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p53 Protein Exhibits 3′-to-5′ Exonuclease Activity

Torsten Mummenbrauer,* Friedemann Janus,* Beate Müller,* Lisa Wiesmüller,* Wolfgang Deppert,* and Frank Grosse†
*Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie
Martinistr. 52
D-20251 Hamburg
Germany
†Institut für Molekulare Biotechnologie
Abteilung Biochemie
Beutenbergstr. 11
D-07745 Jena
Germany

Summary

Highly purified p53 protein from different sources was able to degrade DNA with a 3′-to-5′ polarity, yielding deoxynucleoside monophosphates as reaction products. This exonuclease activity was dependent on Mg^{2+} and inhibited by addition of 5 mM nucleoside monophosphates. This exonuclease activity is intrinsic to the wild-type p53 protein: it copurified with p53 during p53 preparation; only purified wild-type p53, but not identically purified mutant p53 proteins displayed exonuclease activity; the exonuclease activity could be reconstituted from SDS gel-purified and urea-renatured p53 protein and mapped to the core domain of the p53 molecule; and finally, purified p53 protein could be UV cross-linked to GMP. A p53-intrinsic exonuclease activity should substantially extend our view on the role of p53 as a "guardian of the genome."

Introduction

Mutations in the gene of the cellular protein p53 are found in >50% of all human tumors, suggesting that loss of this gene represents an important step in the formation of human cancers (Hollstein et al., 1991; Vogelstein and Kinzler, 1992; Harris and Hollstein, 1993). Despite its central role in the process of carcinogenesis, the gene is dispensable, because p53 double-null mice develop normally; they exhibit, however, a dramatic incidence of tumors in adulthood (Donehower et al., 1992; Harvey et al., 1993). p53 protein has been shown to act as a cell cycle regulator that, when overexpressed, prevents the transition from G1 to S phase of the cell cycle (Mercer et al., 1990; Lin et al., 1992). This action of p53 has been ascribed to its ability to induce expression of genes involved in growth arrest, like the *waf1/cip1* gene that encodes a 21 kDa inhibitor of G1 cyclin-dependent protein kinases (El-Deiry et al., 1993; Harper et al., 1993). An intracellular increase of p53 protein has been reported as result of various genotoxic treatments (Kuerbitz et al., 1992; Fritsche et al., 1993; Hartwell and Kastan, 1994), which in turn induces DNA repair or apoptosis (reviewed in Marx, 1993; Cox and Lane, 1995). This led to the concept of the "guardian of the genome" for p53 (Lane, 1992). However, in addition to the damage-induced p53 functions, evidence for a direct involvement of p53 in cellular DNA replication and DNA repair has accumulated (reviewed in Deppert, 1994). Such an involvement can be deduced from several findings: that p53 colocalizes with PCNA, DNA polymerase α, DNA ligase, and RPA at discrete regions in the nuclei of Herpes virus–infected cells (Wilcock and Lane, 1991); that p53 exerts direct effects on nuclear DNA replication (Cox et al., 1995); that p53 binds to the replication factor RPA (Dutta et al., 1993; Li and Botchan, 1993); and that p53 expression in cells, stimulated to reenter proliferation, closely follows the pattern of cellular DNA synthesis (Mosner and Deppert, 1994; Mosner et al., 1995). Since p53 is not absolutely required for cellular proliferation (see above), one has to assume an auxiliary rather than a mandatory function of p53 in proliferation.

In addition to its functions as transactivator and repressor, several other biochemical activities have been ascribed to the p53 protein. In agreement with its role as a transactivator, p53 has been shown to bind DNA in a sequence-specific manner (Bargonetti et al., 1991; Kern et al., 1991b; Funk et al. 1992; Zambetti et al., 1992; Miyashita and Reed, 1995) and to comprise a peptide domain mediating transactivation from a promotor downstream of the p53 DNA binding motif (El-Deiry et al., 1992; Kern et al., 1992; Schärer and Iggo, 1992). In addition to sequence-specific DNA binding, p53 protein binds nonspecifically to double-stranded (ds) and single-stranded (ss) DNA (Steinmeyer and Deppert, 1988; Kern et al., 1991a). Furthermore, it has been described to reanneal both RNA and DNA (Oberosler et al., 1993; Bakalkin et al., 1994; Brain and Jenkins, 1994). The latter two functions might be important in processes associated with DNA repair, like recognition of DNA damage (Lee et al., 1995) and prevention of unscheduled recombination, as well as in regulation of DNA replication, which could explain its apparent colocalization with replicative complexes. Hence, DNA binding, particularly to single-strand/double-strand junctions, the DNA strand transfer, and the DNA strand annealing activity (Oberosler et al., 1993; Bakalkin et al., 1994; Brain and Jenkins, 1994) might point to an additional role of p53 in processes different from that in transcriptional regulation. To get access to such a postulated function, we characterized the interactions of p53 with DNA. Thereby, we found that a Mg^{2+}-dependent exonuclease activity is intrinsically associated with the p53 protein. Possible physiological functions of this activity in the process of tumor prevention are discussed.

Results

Highly Purified p53 Protein Displays Exonuclease Activity

We have purified wild-type (wt) p53 protein to near homogeneity from four different sources: Sf9 insect cells infected with a mouse wt p53-recombinant baculovirus, clone 6 rat cells that ectopically express a temperature-sensitive mouse p53 protein (Michalovitz et al., 1990), Escherichia coli bacteria that overexpress a soluble form

of human wt p53 protein, and bacteria that produce an insoluble form of mouse wt p53 protein. The baculovirus-produced protein was purified by nickel-chelate affinity chromatography or by affinity chromatography using the immobilized monoclonal antibody PAb421, elution with 1 M KCl, and subsequent elution at pH 12. Alternatively, p53-containing fractions eluted from the PAb421 antibody column were pooled and further purified by column chromatography on heparin-sepharose. The soluble bacterial protein was also purified by PAb421 affinity chromatography and elution with 1 M KCl (pH 12), or by conventional column chromatography according to the method of Hupp et al. (1992). The purified proteins were tested for sequence-specific and unspecific ss- or dsDNA binding under a variety of different assay conditions. Here we observed that, whenever Mg^{2+} had been added to the assay mixture, a substantial part of the DNA substrate became degraded. DNA degradation was dependent on both the time of incubation (Figure 1, lanes 1–3 and 5–10) and the concentration of added p53 protein (see Figures 2 and 5).

Figure 2 and Table 1 demonstrate the copurification of p53 protein and of exonuclease activity from lysates of Sf9 insect cells infected with a mouse wt p53-recombinant baculovirus. Due to the presence of a large amount of not-p53 related exonuclease activity in the lysate (see Table 1), demonstration of the exonuclease activity specifically associated with p53 required substantial prepurification of p53. This was achieved by immunoaffinity chromatography, where the crude lysate was applied to a PAb421 antibody column, and p53 was eluted with high salt (1 M KCl). Aliquots of each fraction were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), followed by silver staining (Figure 2A), and analyzed in parallel for exonuclease activity (Figure 2B) by the filter retention assay as described in Experimental Procedures. The identity of the protein migrating at 53 kDa in Figure 2A with p53 was verified by Western blotting (data not shown). Figure 2A shows a considerable enrichment of p53 in the high salt eluate of the antibody column over p53 in the lysate. Comparison of the p53 elution profile with the profile of exonuclease activity in the corresponding fractions (Figure 2B) demonstrates copurification of the exonuclease activity with the eluted p53, and Table 1 reveals an ~5-fold enrichment of the specific exonuclease activity by immunoaffinity chromatography. The pooled p53 fractions then were subjected to column chromatography on heparin-sepharose. The heparin column was developed with a salt gradient, ranging from 50 mM to 1 M KCl, and individual fractions were analyzed by SDS–PAGE followed by silver staining and for exonuclease activity as described above. p53 was eluted between 350 mM and 450 mM KCl. SDS–PAGE analysis (Figure 2C) shows that the p53 protein in the the p53-containing peak fractions (lanes 5 and 6 in Figure 2C) was essentially pure as judged by silver staining. Figure 2D demonstrates that the exonuclease activity eluted as a single peak from the heparin-sepharose column, and that its activity profile closely followed the p53 elution profile, confirming copurification of p53 with exonuclease activity. Over 80% of the exonuclease activity applied to the heparin-sepharose column was recovered (see Table 1), again

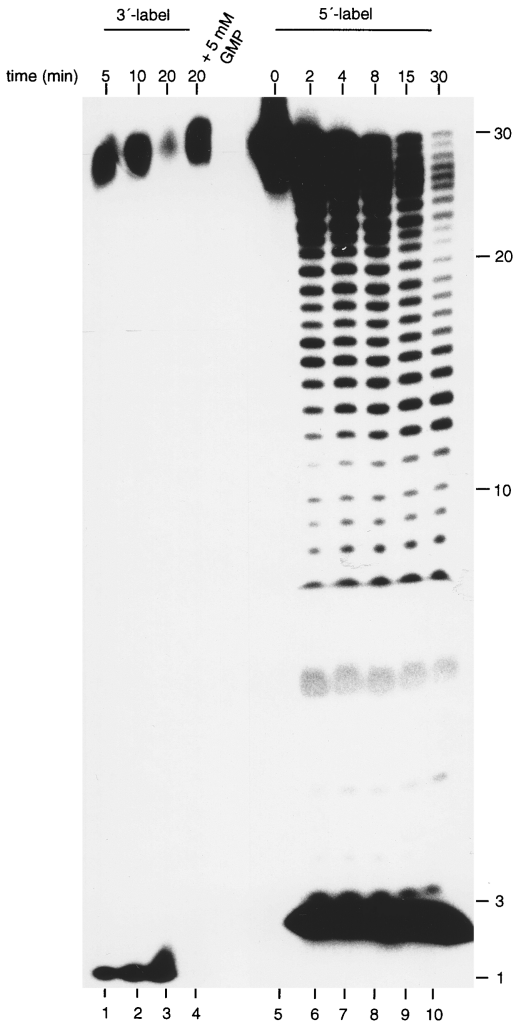

Figure 1. Exonuclease Activity of Purified p53 Protein

Gel electrophoretic measurements of exonuclease activity were carried out with the 30-mer deoxyoligonucleotide 5′-GACACTGGTCAC ACTTGGCTGCTTAGGAAT-3′ (Foord et al., 1993). 3′-Labeling was performed with terminal transferase and [α-^{32}P]ddATP (lanes 1–4) and 5′-labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase (lanes 5–10) as described under Experimental Procedures. The exonuclease activity was measured with ~1 ng p53, here purified from baculovirus-infected Sf9 cells by affinity chromatography with immobilized PAb421 and subsequent elution with 1 M KCl. The reaction products were analyzed on a 20% polyacrylamide gel containing 7 M urea. Lanes 1–3 show the production of ddAMP from the 3′ end of the 3′-labeled oligonucleotide after 5, 10, and 20 min incubation at 37°C; lane 4 displays the same experiment as shown in lane 3, but in the presence of 5 mM GMP. Lanes 5–10 indicate product formation with the 5′-labeled substrate after 0, 2, 4, 8, 15, and 30 min incubation, respectively, at 37°C.

underscoring the tight association of this activity with p53. Furthermore, purification of p53 by heparin-sepharose chromatography led to an additional, ~4-fold increase in specific exonuclease activity of the pooled fractions (Table 1), with the peak fractions in Figure 2C (lanes 5 and 6) containing the highest specific exonuclease activity (1126 U/mg).

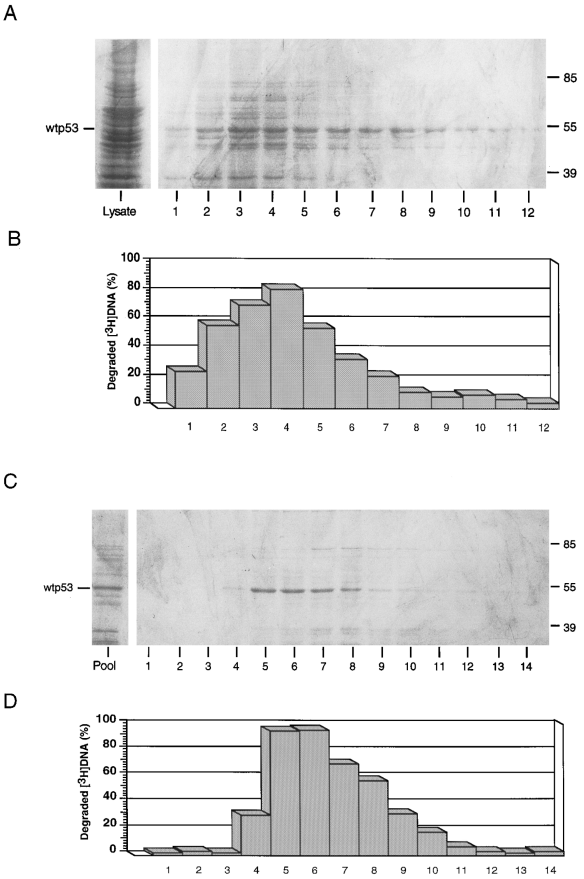

Figure 2. Copurification of p53 and Exonuclease Activity

Murine wt p53 protein was expressed in Sf cells infected with a wt p53-recombinant baculovirus. p53 was purified first by immunoaffinity chromatography on a PAb421 column by high salt (1 M KCl) elution (A and B), followed by further purification of p53-containing fractions on heparin-sepharose column (C and D). Fractions 3–14 (corresponding to lanes 1–12 in [A] and [B]) from high-salt eluate of the PAb421 column were analyzed by SDS–PAGE and silver staining (A), and for exonuclease activity by [^{3}H]-filter retention assay, which is displayed as columns of relative activity (B) as described in Experimental Procedures. Fractions 4–9 (corresponding to lanes 2–7 in [A] and [B]) were pooled, loaded on a 2 ml heparin-sepharose column and eluted with a salt gradient from 0.05 to 1 M KCl. A total of 30 fractions were collected; fractions 1, 5, 7–16, 20, and 25 were analyzed by SDS–PAGE and silver staining (C). All fractions were tested for exonuclease activity as described above (D). Exonuclease activity was seen only in fractions 8–15 (lanes 4–11).

Biochemical Properties of the p53-Associated Exonuclease Activity

The p53 protein-associated exonuclease activity was dependent on the presence of Mg^{2+}, where 10 mM were optimal, and 80% and 40% of the optimal activity were observed at 20 mM and 1 mM $MgCl_2$, respectively (Table 2). Mn^{2+} could replace Mg^{2+}, but its optimal concentration was 0.5 mM. The presence of 0.5 mM Mn^{2+} gave rise to only 10% of the maximal activity seen with 10 mM Mg^{2+} (Table 2). On the other hand, Zn^{2+}, Ca^{2+}, and Ni^{2+} could not replace Mg^{2+} or Mn^{2+} (Table 2). DNA degradation was sensitive to the addition of salt, where NaCl exhibited half-maximal inhibition at ~100 mM, and potassium acetate was half-maximally inhibitory at 125 mM (Table 2). The pH optimum of the degradation reaction was at around pH 8.5, with 80% and 70% residual activity at pH values of 7.0 and 10.0, respectively (Table 2). Furthermore, the exonuclease was inhibited almost completely by 5 mM GMP (Table 2), a concentration that has been shown to inhibit effectively the 3′-to-5′ exonucleases of the mammalian DNA polymerases δ and ϵ and E. coli DNA polymerase I (see: Bialek and Grosse, 1993, and references therein). The p53 associated exonuclease activity was similarly inhibited by dGMP, AMP, and dAMP, but not by nucleoside triphosphates (data not shown). Exonucleolytic activity could be competed most effectively with the template · primer combination poly(dA) · $(dT)_{20}$, whereas the ssRNA poly(rU) and the ssDNA poly(dT) were rather poor competitors for exonucleolytic degradation (Figure 3).

The p53-Associated Exonuclease Exhibits a 3′-to-5′ Polarity and Produces Deoxynucleoside Monophosphates

The directionality of the p53 protein-associated exonuclease was determined by using differently labeled ssDNA substrates. With the 3′-labeled substrate, the p53 protein-associated exonuclease produced labeled nucleoside monophosphate only (Figure 1, lanes 1–3). This finding is in accordance with our data showing that this exonuclease activity is inhibited by nucleoside monophosphates (see above) and argues for a 3′-to-5′ mechanism with the excision of one nucleotide after another. In agreement with this view is the degradation pattern of 5′-labeled DNA substrates. Dependent on the time of incubation a ladder of oligonucleotides was produced (Figure 1, lanes 6–10). Hence, the nuclease removed dNMPs from the 3′-end of the substrate, with trinucleotides as the end product.

Monoclonal Antibodies against p53 Deplete both the p53 Protein and the Associated Exonuclease Activity from Preparations of Purified p53 Protein

A 3′-to-5′ exonuclease activity was found in all preparations of wt p53 protein, regardless of the starting source

Table 1. Purification of p53 Exonuclease Activity

Fraction	Volume (ml)	Total Protein (mg)	Specific Activity (units/mg)	Total Activity (units)
Crude extract	50	2075	48	100000
Immunoaffinity column, fractions 4–10	4	7.6	236	1800
Heparin–Sepharose column, fractions 8–15	6	1.7	882	1500

Immunoaffinity column fractions 4–10 correspond to lanes 2–9 in Figure 2A; Heparin–Sepharose column fractions 8–15 correspond to lanes 4–11 in Figure 2C. 1 Unit corresponds to degradation of 60 pM DNA per 10 min at 37°C.

Table 2. Characterization of the p53 Protein-Associated Exonuclease Activity

Assay Conditions	Degraded DNA[a]
Complete standard assay	1.0
pH 7.0	0.8
pH 8.5	1.0
pH 10.0	0.7
1 mM Mg^{2+}	0.4
10 mM Mg^{2+}	1.0
20 mM Mg^{2+}	0.8
0.5 mM Mn^{2+}	0.1
0.1–5 mM Ca^{2+}, Zn^{2+}, Ni^{2+}	0.0
100 mM NaCl	0.5
125 mM potassium acetate	0.5
5 mM GMP	< 0.05

The complete standard assay contained in a total reaction volume of 20 μl 0.5 nM (2 ng) tetrameric p53 protein overexpressed and isolated from Sf9 cells, and 125 nM (tritiated 3′ ends) poly (dA) • $(dT)_{20}$ – [^{3}H]dT. A 100% loss of tritium label was achieved after 15 min incubation at 37°C. This corresponds to a turnover number of about 0.03 mol [^{3}H]dTMP removed per second and mole of p53 tetramer. Conditions deviating from the standard are indicated in the left-hand column. The right-hand column displays the relative reaction rate in comparison to the optimized standard.
[a]Relative to standard assay.

and the way of purification, providing evidence against an unspecific copurification of a contaminating exonuclease. This conclusion was further supported by demonstrating that the monoclonal anti-p53 antibodies PAb421, PAb248, and PAb1620 completely depleted both p53 protein and the 3′-to-5′ exonuclease activity from the reaction mixture, while an unrelated monoclonal antibody (PAb108) had no effect (Figure 4). Thus, this exonuclease activity either results from a very specific association of an exonuclease, present in eukaryotic as well as in bacterial cells, or it is intrinsic to the p53 protein.

The Exonuclease Activity Copurifies with Wild-Type, but not with Mutant p53 Protein

To further demonstrate that the 3′-to-5′ exonuclease activity is specifically associated with p53, we purified soluble human wt p53 protein, produced in bacteria, by immunoaffinity chromatography on a PAb421-sepharose column as described in Experimental Procedures.

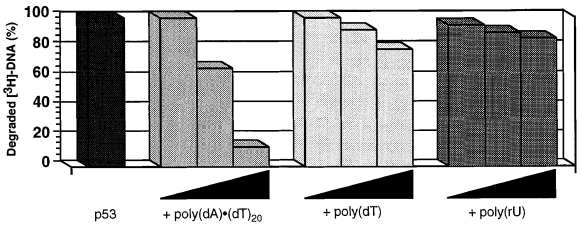

Figure 3. Substrate Requirements of the p53-Associated Exonuclease

p53-associated exonuclease activity was measured with 1 ng p53 protein, purified from Sf9 cells, and 125 nM (3′ ends) poly(dA)·$(dT)_{20}$ – [^{3}H]dT as substrate in a reaction volume of 20 μl as described in Experimental Procedures. Poly(dA)·$(dT)_{20}$, poly(rU), and poly(dT) served as competitors at concentrations of 40 ng (left columns), 400 ng (middle columns), and 4 μg (right columns), respectively.

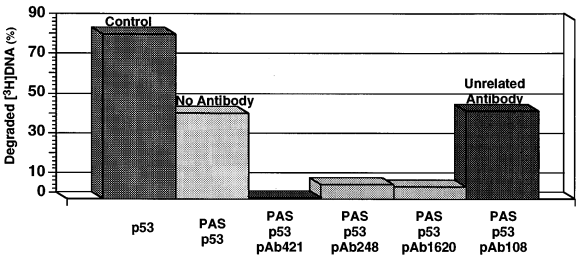

Figure 4. Immunodepletion of Exonuclease by p53-Specific Monoclonal Antibodies

About 1 μg each of the monoclonal anti-p53 antibodies PAb421, PAb248, PAb1620, and the SV40 T antigen-directed antibody PAb108, were added to 50 ng p53 protein, purified from Sf9 cells by Ni^{2+}-NTA affinity chromatography, in 100 μl of 50 mM Tris–acetate (pH 8.5), 100 mM NaCl, 1 mM DTT. Immunoprecipitated p53 protein was removed by the addition of 50 μl protein A–Sepharose (Pharmacia) and subsequent centrifugation. Exonuclease activity was measured in 2 μl of the supernatant with poly(dA)·$(dT)_{20}$ – [^{3}H]dT as DNA substrate, as described under Experimental Procedures. "Control" indicates the loss of [^{3}H] label in 1 μl of the assay mixture without added protein A–Sepharose and without antibodies.

Figure 5A shows that the p53 protein could be effectively purified from the PAb421 column by 1 M KCl elution (fractions 1–6), followed by alkaline elution at pH 12 (fractions 7–16). Salt-eluted p53 starting from fraction 4, and p53 recovered after alkaline elution, migrated as a single band on SDS–PAGE, and was >90% pure as judged by Coomassie blue staining. Comparison of the elution profiles of the p53 protein and the profile of exonuclease activity, as assayed by the filter retention assay (Figure 5B), revealed that the pattern of p53 protein distribution and the pattern of exonuclease activity coincided, providing further evidence for a specific association of the exonuclease activity with p53. This conclusion was also supported by the observation that, like in the copurification experiment described in Figure 2, the peaks of exonucleolytic activity closely paralleled the peaks of p53 protein regardless of the purity of the respective p53 protein fractions. This is exemplified by comparing the exonuclease activity of the comparatively impure salt-eluted p53 fractions 1–3 to that of the relatively pure fractions 4–6. Although fractions 1–3 contained considerably more contaminating proteins than fractions 4–6, the exonuclease activity in all fractions corresponded to the amount of p53 protein present in each of these fractions. In a parallel experiment, in order to further probe the specificity of this association and to get an indication for the biological relevance of the p53-associated exonuclease activity, we purified the human "hot-spot" 273^{His} mutant p53 protein by the same procedures as the corresponding wt p53 protein and analyzed it for exonuclease activity as described above. The 273^{His} mutant was chosen because this DNA contact mutant p53 (Cho et al., 1994) is one of the least defective ones: the 273^{His} mutant p53 protein still is able to assume the wt p53 conformation (PAb1620$^+$, PAb240$^-$, hsc70$^-$) and to bind to wt p53 DNA-consensus elements when present in wt p53 hetero-oligomers (Rolley et al., 1995). Thus the defect of this mutant in sequence-specific DNA binding does not necessarily have a discernable effect on tertiary structure (Friend, 1994; Ory et al., 1994). One

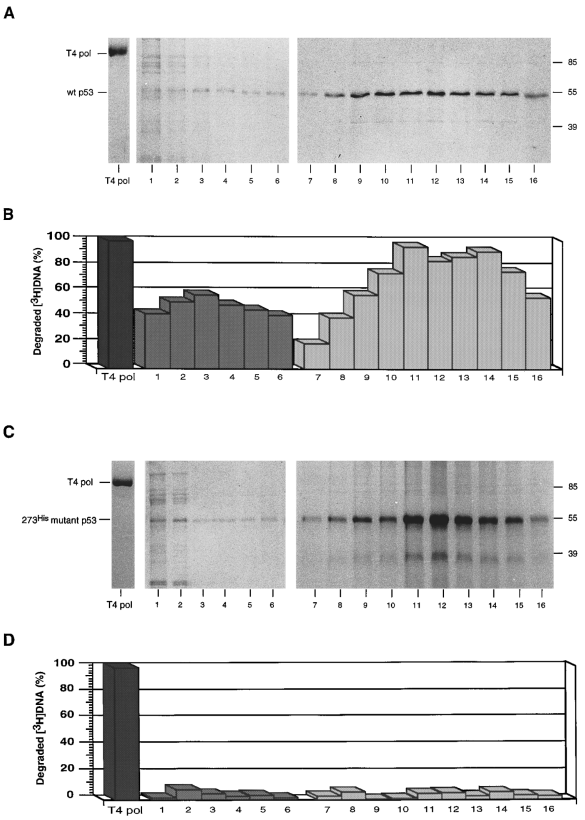

Figure 5. Exonuclease Activity of Immunoaffinity-Purified Wild-Type and 273^{His} Mutant p53

Soluble bacterially expressed human wt p53 (A and B) and 273^{His} mutant p53 (C and D) were immunopurified and tested for exonuclease activity. (A) and (C) show the purification of wt p53 (A) or mutant p53 (C), as monitored by SDS–PAGE analysis of proteins in fractions eluted from the PAb421 column with 1 M KCl (fractions 1–6), followed by alkaline elution at pH 12 (fractions 7–16; see Experimental Procedures). Aliquots from each fraction were acid precipitated and analyzed by SDS–PAGE. A sample (2.5 μl) of each fraction was tested for exonuclease activity by [^{3}H]-filter retention assay; results displayed as columns of relative exonuclease activity as described in Experimental Procedures ([B], wt p53; [D] 273^{His} mutant p53).

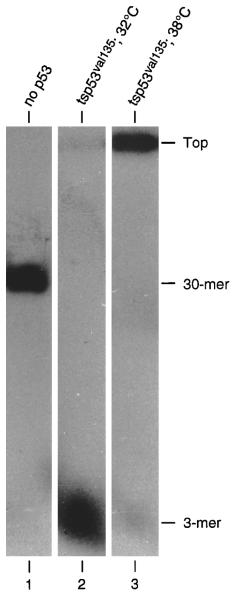

Figure 6. Exonuclease Activity of Thermo-Sensitive tsp53^{val135} Protein

Exonuclease activity was measured with a 5′-labeled oligonucleotide and analyzed by 20% polyacrylamide gel electrophoresis under nondenaturing conditions (Hupp et al., 1992). Lane 1 contained only the oligonucleotide; lane 2 displays reaction products after 20 min incubation at 20°C with 2.5 ng ts p53^{val135} isolated from cells grown at 32°C (wild-type form); lane 3 shows reaction products after 20 min incubation at 20°C with 5 ng ts p53^{val135} isolated from cells grown at 38°C (mutant form).

therefore can assume that this mutant protein most likely is similar to wt p53 in its protein binding properties. Figure 5C shows that the 273^{His} mutant p53 protein was recovered in a higher yield as the wt p53 protein. However, in contrast with the wt p53 protein, the mutant protein was devoid of detectable exonuclease activity. As this human 273^{His} mutant p53 protein was expressed from the same vector, produced in the same bacterial strain, and purified the same way as the corresponding wt p53 protein, this finding strongly supports the notion of a highly specific and biologically relevant association of an exonucleolytic activity with wt p53. We also have analyzed the 175^{His} mutant of p53, which is a structural mutant (Cho et al., 1994), and therefore is in the mutant conformation. As expected, we found it to be negative for exonuclease activity.

To further substantiate our claim that an exonucleolytic activity is specifically associated with the wild-type form of p53, we next purified the temperature-sensitive mouse tsp53^{val135} from rat clone 6 cells. When clone 6 cells are grown at 38°C, the tsp53 protein predominantly assumes a mutant phenotype, whereas in cells grown at 32°C this protein displays wild-type properties (Michalovitz et al., 1990). tsp53 from clone 6 cells grown either at 38°C or at 32°C was purified by immunoaffinity chromatography and tested for exonuclease activity. "Wild-type" tsp53 protein isolated from clone 6 cells kept at 32°C had a prominent exonuclease activity when incubated with the single-stranded 30-mer oligonucleotide (Figure 6, lane 2). In contrast, mutant tsp53^{val135} protein recovered from clone 6 cells kept at 38°C displayed only marginal exonuclease activity when compared to the tsp53 protein in its wild-type form (Figure 6, lane 3). However, the mutant form of the tsp53 protein still bound ssDNA, as judged from the shift of most of the input DNA to the top of the gel in the presence of the mutant form of tsp53 (Figure 6, lane 3).

SDS-PAGE-Purified and Renatured p53 Protein Retains Exonuclease Activity

The data presented so far are compatible with either a very specific association of an exonuclease copurifying with p53 in its wild-type form from bacterial to mammalian cells, or, alternatively, with wt p53 exhibiting an intrinsic exonuclease activity. To rigorously discriminate between these alternatives, we wanted to exclude the possibility of any noncovalent protein–protein interaction during purification. Therefore, we solubilized mouse

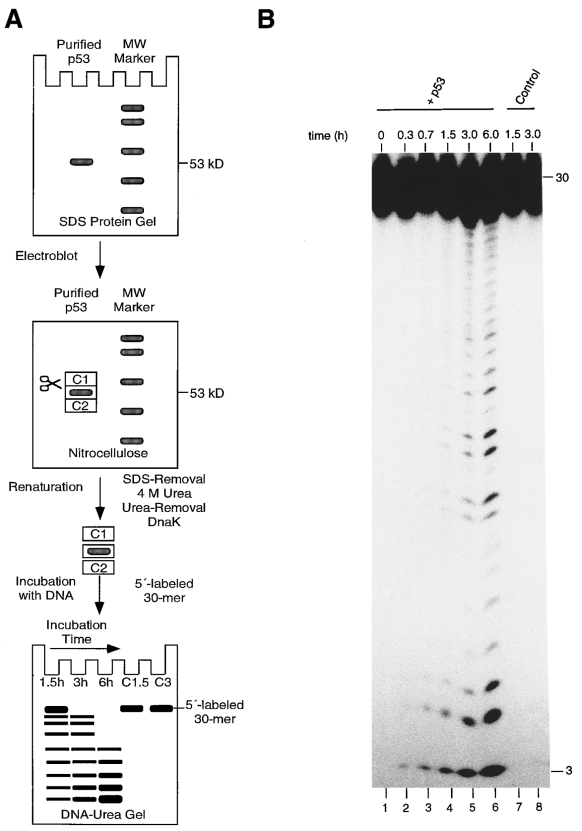

Figure 7. Exonuclease Activity Associated with p53 Protein after SDS–PAGE and In Situ Renaturation

Bacterially expressed murine p53 protein was solubilized from inclusion bodies in the presence of 6 M guanidinium hydrochloride. The solubilized protein was further purified by nickel-NTA chromatography. Guanidinium was removed by subsequent dialysis against buffer containing decreasing concentrations of urea. After this step, the p53 protein was >95% pure but displayed <1% of the exonuclease activity seen with purified p53 protein from Sf9 cells. Depicted in (A) is the experimental design for measuring exonuclease activity of guanidinium-solubilized bacterial p53 after column purification, subsequent purification on an SDS gel, electrotransfer onto nitrocellulose, and in situ protein renaturation. Displayed in (B) is the time-dependent exonuclease activity under the p53 band (lanes 1–6). Lanes 7 and 8 show a control, where a piece of nitrocellulose filter was taken immediately upward of the p53 band.

wt p53 from bacterial inclusion bodies in the presence of 6 M guanidinium chloride, purified it by nickel-chelate chromatography, and renatured it by using a refolding protocol in the presence of decreasing concentrations of urea (see Experimental Procedures). The highly purified p53 protein was loaded onto an SDS polyacrylamide gel and separated from possibly still-copurifying contaminants by gel electrophoresis. Then, we transferred the p53 protein onto a nitrocellulose membrane, renatured it on the membrane, and tested for exonuclease activity. Even after this extremely stringent purification protocol (Figure 7A), the excised band that contained p53 protein showed a time-dependent 3′-to-5′ exonuclease activity (Figure 7B). In contrast, such an activity was not seen with nitrocellulose pieces obtained immediately above or below the position of the p53 band (Figure 7B). Further controls, including similar analyses performed with the 273^{His} mutant p53 and with unrelated

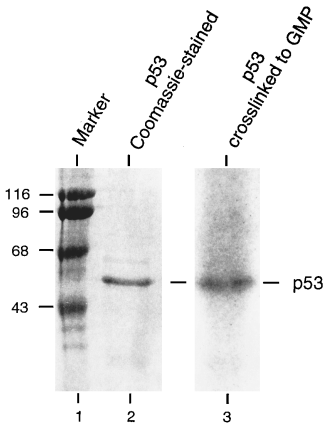

Figure 8. UV Photocross-Linking of GMP to p53 Protein

UV-crosslinked p53 (see Experimental Procedures) and a molecular weight marker were separated by SDS–PAGE. The gel was stained with Coomassie, dried, and analyzed by autoradiography to an X-ray film. The autoradiograph shown in lane 3 corresponds to the Coomassie stain shown in lane 2. Lane 1 contained β-galactosidase (116 kDa), phosphorylase β (96 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa) as molecular weight markers.

proteins such as bovine serum albumin, yielded negative results (data not shown).

UV Photocross-Linking of GMP to p53 Protein

To provide additional independent evidence for an intrinsic exonuclease activity of the p53 protein, we further analyzed the inhibitory effect of GMP on the exonucleolytic activity of p53 (Figure 1, lane 4; Table 2). If the exonuclease activity were intrinsic to the p53 protein, then its inhibition by GMP would have to be due to GMP binding to a nucleoside monophosphate binding site, serving as "exit site" for exonucleolytically released dNMPs. Such a hypothetical site might be detected by photocross-linking GMP to highly purified p53 in solution (see Experimental Procedures). This indeed was the case, as a rather specific and efficient cross-linking of the radioactively labeled GMP to the p53 protein was observed (Figure 8, lane 3). No other hypothetically copurifying protein could be cross-linked to [^{32}P]GMP, further supporting our conclusion that the exonuclease activity associated with purified p53 is intrinsic to the p53 protein.

The p53 Exonuclease Activity Maps to the Core Domain of the p53 Molecule

The p53 molecule can be functionally and structurally divided into three domains, separated by hinge regions (Bargonetti et al., 1993; Pavletich et al., 1993; Wang et al., 1993): the N-terminal transactivator domain (amino acids 1–42; Lin et al., 1994); the core domain (amino acids 120–290), which is the sequence-specific binding domain of p53 (Bargonetti, et al., 1993; El-Deiry et al., 1992); and the C-terminus (amino acids 311–390), which contains the p53 oligomerization domain (Stürzbecher et al., 1992), the nuclear localization signals (Shaulsky et al., 1990), several regulatory phosphorylation sites (Hupp and Lane, 1994; Meek, 1994), and a non-sequence-specific DNA/RNA binding domain (Foord et al., 1991; Wang et al., 1993; Mosner et al., 1995). Although

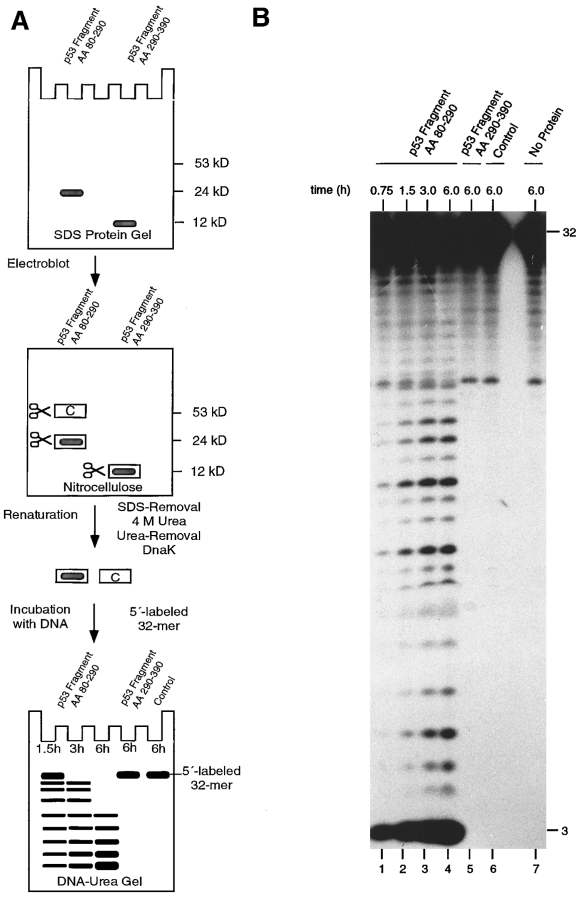

Figure 9. The Core Domain of the p53 Protein Displays Exonuclease Activity.

Bacterially expressed protein fragments of the murine p53 protein (the core domain consisting of amino acids 80–290 and a C-terminal fragment comprising the amino acids 290–390) were treated as shown in (A). Displayed in (B) is the time dependent exonuclease activity under the band corresponding to the core domain of p53 (lanes 1–4). No exonuclease activity could be detected either under the band of the C-terminal fragment (lane 5) or the control piece corresponding to a protein size of 53 kDa (lane 6). The heterogeneity of the DNA used in this experiment was due to incomplete purification of the chemically synthesized oligonucleotide (lane 7). Note that a nitrocellulose piece cut out from the 53 kDa region of the nitrocellulose blot above the 24 kDa p53 core fragment (control in [A] and [B]) did not exhibit exonuclease activity, further excluding the possibility that an exogenous 53 kDa exonuclease copurifies with p53.

our finding that the 273^{His} mutant was devoid of exonucleolytic activity (Figure 5) already suggested an involvement of the central core domain in this activity, this did not unequivocally exclude the possibility that the p53-intrinsic exonuclease activity was mediated by the p53 C-terminal nonspecific DNA binding domain. To discriminate between these possibilities, bacterially expressed p53 fragments comprising the core domain (amino acids 80–290) or the C-terminus of p53 (amino acids 290–390) (Müller et al., 1996) were purified by the denaturation/renaturation protocol as described above and analyzed for exonuclease activity (Figure 9). Figure 9B shows that only the p53 core domain, but not the p53 C-terminus exhibited exonuclease activity, thereby localizing the p53 exonucleolytic activity to the central DNA binding domain of p53. As this domain is the main target for mutational inactivation of p53's tumor suppressor function, this finding suggests that mutations in the core domain, like in 273^{His} mutant p53, may also inactivate p53's exonuclease activity. Our finding, that the purified 24 kDa core domain of p53 displayed exonuclease activity, also eliminates the remote possibility that a bacterial exonuclease, comigrating with the p53 protein in SDS–PAGE, might be responsible for the exonucleolytic activity observed with full-length p53, shown in Figure 7B.

Discussion

The present view of the molecular action of the tumor suppressor p53 implies a function as a cell cycle regulator in response to DNA damage (reviewed in Cox and Lane, 1995). In this study we provide stringent evidence, derived from several independent experimental approaches, that wild-type p53 protein displays an intrinsic exonuclease activity. Mammalian cells contain a variety of different exonucleases with functions in DNA replication, DNA recombination, and DNA repair (see e.g., Kornberg and Baker, 1991; Linn et al., 1993). Thus, the exonuclease activity associated with p53 might act in all these processes.

The exonuclease activity of p53 could be mapped to the central core of the p53 molecule, which is the target for most of the missense mutations inactivating the tumor suppressor function of p53, like in the human hotspot 273^{His} mutant of p53, which also lacked exonuclease activity. So far, the most prominent feature of the wild-type p53 core domain is its ability to bind to p53 DNA consensus elements in a sequence-specific manner. Sequence-specific DNA binding of the core domain seems to be regulated by the p53 C-terminus and can be activated by a variety of posttranslational modifications (Hupp and Lane, 1994) and/or by recognition of DNA bulges resulting from DNA deletion/insertion mismatches (Lee et al., 1995). In addition, the p53 core domain has been shown to bind nonspecifically to internal regions of single-stranded DNA (Bakalkin et al., 1994). The 3′-to-5′ exonuclease activity described for the core domain in this study defines yet another function for this domain. Such multiple functions might be considered unusual for a single protein domain. However, one has to bear in mind that the DNA binding domain of p53, as defined by X-ray crystallography, represents a hitherto unknown binding domain for the sequence-specific interaction of DNA binding proteins with DNA, marked by the compaction of several individual DNA binding elements (Cho et al., 1994). Of particular interest in this regard is the recently demonstrated similarity between the DNA binding domain of p53 and the catalytic domain of the major apurinic/apyrimidinic (AP) repair endonuclease in E. coli, exonuclease III (Mol et al., 1995). The catalytic domain in this multifunctional enzyme is made up by an $\alpha\beta$-sandwich scaffold encompassing a single divalent metal cation site. This $\alpha\beta$-sandwich scaffold positions the structural elements, including a protruding basic helix, required for DNA interaction. A similar $\alpha\beta$-sandwich scaffold has been found for the p53-DNA complex (Cho et al., 1994).

p53 protein levels are rapidly induced after γ-irradiation of living cells. This type of radiation preferentially induces dsDNA breaks (Kastan et al., 1992; Nelson and Kastan, 1994), which subsequently are repaired by recombination processes (Szostak et al., 1983). Double-strand break repair is thought to require DNA helicases, such as the Ku autoantigen (Suwa et al., 1994; Tuteja et al., 1994), and exonucleases. Hence, the exonuclease activity of p53 might be directly involved in repair processes such as dsDNA break repair. Another type of error avoidance mechanism requiring exonuclease activities is the so-called postreplicative mismatch repair pathway. In bacteria this system invokes the coordinated action of the MutSLH damage recognition/endonuclease complex along with the UvrD helicase, DNA polymerase III, DNA ligase, single-strand DNA binding protein, and any one of the exonucleases ExoI (3′-to-5′ exo), ExoVII (3′-to-5′ and 5′-to-3′ exo) or RecJ (5′-to-3′ exo) (Modrich, 1987; Modrich, 1989). An involvement of p53 protein's exonuclease in the postreplicative mismatch repair pathway would be of particular interest because this would link one of the enzymological functions of p53, i.e. its exonuclease activity, to the tumor suppressor function of the mammalian MutSLH homologs hMSH2, hMLH1, hPMS1, and hPMS2. Functional loss of either of these proteins leads to an increased incidence of colon carcinomas (for reviews, see Radman and Wagner, 1993; Bodmer et al., 1994; Modrich, 1994). Furthermore, mutations in any of these genes display a generalized increase in spontaneous mutation rates, a replication error–positive (RER^+) phenotype, and a resistance to alkylating agents (Leach et al., 1993; Parsons et al., 1993; Bhattacharyya et al., 1994). In this respect, it is intriguing that a relative resistance to alkylating agents was also observed with p53-deficient animals (Harvey et al., 1993). Furthermore, there is also some evidence that, like wild-type p53 protein itself (Wiesmüller et al., 1996), an intact mammalian MutSHL system displays an "anti-recombinogenic" effect (Fishel and Kolodner, 1995). In line with the possibility of a direct involvement of p53 in mismatch repair is the recent demonstration that wt p53 can "sense" DNA mismatch lesions by tightly binding to DNA-containing insertion/deletion mismatches (Lee et al., 1995).

Last but not least, the biochemical similarity between proofreading exonucleases of various DNA polymerases and p53's exonuclease provokes the tempting speculation that p53 might act as external proofreader for errors produced by some of the cellular DNA polymerases. In eukaryotes, two of the replicative DNA polymerases, namely the PCNA-dependent polymerases δ and ϵ, contain an intrinsic 3′-to-5′ exonuclease activity that has been shown to act as proofreader for errors produced by either enzyme. In contrast, two further polymerases, DNA polymerases α and β, do not possess such an intrinsic proofreading function (Wang, 1991). However, DNA polymerase α is certainly involved in nuclear DNA replication, whereas DNA polymerase β has been assigned to DNA repair processes (Wang, 1991). When DNA damage has occurred, there is an apparent shutoff of PCNA-dependent DNA replication (Flores-Rozas et al., 1994; Waga et al., 1994). Although PCNA-dependent DNA-repair synthesis still seems to continue under conditions of $p21^{CIP1/WAF1}$ induction (Li et al., 1994), DNA repair synthesis might also be performed by the PCNA-independent and proofreader-free DNA polymerases β and/or α. Thus, the increase of p53 protein levels after damage induction might not only shut off PCNA-dependent DNA replication, but might also increase the accuracy of DNA synthesis performed by the more error-prone DNA polymerases β and α due to the active contribution of a 3′-to-5′ proofreading exonuclease. Regardless in which of these proposed functions the intrinsic 3′-to-5′ exonuclease would participate, the demonstration of this catalytic activity for p53 should expand the role of p53 from a protein acting primarily upon exogeneously inflicted DNA damage to an active participant in a variety of DNA repair processes, including replication-associated DNA repair.

Experimental Procedures

Bacteria and Cells

Human wt p53 cDNA, human 273^{His} mutant p53, and human 175^{His} mutant p53 cDNA (mutant cDNAs were provided by H.-W. Stürzbecher, Heinrich-Pette-Institute, Hamburg, Germany) were inserted into the pET3A plasmid, and expressed in BL21(DE3) bacteria (Studier et al., 1990). About 50% of the overexpressed wt p53 protein and about 10% of the overexpressed mutant p53 were soluble. Furthermore, a murine wt p53 cDNA, containing sequence information for six histidine residues (His_6) at the N-terminus, was inserted into the pH6EX3 vector and expressed in DH5α bacteria, but delivered p53 protein as insoluble inclusion bodies. A recombinant baculovirus that overexpressed the wild-type form of murine p53 protein with coding information for a His_6 tag at the N-terminus (Wang et al., 1993) was provided by P. Tegtmeyer (SUNY, Stony Brook). Clone 6 cells, i.e. rat embryo cells transformed with $p53^{val135}$ and activated ras oncogene (Michalovitz et al., 1990), were provided by M. Oren (Weizmann Institute, Rehovot, Israel).

Preparation of Crude Cell Lysates

p53 from Recombinant Baculoviruses

About 10^9 Sf9 insect cells were infected with the corresponding recombinant baculovirus (Wang et al., 1993). Cells were harvested, 48 hr after infection, with a rubber policeman and lysed by the addition of 50 ml ice-cold lysis buffer (20 mM Tris [pH 8.0], 2 mM EGTA [pH 8.0], 0.5% Lubrol, 500 mM NaCl, 10 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM $Na_2S_2O_5$, 50 μg/ml Leupeptin, 1% [v/v] Trasylol [Bayer, Leverkusen, FRG]).

Bacterially Expressed p53

Human wt p53 protein and 273^{His} or 175^{His} mutant p53 were prepared from the soluble fraction of an E. coli overexpressing strain. Bacterial cells were pelleted and lysed by addition of 50 ml ice-cold lysis buffer as described above.

p53 from Clone 6 Cells

About 10^9 cells were grown at 32°C as well as at 38°C, harvested by rubber policeman, washed and lysed by addition of 50 ml ice-cold lysis buffer as described above.

Immunoaffinity Chromatography

Crude lysate was centrifuged at 200,000 g for 1 hr to remove cellular debris. The clarified extract was diluted 5-fold with 20 mM Tris (pH 8.0), 2 mM EGTA (pH 8.0), 0.5% Lubrol, 2 mM PMSF, 5 mM $Na_2S_2O_5$, 50 μg/ml Leupeptin, 1% (v/v) Trasylol (Bayer, Leverkusen, FRG), and centrifuged for 20 min at 20,000 g to remove precipitated proteins. The cleared extract was loaded onto a 1 ml column containing 1 mg purified PAb421 coupled to CNBr-activated Sepharose 4B-CL (Pharmacia) and washed with 200 ml buffer A (30 mM potassium phosphate [pH 8.0], 50 mM KCl, 1 mM EDTA, 2 mM DTT).

Salt Elution

The column was eluted with 15 ml 1 M KCl in buffer A, and 1 ml fractions were collected. This step eluted ~25% of the loaded p53 protein with a purity of >85% as judged from SDS–PAGE.

Alkaline Elution

The remaining p53 protein was eluted with 10 ml buffer B (100 mM potassium phosphate [pH 12.0], 1 M KCl, 1 mM EDTA, 2 mM DTT, and 20% glycerol) and immediately neutralized by the addition of an appropriate amount of KH_2PO_4 (Nasheuer and Grosse, 1987). p53-containing fractions were concentrated by dialysis against solid polyethylene glycol 40,000 (Merck, Darmstadt, Germany) to a concentration of about 50–100 μg/ml. The concentrated fraction could be stored for about 3 weeks at 4°C without considerable loss of exonuclease activity.

Heparin-Sepharose Column Chromatography

p53 was partially purified by immunoaffinity chromatography and high salt elution as described above. p53-containing fractions were pooled, diluted to a salt content of 50 mM KCl, and loaded on a 2 ml heparin-sepharose column. After washing with 25 ml buffer A (30 mM potassium phosphate [pH 8.0], 50 mM KCl, 1 mM EDTA, 2 mM DTT), proteins were eluted in a total volume of 30 ml by a salt gradient from 0.05–1 M KCl in buffer A.

Affinity Purification of Histidine-Tagged Protein

When histidine-tagged p53 protein was overexpressed, the crude cellular extract was passed over a 1 ml nickel-NTA column (Qiagen) (Wang et al., 1993). The column was washed with 50 ml of buffer C (50 mM imidazole [pH 7.5], 100 mM Tris [pH 7.0], 150 mM NaCl, 1 mM PMSF, 0.5% Lubrol) and eluted with buffer D (250 mM imidazole [pH 7.5], 100 mM Tris [pH 7.0], 150 mM NaCl, 1 mM PMSF, 0.5% Lubrol). Protein prepared in this manner was exonuclease proficient and >90% pure. Concentration and storage were as described above.

Purification of p53 by Affinity Chromatography, SDS–PAGE, and Renaturation

Insoluble murine p53 was collected by centrifugation of bacterial crude extracts and solubilized in 6 M guanidinium chloride. Solubilized p53 was purified in the presence of 6 M guanidinium hydrochloride by chromatography on a nickel-chelate column, essentially as described above. Guanidinium was removed by subsequent dialysis against buffers containing decreasing concentrations of urea (1 M, 0.8 M, 0.5 M, 0.1 M, and no urea). Guanidinium hydrochloride–solubilized p53 protein was to >95% pure but after renaturation typically displayed <1% of the exonuclease activity observed with purified soluble protein from the other sources. This protein was loaded onto a 10% SDS–PAGE (Laemmli, 1970) and separated by electrophoresis at 10 V/cm at room temperature. The protein was transferred electrophoretically onto a nitrocellulose membrane (Hybond C super, Amersham). Protein standards, including a p53 standard, were visualized with amido black. Nitrocellulose slices containing unstained p53 as well as control slices taken from the neighborhood of the p53 band (see Figures 7A and 7B) were excised from the membrane at their corresponding positions. Renaturation of the protein was performed by subsequent incubation in 50% isopropanol as follows: for 30 min in renaturation buffer 1 (50 mM NaCl, 10 mM Tris–HCl [pH 7.0], 2 mM EDTA, 1 mM DTT, 4 M urea, 1% Triton X-100), two times for 30 min each; in renaturation buffer 1 without Triton X-100, two times for 30 min each; and finally for 30 min in renaturation buffer 2 (50 mM NaCl, 10 mM Tris–HCl [pH 7.0], 1 mM EDTA, 1 mM DTT, 6 mM $MgCl_2$, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrolidon, 1 mM ATP, and 1 μg/ml DnaK protein (Boehringer Mannheim)). Thereafter, the nitrocellulose membrane with renatured p53 protein and controls were incubated with a 5′-labeled oligonucleotide (see below) for the indicated times. The reaction products were analyzed on a 20% polyacrylamide gel containing 7 M urea (Hohn and Grosse, 1987).

Exonuclease Assays

Preparation of 3′-Terminally Labeled Substrates for the Exonuclease

$(dT)_{20}$ (3′ ends, 5 μM) was incubated with 25 μCi [^{3}H]dTTP in 100 μl reaction buffer consisting of 100 mM potassium cacodylate [pH 7.0], 1 mM $CoCl_2$, and 0.2 mM DTT (Grosse and Manns, 1993). The reaction was started by the addition of 50 U terminal transferase (Stratagene); incubation was for 30 min at 30°C. After heat-inactivation for 10 min at 65°C, 0.2 mM poly(dA) was added and the reaction mixture was allowed to cool down slowly to room temperature. The reaction mixture was passed through a 1 ml spin column filled with Sephadex G-50 (Pharmacia) that was equilibrated with 50 mM Tris–acetate [pH 7.3], 1 mM EDTA. This step removed unincorporated dNTPs and the reaction buffer. The labeled product was pipetted onto the hydrophilic side of a microdialysis membrane (Type VS, pore size 0.025 μm, Millipore, Eschborn, FRG) that swam on 20 ml 10 mM Tris–HCl [pH 8.0], 0.1 mM EDTA. Dialysis was carried out for at least 2 hr at room temperature. After dialysis, the specific activity was typically 160 cpm/fmol.

Preparation of 5′-Labeled Oligonucleotides

Oligonucleotides were 5′-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase according to a standard procedure (Sambrook et al., 1989). Unincorporated radioactivity was removed by separation over a 20% polyacrylamide gel; the labeled oligonucleotides were detected by an autoradiography. Corresponding gel slices were cut out and extracted with Tris–EDTA (pH 8.0) overnight at room temperature.

Measurement of 3′-to-5′ Exonuclease Activity by Filter Binding Assays

The 3′-to-5′ exonuclease activity was assayed by measuring the loss of 3′-labeled nucleotides after incubation with enzyme. The reaction mixture (20 μl) contained 50 mM Tris–acetate (pH 8.5), 10 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml bovine serum albumin, and 125 nM (tritiated 3′ ends) of labeled poly(dA) · $(dT)_{20}$-[^{3}H]dT (~10,000 cpm). The reaction was started by the addition of 1–2 ng p53 and performed for 15 min at 37°C. The exonuclease activity of T4 DNA polymerase (donated to us by L. Reha-Krantz, University of Alberta, Edmonton, Canada) served as a positive control. The mixture was spotted onto DE81 filters (Whatman, Maidstone, UK), the filters were washed (2 ml, five times) with 0.5 M $(NH_4)HCO_3$ and then dried under infrared light. Radioactivity that remained on the filters was measured by liquid scintillation counting.

Measurement of Exonuclease Activity by Gel Electrophoresis

Gel electrophoretic measurements of exonuclease activity were performed with 1–2 ng of the 30-mer deoxyoligonucleotide 5′-GACACT GGTCACACTTGGCTGCTTAGGAAT-3′ (Foord et al., 1993). The oligonucleotide was chemically synthesized by using the phosphoroamidite method and purified by reversed phase HPLC and subsequent gel electrophoresis. 5′-labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase was according to a standard procedure. 3′-labeling was performed with terminal transferase and [α-^{32}P]ddATP (Amersham) according to the instructions of the manufacturer of the 3′-labeling kit (Boehringer). In both cases, the reaction products were analyzed on a 20% polyacrylamide gel containing 7 M urea.

UV Cross-Linking of GMP to the p53 Protein

A total of 1 μg of p53 protein, overexpressed from Sf9 cells, was incubated with 50 μCi of [^{32}P]GMP (3000 Ci/mmol; Hartmann Analytik GmbH, Braunschweig, FRG) in 25 mM Tris–HCl (pH 8.5), 10 mM $MgCl_2$, 75 mM potassium acetate. Samples were irradiated with 0.3 J/cm^2 at 254 nm (Nasheuer and Grosse, 1988). UV-crosslinked p53 and a molecular weight marker were separated by SDS–PAGE. The gel was stained with Coomassie, dried, and analyzed by autoradiography.

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