p53 accumulation due to down-regulation of ubiquitin: relevance for neuronal apoptosis

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Abstract

The p53 tumor suppressor protein is a major regulator of cell growth arrest and apoptosis in response to DNA damage. Both p53 function and stability are tightly controlled by Mdm2, which binds to the p53 N-terminus and targets p53 for ubiquitin-mediated proteolysis. Previous studies suggest that adrenalectomy-induced neuronal apoptosis is p53-dependent. Here we demonstrate both nuclear accumulation and functional activation of p53 protein in apoptotic hippocampal neurons from adrenalectomized rats. Increased p53 expression occurred despite the accumulation of its negative regulator, Mdm2, and the formation of p53-Mdm2 complexes. The persistence of p53 expression was explained by a striking decrease in free ubiquitin in p53-positive neurons. The addition of exogenous ubiquitin to p53-Mdm2 complexes from apoptotic neurons restored p53 degradation. These findings demonstrate a novel mechanism of p53 stabilization mediated by decreased ubiquitin levels. Regulation of free ubiquitin may therefore be an effective way to modulate p53-dependent apoptosis in certain cell types. *Cell Death and Differentiation* (2000) **7**, 675–681.

Keywords: p53; ubiquitin; Mdm2; neuronal; apoptosis

Abbreviations: ADX, adrenalectomy; CNS, central nervous system; DG, dentate gyrus; PC, pyramidal cell

Introduction

The p53 tumor suppressor protein is a major regulator of cell growth arrest and apoptosis in response to DNA damage and other cellular stressors.[1-3] In addition to post-translational modifications p53 function is tightly controlled by protein-protein interactions.[1,2] In this regard, the Mdm2 oncoprotein, a product of a p53-responsive gene, is a major inhibitor of p53 function and abundance.[4,5] Mdm2 binds to the N-terminal transcriptional activation domain of p53 protein and blocks p53-regulated gene expression.[4,5] Importantly, Mdm2 also has ubiquitin ligase activity, and through ubiquitination targets p53 for proteasome-mediated degradation.[6,7] Thus, Mdm2 plays a key role in an autoregulatory feedback loop that maintains low p53 levels in normal cells and limits the magnitude of the p53 response following DNA damage.[6-9]

Bilateral adrenalectomy (ADX) in adult rats results in the cumulative loss of dentate gyrus granule neurons of the hippocampus through an apoptotic pathway (Figure 1a, inset).[10,11] Recent evidence suggests that neuronal apoptosis may be triggered by abnormal activation of the cell cycle.[12,13] In this regard, we previously demonstrated both DNA fragmentation and p53 induction in apoptotic hippocampal granule cells following ADX.[14] Additional evidence supporting a causal relationship between p53 activation and neuronal apoptosis comes from studies demonstrating that p53 overexpression results in apoptosis of hippocampal neurons in culture, while granule cells from p53-deficient mice are protected against ADX-induced death.[15-17] Thus activation of the p53-DNA damage response pathway may play a central role in certain types of neuronal degeneration.

Although a regulatory relationship between p53 and Mdm2 has been well-documented outside the central nervous system (CNS), i.e., Mdm2 inhibits p53 activity and promotes p53 degradation, there is a profound lack of information regarding p53-Mdm2 interactions in the CNS. Here we demonstrate the unexpected finding of increased Mdm2 levels in p53-positive neurons undergoing apoptosis. Further investigation of the p53 proteolytic pathway revealed that apoptotic neurons exhibited a marked decrease in ubiquitin expression. Following addition of exogenous ubiquitin to p53-Mdm2 complexes isolated from adrenalectomized rats there was almost complete restoration of p53 degradation. These findings demonstrate a novel mechanism of p53 stabilization that is mediated by decreased levels of free ubiquitin. Thus down-regulation of ubiquitin expression may play a major role in the p53 response to DNA damage and apoptosis in certain cell types.

Results

ADX-induced neuronal apoptosis is associated with increased expression and nuclear translocation of p53 protein

Since p53 mRNA is induced in apoptotic granule neurons following ADX,[14] we determined whether there is a
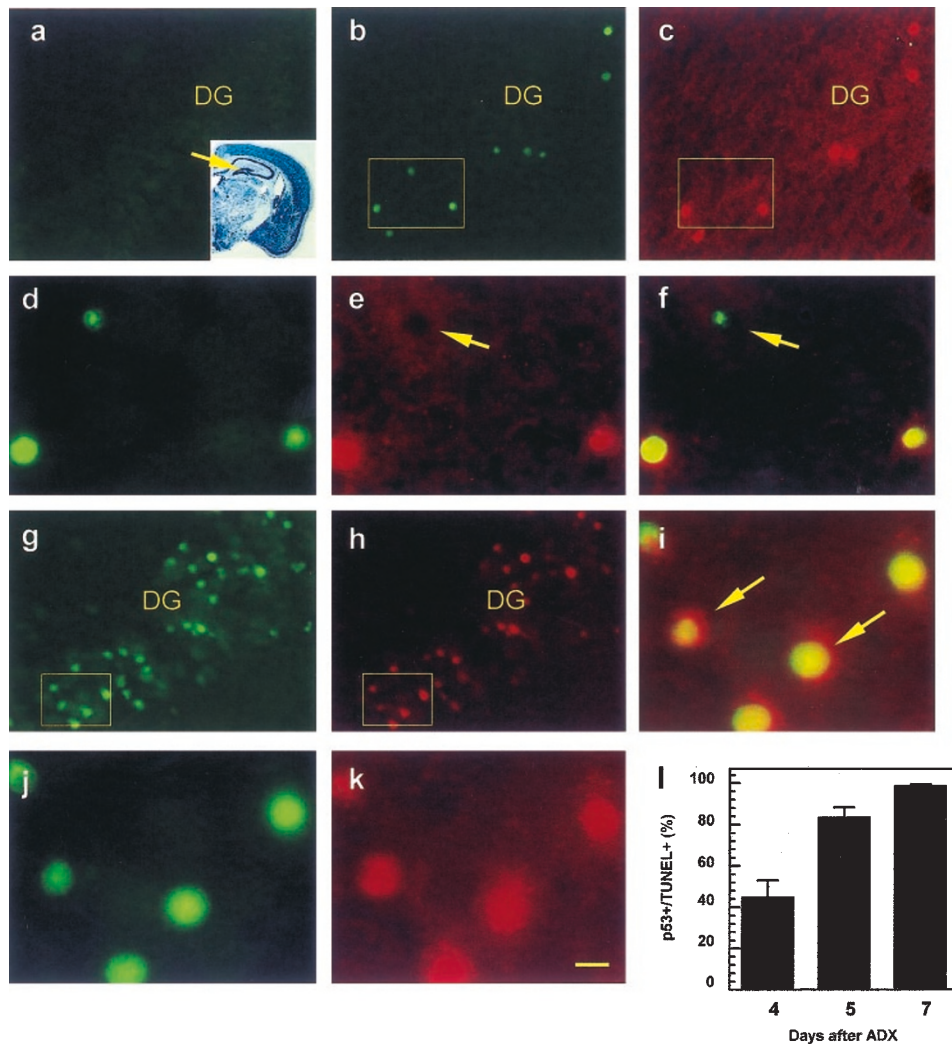
Figure 1 p53 and DNA damage are co-localized in apoptotic granule cells after ADX. Dual immunofluorescence labeling for both p53 (red) and TUNEL (green) was performed as described in Materials and Methods. (**a**) Section from a sham-operated rat shows no TUNEL-positive cells in the dentate gyrus granule cell layer (DG). Similar results were obtained for p53 (see Figure 3a). Inset, coronal hemisection through the hippocampus of an adult rat brain; arrow denotes the dentate gyrus granule cell layer (modified from www.nervenet.org/mbl). (**b**) Distribution of TUNEL-positive granule cells in the dentate gyrus 5 days after ADX. (**c**) The same section stained with anti-p53 antibody. Areas highlighted in (**b**) and (**c**) are shown at higher magnification in (**d**) and (**e**), respectively. Arrow in (**e**) denotes a p53-negative cell. (**f**) Overlap of (**d**) and (**e**) shows that p53 and DNA damage co-localize in most cells (yellow). Some p53-negative cells are TUNEL-positive (arrow). (**g**) Distribution of TUNEL-positive granule cells in the dentate gyrus 7 days after ADX. (**h**) The same section stained with anti-p53 antibody. Areas highlighted in (**g**) and (**h**) are shown at higher magnification in (**j**) and (**k**), respectively. (**i**) Overlap of (**j**) and (**k**) shows co-localization of p53 and DNA damage (arrows). (**l**) The number of p53- and TUNEL-positive cells were counted (four rats per time point, three sections per rat), and the ratio [p53+/TUNEL+] plotted as a function of time after ADX. Data are depicted as mean $\pm$ S.D. Scale bar: **a – c, g, h**: 80 μm; **d – f, i – k**: 16 μm

corresponding change in p53 protein. Activated p53 protein typically undergoes translocation to the nucleus where it functions as a transcription factor.[1–3] Immunohistochemistry revealed low levels of cytoplasmic p53 in granule cells from sham-operated control rats (Figure 1a). By contrast, following ADX there was a cumulative increase in nuclear p53-immunoreactivity as well as DNA damage that was detected by TUNEL (Figure 1b–k). The ADX-induced alteration in p53 expression was confirmed by Western blotting (Figure 3o, lanes 1, 2). In addition, dual immunofluorescence revealed that there was complete overlap between the distributions of TUNEL- and p53-positive cells at 7 days after ADX (Figure 1f,i). At 4 and 5

days after ADX a subpopulation of TUNEL-positive cells was observed to be p53-negative (Figure 1d,e,l).

ADX activates endogenous neuronal p53 protein

To further evaluate the mechanism of p53-mediated neuronal apoptosis we investigated p53 sequence-specific DNA binding activity in granule cell nuclear extracts from adrenalectomized rats. A significant increase in DNA binding activity was detected by 5 days (Figure 2a) consistent with the induction of p53 mRNA and protein expression. These findings were supported by an RT – PCR analysis revealing increased expression of Mdm2, a transcriptional target of p53 (Figure 2b).
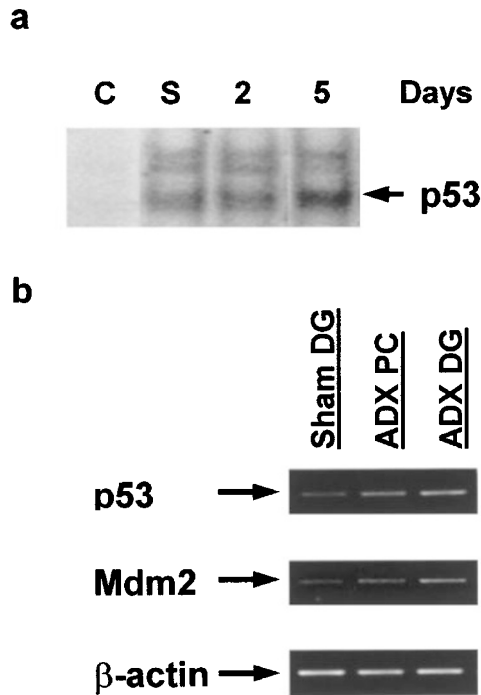
a

b

Figure 2 Effects of ADX on p53 DNA binding activity and transcriptional activation. (**a**) EMSA was performed as described using granule cell extracts prepared from either sham-operated rats (S) or at specified times after ADX. C, oligonucleotide probe only. (**b**) Total RNA from the dissected dentate gyrus (DG) or the adjacent pyramidal cell (PC) layer of either sham-operated or adrenalectomized rats, was analyzed semi-quantitatively by RT–PCR as described. An increase in amplified products from ADX PC is due to incomplete dissection

Mdm2 is upregulated and interacts with p53 after ADX

Although Mdm2 has been clearly shown to regulate p53 expression and activity in non-neural cells,[6–9] the functional importance of p53-Mdm2 interactions in the CNS has not been explored. We therefore sought to determine the relationship between p53 and Mdm2 *in vivo* following ADX. Increased expression of p53 and Mdm2 mRNA and protein was observed in apoptotic granule neurons (Figures 2b and 3o,p), and co-localization (Figure 3c–j) implied that there was a significant physical interaction. This was confirmed by the isolation of p53-Mdm2 complexes from granule cell lysates prepared from adrenalectomized rats (Figure 3o, lanes 3, 4).

p53 accumulation in apoptotic neurons is associated with downregulation of ubiquitin

The presence of p53-Mdm2 complexes in granule cell extracts indicated that Mdm2 binding was functionally intact, and suggested that other alterations in the p53-Mdm2 proteolytic pathway were responsible for stabilizing p53. As Mdm2 regulates p53 degradation through its ubiquitin ligase activity,[18] we investigated the possibility that modulation of ubiquitin levels played a role in regulating p53 abundance following ADX. Notably, there was a striking decrease in

ubiquitin immunoreactivity only in p53-positive neurons, while adjacent p53-negative neurons exhibited abundant amounts of ubiquitin (Figure 4c,d,f,g). This result was confirmed by Western blot analysis of free ubiquitin levels in granule cell lysates (Figure 4i).

Exogenous ubiquitin restores the p53 degradation pathway

Although ubiquitin levels were decreased it was important to determine whether activation of ubiquitin-mediated proteolysis was still possible. Initial studies using ubiquitin-depleted granule cell extracts from naïve rats demonstrated that p53 degradation could be reconstituted by adding exogenous ubiquitin (Figure 4j, lanes 1, 2). Similarly, p53 degradation was enhanced following the addition of exogenous ubiquitin to p53-Mdm2 complexes isolated from the hippocampi of adrenalectomized rats (Figure 4j, lanes 3, 4).

Discussion

Among the multiple mechanisms regulating p53 function protein-protein interactions play a dominant role.[1,2] In this regard, the Mdm2 protein, encoded by a p53-target gene, is a critical regulator of p53 activity in a variety of cell types.[4,5] Previous studies have shown that Mdm2 binds to and blocks the N-terminal transactivation domain of p53, and also promotes the rapid degradation of p53 through a ubiquitin-dependent pathway.[6–9] Evidence for the functional importance of Mdm2 binding is also provided by reports demonstrating that overexpression of Mdm2 blocks both p53-mediated cell cycle arrest and apoptosis in cultured cells.[19–21] Interestingly, inhibition of p53 degradation by proteasome inhibitors, which act downstream from Mdm2, enhances both p53 accumulation and apoptosis.[22]

The results of our study extend those obtained in cultured cells by demonstrating a significant accumulation of endogenous p53 in the nuclei of apoptotic neurons *in vivo*. While p53 immunoreactivity and TUNEL staining were typically co-localized, the presence of p53-negative cells containing fragmented DNA at the earliest time point studied is consistent with the idea that DNA damage precedes and triggers p53 activation. The increase in p53 DNA binding activity and Mdm2 gene transcription indicate that p53 is functionally active in apoptotic neurons. As DNA damage, increased p53 expression and DNA binding also occur during excitotoxic cell death,[23,24] activation of the p53 pathway may be a common response of mature CNS neurons exposed to DNA-damaging, apoptotic stimuli.

Until now there have been no investigations into the relationship between p53 and Mdm2 in the CNS. In the present study, increased expression and co-localization of both p53 and Mdm2, as well as formation of p53-Mdm2 complexes were observed in apoptotic neurons. Although differences between the relative amounts of free *versus* bound p53 were not investigated, our findings suggest that there was an initial activation of the p53-Mdm2 autoregulatory loop following ADX. However, the persistently elevated p53 levels in the presence of increased Mdm2 expression suggested that there was an impairment of
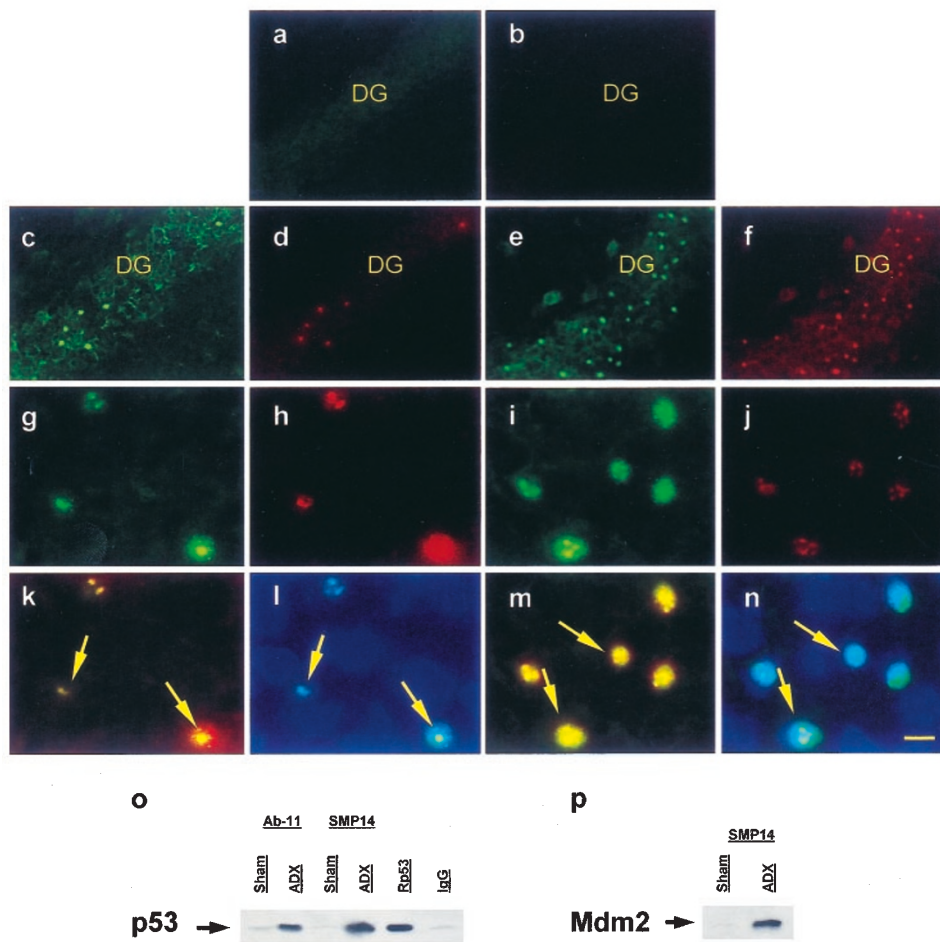
Figure 3 Expression and co-localization of p53 and Mdm2, and formation of p53-Mdm2 complexes after ADX. Dual immunofluorescence labeling for both p53 (green) and Mdm2 (red) was performed as described in Materials and Methods. (**a**) Section from a sham-operated rat stained with anti-p53 antibody. (**b**) The same section stained with anti-Mdm2 antibody. (**c**) Distribution of p53-positive cells 5 days after ADX. (**d**) The same section stained with anti-Mdm2 antibody. (**e**) Distribution of p53-positive cells 7 days after ADX. (**f**) The same section stained with anti-Mdm2 antibody. DG, dentate gyrus. (**g**) to (**j**) Higher power views of cells in (**c**–**f**), respectively. (**k**) and (**m**) Overlap reveals co-localization (yellow) of p53 and Mdm2 in cells at 5 and 7 days post-ADX, respectively (arrows). (**l** and **n**) p53 also co-localizes with the nuclear marker, DAPI, in the same sections (arrows). (**o**) Granule cell lysates were prepared from either sham or adrenalectomized rats, immunoprecipitated using either a p53-specific (Ab-11) or anti-Mdm2 (SMP14) antibody, and p53 detected using the anti-p53 antibody, CM5. Rp53, recombinant p53 standard; IgG, heavy chain immunoglobulin used in immunoprecipitation. (**p**) Immunoprecipitation of Mdm2 in granule cell lysates from sham and ADX groups. Equal amounts of lysates were used in first 2 lanes of (**o**) and (**p**). Scale bar: **a**–**f**: 100 μm; **g**–**n**: 16 μm

either Mdm2 function or p53 degradation downstream from Mdm2 binding. Co-localization studies demonstrated reduced levels of ubiquitin in p53-positive neurons providing strong evidence for a novel mechanism of p53 stabilization. Since the ubiquitin ligase function of Mdm2 was preserved as demonstrated by reconstitution experiments, we conclude that the accumulation of p53 protein was due to a marked decrease in free ubiquitin.

Ubiquitin has long been recognized to play a central role in protein degradation under various physiological conditions, and has also been implicated in malignant transformation and other pathological states.[25] Abnormal aggregates containing ubiquitinated proteins are commonly observed in neurodegenerative diseases.[26] By contrast, the functional significance of decreased ubiquitin levels is unknown. Previous studies have demonstrated down-

regulation of ubiquitin in vulnerable CA1 pyramidal neurons following transient cerebral ischemia.[27–29] Notably, the distribution of cells with low levels of ubiquitin closely matched that of ischemia-induced neuronal degeneration. Increased p53 expression has been recently demonstrated following transient cerebral ischemia.[30] Combined with our results, these findings suggest a link between decreased ubiquitin, p53 accumulation and ischemic cell death. Outside the CNS, low levels of free ubiquitin may contribute to the sustained p53 response reported to occur in human fibroblasts following DNA damage, as well as the accumulation of p53 protein in certain tumor cell lines expressing increased amounts of Mdm2.[31–33]

In conclusion, our results suggest that ubiquitin may be a key determinant of p53 stabilization and apoptotic cell
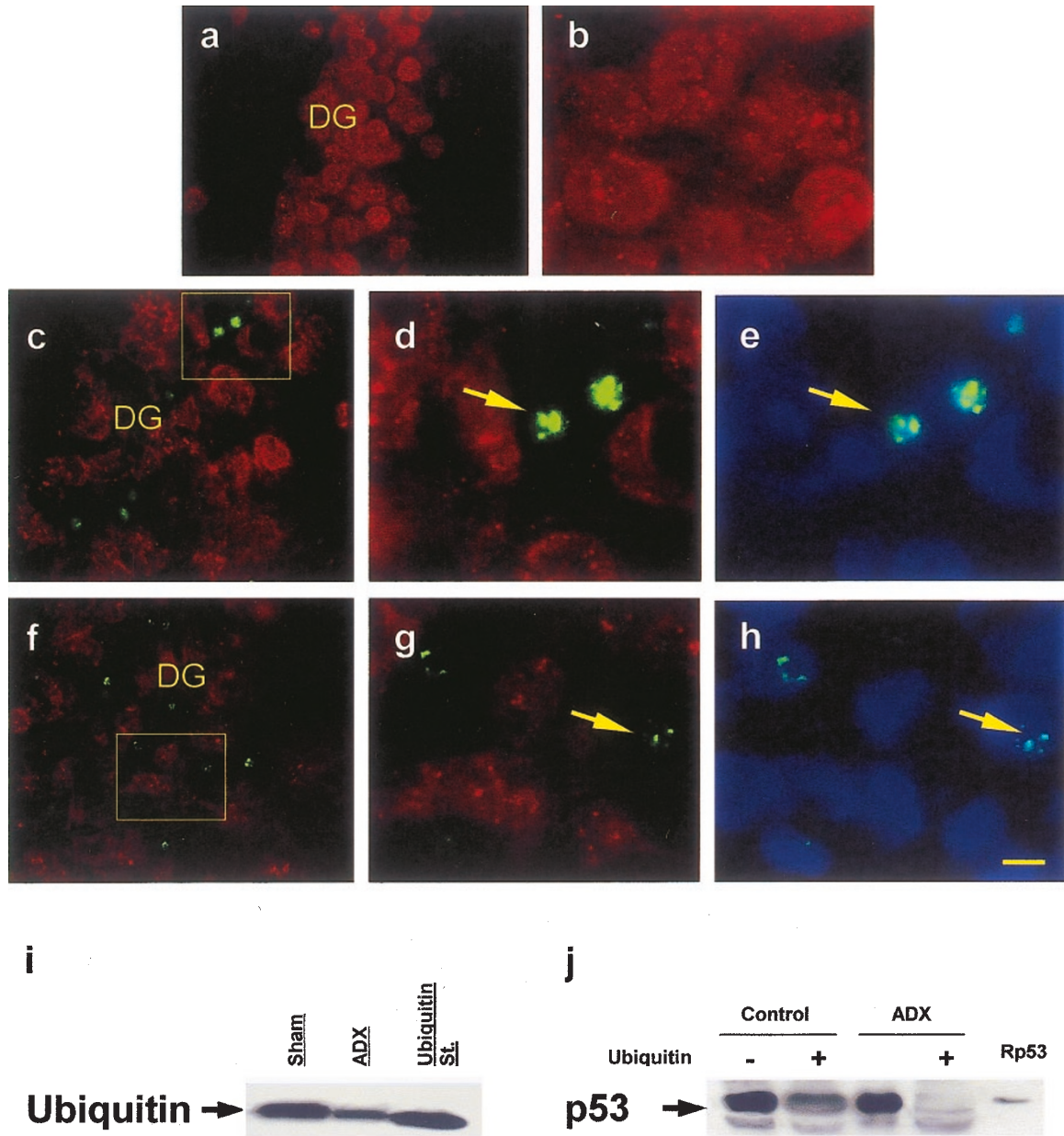
Figure 4 Decreased ubiquitin expression is associated with p53 stabilization in apoptotic granule cells. Dual immunofluorescence labeling for both p53 (green) and ubiquitin (red) was performed as described in Materials and Methods. (**a**) Section from a sham-operated rat stained with anti-p53 and anti-ubiquitin antibodies. DG, dentate gyrus. (**b**) Higher power view of cells in (**a**). (**c**) p53-positive cells exhibit reduced ubiquitin immunoreactivity 5 days after ADX. (**d**) p53-positive cells in (**c**) are shown at higher magnification (arrow). (**e**) Nuclear p53 co-localizes with DAPI in these cells (arrow). (**f**) At 7 days post-ADX, p53-positive cells also have decreased ubiquitin. (**g**) A p53-positive cell is shown at higher magnification and (**h**) is also DAPI-positive (arrows). (**i**) Western blot analysis of granule cell extracts from sham or adrenalectomized rats using anti-ubiquitin antibody. Ubiquitin st., ubiquitin standard. (**j**) p53-Mdm2 complexes from adrenalectomized rats were immunoprecipitated and incubated with either ubiquitin-depleted lysates from naive rats or granule cell extracts from adrenalectomized rats, with or without exogenous ubiquitin. Rp53, recombinant p53 standard. Scale bar: **a**: 80 μm; **c, f**: 60 μm; **b, d, e, g, h**: 16 μm

death in the CNS and perhaps other organ systems. Additional investigations are necessary to establish a clear role for ubiquitin and its related proteolytic cascade in neuronal apoptosis, as well as whether modulation of ubiquitin abundance can be an effective way to regulate p53-mediated apoptosis in the CNS.

Materials and Methods

Animal surgery and tissue preparation

All of the rats used in this study were treated in accordance with guidelines issued by the National Institutes of Health and Institutional Animal Care and Use Committee of the University of Southern

California. Adult male Fisher 344 rats were adrenalectomized or sham-operated under pentobarbital anesthesia.[14] At either 4, 5 or 7 days after ADX rats were decapitated under methoxyfluorane anesthesia and half the brain rapidly frozen for cryostat sectioning at 10 μm (n=4 rats per time point). The dentate gyrus was dissected from the other half-brain and stored at -80°C. Serum corticosterone was non-detectable in cardiac blood samples from adrenalectomized rats; the average corticosterone level in sham-operated rats was 629 ± 130 ng/ml.[14]

Immunohistochemistry

Frozen coronal brain sections from both adrenalectomized and sham-operated rats were fixed in 95% methanol for 1 h, followed by microwave antigen retrieval in 10 mM citrate buffer (pH 6.4) and incubation in a blocking buffer developed in our laboratory for 30 min at room temperature. Sections were incubated with a rabbit polyclonal anti-p53 antibody (CM5, Novocastra, Newcastle upon Tyne, UK) overnight at 4°C, followed by several brief washes with phosphate-buffered saline (PBS), and either a biotinylated (see below) or FITC-conjugated secondary antibody for 30 min. For double-labeling, sections were incubated with CM5 and either a mouse monoclonal anti-Mdm2 (SMP14, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse monoclonal anti-ubiquitin (Ubi-1, Zymed Laboratories, San Francisco, CA, USA) antibody overnight at 4°C, followed by incubation with FITC-conjugated sheep anti-rabbit and Texas Red-conjugated goat anti-mouse secondary antibodies for 30 min. After several PBS washes, sections were coverslipped with Vectorshield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and analyzed by fluorescence microscopy.

TdT-mediated dUTP nick end labeling (TUNEL)

Frozen sections were fixed and microwaved as described above, labeled with an In Situ Cell Death Detection kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol, and analyzed by fluorescence microscopy. For double-labeling sections were re-microwaved after TUNEL staining, incubated in blocking buffer for 30 min at room temperature, washed in PBS and incubated with CM5 followed by a biotinylated secondary antibody. After several PBS washes, p53 immunoreactivity was detected using streptavidin-conjugated Texas Red (Amersham, Piscataway, NJ, USA).

Electrophoretic gel mobility shift assay (EMSA)

Nuclear protein extracts were prepared from the dissected dentate gyrus of adrenalectomized and sham-operated rats as previously described.[24] A double-stranded oligonucleotide coding for the p53 consensus sequence, 5′-TACAGAACATGTCTAAGCATGCTGG-3′, was end-labeled with ^{32}P-ATP and incubated with 10 μg nuclear proteins in binding buffer. DNA-protein complexes were resolved by non-denaturing polyacrylamide gel electrophoresis.[24]

Reverse transcription-polymerase chain reaction (RT−PCR)

Total RNA was extracted from the dissected dentate gyrus 5 days after ADX using TRIZOL (Gibco BRL, Gaithersburg, MD, USA), and 1.5 μg total RNA was reverse transcribed with 1 μg oligo(dT) in 20 μl of reaction buffer [1 mM each dNTPs, 25 mM Tris-HCl (pH 8.3), 25 mM KCl, 5 mM MgCl$_2$, 5 mM dithiothreitol, 0.25 mM spermidine, 10 U RNAse inhibitor (Boehringer Mannheim, Indianapolis, IN, USA), 10 U

avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA)] for 1 h at 42°C and 10 min at 75°C. The reaction was stopped by addition of 100 μl of 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. PCR was performed with 10 μl of RT reaction mixture with 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.1% Triton X-100, 1 mM MgCl$_2$, 400 nM each primer, 200 μM each dNTP, 5 U Taq DNA polymerase (Promega) in a final volume of 50 μl. The sample was subjected to 30 cycles (94°C, 1 min; 52°C, 1 min; 72°C, 1 min) in a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT, USA). Rat primer sequences: p53 antisense, 5′-GACTTCTTGTAGATGGCCATGG-3′; p53 sense, 5′-ATGGAGGAGTCACAGTCGGATA-3′; Mdm2 antisense, 5′-TTATCGTCTGGAAGCCAG-3′; Mdm2 sense, 5′-TGCAAGCACCTCA-CAGAT-3′. PCR using primers for rat β-actin (Promega) was performed simultaneously and the results were used to normalize cDNA amounts.

Immunoprecipitation and Western blotting

Dissected dentate gyri were sonicated in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40 plus 1 mM phenylmethylsulfonylfluoride and 1 μg/ml leupeptin. For immunoprecipitation 400 μg total protein was incubated with either a p53-specific antibody (CM5 or Ab-11) or SMP14 conjugated to protein A/G agarose beads (Santa Cruz) for 4 h at 4°C with gentle mixing. The pellets were washed in lysis buffer, resuspended and resolved on a denaturing polyacrylamide gel containing SDS and transferred to a nitrocellulose membrane overnight at 4°C. Membranes were incubated for 45 min at room temperature in Tris blocking buffer containing 8% nonfat dry milk and 0.1% Tween 20. Proteins were detected by incubation with primary antibody for 2 h followed by a horseradish peroxidase-conjugated secondary antibody for 30 min and visualization by ECL (Amersham). For ubiquitin Western blotting 100 μg total protein was loaded per lane and confirmed by Coomassie blue staining. The Ab-11 antibody was obtained from Calbiochem (San Diego, CA, USA).

p53 degradation assay

Employing a modification of the method described by Shkedy *et al*,[34] granule cell lysates were prepared from the hippocampi of either naïve rats or at 7 days after ADX by successive freezing and thawing, and supernatants were frozen in ice-cold distilled water at -80°C. For ubiquitin depletion, 20 μl Ubi-1 was added to protein lysates (500 μg) from naïve rats and gently mixed for 3 h at 4°C, followed by addition of 50 μl protein A/G agarose beads (Santa Cruz) and mixing for an additional 4 h. After centrifugation the supernatant was stored at -80°C. Lysates were incubated with immunoprecipitated p53-Mdm2 complexes from adrenalectomized rats in a buffer (25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl$_2$, 10 mM DTT, 10 mM ATP) for 1 h at 30°C, with or without the addition of 6 μg ubiquitin (Sigma). Following addition of sample buffer immunoprecipitates were electrophoretically separated and evaluated by Western blotting.

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