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Interaction of heat-shock protein 70 with p53 translated *in vitro*: evidence for interaction with dimeric p53 and for a role in the regulation of p53 conformation

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In intact cells, hsp70 proteins selectively complex with mutant p53. We report here that rabbit reticulocyte lysate contains hsp70 which selectively complexes with the mutant p53 translated *in vitro*. Hsp70 complexes with dimers and possibly monomers of p53 in a manner that requires the terminal 28 amino acids of p53. Using murine $p53^{Val135}$, which is temperature-sensitive for phenotype, we demonstrate that p53-hsp70 complexes can occur after post-translational switching from wild-type to mutant p53 phenotype. Moreover, the temperature-induced switch of full-length $p53^{Val135}$ from wild-type to mutant phenotype is ATP-independent, whereas the switch from mutant to wild-type form requires ATP hydrolysis and involves hsp70. These results imply that hsp70 is involved in the regulation of p53 conformation.

Key words: cell growth control/heat-shock proteins/hsp70/p53

Introduction

The p53 protein plays a role in the control of cell proliferation and the p53 gene is the most commonly affected gene in human cancer (Hollstein *et al.*, 1991; Caron de Fromentel and Soussi, 1992). The p53 gene is a tumour suppressor gene, and mice homozygous for a null p53 allele introduced by homologous recombination appear to be developmentally normal but prematurely susceptible to a variety of neoplasms (Donehower *et al.*, 1992). Point mutations within conserved domains can inactivate p53 suppressor function and some may also activate p53 as an oncogene and promote cell proliferation (see Marshall, 1991 and Levine *et al.*, 1991 for reviews). Point mutations can affect the tertiary structure of p53 protein, altering its reactivity with specific anti-p53 monoclonal antibodies: the wild-type phenotype is reactive with monoclonal antibodies PAb246 and PAb1620 but not with PAb240. Conversely, the mutant phenotype is reactive with PAb240 but not with PAb246 and PAb1620 (Cook and Milner, 1990; Gannon *et al.*, 1990).

Most transforming p53 mutants form stable complexes with both stress-inducible and constitutively expressed members of the 70 kDa family of heat-shock proteins (Pinhasi-Kimhi, 1986; Hinds *et al.*, 1987; Sturzbecher *et al.*, 1987, 1988; Finlay, 1988). Heat-shock proteins (hsps) belong to a class of proteins broadly defined as 'molecular chaperones', involved in facilitating the transport, folding and assembly of many proteins (for reviews, see Gething and Sambrook, 1992; Rothman, 1989). In the case of p53, interaction with hsps appears to involve remarkably conserved structures, since p53 also interacts with a bacterial hsp, dnaK, when expressed in *Escherichia coli* (Clarke *et al.*, 1988). Moreover, both purified p53-hsp70 and p53-dnaK complexes dissociate *in vitro* when incubated with micromolar levels of ATP, suggesting that the stability of the complex is regulated by the intrinsic ATPase activity of hsps (Clarke *et al.*, 1988). However, more detailed study of p53-hsp70 complexes under defined conditions has been hampered by lack of a suitable *in vitro* association assay.

We now demonstrate the interaction of hsp70 protein present in rabbit reticulocyte lysate with p53 proteins translated in the same lysate. Translation of p53 RNA in reticulocyte lysate is a useful system for studying p53 conformation under defined conditions (Milner *et al.*, 1991; Milner and Medcalf, 1991). For example, a mutant allele of murine p53 ($p53^{Val135}$) which is temperature-sensitive for function (Michalovitz *et al.*, 1990) has been shown to be temperature-sensitive for conformation when expressed *in vitro* (Milner and Medcalf, 1990). $p53^{Val135}$ adopts the wild-type phenotype at 30°C and the mutant phenotype at 37°C. Moreover, a simple post-translational shift of temperature is sufficient to induce the protein to switch from wild-type to mutant phenotype, and vice versa (Milner and Medcalf, 1990). Using this p53 mutant we now show that hsp70 present in reticulocyte lysate complexes with p53 during the translation of mutant, but not wild-type form of p53. Complex formation also occurs when p53 is induced to switch from wild-type to mutant phenotype by a post-translational shift in temperature. This finding allowed us to investigate the role of ATP and of hsp70 binding during the interconversion between wild-type and mutant p53 phenotypes.

Results

Binding of in vitro translated murine p53 to hsp70 present in rabbit reticulocyte lysate

The presence of hsp70 in rabbit reticulocyte lysate was revealed by immunoblotting with a rabbit antiserum against the carboxyl terminus of mammalian hsp70 (data not shown). This antiserum, kindly given by Dr Stephen Ullrich (NCI), recognizes both stress-inducible and constitutively expressed members of the hsp70 family (see Materials and methods). To investigate the association between p53 and lysate hsp70, both wild-type and mutant p53 ($p53^{Val135}$) were translated *in vitro* at 37°C in the presence of [^{35}S]methionine. Immunoprecipitations were carried out with antibodies to p53 and to hsp70, using normal rabbit serum as a negative control. Figure 1A clearly shows that ^{35}S-labelled mutant p53, but not wild-type, co-precipitates with lysate hsp70. Under these conditions the amount of p53 complexed with hsp70 represents ~5-10% of total translated p53.

Comparable results were obtained with other p53 mutants (not shown).

The specificity of p53−hsp70 interaction was further demonstrated by sequential immunoprecipitations. *In vitro* translated mutant p53 was immunoprecipitated with monoclonal antibodies PAb248 (which reacts with both wild-type and mutant p53), PAb240 (specific for mutant phenotype), PAb246 (specific for wild-type phenotype) and with rabbit serum to hsp70. The supernatant of immunoprecipitations with antibodies to hsp70 was then re-immunoprecipitated with antibodies to p53 or to hsp70 (Figure 1B). When translated at 37°C, $p53^{Val135}$ adopted a mutant phenotype (reactive with PAb240 and only marginally detected by PAb246), and ~5−10% p53 was complexed with hsp70. Importantly, re-immunoprecipitation with antibodies to hsp70 detected no residual p53 (Figure 1B), ruling out the possibility that antibodies to hsp70 react non-specifically with mutant p53. Time-course analysis of binding showed that p53−hsp70 complex formation is an early event during translation of mutant p53, suggesting that interaction with hsp70 occurs during or immediately after translation of mutant p53 *in vitro*, and is not a result of accumulation of mutant p53 protein (data not shown).

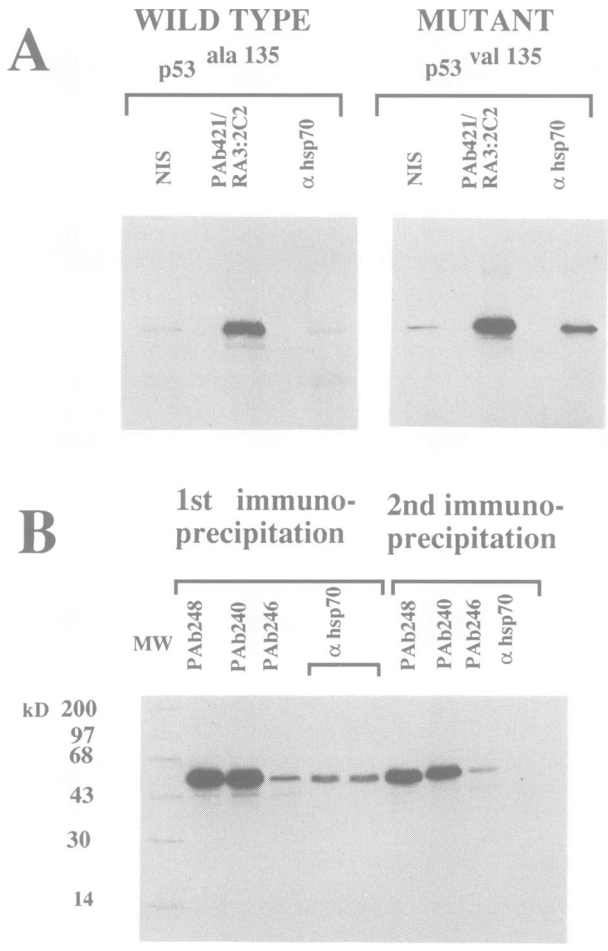

Fig. 1. Co-immunoprecipitation of ^{35}S-labelled p53 with hsp70 following translation *in vitro*. (**A**) Wild-type ($p53^{Ala135}$) or mutant ($p53^{Val135}$) p53 were translated at 37°C in the presence of ^{35}S-labelled methionine. Equivalent aliquots of translated products were incubated with anti-p53 monoclonal antibodies as indicated, or with anti-hsp70 serum. Non-immune rabbit serum (NIS) was used as a negative control. Precipitation was achieved by adding 10 μl of formalin-fixed *S.aureus* (10%) and proteins were analysed by SDS−PAGE, followed by autoradiography (see Materials and methods). (**B**) Sequential immunoprecipitations: equivalent amounts of *in vitro* translated mutant p53 ($p53^{Val135}$) were immunoprecipitated by anti-p53 monoclonal antibodies PAb248, PAb240 and PAb246, or with anti-hsp70 serum. The supernatants of the latter precipitations were pooled, and the presence of residual p53 after immunoprecipitation with anti-hsp70 was examined by re-immunoprecipitating with anti-p53 or anti-hsp70 antibodies, as indicated. Analyses are as detailed in panel A. MW: molecular weight markers (in kilodaltons).

Post-translational binding of p53 to hsp70

Temperature exerts a post-translational effect on the phenotype of $p53^{Val135}$ (Milner and Medcalf, 1990). The protein adopts the wild-type phenotype when translated at 30°C, but undergoes a conversion to mutant phenotype within 2 min of temperature shift from 30°C to 37°C. Using $p53^{Val135}$, we next asked whether binding of p53 to hsp70 could occur as a result of post-translational switching from wild-type to mutant phenotype.

$p53^{Val135}$ was translated for 1 h at 30°C and further protein synthesis was blocked by adding anisomycin. The translated lysate was then incubated for 5 min at 37°C. As predicted, $p53^{Val135}$ adopted a wild-type phenotype (reactive with PAb246 and not with PAb240) at 30°C, and switched to a mutant phenotype (reactive with PAb240 and not with PAb246) at 37°C. The change in conformation was accompanied by an increase in co-precipitation of p53 with hsp70 (Figure 2). This indicates that hsp70 can associate post-translationally with full-length p53 when $p53^{Val135}$ is induced to switch from the wild-type to the mutant conformation.

Determination of the size of p53−hsp70 complexes

After size fractionation (by gel filtration), *in vitro* translated p53 resolves into peaks compatible with tetrameric, dimeric and monomeric forms of p53 (Milner *et al.*, 1991; P.Hainaut and J.Milner, unpublished observations). To determine the size of p53−hsp70 complexes, $p53^{Val135}$ was translated at 30°C, shifted to 37°C for 5 min and subjected to size fractionations as described previously in Milner *et al.* (1991). TCA-precipitation of fractionated material shows that translated p53 forms dimers (Figure 3A, peak 2) and

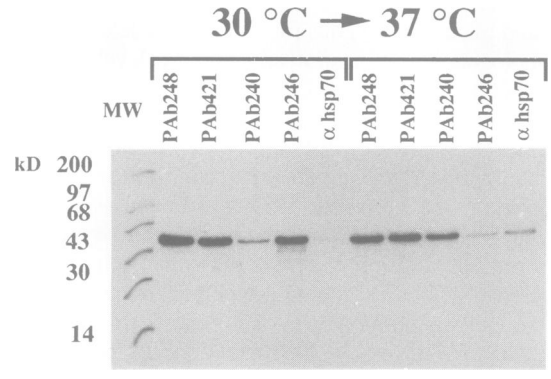

Fig. 2. Post-translational effect of temperature on complex formation between *in vitro* translated $p53^{Val135}$ and lysate hsp70. $p53^{Val135}$ was translated for 1 h at 30°C. Anisomycin (final concentration: 2 μg/μl) was then added to stop translation and an aliquot of lysate was taken for immunoprecipitation. The remaining lysate was further incubated for 5 min at 37°C. Immunoprecipitations with either anti-p53 or anti-hsp70 antibodies and analysis of precipitated p53 were carried out as described in the legend to Figure 1. MW: molecular weight markers (in kilodaltons).

monomers (Figure 3A, peak 1). High molecular weight forms were represented as a shoulder (Figure 3A, peak 4, compatible with tetramers of p53). Each fraction was immunoprecipitated with antibodies to hsp70 and analysed by SDS–PAGE. From the size fractions p53 co-immunoprecipitated with hsp70 in a single peak (fractions 4–9, Figure 3B) with a maximum in fraction 7 (apparent molecular weight: 120–180 kDa). These results are consistent with one or two p53 molecules complexed to one hsp70 molecule (1:1 and 2:1 complexes). Identical results were obtained with $p53^{Val135}$ translated at 37°C (not shown). It is important to note that since p53–hsp70 complexes represented only 5–10% of total p53, they did not appear as a detectable TCA-precipitable peak against the background of non-complexed p53 in Figure 3A.

Specific association of hsp70 with dimers of p53

Size fractionation studies indicate that p53 forms high affinity oligomeric complexes (Kraiss *et al.*, 1988; Milner *et al.*, 1991) and it is likely that quaternary structure is important for p53 function. The above results indicate that hsp70 may target p53 monomers and dimers and we were interested to determine more precisely whether hsp70 complexes with dimers of p53. For this purpose human p53 was co-translated with murine p53. The two proteins can be distinguished by their different mobilities on SDS–PAGE and by species-specific monoclonal antibodies (Milner and Medcalf, 1991). For example, PAb246 and PAb248 are mouse-specific and have been used to demonstrate the formation of murine–human p53 complexes when the two proteins are translated *in vitro* (Milner *et al.*, 1991). When co-translated at 30°C, wild-type human p53 complexes with murine $p53^{Val135}$ and both proteins adopt the wild-type phenotype. When such complexes are subjected to temperature shift (up to 37°C), the temperature-sensitive $p53^{Val135}$ drives the human p53 into the mutant phenotypic form (Milner and Medcalf, 1991; see also Figure 4A). In the present studies we exploited this phenomenon. Since human wild-type p53 changes conformation to the mutant phenotype only when complexed with $p53^{Val135}$ (i.e. a minimum of dimeric structure), we reasoned that detection of human p53–hsp70 complexes after temperature shift would reflect the presence of murine–human p53 oligomers.

Human p53 plus murine $p53^{Val135}$ were co-translated at 30°C for 1 h. After addition of anisomycin to block further protein synthesis, temperature was shifted to 37°C for 5 min and the presence of p53–hsp70 complexes was detected by immunoprecipitation with anti-hsp70 antiserum. Detection of a doublet of p53 proteins on SDS–PAGE (indicated by two arrows in Figure 4B, lower panel), demonstrates that both human (upper arrow) and murine (lower arrow) p53 molecules complexed with hsp70. Since wild-type human p53 translated alone does not bind to hsp70 at 37°C (Figure 4B, middle panel), this result reveals the targeting of mixed human + mouse p53 oligomers by hsp70. The minimal p53 oligomeric form required to explain these results is a dimer containing one murine and one human p53 molecule. Size fractionation analyses (see above) indicated that hsp70 does not complex with more than two molecules of p53 and we therefore conclude that hsp70 can complex with murine–human p53 dimers.

The carboxyl terminus of p53 is required for complex formation with hsp70

The carboxyl terminus of p53 is necessary for the formation of stable p53 oligomers when the protein is translated *in vitro* (Milner *et al.*, 1991). A truncated form of mutant p53 comprising residues 1–343 ($p53^{ValStu}$) is predominantly monomeric (Milner and Medcalf, 1991), whereas p53-M-8, a mutant p53 in which the 28 carboxyl-terminal residues are replaced by 17 unrelated amino acids (Arai *et al.*, 1986), forms monomers and dimers, but not higher molecular weight oligomers (J.Milner and E.A.Medcalf, submitted). To investigate the role of the carboxyl terminus of p53 in hsp70 binding, $p53^{ValStu}$ and p53-M-8 were translated at 37°C and subjected to size fractionation on Superose 6 as described above (see Figure 3). Each fraction was analysed by immunoprecipitation with anti-hsp70 antiserum. Although $p53^{ValStu}$ and p53-M-8 both adopted a predominantly mutant phenotype (not shown), neither formed specific complexes

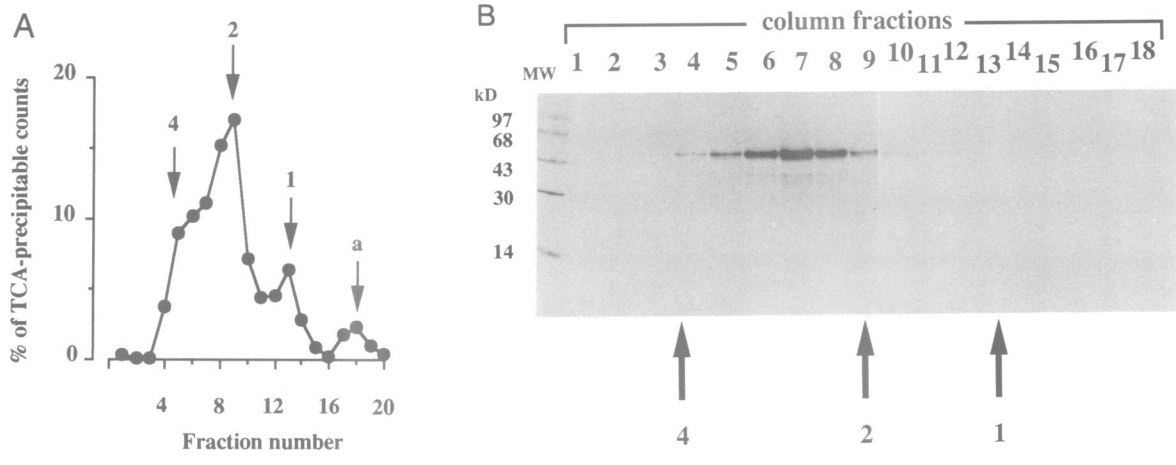

Fig. 3. Determination of the size of p53–hsp70 complexes formed after *in vitro* translation of $p53^{Val135}$. (**A**) Size fractionation of ^{35}S-labelled $p53^{Val135}$. Translation was carried out at 30°C and temperature was shifted to 37°C for 5 min before fractionation on Superose 6 (Pharmacia). Results are expressed as the percentage of total TCA-precipitable counts associated with each column fraction. The size of peaks containing p53 was evaluated by comparing with markers of known molecular weight. Peak 4: 200–300 kDa (p53 tetramers); peak 2: 90–130 kDa (p53 dimers); peak 1: 50–70 kDa (p53 monomers); peak a: 20 kDa (mainly labelled tRNA). The presence of p53 in peaks 1, 2 and 4 has been described previously (see Milner *et al.*, 1991). (**B**) Each size fraction was immunoprecipitated with anti-hsp70 serum, and co-precipitating ^{35}S-labelled p53 was analysed on SDS–PAGE as described in the legend to Figure 1. MW: molecular weight markers (in kilodaltons).

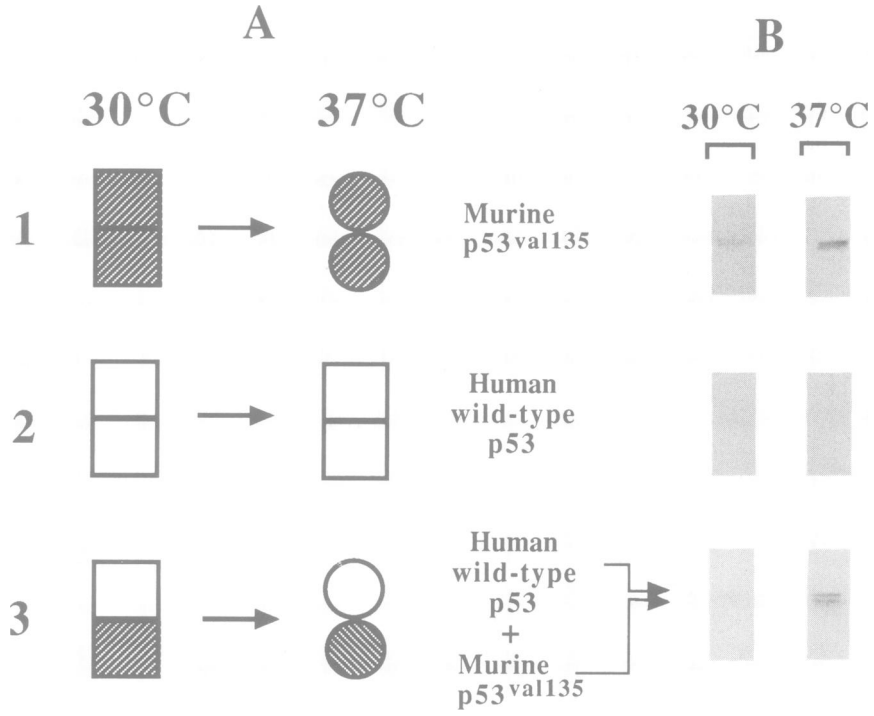

Fig. 4. Association of hsp70 with mixed oligomers formed by co-translation *in vitro* of murine $p53^{Val135}$ and human wild-type p53. (**A**) Schematic representation of the effect of temperature on the conformational phenotype of murine $p53^{Val135}$ and human wild-type p53. Phenotypically wild-type and mutant p53 are represented by a square and a circle, respectively. p53 is represented as a dimer. Murine $p53^{Val135}$ (hatched symbols) is temperature-sensitive for conformation, adopting the wild-type phenotype at 30°C and switching to the mutant phenotype at 37°C. In contrast, human wild-type p53 (white symbols) is phenotypically wild-type at 30°C and at 37°C. Co-translation of murine $p53^{Val135}$ and human wild-type p53 yields mixed oligomers that adopt the wild-type phenotype at 30°C. Upon shifting the temperature to 37°C, mutant murine p53 drives human wild-type to adopt the mutant phenotype (see Milner and Medcalf, 1991). (**B**) Co-immunoprecipitation of p53 with anti-hsp70 serum at 30°C and 37°C. The double arrow in the lower panel indicates human p53 (upper arrow) and murine p53 (lower arrow). Immunoprecipitations were carried out as in the legend to Figure 1.

with hsp70 (Figure 5). Note that the small amount of $p53^{ValStu}$ found in fractions 11–14 corresponds to monomeric p53 and does not represent complexes of p53 with hsp70. This result indicates that the carboxyl terminus of p53 is required for complex formation with hsp70. Moreover, since p53-M-8 can form dimers, its inability to complex with hsp70 is unlikely to be due to lack of oligomerization. Overall, the above results suggest that sequences in the carboxyl terminus of p53 are essential for the formation of a stable complex with hsp70.

ATP hydrolysis is not required for conversion of wild-type to mutant phenotype of $p53^{Val135}$ following temperature shift

The temperature sensitivity of $p53^{Val135}$ for conformational phenotype provides us with a unique system for studying a possible role for hsp70 in the regulation of p53 conformation. Since the ATPase activity of hsps may be required for their function as 'chaperones' (see Gething and Sambrook, 1992), we analysed the requirement for ATP in hsp70–p53 interactions and in changes in p53 conformation using a non-hydrolysable analogue of ATP, adenylyl imidodiphosphate (AMP-PNP). Excess amounts of AMP-PNP (4 mM) were used to overcome the effects of ATP and ATP-regenerating system present in reticulocyte lysate. In control experiments, we found that ATP at 4 mM affected neither the conformation of $p53^{Val135}$ nor the phenotypic shift induced by temperature (not shown).

$p53^{Val135}$ was translated at 30°C for 1 h. After addition of anisomycin, the lysate was supplemented with either AMP-PNP or ATP as a control and incubated for a further 10 min at 30°C; an aliquot was then taken for immunoprecipitation. The temperature was then shifted to 37°C, and

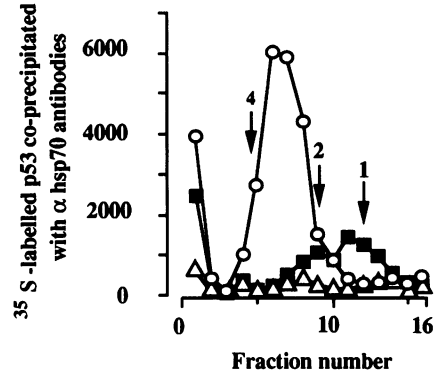

Fig. 5. Binding of hsp70 to $p53^{ValStu}$ (truncated p53) and p53-M-8. $p53^{ValStu}$ and p53-M-8 were translated at 37°C and equivalent amounts of radioactive p53 were analysed by size fractionation on Superose 6 (Pharmacia) as in Figure 3. Each column fraction was immunoprecipitated with anti-hsp70 serum. Results are expressed as the amount of immunoprecipitated ^{35}S-labelled material (in d.p.m.) per 200 μl of fraction. Fractionation of p53–hsp70 complexes formed after shifting $p53^{Val135}$ at 37°C is shown for comparison. Note that the radioactive material detected in fraction 1 does not correspond to p53. Arrows 1, 2 and 4 identify the expected position of, respectively, p53 monomers, dimers and tetramers. Squares, $p53^{ValStu}$; triangles, p53-M-8; circles, $p53^{Val135}$ at 37°C.

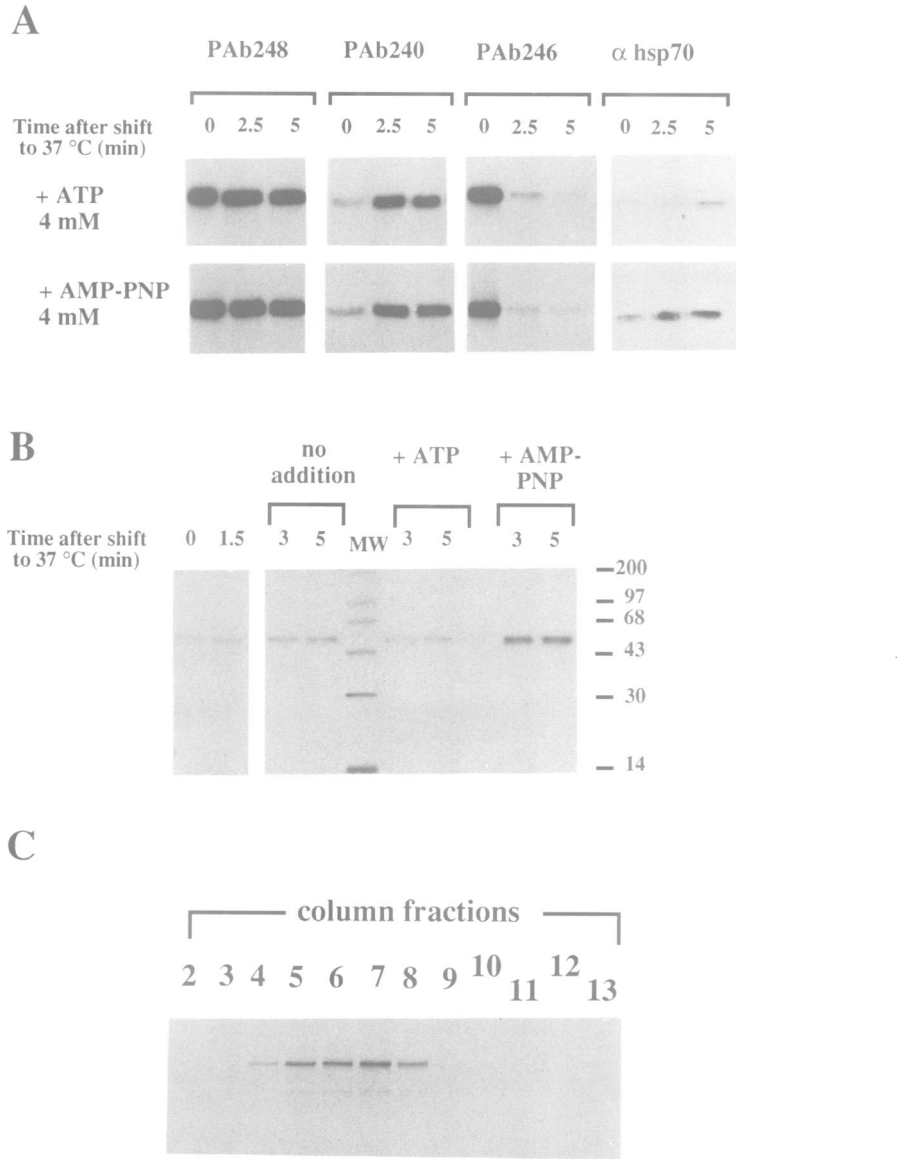

Fig. 6. Effect of ATP on the shift of $p53^{Val135}$ from wild-type to mutant phenotype, induced by raising the temperature from 30°C to 37°C. **A.** $p53^{Val135}$ was translated at 30°C for 1 h, and the temperature was shifted to 37°C after the addition of anisomycin and of either ATP (4 mM) or the non-hydrolysable ATP analogue AMP-PNP (4 mM). Aliquots were taken 0, 2.5 and 5 min after temperature shift, immunoprecipitated with antibodies as indicated and analysed on SDS–PAGE as in the legend to Figure 1. **B.** $p53^{Val135}$ translated at 30°C was shifted up to 37°C. After 1.5 min at 37°C, ATP (4 mM) or AMP-PNP (4 mM) were added. Supplemented and control (no addition) lysates were further incubated at 37°C and aliquots were taken for immunoprecipitation with anti-hsp70 serum at 3 and 5 min after the shift to 37°C. **C.** Determination of the size of p53–hsp70 complexes formed after shift to 37°C in the presence of AMP-PNP. Proteins were fractionated on Superose 6 and each column fraction was analysed as described in the legend to Figure 3B.

after 2.5 or 5 min at 37°C, aliquots of the lysate were diluted on ice and immunoprecipitated. In the presence of ATP, temperature induced a rapid conversion from wild-type to mutant phenotype, within 2.5 min at 37°C (Figure 6A, upper panel, compare the increase in PAb240 reactivity with the decrease in PAb246). However, increase in p53 bound to hsp70 was not detected until 5 min at 37°C, suggesting that hsp70 binding is subsequent to the change from wild-type to mutant phenotype.

Addition of AMP-PNP had no effect on the conversion from wild-type to mutant phenotype (Figure 6A, lower panel). However, we consistently observed a 2- to 4-fold increase in p53 bound to hsp70, suggesting that AMP-PNP stabilized p53–hsp70 complexes. We tested this further by adding AMP-PNP or ATP after shifting the temperature from 30°C to 37°C. After 1.5 min at 37°C, an aliquot was taken for immunoprecipitation, and the rest of the lysate was supplemented with either ATP or AMP-PNP and analysed at 3 and 5 min after temperature shift (Figure 6B). Addition of AMP-PNP resulted in a marked increase in the amount of p53–hsp70 complexes, whereas addition of ATP led to a small but reproducible decrease in the amount of p53 bound to hsp70. Size fractionation on Superose 6 showed that p53–hsp70 complexes formed in the presence of AMP-PNP were consistent with monomers and/or dimers of p53 complexed to one hsp70 molecule (1:1 and 2:1 complexes, Figure 6C). Similar results were obtained with another ATP analogue, ATPγS (data not shown). These data are in

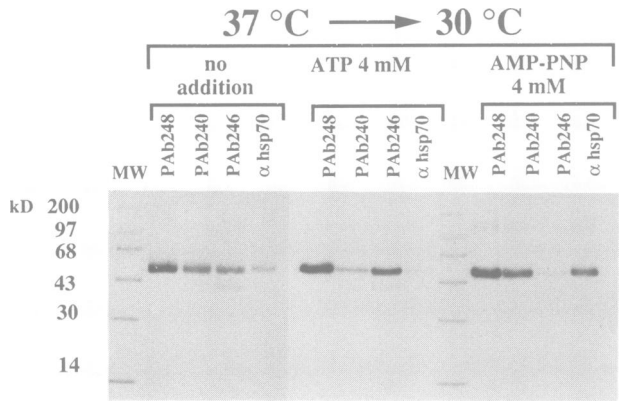

Fig. 7. Effect of ATP on the refolding of $p53^{Val135}$ from mutant to wild-type phenotype, induced by lowering the temperature from 37°C to 30°C. $p53^{Val135}$ was translated at 30°C and the temperature was shifted to 37°C for 3 min. The lysate was then divided into three aliquots, one serving as a control (no addition) and the others being supplemented with either ATP (4 mM) or AMP-PNP (4 mM). Temperature was shifted back to 30°C. In each aliquot, one volume of fresh lysate (supplemented with either ATP or AMP-PNP as required) was added after 2 h and lysates were incubated further at 30°C for another 2 h. Equivalent aliquots of lysates were then diluted and immunoprecipitated with antibodies as indicated. Immunoprecipitation and SDS–PAGE analysis were carried out as described in the legend to Figure 1. MW: molecular weight markers (in kilodaltons).

agreement with the observations of Clarke *et al.* (1988) showing that ATP regulates the stability of p53–hsp70 complexes isolated from cellular extracts. In addition, our results indicate that ATP hydrolysis is not required for the temperature-induced shift from wild-type to mutant forms of $p53^{Val135}$, and that binding to hsp70 appears to be a consequence of the acquisition of mutant p53 phenotype. Therefore, the ATPase activity of hsp70 is not involved in the shift from wild-type to mutant phenotype.

ATP hydrolysis is required for conversion of the mutant to the wild-type phenotype of $p53^{Val135}$

The temperature-induced switching of $p53^{Val135}$ conformation is reversible, and lowering the temperature from 37°C to 30°C leads to a partial refolding from mutant to wild-type form after 30 min (Milner and Medcalf, 1990). We found that this conversion was improved by the addition of freshly thawed, untranslated lysate and longer incubations (4 h) at 30°C (data not shown). To determine the requirements for ATP hydrolysis in this process, $p53^{Val135}$ was first translated at 30°C and driven to adopt the mutant phenotype by raising the temperature to 37°C for 3 min. ATP or AMP-PNP was then added and temperature was shifted back to 30°C. After 2 h, one volume of freshly thawed lysate (supplemented with either ATP or AMP-PNP as required) was added, and lysates were incubated for another 2 h at 30°C. In the absence of either ATP or AMP-PNP, only partial phenotypic reversion was induced by lowering the temperature to 30°C. The protein became reactive with PAb246, but also retained some activity with PAb240, indicating that both wild-type and mutant p53 were present in approximately equal amounts (Figure 7, left panel). Addition of ATP resulted in full conversion of $p53^{Val135}$ to wild-type phenotype (Figure 7, middle panel). In contrast, the phenotypic reversion was completely blocked in the presence of AMP-PNP, and there was a remarkable accumulation of p53–hsp70 complexes (representing ~40% total p53, Figure 7, right panel). The size of these complexes was consistent with monomers and/or dimers of p53 complexed with one molecule of hsp70 (not shown).

These results indicate that dissociation of p53–hsp70 complexes and adoption of wild-type p53 phenotype are both prevented in the presence of an excess of a non-hydrolysable analogue of ATP. To investigate further the requirement for hsp70 during phenotypic reversion, we set out to examine reversion in the effective absence of p53–hsp70 complex formation. Our first approach, which was to immunodeplete the reticulocyte lysate of hsp70, proved unsatisfactory due to the inability of the anti-hsp70 serum to remove hsp70 quantitatively. Therefore, as an alternative approach, experiments identical to those described above were carried out with $p53^{ValStu}$, a truncated form of mutant p53 that does not bind hsp70 but is temperature-sensitive for conformational phenotype. We found that $p53^{ValStu}$ refolded from mutant to wild-type phenotype in an ATP-dependent manner, but in the absence of detectable p53–hsp70 complex formation (data not shown). This result shows that truncated $p53^{ValStu}$, which does not bind hsp70, still retains the ability to refold in an ATP-dependent manner, indicating that additional ATP-dependent steps, distinct from hsp70 ATPase, are required for refolding p53 into the wild-type phenotype.

In summary, our results indicate that ATP hydrolysis is required for refolding $p53^{Val135}$ from mutant to wild-type phenotype. With full-length p53, blocking this process leads to an accumulation of mutant p53–hsp70 complexes, suggesting that hsp70 could play a regulatory role during the folding of p53 into the wild-type phenotype.

Discussion

This report describes the interactions between *in vitro* translated p53 and hsp70 proteins present in rabbit reticulocyte lysate, and is the first demonstration of complex formation between p53 and hsp70 in a cell-free system. The exact nature of the hsp70 protein(s) involved is unknown, since the antiserum we used recognizes both stress-inducible and constitutively expressed members of the hsp70 family. This system allowed us to determine the structural specificity of p53–hsp70 complex formation at the level of primary, tertiary and quaternary p53 structures. In addition, using a form of mutant p53 that is temperature-sensitive for conformation, we were able to modulate p53 conformation *in vitro* and to analyse the requirements for ATP hydrolysis and interactions with hsp70 during the interconversion between conformational phenotypes of p53.

Lysate hsp70 complexes with *in vitro* translated mutant, but not wild-type p53, in agreement with the specificity of complex formation described in intact cells (Sturzbecher *et al.*, 1987, 1988; Finlay *et al.*, 1988). *In vitro* complexes are formed during or immediately after translation, suggesting that binding of hsp70 to p53 does not result from mere accumulation of mutant protein. Moreover, using the temperature-sensitive mutant $p53^{Val135}$, we found that complexes can also form after post-translational switching from wild-type to mutant p53 phenotype. This result indicates that hsp70 can target full-length p53 and that complex formation can result from a change in the tertiary structure of pre-existing p53.

We further show that the mutant phenotype is not the only structural requirement for p53–hsp70 complex formation. For example, hsp70 selectively complexes with p53 dimers and possibly monomers, but not with higher molecular weight forms of mutant p53. This observation is based (i) on size estimates after gel filtration, and (ii) on the actual demonstration of complexes between hsp70 and murine–human p53 oligomers (see Results). Our results are at variance with those of Clarke *et al.* (1988), who found that cellular p53–hsp70 complexes resolved into two peaks at 125 and 660 kDa when fractionated on Sephacryl S-300. It is possible that cellular p53–hsp70 complexes contain components that are absent in reticulocyte lysate. However, gel filtration of mutant p53–hsp70 complexes from 3T3tx cells (under conditions identical to those used here) reveals an estimated size identical to that observed in reticulocyte lysate (P.Hainaut and J.Milner, unpublished results) and we believe that complex formation in rabbit reticulocyte lysate is a useful and meaningful model for studying p53–hsp70 interactions.

The carboxyl terminus of p53 is also necessary for association with hsp70, as demonstrated by the failure of hsp70 to complex with two p53 mutants with altered carboxyl termini, $p53^{ValStu}$ (truncated $p53^{Val135}$) and p53-M-8. We can rule out the possibility that the absence of hsp70 binding is due to an effect of the carboxyl terminus on p53 conformation (since both proteins have a predominantly mutant phenotype) or on p53 oligomerization (since p53-M-8 is able to form dimers).

The possible involvement of the p53 carboxyl terminus in binding with hsp70 has previously been studied by Sturzbecher *et al.* (1988), using p53 constructs transfected into COS cells. Double mutants of p53 were used with carboxyl-terminal deletions and with the carboxyl terminus replaced by 'stuffer' fragments of a fusion protein. On the basis of their results, Sturzbecher *et al.* concluded (i) that hsp70 binding to p53 is dependent upon p53 protein conformation and (ii) that the sequence downstream of residue 274 of p53 was unimportant for hsp70 binding. In contrast, our results demonstrate that the carboxyl-terminal 28 amino acids of p53 are required for association with hsp70. As an alternative explanation for the results of Sturzbecher *et al.* (1988), we suggest that replacement of p53 carboxyl terminus by an irrelevant sequence may either have introduced hsp70 binding motifs, or induced a grossly aberrant form of p53 recognized by hsp70.

The carboxyl terminus of p53 appears to be involved in several biologically significant processes, including p53 oligomerization (Milner *et al.*, 1991) and proteolysis by the ubiquitin-mediated degradation system (J.Milner and E.A. Medcalf, submitted). In addition, it contains major nuclear localization signals and phosphorylation sites for cdc2 (Addison *et al.*, 1990; Bischoff *et al.*, 1990) and casein kinase II (Meek *et al.*, 1990). Conformation-specific binding of hsp70 to this region stresses that the carboxyl terminus of p53 is likely to be a major regulatory domain for the functioning of p53.

The biological significance of p53–hsp70 complexes is poorly understood. It has been proposed that p53–hsp70 complexes merely consist of a subclass of aberrant p53 polypeptides whose appropriate folding and transport programme has been disrupted by the encoded p53 mutation (Sturzbecher *et al.*, 1988). Others have suggested that hsp70 might be involved in the assembly of p53 oligomers, and that accumulation of p53–hsp70 complexes might reflect the trapping of p53 in long-lived, unproductive complexes (Weinberg, 1991). It should be noted that we did not find any evidence for a role of hsp70 in assembly or disassembly of p53 oligomers (unpublished data). The results presented here suggest that binding of hsp70 to p53 could be part of a regulated process linked with changes in p53 conformation. Using the temperature-sensitive mutant $p53^{Val135}$, we found that the shift from wild-type to mutant phenotype is rapid and ATP-independent, and that it precedes association with hsp70 (see Results). In contrast, the reverse shift from mutant to wild-type phenotype is slower and requires ATP hydrolysis. In the presence of a non-hydrolysable analogue of ATP, p53 retains the mutant phenotype and there is an accumulation of p53–hsp70 complexes. Thus, complex formation could represent an intermediate step during reversion from mutant to wild-type phenotype. However, using $p53^{Val135}$ with a deleted carboxyl terminus, we found that refolding into the wild-type phenotype could occur in the absence of hsp70 binding. Nonetheless, ATP hydrolysis was required for refolding into the wild-type phenotype, indicating the involvement of one (or more) additional ATP-dependent steps. We propose that during refolding of full-length $p53^{Val135}$ into the wild-type phenotype, hsp70 interacts with the carboxyl terminus of p53 and may stabilize intermediate form(s) of p53 in the folding pathway. Activation of hsp70 ATPase activity, accompanied by release of hsp70 from the complex, could allow p53 to complete its folding, a process that is itself also ATP-dependent.

A conformational hypothesis proposes that normal p53 may function both to suppress and promote cell proliferation, a given function depending upon the conformation of the p53 polypeptide (see review in Milner, 1991). The suppressor form of p53 is equivalent to wild-type phenotype, and may be inactivated by cell growth stimulation which induces transient loss of PAb246 immunoreactivity (Milner and Watson, 1990). The promoter form resembles mutant p53 phenotype. Recently, the promoter form of p53 (reactive with PAb240) has been identified in normal human lymphocytes (Rivas *et al.*, 1992), reinforcing the idea that genotypically wild-type p53 can adopt alternative conformations important for cell growth control. Therefore, any mutation that deregulates the normal control of p53 conformation may lead to cancer (Milner, 1991). In keeping with this hypothesis, our results suggesting a role for hsp70 in the regulation of p53 conformation implies that hsp70 may exert a crucial control on p53 function(s). According to this idea, hsp70, by regulating the folding from mutant (promoter) to wild-type (suppressor) p53 phenotype, might be an important effector in the cellular pathways controlling normal cell growth. Oncogenic mutations of p53 could result in a decreased ability of p53 to stimulate hsp70 ATPase activity, thus causing an irreversible 'lock' in the mutant conformation and accumulation of p53–hsp70 complexes.

Materials and methods

Plasmids

Plasmids $pSP6p53^{Ala135}$ and $pSP6p53^{Val135}$ are used for translation of wild-type and mutant p53 respectively. The construction of these plasmids is detailed in Milner *et al.* (1991). $pSP6p53^{Ala135}$ contains wild-type murine cDNA (with alanine at amino acid residue 135), whereas $pSP6p53^{Val135}$ contains an activated mutant of murine p53 cDNA with valine instead of alanine at residue 135 (Hinds *et al.*, 1989; Eliyahu *et al.*, 1989). The translation product of $pSP6p53^{Val135}$ is temperature-sensitive for

conformation (Milner and Medcalf, 1990). $p53^{ValStu}$ (truncated $p53^{Val135}$) was obtained by linearization of $pSP6p53^{Val135}$ with *StuI*, followed by transcription and translation (Milner and Medcalf, 1991). p53-M-8 is a cDNA clone of mouse origin that corresponds to an abnormal splicing product of a mutant p53 allele (Arai *et al.*, 1986). For human wild-type p53, the plasmid pSP6p53-H8 was used. H8 is equivalent to the H1 form of wild-type p53 (Harris *et al.*, 1986).

Transcription and translation

Plasmids were amplified in *E.coli* TG1, purified and used for transcription and translation as detailed in Gamble and Milner (1988). Rabbit reticulocyte lysates were from Promega. Since the immunological phenotype of *in vitro* translated p53 may vary between different batches of lysates (Cook and Milner, 1990), a single batch of lysate was used for all experiments involving temperature shifts. Translations were carried out for 1 h at either 30°C or 37°C as indicated, in the presence of 0.75 μM ^{35}S-labelled methionine (40.5 TBq/mmol, Amersham). For experiments involving temperature shifts, translations were stopped prior to temperature shift by the addition of anisomycin (diluted at 10 μg/μl in RPMI; final concentration 2 μg/μl). Otherwise, translations were stopped by diluting 1:100 in immunoprecipitation buffer (see below) and chilling on ice. ATP (Sigma) and AMP-PNP (Boehringer Mannheim) were kept at −20°C as 40 mM stock solutions.

Antibodies

To detect hsp70, a rabbit anti-hsp70 antiserum was used at a final concentration of 1:100. This serum was raised against a synthetic peptide corresponding to the carboxyl terminus of human hsp70 (sequence GFGAQGPKGGSGSGPTIEEVD). It recognizes both constitutively expressed and stress-inducible hsp70 from various species (Ehrhardt *et al.*, 1988; S.Ullrich, personal communication). To detect p53, the following monoclonal antibodies were used: RA3:2C2 (Wade-Evans and Jenkins, 1985), PAb421 (Harlow *et al.*, 1981), PAb246 and PAb248 (Yewdell *et al.*, 1986), PAb1620 (Ball *et al.*, 1984; Milner *et al.*, 1987) and PAb240 (Gannon *et al.*, 1990).

Immunoprecipitation and SDS−PAGE

Immunoprecipitations were carried out as described previously (Milner and Medcalf, 1991), except that the buffer used for dilutions and washings was 10 mM Tris−HCl, 140 mM NaCl, 0.1% NP-40 (pH 8.0). Briefly, aliquots of lysate were diluted 1:100 with ice-cold buffer and incubated for 1 h on ice with either anti-p53 monoclonal antibodies (1:4) or rabbit anti-hsp70 serum (1:100). For immunoprecipitation with monoclonal antibodies, rabbit anti-mouse immunoglobulins (Dako) were added at a final concentration of 1:30 for 30 min in order to ensure quantitative immunoprecipitation. Immune complexes were collected on formalin-fixed *Staphylococcus aureus* (Staph A, 10%), pre-washed with immunoprecipitation buffer before use. Staph A pellets were washed by resuspension in 200 μl ice-cold buffer with vigorous shaking, followed by dilution with 1 ml buffer and centrifugation. This procedure was repeated twice. Immunoprecipitated material was recovered by resuspension of pellets in SDS−PAGE sample buffer. An aliquot of each immunoprecipitation was counted in a Packard Tri-Carb counter. SDS−PAGE was carried out as described, using 15% minigels with 5% stacking gel (Milner and Medcalf, 1990). After treatment with Amplify (Amersham), fixed gels were dried and exposed to Kodak X-Omat S X-ray film at −70°C, using aluminium foil to improve radiographic resolution (Milner *et al.*, 1991). For Western blots, unfixed gels were electrotransferred onto 0.22 μm nitrocellulose membranes (Schleicher and Schüll) and ovalbumin (2%) was used as a blocking agent. Bound antibodies were revealed using ^{125}I-labelled protein A (Amersham).

Size fractionation

Freshly translated ^{35}S-labelled p53 was fractionated by gel filtration on a Superose 6 column using flow pressure liquid chromatography (Pharmacia) as described by Milner *et al.* (1991).

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