Accumulation of soluble and nucleolar-associated p53 proteins following cellular stress

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INTRODUCTION

The tumor suppressor p53 is a nucleocytoplasmic shuttling protein that accumulates in the nucleus of cells exposed to various cellular stresses. One important role of nuclear p53 is to mobilize a stress response by transactivating target genes such as the p21WAF1 gene. In this study, we investigated more closely the localization of p53 in cells following various stresses. Immunocytochemistry of fixed human fibroblasts treated with either UV light, the kinase and transcription inhibitor DRB or the proteasome inhibitor MG132 revealed abundant p53 localized to the nucleus. When cells treated with UV or DRB were permeabilized prior to fixation to allow soluble proteins to diffuse, the nuclear p53 signal was abolished. However, in cells treated with MG132, residual p53 localized to distinct large foci. Furthermore, nucleolin co-localized with p53 to these foci, suggesting that these foci were nucleolar structures. Interestingly, the MDM2 protein was found to co-localize with p53 to nucleolar structures following proteasome inhibition. Our results suggest that the p53 proteins accumulating in the nucleus following UV-irradiation or blockage of transcription are freely soluble and, thus, should be able to roam the nucleus to ensure high occupancy of p53 binding sites. However, inhibition of proteasome activity may be a unique stress in that it leads to the sequestering of p53 proteins to the nucleolus, thereby blunting the p53-mediated transactivation of target genes.

SUMMARY

The tumor suppressor p53 is a nucleocytoplasmic shuttling protein that accumulates in the nucleus of cells exposed to various cellular stresses. One important role of nuclear p53 is to mobilize a stress response by transactivating target genes such as the p21WAF1 gene. In this study, we investigated more closely the localization of p53 in cells following various stresses. Immunocytochemistry of fixed human fibroblasts treated with either UV light, the kinase and transcription inhibitor DRB or the proteasome inhibitor MG132 revealed abundant p53 localized to the nucleus. When cells treated with UV or DRB were permeabilized prior to fixation to allow soluble proteins to diffuse, the nuclear p53 signal was abolished. However, in cells treated with MG132, residual p53 localized to distinct large foci. Furthermore, nucleolin co-localized with p53 to these foci, suggesting that these foci were nucleolar structures. Interestingly, the MDM2 protein was found to co-localize with p53 to nucleolar structures following proteasome inhibition. Our results suggest that the p53 proteins accumulating in the nucleus following UV-irradiation or blockage of transcription are freely soluble and, thus, should be able to roam the nucleus to ensure high occupancy of p53 binding sites. However, inhibition of proteasome activity may be a unique stress in that it leads to the sequestering of p53 proteins to the nucleolus, thereby blunting the p53-mediated transactivation of target genes.

Key words: UV light, Proteasome inhibitors, Transcription, Nucleolus, MDM2

INTRODUCTION

The ability of cells to arrest at cell-cycle checkpoints following exposure to various cellular stresses is crucial for the avoidance of mutagenesis and to ensure genomic stability. The tumor suppressor p53 plays a critical role in the regulation of many aspects of cellular stress response, including DNA repair and apoptosis. p53 is a member of the p53 family, which plays a key role in the cellular response to DNA damage and genomic instability. p53 is a transcription factor that can bind to DNA and activate the transcription of genes involved in cell cycle arrest, apoptosis, and DNA repair.

MDM2 is thought to regulate nuclear export of p53 (Boyd et al., 2000; Geyer et al., 2000; Roth et al., 1998; Tao and Levine, 1999). Under non-stressed condition there is a low level of p53 in the nucleus. Some of these nuclear p53 proteins are soluble, whereas others are anchored to either nucleoplasmic or nucleolar structures (Rubbi and Milner, 2000). The nuclear attachment of p53 is primarily RNA-dependent because RNase treatment of permeabilized nuclei was shown to release most of the anchored p53 (Rubbi and Milner, 2000). In the present study we investigated whether the p53 proteins accumulating in the nucleus of human fibroblasts following specific cellular stresses could be solubilized by cell permeabilization. Our results show that p53 proteins accumulating in the nucleus following exposure to UV light or DRB are readily solubilized by permeabilization. We propose that by flooding the nucleus with freely diffusible p53 proteins following cellular stress, the cell increases the odds of p53 occupancy at p53 response elements in the chromatin and ensures high transactivation of target genes. Following proteasome inhibition however, a subpopulation of p53 proteins accumulating in the nucleus was found to be firmly attached a few large nuclear foci. Both nucleolin and MDM2 co-localized with p53 to these foci. Furthermore, no increase in transactivation of the p53 target gene p21WAF1 was observed following accumulation of p53 by proteasome inhibition. These results suggest that different cellular stresses cause

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nuclear accumulation of p53 by different mechanisms and that the ability of p53 to transactivate target genes may depend on the ability of p53 to freely diffuse within the nucleus.

MATERIALS AND METHODS

Cell cultures and treatments
Human neonatal diploid fibroblasts (a gift from Mary Davis, University of Michigan) were grown as monolayers in dishes or on coverslips in MEM supplemented with 15% fetal calf serum, 2% vitamins, 2x amino acids and 1x antibiotics. Cells were grown to confluence before the start of each experiment. Cells were irradiated with 30 J/m² UV light (254 nm) at room temperature at a fluency of 0.6 J/m²/second (UVX radiometer, UVP Inc) and then incubated at 37°C for indicated periods of time. For the chemical treatments, cells were incubated with either 100 μM DRB or 10 μM MG132 at 37°C for indicated periods of time.

Immunocytochemistry
For immunocytochemistry, cells were washed once with PBS, fixed for 30 minutes in 1:1 methanol/acetic at −20°C followed by three rinses in PBS. After aspiration, 90 μl of primary antibody solution (see below) was quickly added to the coverslips. After four hours of incubation at room temperature, the antibody solutions were aspirated and the coverslips were washed three times for 5 minutes each with PBSBT (PBS, 5 g bovine serum albumin, 500 g/metric ton Tween 20 per litre PBS) on a slow moving rocker. The coverslips were then incubated for 1 hour with 90 μl of secondary anti-rabbit or anti-mouse antibody (Sigma) conjugated to FITC and diluted 1:100 in PBSBT. Following completion of incubation, the antibody solutions were aspirated and the samples were incubated with a propidium iodide solution (1 μg Pi/ml PBSBT) for 5 minutes on a rocker. The coverslips were then washed twice with PBSBT and briefly dried off and mounted on a microscope slide with 1 drop of Vectashield (Vector Laboratories Inc.). A Zeiss Akioskop microscope with Plan-NEUFLUAR 63×1.25 oil lens and Microimage i308 digital camera was used for viewing and for digital photography. Contrast of digital images was adjusted with Adobe PhotoShop software. Primary antibodies were as follows: supernatants from hybridoma cells expressing either anti-p53 mAb 1801 or anti-p53 Ab1 were used undiluted, and the FL-393 anti-p53 rabbit antibody (Santa Cruz Biotechnology Inc.) was used in a dilution of 1:500 in PBSBT. For nucleolin, C23 (MS-3) (Santa Cruz) anti-rabbit antibody (Santa Cruz Biotechnology Inc.) was used in a dilution of 1:500 in PBSBT. For MDM2, a monoclonal antibody supplied by Jiayuh Lin, University of Michigan was used undiluted. Permeabilization of cells was carried out as previously described (Rubbi and Milner, 2000). Briefly, cells were washed with TBS (150 mM NaCl, 10 mM Tris, 5 mM MgCl₂, pH 7.4), aspirated and then briefly washed with TBS-G (TBS, 25% glycerol, 0.5 mM EGTA). Following aspiration, the cells were incubated for 4 minutes in TBS-G-TX (TBS-G, 0.05% Triton X-100). Finally, after aspiration the cells were incubated for 20 minutes in solution S (100 mM KCl, 50 mM Tris, 10 mM MgCl₂, 0.5 mM EGTA, 25% glycerol) on a slow moving rocker. For RNase treatment, 200 U/ml RNase A were added to the S buffer as previously described (Rubbi and Milner, 2000). After permeabilization, cells were washed once with PBS and fixed as described above.

Northern blot
Human fibroblasts were either mock-treated or treated with 30 J/m², 100 μM DRB or 10 μM MG132. Sixteen hours later, total RNA was isolated from these cells using Trizol (GibcoBRL). The RNA samples were loaded 10 μg/lane on a 1.2% agarose/formaldehyde gel and, following electrophoresis, the RNA was transferred onto Nitro nylon membranes (Schleicher&Suell). Blots were hybridized with p21WA F1 and β-actin cDNA probes radioactively labelled in a RT-PCR procedure with [α-32P]deoxyctydine triphosphate (dCTP) (Amersham Pharmacia Biotec). Following exposure to X-ray film (6-12 hours), the film was scanned and the intensities of the p21WA F1 bands were analyzed using NIH Image 1.62 software. The intensities of the p21WA F1 bands were then normalized to actin expression and expressed relative to mock-treated control cells.

RESULTS
To study whether the p53 proteins accumulating in the nucleus following cellular stresses are soluble or attached to nuclear structures, diploid human fibroblasts were mock treated or exposed to either UV light (30 J/m²), the RNA polymerase II inhibitor DRB (100 μM) or the proteasome inhibitor MG132 (10 μM). Following a 16-hour incubation, the cells were either directly fixed in ice-cold methanol/acetone (1:1) or permeabilized prior to fixation to remove any soluble proteins. The fixed cells were then incubated with anti-p53 antibodies followed by FITC-conjugated secondary antibodies.

It was found that untreated cells stained very weakly for p53, whereas exposure to UV light, DRB or MG132 resulted in a strong nuclear p53 staining (Fig. 1A-D). However, when cells
were permeabilized prior to fixation, the p53 staining was abolished in cells treated with UV light or DRB, while some residual distinct nuclear staining was found in MG132-treated cells (Fig. 1E-H). These results suggest that most of the p53 proteins accumulating in the nucleus following these stresses are not tightly attached to nuclear components but rather, may be free to diffuse throughout the nucleus. However, a sub-population of p53 localized and were firmly attached to a few bright nuclear foci following proteasome inhibition.

We next investigated the time course for the formation of the p53 foci following proteasome inhibition. Cells were treated with 10 μM MG132 for 2, 4 or 8 hours followed either by direct fixation or permeabilization prior to fixation. The results show that nuclear accumulation of p53 was detectable after only 2 hours of MG132 treatment (Fig. 2A-D). Following permeabilization, the formation of stable nuclear p53 foci was observed in some cells after 4 hours of MG132 treatment while these foci had formed in the majority of the cells after 8 hours (Fig. 2E-H). These observations suggest that nuclear accumulation of p53 preceded the attachment of p53 to the nuclear foci in cells treated with MG132.

Next we examined the staining pattern of another nucleocytoplasmic shuttling protein, nucleolin, in cells exposed to UV light, DRB or MG132. It was found that mock and MG132-treated cells showed nucleolin staining of nuclear foci (Fig. 3A,D) which probably represent nucleolar structures (Ginisty et al., 1999). The nuclear staining of nucleolin was somewhat weakened following UV-irradiation (Fig. 3B) and was almost completely lost in cells treated with DRB (Fig. 3C). This attenuated nucleolin staining may be related to the deterioration of nucleolar structures which has been shown to occur following inhibition of transcription by DRB (Brasch, 1990; Haaf and Ward, 1996). When cells were permeabilized prior to fixation the nucleolar staining of nucleolin disappeared (Fig. 3E). This suggests that nucleolin is normally free to diffuse in cells as has been previously observed for another nucleolar protein, fibrillarin (Phair and Misteli, 2000). Similar to p53, nucleolin was found to be firmly anchored to nucleolar structures in MG132-treated cells even following permeabilization (Fig. 3H).

To explore whether the localization of p53 to the nucleolus in MG132-treated cells involved binding to RNA, we permeabilized MG132-treated cells and incubated the cells for 30 minutes with RNase followed by fixation and subsequent

![Fig. 2. Time course for MG132-induced p53 nuclear foci formation.](image)

Human fibroblasts were treated with 10 μM MG132 for different periods of time before being either directly fixed in methanol/acetone (A-D) or permeabilized prior to fixation (E-H). Staining was performed as in Fig. 1, except PI was applied only to permeabilized cells (E-H).

![Fig. 3. MG132 stabilizes the association of nucleolin with nucleolar structures whereas DRB abolishes nucleolin staining.](image)

Human fibroblasts were treated as described in Fig. 1 and either directly fixed in methanol/acetone (A-D) or permeabilized prior to fixation (E-H) and stained with anti-nucleolin antibodies and secondary anti-mouse antibodies conjugated with FITC (green). DNA was stained with PI (red).
immunocytostaining of p53 (Rubbi and Milner, 2000). The results show that the staining of p53 in the nucleoli was not diminished by RNase-treatment, suggesting that the tethering of p53 to the nucleolus in MG132-treated cells was not mediated by RNA-containing structures (Fig. 4A-C). To confirm that the staining observed in the nucleolus was specific for p53, we used additional anti-p53 antibodies for staining. We observed a similar staining pattern of p53 localization to nuclear foci with these alternative antibodies, confirming that a subpopulation of p53 proteins firmly associates to distinct nuclear foci following proteasome inhibition (Fig. 4D,E).

To examine whether p53 and nucleolin co-localized to the nuclear foci following MG132 treatment, cells were immunostained with both anti-p53 and anti-nucleolin antibodies. It was found that p53 and nucleolin co-localized following proteasome inhibition both in directly fixed cells (Fig. 5A-C) and in permeabilized cells (Fig. 5D-F). These results indicate that the nuclear foci that p53 proteins localize to following proteasome inhibition are probably nucleoli. We also investigated the localization of the MDM2 protein following MG132 treatment. MDM2 has been suggested to regulate the nuclear export of p53 (Roth et al., 1998; Tao and Levine, 1999). Immunostaining of both p53 and MDM2 in MG132-treated cells showed that some MDM2 proteins co-localized with p53 to nuclear foci both in cells directly fixed (Fig. 5G-I) and in cells permeabilized prior to fixation (Fig. 5J-L). These results suggest that proteasome function is required for proper nuclear export of both p53 and MDM2. Furthermore, the accumulation of p53 and MDM2 to nucleolar structures suggests that the nuclear export of p53 and MDM2 occurs via the nucleolus.

To investigate whether the p53 proteins accumulating in the cells following MG132 treatment were able to transactivate the target gene p21WAF1, we performed northern blot experiments. It was found that the steady state levels of p21WAF1 mRNA were slightly elevated in cells treated with 30 μM or 100 μM DRB (Fig. 6). However, we did not detect any major increase in the steady state level of p21WAF1 mRNA in MG132-treated cells despite the finding that MG132 induced a significant increase in the level of p53 in the nucleus of these cells. This result is in agreement with previous reports showing that proteasome inhibitors only modestly increase p21WAF1 mRNA levels (about twofold) in diploid human fibroblasts (Chen et al., 2000; Maki et al., 1996). By contrast, studies using the breast cancer cell line MCF-7 have shown that p21WAF1 mRNA levels are increased significantly following proteasome inhibition (Blagosklonny et al., 1996; Zimmermann et al., 2000), suggesting that the regulation of p21WAF1 differs between normal and cancer cells.
Northern blot examination of light or DRB and not induced by MG132 in diploid fibroblasts. The intensities of the p21 \textit{WA F1} bands were normalized to the intensities of the actin bands, which were used as loading controls. The values shown in the figure are expressed relative to control cells.

\section*{DISCUSSION}

In this study, we investigated whether p53 proteins accumulating in the nucleus following various cellular stresses are anchored to nuclear structures or whether they are free to diffuse in the cell. We found that p53 proteins accumulating following exposure to UV light or the transcription inhibitor DRB were readily solubilized from the nucleus following cell permeabilization. In cells treated with the proteasome inhibitor MG132, however, some of the nuclear p53 proteins were firmly attached to a few large foci. The attachment of p53 to these structures did not appear to involve RNA because treatment with RNase did not remove the p53 staining. Furthermore, two other nucleocytoplasmic shuttling proteins, nucleolin and MDM2, were found to become firmly attached to these nuclear foci following proteasome inhibition.

The mechanism by which p53 accumulates in the cell nucleus after exposure to various cellular stresses is not fully understood (Ljungman, 2000). It has been suggested that the MDM2 protein is important in directing the nuclear export and degradation of p53 under non-stressed conditions (Boyd et al., 2000; Freedman et al., 1999; Geyer et al., 2000). Following some types of stresses, however, the p53 protein is modified so that the interaction between p53 and MDM2 is attenuated (Giaccia and Kastan, 1998; Jayaraman and Prives, 1999). Phosphorylations of the Ser15, Ser20 and Thr18 sites of p53 have been shown to activate the sequence-specific DNA-binding activity, whereas the Ser15 and Lys382 sites of p53. Thus, our results do not support a model in which modifications of the p53 at Ser15 and Lys382 determine the localization of p53 following stress. However, p53 can be modified at many sites and therefore our limited data do not allow us to rule out that modifications of other sites may play a role in p53 localization. It is tempting to speculate, based on the finding that p53 and MDM2 co-localized to nucleolar structures following proteasome inhibition, that these proteins normally exit the nucleus via the nucleolus in a proteasome-dependent manner. However, our observation that the association of p53 proteins with nucleolar structures did not occur until after appreciable amounts of p53 had already accumulated in the nucleus suggests that blockage of nuclear export via the nucleolus is not chiefly responsible for the nuclear accumulation of p53 following proteasome inhibition.

Under normal conditions, p53 is actively exported out of the nucleus and degraded in a proteasome-dependent manner (Ljungman, 2000). To fully activate p53-inducible genes following cellular stress, p53 proteins need to become stable, their sequence-dependent DNA-binding domains must be activated and they need to localize to the cell nucleus. Treatment of cells with either DRB or MG132 leads to nuclear accumulation of p53 proteins but because the p53 proteins are not modified at certain key residues, the sequence-specific DNA-binding activity may not be fully activated. However, the brute increase in p53 protein levels in the nucleus is expected to be sufficient to increase transactivation of downstream genes to a certain extent (Blagosklonny et al., 1996; Chen et al., 2000; Ljungman et al., 1999; Maki et al., 1996; Siliciano et al., 1997; Zimmermann et al., 2000). By contrast, UV-irradiation induces nuclear accumulation of p53 proteins modified at many sites that have been shown to activate the sequence-specific DNA

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig6.png}
\caption{The p53 target gene \textit{p21 WAF1} is induced only weakly by UV light or DRB and not induced by MG132 in diploid fibroblasts. Northern blot examination of \textit{p21 WAF1} mRNA levels were performed either 16 hours after UV irradiation (30 J/m²), or following 16 hours of incubation in the presence of DRB (100 μM) or MG132 (10 μM). The intensities of the \textit{p21 WAF1} bands were normalized to the intensities of the actin bands, which were used as loading controls. The values shown in the figure are expressed relative to control cells.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig7.png}
\caption{Nuclear accumulation of p53, protein modifications and the ability of p53 to freely diffuse in the nucleus regulate p53-mediated transactivation in cells. Under normal conditions, the low level of p53 (small circles) amounts to only a low basal level of transactivation. However, following exposure to DNA-damaging agents (UV light), transcription inhibitors (DRB and UV light) or proteasome inhibitors (MG132), p53 accumulates to high levels in the nucleus. UV light induces p53 protein modifications (filled small circles) that stimulate its sequence-specific DNA-binding activity, whereas DRB and MG132 do not. Furthermore, p53 is freely diffusible in cells treated with UV light or DRB but a population of p53 proteins is sequestered to nucleolar structures (filled large circles) in MG132-treated cells. Both UV light and DRB will at higher doses inhibit transcription, whereas MG132 sequester p53 resulting in a blunted p53 response.}
\end{figure}
binding of p53 (Dumaz and Meek, 1999; Sakaguchi et al., 1998; Shieh et al., 1997; Siliciano et al., 1997). As expected, UV-irradiation induces a strong transactivation of p53 response genes especially in reporter assays where the target gene is small (Lu and Lane, 1993). However, expression of endogenous p53-inducible genes following UV-irradiation is not that impressive, especially at higher doses where transactivation is expected to be interfered with by transcription-blocking lesions (Ljungman and Zhang, 1996; Ljungman et al., 1999; Lu et al., 1996; McKay et al., 1998; Perry et al., 1993; Reinke and Lozano, 1997). Similarly, the transactivation by p53 in DRB-treated cells becomes limited by the effects DRB has on general transcription (Ljungman et al., 1999). Finally, our finding that a subpopulation of p53 proteins becomes sequestered to nucleolar structures following proteasome inhibition may explain the limited transactivation of the p53 target gene p21WAF1 by MG132 observed in this study. Alternatively, p53 proteins accumulating during proteasome inhibition may be incorrectly folded and therefore non-functional (Magae et al., 1997).

In summary, we have shown that p53 proteins accumulating in the nucleus of human fibroblasts following exposure to UV light or the transcription inhibitor DRB appear to be freely diffusible. This may ensure high occupancy of p53 response elements and, if combined with specific modifications of the p53 protein, would be expected to result in a strong transactivation of p53 target genes. However, agents that inhibit transcription, such as UV light and DRB, or that cause the sequestering of the p53 protein, such as proteasome inhibitors, will not induce p53 transactivation fully. Future studies will address the mechanism and functional significance of the sequestering of p53 proteins to the nucleolar following proteasome inhibition.

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