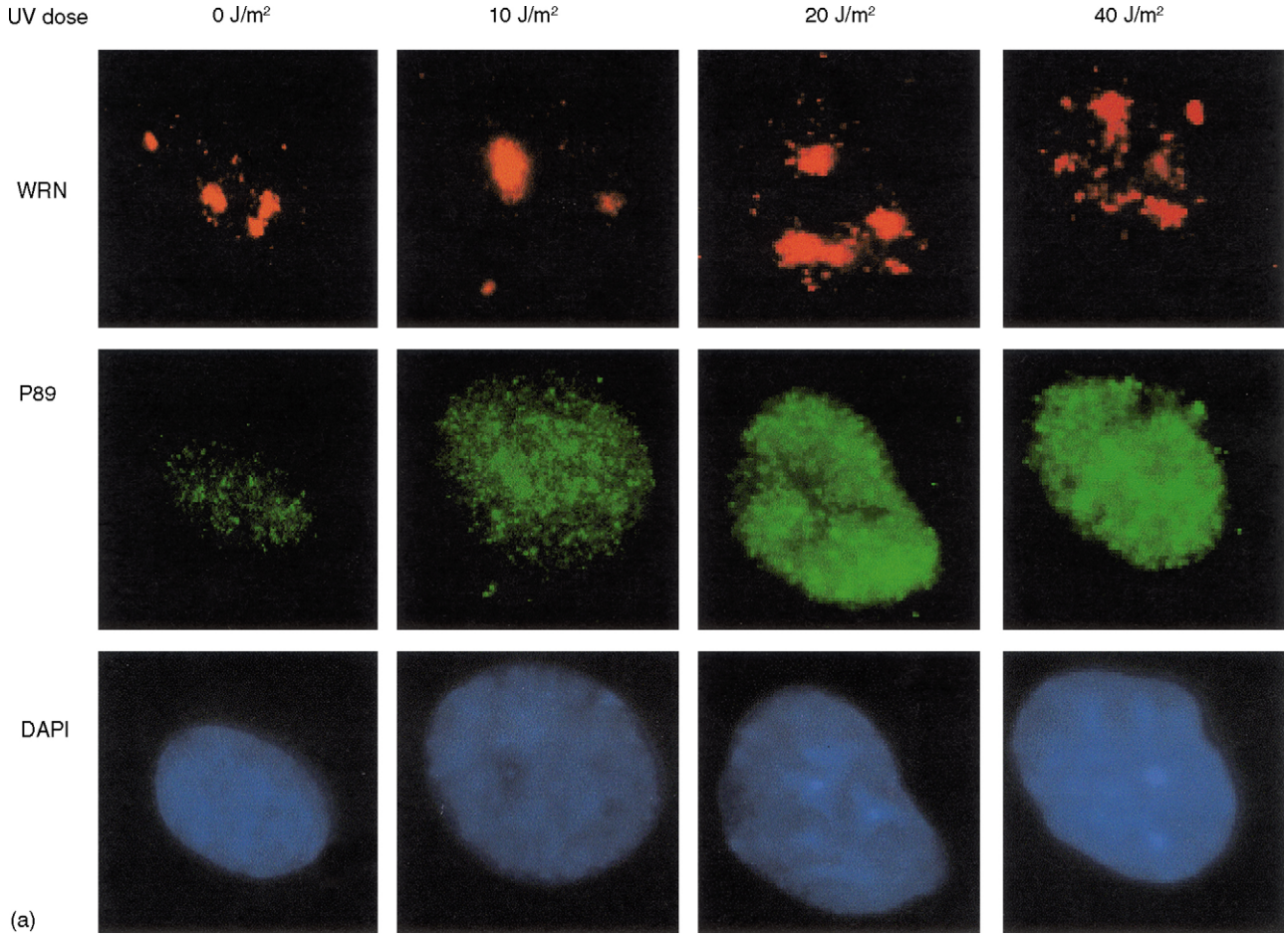


Fig. 3. WRN is not associated with UV induced damage processing (a) Human primary fibroblast cells (MRC 5) were irradiated with different doses of UV and after 30 min incubation cells were immunolabeled simultaneously for cellular localization of WRN and P89 (top panel). Lower panel shows the DAPI staining of the same cells. (b) Cells were incubated in fresh medium after UV irradiation (20 J/m^2) to allow repair. At different time point cells were collected and immunolabeled simultaneously for cellular localization of WRN (top) and p89 (middle). In the bottom panel WRN deficient primary fibroblasts AG03141C were immunolabeled for p89. WRN primary fibroblast cells were irradiated with UV (20 J/m^2) and incubated in the fresh medium. At different time point cells were collected and immunolabeled for p89. DAPI staining of the same cells is shown. (c) Colocalization of nucleolin (c23) and WRN in G1 arrested human HeLa ceils. Cells were irradiated with UV (10 J/m^2 and 20 J/m^2) or treated with mytomycin C, and then processed for simultaneous detection of c23 (green) and WRN (red). The right vertical panel shows the merged picture of c23 and WRN.

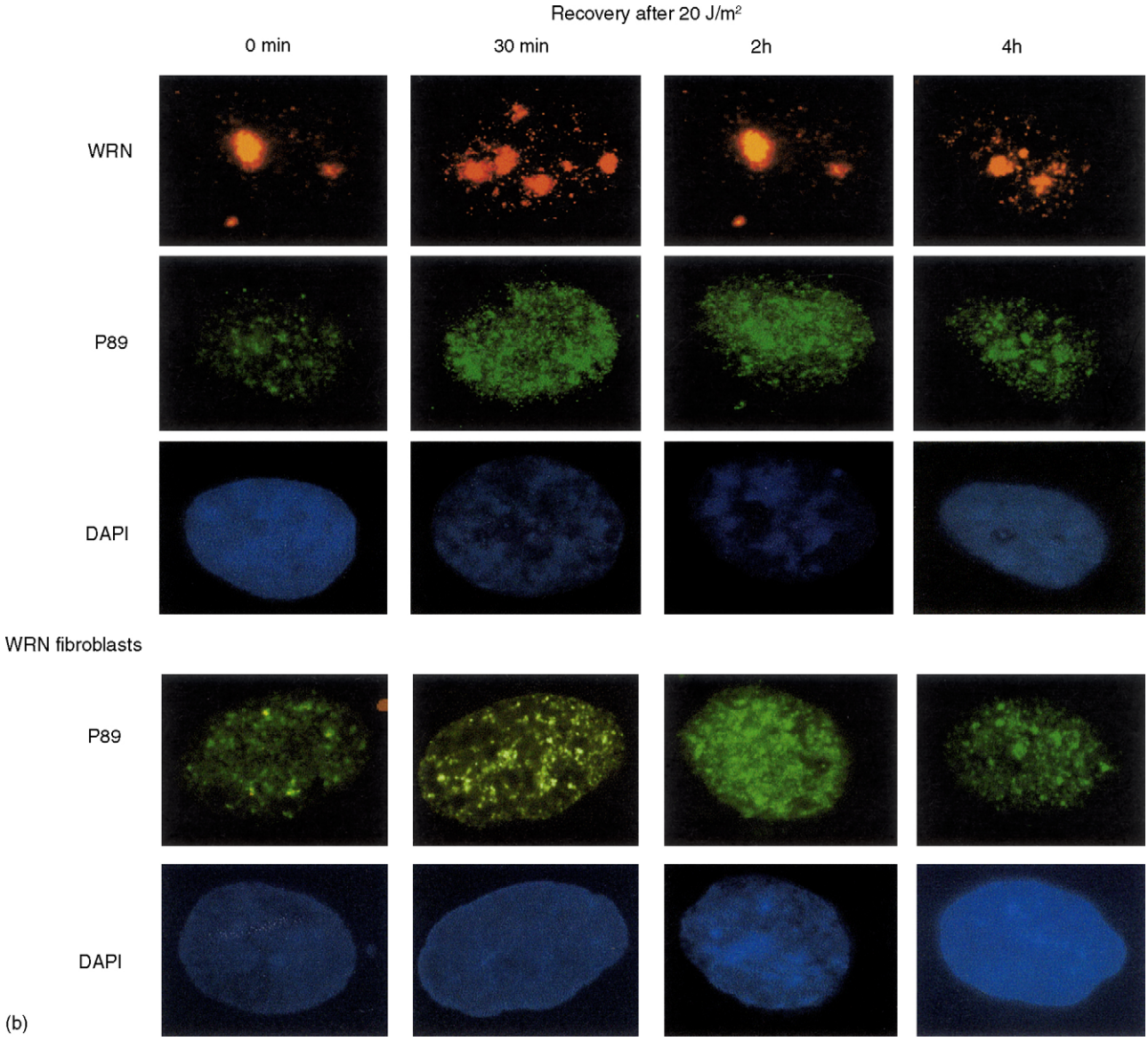


Fig. 3. (*Continued*).

WRN seemed to be associated with DNA after DNA damage induced by DNA damaging agents and again, much less so after UV and in undamaged cells. In Fig. 4b, the intensity of WRN foci were calculated in the DNAse treated and non-treated cells. It is evident that the total intensity of WRN foci in the DNAse treated cells decreased significantly in all cases except for undamaged and UV treated cells. At least 50 cells were analyzed for each measurement. Thus, the foci formed by WRN in the cells damaged with agents other than UV are DNAse sensitive. This supports the hypothesis that WRN is involved with DNA damage metabolism.

To further characterize this observation we used a biochemical approach. Cells were arrested at G1 and treated with different damaging agents as mentioned in the experimental procedure section. Total cellular soluble and insoluble protein fractions were isolated and immunoblotted with WRN antibody. As seen in Fig. 4c, WRN was found in both the soluble and insoluble fraction of undamaged and UV irradiated cells. This is in contrast to the situation in MMC and MMS treated cells, where there was relatively less WRN in the soluble compared to the insoluble fraction. As a control we probed the same blot with PCNA. PCNA was associated to a greater extent with the insoluble fraction in all the damaged cells (UV, MMC or MMS). The ratio of WRN in the soluble:insoluble fractions was quantified as 1:1.5 for undamaged cells, 1:1.7 for UV-irradiated cells, 1:2.5 for MMC treated cells and 1:3 for MMS treated cells (Fig. 4d). In undamaged cells, PCNA was found both in the soluble and insoluble fraction. To check the purity of the insoluble fraction, the blot was probed with lamin A/C, which is present only in the insoluble fraction. Normalized to the lamin A/C in each lane, the values were 0.85 for

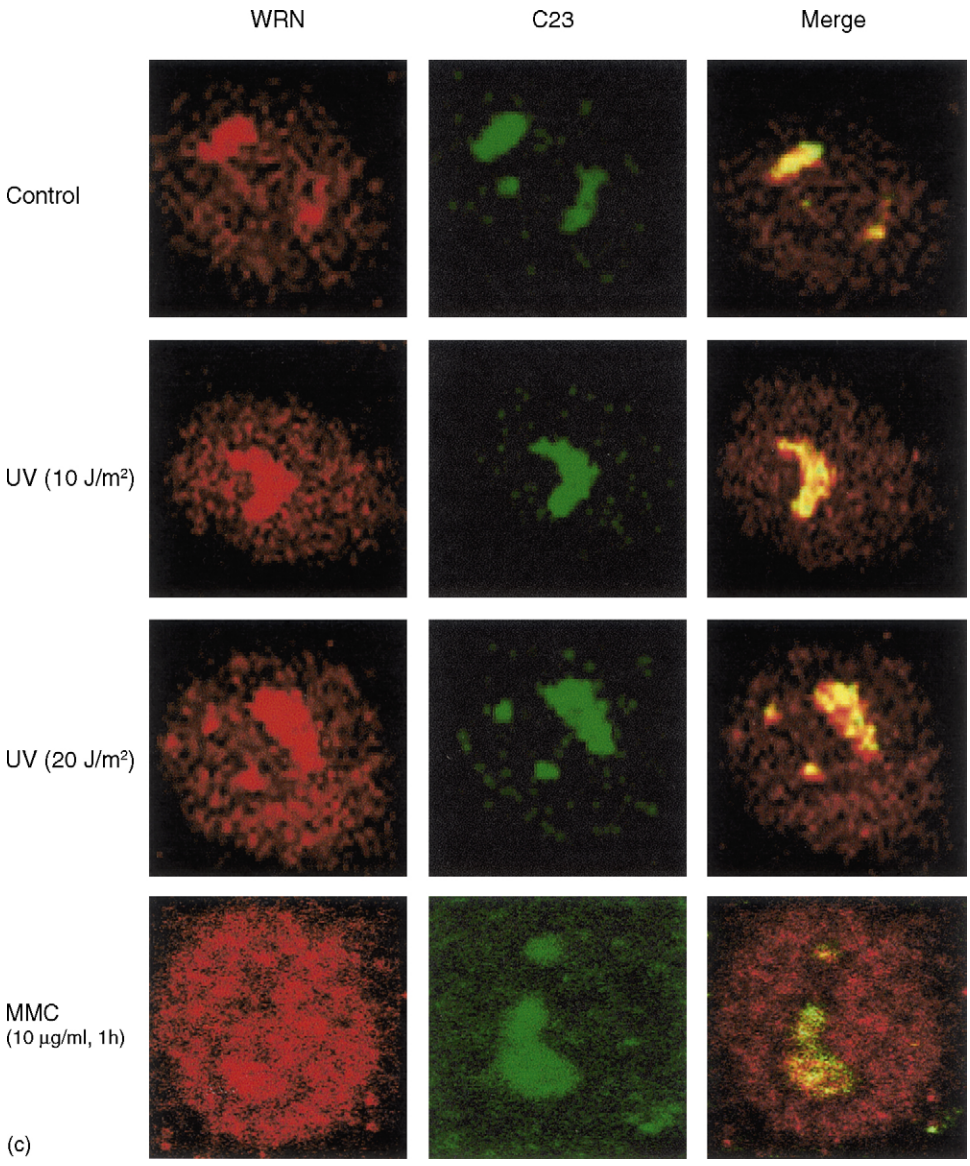


Fig. 3. (*Continued*).

undamaged cells, 0.93 for UV irradiated cells, 1.17 for MMC treated cells and 1.43 for MMS treated cells. Again, the ratio for UV resembles that in undamaged cells, whereas it is higher for the other kinds of DNA damage.

3.5. WRN trafficking may be regulated by acetylation and DNA damage specific phosphorylation

It has been observed that post-translational modulation of different proteins can regulate their catalytic activities. Since WRN relocalization to the nucleoplasm is DNA damage specific, we next examined whether post-translational modulation of WRN might be involved in the regulation of the dynamic changes in WRN localization. We immunoprecipitated WRN from whole cell lysates and immunoblotted with specific antibodies against phosphorylated ser/thr, phosphorylated tyrosine or lysine acetylation. As seen in Fig. 5, WRN was acetylated after MMC or MMS treatment but not after UV or in undamaged cells (middle panel). This is in general agreement with a previous study (Blander et al., 2002). Also, MMC induced more tyrosine phosphorylation compared with other treatments (top panel). Moreover, we observed that WRN was further phosphorylated after MMC and MMS treatment at ser/thr residues, but not after UV. Thus, the catalytic function of WRN may require post-translational modulation, which in turn is closely associated with specific DNA repair pathways.

3.6. Wortmannin regulates WRN restoration

We wanted to further explore how phosphorylation regulated WRN distribution. Cells were treated with the PI3 kinase inhibitor wortmannin for 1 h prior to exposure to

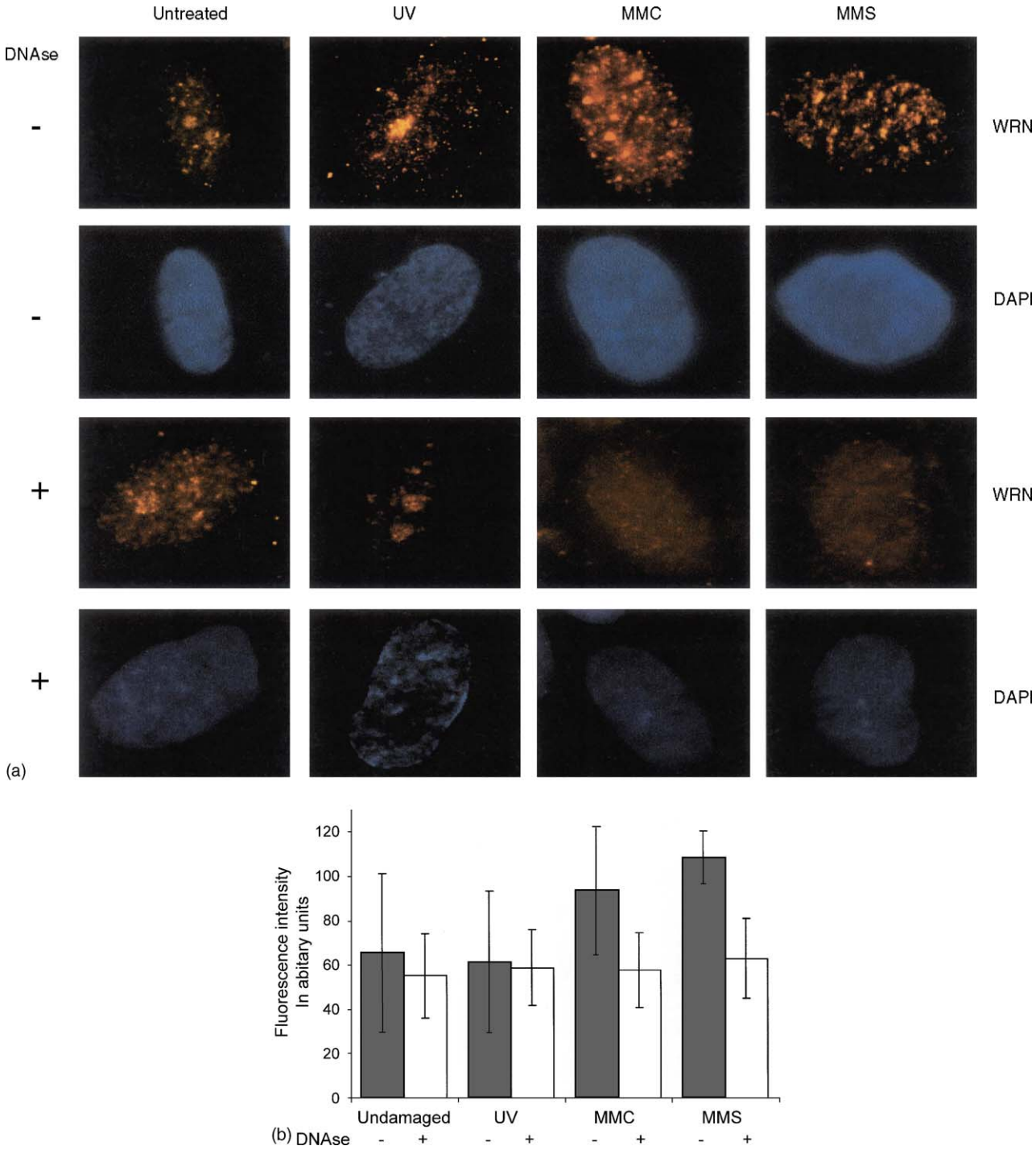


Fig. 4. (a) WRN foci are DNAse sensitive. HeLa cells were mock treated or treated with UV, MMC or MMS. Cells were permeabilzed with 0.5% Triton X-100 in PBS and treated with RNAse free DNAse (10 U) in 100 mM in 0.1 M sodium acetate, 5 mM $MgSO_4$, at 37 °C for 10 min before fixing. The cells were then immunolabeled for WRN protein. Top panels show the distribution of WRN along with the DAPI staining in cells not treated with DNAse. Bottom panels show the WRN and DAPI staining in the DNAse treated cells. (b) Total fluorescence intensity of the cells staining with WRN was calculated using Adobe photoshop software. At least 50 cells were scored at each time point. (■) Control, (□) DNAse treated. (c) Western blot analysis of distribution of WRN. Synchronized HeLa cells were mock-treated or treated with DNA damaging agents. After incubation, cellular soluble and insoluble proteins were isolated as described in Section 2. Approximately 50 μg of protein from each fraction was loaded on a 4–15% Tris–glycine polycrylamide gel and transferred to a PVDF membrane. The membrane was immunoblotted with mouse monoclonal anti-WRN (PharMingen, 1:500, top panel), lamin A/C (Santa Cruz, 1:1000, middle panel) or PCNA (Santa Cruz, 1:500, lower panel). The resulting signal was visualized with ECL plus Western blotting detection system (Amersham Biosciences). S: soluble; I: insoluble. (d) Ratio of insoluble to soluble WRN protein after DNA damage. Total soluble and insoluble proteins were isolated (see Section 2) and immunobloted with WRN antibody. The intensity of the WRN band was measured by ImageQuant software (Molecular Dynamics).

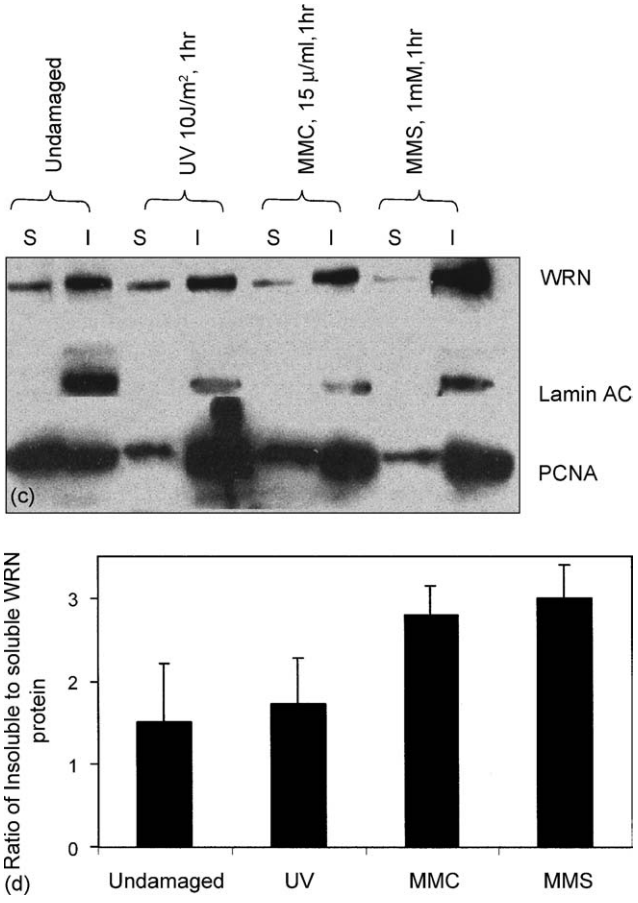


Fig. 4. (*Continued*).

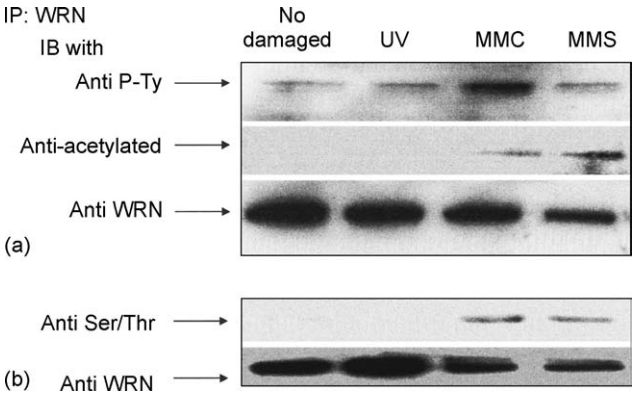


Fig. 5. (a) Post-translation modulation of WRN after DNA damage. Total cellular lysate was imunoprecipitated with WRN antibody (H 300, Santa Cruz) and the immunoprecipitated complex was isolated in a 4–15% Tris–glycine polycrylamide gel and transferred to PVDF membrane. The membrane was immunobloted with mouse monoclonal anti-phospho tyrosine (Sigma, 1:5000, top panel). The resulting signal was visualized with ECL plus a Western blotting detection system (Amersham Biosciences). The membrane was stripped and re-blotted with rabbit polyclonal anti-acetylated lysine (Cell Signaling, 1:1000, middle panel) and finally with mouse monoclonal anti-WRN (PharMingen, 1:500, top panell). (b) Ser/thr phosphorylation status of WRN after DNA damage. Total cell lysate was immunoprecipitated with WRN antibody and immunoblotted with anti-phospho ser/thr (Cell Signaling, 1:1000, top panel). The membrane was stripped and re-blotted with anti-WRN antibody (bottom panel).

different DNA damaging agents. After 1 h exposure, the damaging agents were removed and the cells were further incubated with complete medium in the presence or absence of wortmannin. As seen in Fig. 6a, wortmannin alone did not affect WRN distribution over the 24 h time period. In the MMC (column b) or MMS treated (column d) cells, WRN distribution is restored after 24 h, but in the presence of wortmannin (columns c and e, respectively), WRN relocalized from the nucleolus to the nucleoplasm and restoration of WRN after 24 h was absent. Thus, this suggests that PI3 kinase activity may not be involved in redistribution of WRN from the nucleolus to the nucleoplasm whereas inhibition of this kinase activity after DNA damage prevented restoration of WRN distribution from the nucleoplasm to the nucleolus. In Fig. 6b, we quantified the number of cells with WRN localization in the nucleolus after DNA damage in the presence and absence of wortmannin. At least 100 cells were counted from random fields. The number of cells containing WRN in the nucleolus increased after damage with MMC or MMS in the absence of wortmannin. Incubation with wortmannin for 24 h did not change WRN localization significantly (72% compared to 76%) in undamaged cells. Thus, inhibition of phosphorylation inhibited the restoration of WRN localization from nucleoplasm to the nucleolus.

4. Discussion

Enhanced genomic instability of cells derived from WS patients and the increased incidence of cancer in these patients supports a role of WRN in the maintanance of genomic integrity. Here, we report that several types of DNA damage mobilize WRN and cause its translocation from the nucleolus into discrete nucleoplasmic foci in a manner that depends on the nature of the DNA damage. While this relocation has been reported previously, characterization of WRN cellular functions in relation to the exposure of cells to different types of stress has not been examined. This relocation suggests a role for WRN in the DNA damage response, at least after some types of DNA damage. Different types of DNA damage are repaired by distinct DNA repair pathways. DNA base lesions are repaired by BER, interstrand crosslinks in DNA are repaired by recombination repair, and UV induced DNA photo products are removed by NER. WRN protein has been shown to be involved in processing DNA damage induced by agents that mainly cause DNA base damage (Blank et al., 2004) and DNA ds breaks (Chen et al., 2003), but not after UV. In extension of these past results, our present observations suggest a preferred role of WRN in BER and DNA ds repair compared to NER.

One way to investigate the DNA repair pathways in which WRN participates is to examine functional protein interactions. Since WRN has been found to bind to many proteins, we are primarily interested in those protein

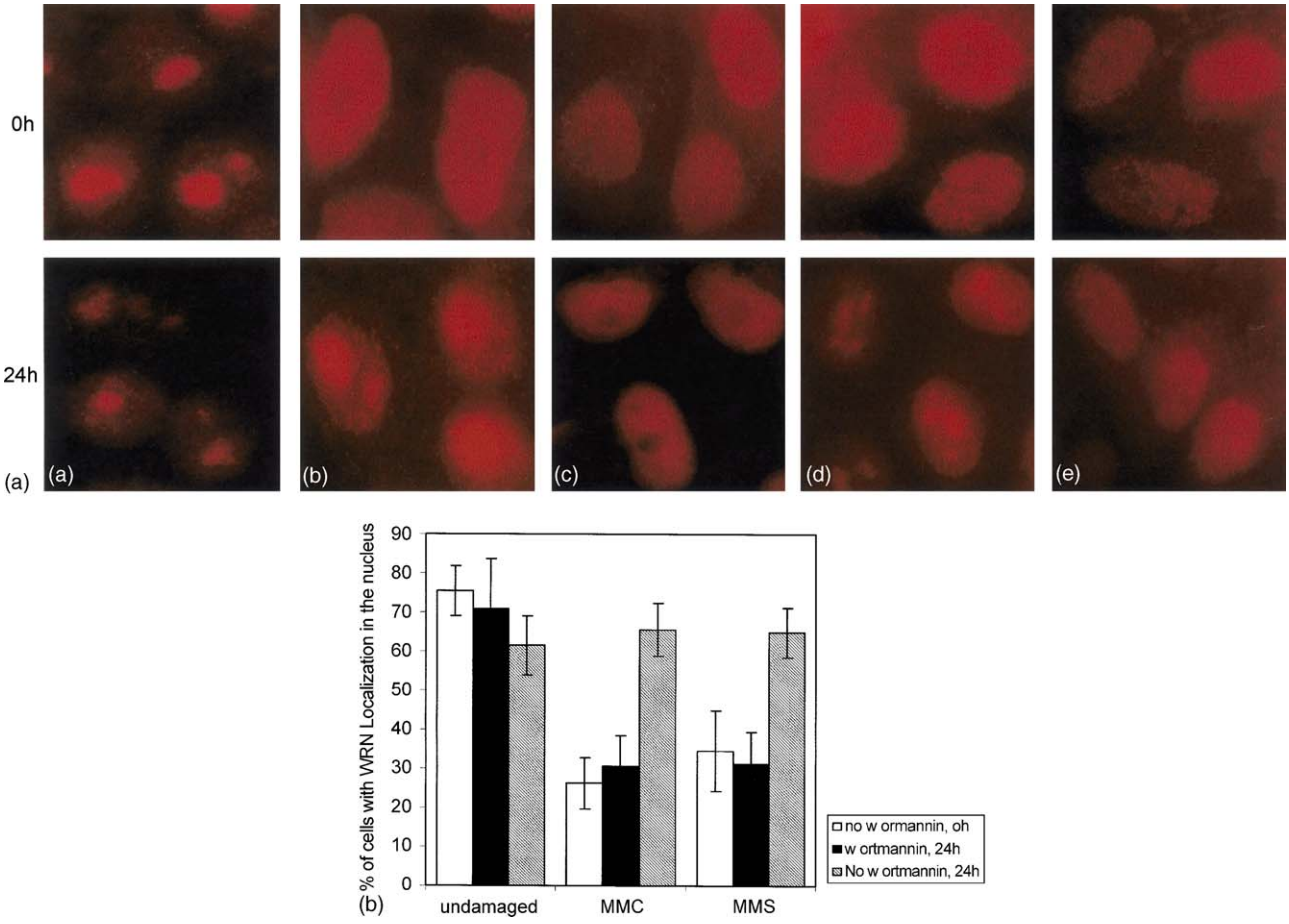


Fig. 6. (a) Cellular distribution of WRN in the presence of PI3 kinase inhibitor wortmannin (25 μM). Cells were treated with different DNA damaging agents in the presence (top panel, a, c, and e) or absence (top panel, b and d) of kinase inhibitors and processed for immunolabeling. Another sets of cells were incubated further in the presence (bottom panel, a, c, and e) or absence (panel, b and d) of kinase inhibitor for 24 h. After 24 h cells were processed for immunolabeling to visualize WRN localization (a) undamaged cells + wortmannin, (b) MMC treated cells, (c) MMC + wortmannin treated cells, (d) MMS treated cells and (e) MMS + wortmannin treated cells. (b) Bar diagram showing the effect of WRN localization in the presence of the kinase inhibitor wortmannin. HeLa cells were treated with MMC or MMS in the presence or absence of wortmannin for 1 h. After 1 h cells were washed and further incubated in the presence or absence of wortmannin. At each time point cells were processed for indirect immunolabeling for the visualization of WRN location. Percentage of cells showing nucleolar localization of WRN was calculated. At least 100 cells were scored at each time. The average of two independent experiments with standard deviation is plotted.

interactions that are also functional. Several reports are available about the interaction of WRN with DNA repair proteins mainly involved in BER and DNA DSB repair (Harrigan et al., 2003; Cooper et al., 2000). WRN is believed to play an active role in the processing of non-homologous end joining and its catalytic activities are regulated by the phosphorylation of WRN by DNA dependent protein kinase C (DNA–PKC) (Yannone et al., 2001; Karmakar et al., 2002). Thus, after the formation of DNA DSB, WRN becomes a part of the DNA–PK complex. Also, it has been proposed that WRN participates in the homologous recombination process through its interaction with RAD52 (Baynton et al., 2003) and the MRE11 complex (Cheng et al., 2004). WRN also interacts functionally with the BER proteins DNA polymerase β and Fen-1 (Harrigan et al., 2003; BroshJr. et al., 2001). Whereas WRN has protein partners in BER and recombination, there are no observations of protein interactions with NER proteins.

A primary localization of WRN in the nucleolus has been reported from different laboratories. After DNA damage (Sakamoto et al., 2001) or replication arrest (Constantinou et al., 2000a), WRN relocates from the nucleolus to the nucleoplasm and forms distinct foci. These foci colocalize with proteins involved in DNA repair or replication suggesting a potential role of WRN in DNA damage response pathways or in the restoration of arrested DNA replication forks. Thus, relocalization of WRN is the consequence of either arrested replication forks or other aspects of the DNA damage response. We have used HeLa cells in G1 phase or confluent primary fibroblasts in our study and thus, chances of ongoing replication are limited. Moreover, we have seen that the movement of WRN from the nucleolus to the nucleoplasm is DNA damage specific. UV irradiation does not affect the cellular distribution of WRN significantly (Fig. 1), When we labeled cells simultaneously for WRN and TFIIH, we observed that

TFIIH was redistributed to smaller foci throughout the nucleus in a dose dependent manner while WRN distribution was unaffected (Fig. 3a). TFIIH is the basal transcription factor also associated with NER (Araujo and Wood, 1999). Our data oppose a previous observation where it was suggested that WRN redistributed after UV irradiation (Blander et al., 2002). The dose of UV used in the other study was high (40 J/m^2), which may shut down transcription completely. It has been reported that WRN stimulates RNA polymerase II mediated transcription (Balajee et al., 1999) and possibly is also involved in RNA polymerase I mediated transcription (Shiratori et al., 2002). We have also seen that after inhibition of transcription by α-amanitin or actinomycin D, WRN relocalizes from the nucelolus to nucleoplasm (data not shown). Thus, WRN may relocalize from the nucleolus to the nucleoplasm after cellular transcription is inhibited even though there is no DNA damage. We have also demonstrated that WRN is acetylated after DNA base damage or after DNA DSB but not after UV (Fig. 5a), further supporting a lesser involvement of WRN in NER, if any at all. Taken together, WRN acetylation may be responsible for the relocation of WRN from nucleolus to nucleoplasm (Blander et al., 2002). We have also found that WRN is phosphorylated at the ser/thr residues when the cells were treated with MMC or MMS, but not after UV (Fig. 5b).

It has been shown that several nuclear proteins involved in DNA repair change their subnuclear locations after cellular stress and are concentrated at discrete foci. Such foci are believed to be active parts of DNA metabolism and consist of many protein complexes. Proteins involved in these foci are associated with damaged DNA and are resistant to detergent and sensitive to DNAse. For example, PCNA was found to be associated with damaged DNA in a detergent-resistant form after UV irradiation (Pagano et al., 1994) and the chromatin-bound form of PCNA was released by the use of DNAse I (Savio et al., 1996). In this study, we have seen that the association of cellular WRN with DNA is enhanced after particular types of DNA damage excluding UV treatment.

Post-translational modification of certain proteins act as a molecular signal which may be responsible for their dynamic behavior. Following DNA damage, p53 is phosphorylated on serine residues 15, 20, 33, and 37 within the amino-terminal domain. These phosphorylation events are thought to play a key role in regulating p53 stability and activity (Ashcroft et al., 1999). Recently, it was suggested that deacetylated WRN is localized in the nucleolus and that accetylation is a necessary step for its recruitment to the nucleoplasm (Blander et al., 2002). Consistent with this observation, we have also seen that WRN is acetylated in cells treated with MMC or MMS but not after UV irradiation (Fig. 5). Similarly, we have seen previously that after bleomycin and 4-NQO treatment WRN is phosphorylated at the ser/thr residues by DNA–PKCs (Karmakar et al., 2002). In the present study, we have further seen that WRN is phosphorylated after MMC or MMS treatment but not after UV. Taken together it is likely that cellular WRN trafficking and catalytic activity is regulated by acetylation and phosphorylation, respectively, which in turns may be related to specific DNA damage response pathways.

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