RESEARCH COMMUNICATION

Increased salt concentration reversibly destabilizes p53 quaternary structure and sequence-specific DNA binding

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Growth suppression by p53 correlates with sequence-specific DNA binding and is determined by tertiary and quaternary protein structures. Exposure to 300 mM NaCl did not affect p53 tertiary structure, but dissociated high-molecular-mass complexes with concomitant loss of specific DNA binding. Both effects were reversible. We conclude that high salt can reversibly destabilize the quaternary structure of p53 that is most efficient for sequence-specific DNA binding.

INTRODUCTION

The tumour suppressor p53 is a transcriptional regulator which binds specific DNA sequences and *trans*-activates the expression of genes under promoters containing p53 binding sites (reviewed by Oren, 1992; Donehower and Bradley, 1993). The ability to interact with specific DNA targets depends on p53 tertiary and quaternary structures. Thus sequence-specific DNA binding is most efficient for oligomeric structures corresponding to p53 tetramers and/or higher-order structures (Hainaut et al., 1994), whereas monomeric p53 does not bind DNA (Shaulian et al., 1993; Tarurina and Jenkins, 1993). Analysis of p53 tertiary structure with conformation-specific monoclonal antibodies has revealed that reactivity with the monoclonal antibody PAb1620 is a pre-requisite for DNA binding (Halazonetis et al., 1993; Hainaut and Milner, 1993a,b; Hainaut et al., 1994; A. Hall and J. Milner, unpublished work).

Protein–DNA binding interactions are highly dependent on salt concentration, and in general binding affinities decrease with increasing salt concentrations (see review by Record et al., 1991). Salt concentration may influence protein conformation and subunit polymerization as well as the rate of association/dissociation of non-specific and specific protein–DNA complexes (Record et al., 1991). In the present study we have examined the effects of univalent-salt concentration on sequence-specific DNA binding by p53 in relation to (i) p53 tertiary structure (determined by reactivity with conformation-specific monoclonal antibodies) and (ii) p53 quaternary structure (determined by size fractionation on gel-filtration columns).

p53 was expressed *in vitro* by using rabbit reticulocyte lysate for translation. Salt concentration dramatically affected DNA binding of p53, apparently due to reversible dissociation of the p53 quaternary structure required for specific DNA binding.

EXPERIMENTAL

Transcription, translation and immunoprecipitation of p53

The plasmid pSP6p53^{Ala135} was linearized with *Hin*dIII and transcribed *in vitro* to produce RNA for translation of wild-type murine p53 (Milner et al., 1991). Translations in rabbit reticulocyte lysate (Promega) were carried out for 1 h at 37 °C, in the presence of 0.75 μM added [^{35}S]methionine (40.5 TBq/mmol; Amersham). Portions of lysate were diluted 1:100 in 10 mM Tris/HCl, pH 8, containing 0.1% Nonidet P-40 (NP40) (Tris/NP40 buffer) and the indicated concentration of NaCl (5–500 mM), and were immunoprecipitated as described elsewhere with antibodies PAb240, PAb1620, PAb246, PAb248 and PAb421, with PAb416 as negative control (Cook and Milner, 1990; Milner et al., 1991). Immunoprecipitates were analysed by SDS/PAGE. Densitometric analysis of autoradiograms was performed with a LKB Ultroscan XL densitometer.

DNA-binding assays

Samples of lysate were diluted 1:100 in Tris/NP40 buffer containing various concentrations of NaCl as described above. For electro-mobility shift assay (EMSA), 100 μl portions of diluted lysate were supplemented with 20 μl of glycerol (50% in Tris/NP40 buffer, containing NaCl at a concentration identical with that in the dilution buffer), 0.1 μg of salmon sperm DNA as a non-specific competitor, 5 mM dithiothreitol, antibody PAb421 (30 μl of hybridoma supernatant) and 5 ng of ^{32}P-end-labelled double-stranded oligonucleotide 5′-GGACATGCCCGGGC-ATGTCC-3′ as a specific p53 binding site (p53CON; Funk et al., 1992). Samples were incubated for 30 min at room temperature. Reaction products were run on PAGE (4% gel) at 122 V under cooling, in Tris/borate/EDTA buffer containing 1 mM EDTA.

Fractionation by gel filtration

Samples of translated lysate were diluted in Tris/NP40 buffer containing various concentrations of NaCl (see above), and 200 μl samples of diluted lysate were applied on to either a TSK3000 (Anachem) or a Superose 6 (Pharmacia) gel-filtration column equilibrated in 10 mM Tris/HCl, pH 7.6, containing 0.1% NP40 and a concentration of NaCl identical with that in the dilution buffer. Samples were run on a Gilson h.p.l.c. system at flow rates of 1 ml/min for TSK3000 or 0.5 ml/min for Superose 6. The fractionation profiles of murine wild-type p53 on these columns, and their relation to known molecular-mass markers, are detailed elsewhere (Milner et al., 1991; Hainaut et al., 1994). The presence of radiolabelled p53 in column fractions was determined by scintillation counting and by SDS/PAGE.

Abbreviations used: EMSA, electro-mobility shift assay; NP40, Nonidet P-40.

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RESULTS AND DISCUSSION

Influence of univalent cation on sequence-specific DNA binding by p53

To evaluate the effect of salt concentration on DNA binding, murine wild-type p53 was translated *in vitro* and samples of lysate were diluted 1:100 in a DNA-binding buffer containing NaCl at concentrations in the range 5–500 mM (see the Experimental section). Binding assays were carried out in the presence of the monoclonal antibody PAb421, which reacts with an epitope located in the C-terminus of the p53 polypeptide (residues 368–378 of murine p53), and stabilizes specific p53–DNA complexes detected by EMSA (Funk et al., 1992; Hupp et al., 1992; Hainaut et al., 1994).

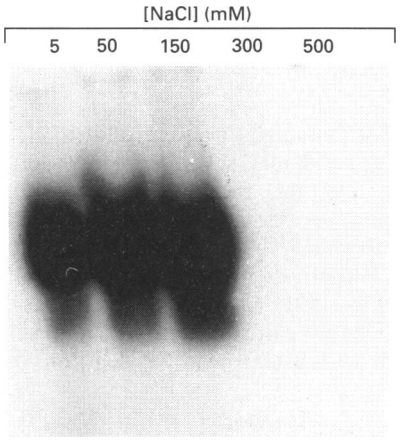

Figure 1 Influence of the concentration of NaCl on the specific binding of p53 to the consensus DNA sequence p53CON

Wild-type murine p53 translated *in vitro* was diluted 1:100 in DNA-binding buffer containing NaCl as indicated and assayed for binding to ^{32}P-labelled p53CON (5′-GGACATGCCCGGGCATGTCC-3′) (see the Experimental section). All reactions were carried out in the presence of PAb421, which super-shifts and stabilizes p53–DNA complexes (Funk et al., 1992; Hupp et al., 1992). EMSA was performed by PAGE on 4% gels (see the Experimental section).

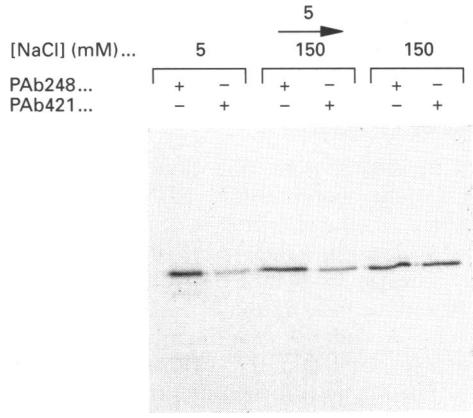

Figure 2 Effect of NaCl on the presentation of the PAb421 epitope

Murine wild-type p53 was translated *in vitro* in the presence of [^{35}S]methionine and subsequently diluted 1:100 in buffer containing NaCl at 5 mM, 150 mM, or 5 mM followed by addition of salt to 150 mM (indicated by horizontal arrow). The labelled protein was precipitated with either PAb248 (as a control) or PAb421. Immunoprecipitates were analysed by SDS/PAGE.

Optimal DNA binding was observed at concentrations of NaCl between 50 and 150 mM, compatible with physiological salt concentrations. In contrast, binding was decreased by about 40% at 5 mM (Figure 1). Increasing salt concentration from 5 to 150 mM restored optimal DNA binding (results not shown). High salt concentrations had a dramatic effect on p53–DNA complexes, and no binding was detectable at concentrations of NaCl equal to or greater than 300 mM (Figure 1). However, DNA binding could be recovered by dilution from 300 mM to 150 mM NaCl (see Figure 4b). Similar results were obtained with KCl (results not shown), suggesting that these effects are independent of the chemical nature of the univalent cation involved. These results indicate that specific interactions between p53 and its target DNA *in vitro* are crucially dependent on the concentration of univalent cations. We have previously demonstrated a requirement for specific bivalent cations, in particular Zn(II), for the stabilization of p53 in the wild-type conformation capable of specific DNA binding (Hainaut and Milner, 1993a).

Effect of salt concentration on p53 tertiary structure

In physiological conditions, wild-type p53 translated *in vitro* is reactive with PAb248, PAb246 and PAb1620 (PAb246 and PAb1620 recognize conformation-dependent epitopes), but reacts weakly, if at all, with PAb240 (Cook and Milner, 1990; Milner and Medcalf, 1990). This immunoreactivity was not significantly affected by exposure to either low or high salt concentrations (results not shown).

Specific DNA binding correlates with the 246$^+$ form of murine p53 (equivalent to 1620$^+$) (Hainaut and Milner, 1993a,b). Since increasing salt concentration inhibits DNA binding without disrupting the 1620$^+$ conformation of p53, we conclude that the effect of salt on the formation of stable p53–DNA complexes involves factor(s) other than p53 tertiary structure.

Reactivity with the C-terminal antibody PAb421 was decreased at 5 mM NaCl as compared with 150 mM, an effect which could be partially reversed by addition of NaCl to 150 mM (Figure 2). This indicates that low salt specifically affected the presentation of the PAb421 epitope. Since PAb421 is included in the DNA-binding reaction to stabilize p53–DNA complexes (see above), the decrease in DNA-binding activity at 5 mM NaCl (see Figure 1) may reflect the decreased availability of the PAb421 epitope rather than a decreased affinity of p53 for target DNA. Indeed, in the absence of other variables, decreasing salt concentrations are expected to promote protein–DNA interactions (Record et al., 1991). Specific DNA binding in the absence of PAb421 can be detected by using a biotinylated DNA target to bind ^{35}S-labelled p53, followed by collection of p53–DNA complexes on avidin–agarose (Hainaut et al., 1994). Using this approach, we found that exposure to low salt resulted in an increase in DNA–p53 complex-formation (results not shown).

Taken together, these results indicate that low salt (i) promotes p53–DNA interactions, but (ii) also affects the folding of a C-terminal domain in p53, modulating the presentation of the PAb421 epitope. This, in turn, affects the detection of stable p53–DNA complexes by EMSA. An effect on PAb421 reactivity has been previously noted by Halazonetis et al. (1993), who reported that the PAb421 epitope was masked in 5 mM NaCl but exposed after interaction of p53 with a specific DNA target. However, in our hands, p53 was still partially reactive with PAb421 in 5 mM NaCl (Figure 2; 40% of PAb421 reactivity at 150 mM, as determined by densitometry), and this reactivity was

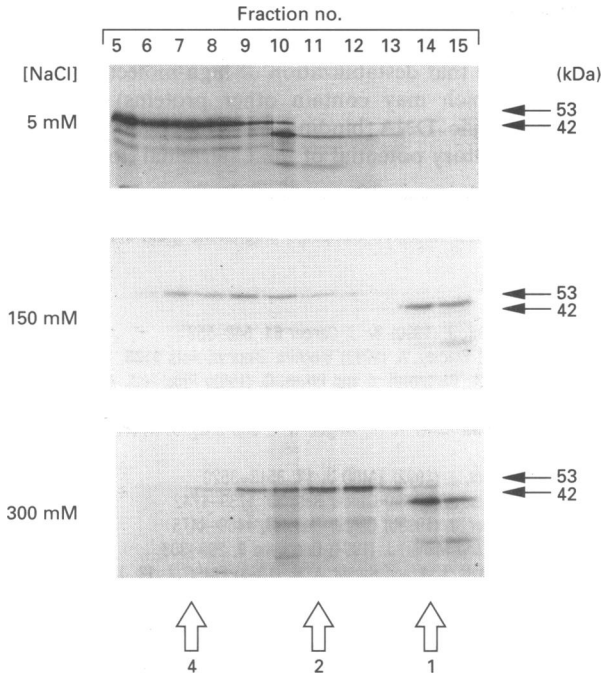

Figure 3 Effect of salt concentration on the quaternary structure of p53

Wild-type p53 translated *in vitro* was diluted 1:100 in buffer containing NaCl as indicated, fractionated on Superose 6 equilibrated in a buffer containing NaCl at a concentration identical with that in the dilution buffer, and eluted with the same buffer give 20 1-ml fractions at a flow rate of 0.5 ml/min. Equivalent samples of fractions 5–15 were analysed by SDS/PAGE on 15% gels. White arrows (bottom) indicate the position of putative p53 tetramers (4), p53 dimers (2) and monomers (1) (Milner et al., 1991; Hainaut et al., 1994). Black arrows on the right indicate the position of the 53 kDa and 42 kDa forms. The significance of other faster-migrating forms is not precisely known.

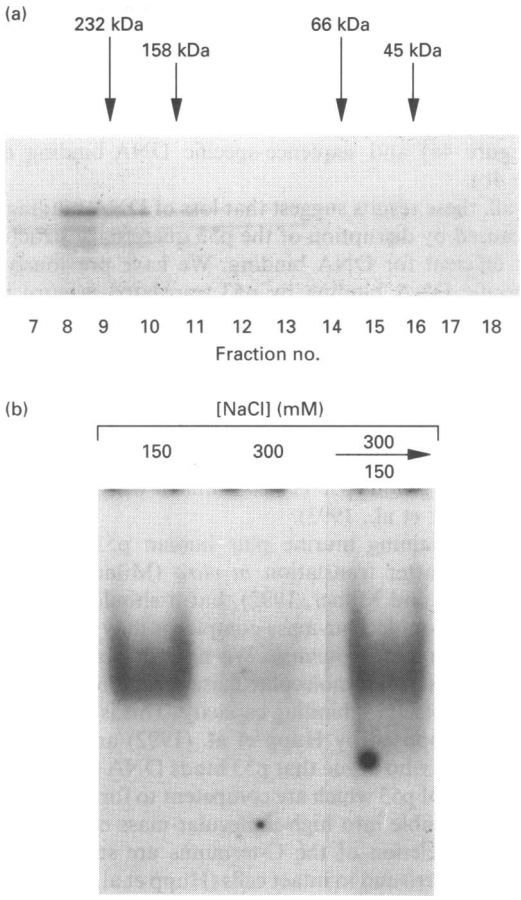

Figure 4 Reversibility of the effect of 300 mM NaCl on p53 quaternary structure and DNA-binding activity

(**a**) p53 translated *in vitro* was exposed to 300 mM NaCl and fractionated on TSK3000 equilibrated in 300 mM NaCl (results not shown). p53 material with a size consistent with dimers (fractions 10–11) was diluted to 150 mM NaCl and re-fractionated on TSK3000 (equilibrated in 150 mM NaCl) and analysed by SDS/PAGE. Arrows at the top indicate the position of protein molecular-mass standards (45 kDa, ovalbumin; 66 kDa, BSA; 158 kDa, aldolase; 232 kDa, catalase). (**b**) p53 translated *in vitro* was exposed to 150 mM NaCl, 300 mM NaCl, or 300 mM NaCl followed by dilution to 150 mM NaCl (as indicated). DNA binding to the specific sequence p53CON was assayed by EMSA as described in the legend to Figure 1.

only marginally increased by addition of specific DNA (to 58%; results not shown).

The C-terminus of p53 is regulatory for DNA binding (Hupp et al., 1992) and contains a substrate site for casein kinase II (Meek et al., 1990). C-terminal phosphorylation by this kinase activates DNA binding *in vitro* (Hupp et al., 1992) and modulates p53 suppressor activity in intact cells (Milne et al., 1992). That presentation of the C-terminal domain containing the PAb421 epitope is dependent on ionic conditions *in vitro* is particularly interesting, since, in primary lymphocytes cultured in serum-free medium, wild-type p53 is non-reactive with PAb421. Mitogenic stimulation exposes the PAb421 epitope, with reciprocal masking of the epitope for PAb248 (Milner, 1984; reviewed by Milner, 1991, 1994). Thus the PAb421-negative form of wild-type p53 may represent a functionally important form of wild-type p53 associated with cell quiescence. Although the ionic effects reported here are observed *in vitro*, they may mimic the effect of cellular regulatory factor(s) responsible for loss of PAb421 reactivity in quiescent cells.

Effects of salt concentration on p53 quaternary structure

Sequence-specific DNA binding is essentially restricted to fractions equivalent in size to tetramers and higher-molecular-mass forms (column fractions 6–9); dimeric p53 showed only weak binding, whereas a monomeric 42 kDa form did not bind DNA at all (Hainaut et al., 1994). In 5 mM NaCl, all p53 forms fractionated as high-molecular-mass oligomers with a size equal to or greater than 200 kDa (Figure 3, top panel). In contrast, in 300 mM NaCl, p53 predominated in size fractions compatible with p53 dimers and monomers (Figure 3, bottom panel).

Our results indicate that assembly of p53 into high-molecular-mass forms is dependent on ionic interactions that are titrated by increasing salt concentrations. In contrast, assembly of p53 into dimers appears to be salt-resistant, suggesting that they depend on non-ionic contacts between competent p53 monomers. This is in agreement with the observation that dimer formation may involve hydrophobic interactions between residues exposed by an amphipathic α-helix spanning amino acids 334–348 of murine p53, whereas formation of higher-order structures may require ionic interactions between other residues, including basic residues in the extreme C-terminus of the polypeptide (Milner et al., 1991; Sturzbecher et al., 1992; Medcalf and Milner, 1993).

To determine the reversibility of the effect of high salt on p53 quaternary structure, p53 was first exposed to 300 mM salt and fractionated on TSK3000, which separates p53 oligomeric forms in a manner essentially comparable with Superose 6. Fractions 10–11 from TSK3000, containing material consistent with p53

dimers, were diluted to 150 mM NaCl and submitted to a second fractionation. The presence of p53 in each fraction was determined by analysis by SDS/PAGE (Figure 4a). Decreasing the salt concentration from 300 mM to 150 mM restored both the capacity of p53 to form high-molecular-mass structures (fractions 8–9, Figure 4a) and sequence-specific DNA-binding capacity (Figure 4b).

Overall, these results suggest that loss of DNA binding in high salt is caused by disruption of the p53 quaternary structure that is most efficient for DNA binding. We have previously shown that specific DNA binding by p53 translated *in vitro* involves quaternary structures consistent with tetramers and/or high-molecular-mass forms of the protein (Hainaut et al., 1994). The size-fractionation profile of p53 expressed *in vitro* is similar to that of endogenous p53 expressed in cells (Milner et al., 1993; Kraiss et al., 1992) and to p53 expressed via a baculoviral vector (Friedman et al., 1993). Moreover, abnormal quaternary assembly of endogenous p53 *in vivo* is linked with transformed cell growth (Milner et al., 1993).

Dimers containing murine plus human p53 can clearly be demonstrated after translation *in vitro* (Milner and Medcalf, 1991; Hainaut and Milner, 1992), but it should be emphasized that the higher-molecular-mass complexes may represent p53 in complex with other protein(s). We now demonstrate that disruption of these high-molecular-mass structures results in the complete loss of DNA-binding capacity. This is at variance with the models proposed by Hupp et al. (1992) and Tarurina and Jenkins (1993), who argue that p53 binds DNA as a homodimer. Indeed, forms of p53 which are competent to form dimers but are unable to assemble into high-molecular-mass complexes due to mutation or deletion of the C-terminus are still competent to bind DNA *in vitro* and in intact cells (Hupp et al., 1992; Tarurina and Jenkins, 1993). One possible explanation for these apparently contradictory results is that the high-molecular-mass form of p53 which most effectively binds DNA could represent p53 dimers in complex with other protein(s) (Milner, 1994). Rabbit reticulocyte lysate contains proteins that can complex with p53 translated *in vitro* (Hainaut and Milner, 1992), and cellular p53 interacts specifically with proteins such as hsp70, MDM2, TBP, Sp1 and WT-1 (reviewed by Donehower and Bradley, 1993). Our present results indicate that destabilization of high-molecular-mass p53 complexes (which may contain other proteins) in high salt abolishes specific DNA binding, possibly by unmasking the negative regulatory potential of the C-terminal domain of p53.

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