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Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53-mediated growth arrest

Yaacov Barak and Moshe Oren[1]

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel
[1]Corresponding author

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To explore the biochemical functions of p53, we have initiated a search for cellular p53-binding proteins. Co-precipitation of three polypeptides was observed when cell lines overexpressing a temperature-sensitive (ts) p53 mutant were maintained at 32.5°C (wild-type p53 activity, leading to growth arrest) but not at 37.5°C (mutant p53 activity). One of these three proteins, designated p95 on the basis of its apparent molecular mass, was highly abundant in p53 immune complexes. We demonstrate herein that p95 is a p53-binding protein, which exhibits poor p53-binding in cells overproducing several distinct mutant p53 proteins. Yet, p95 associates equally well with both the wild-type (wt) and the mutant conformations of the ts p53 in transformed cells growth-arrested at 32.5°C. On the basis of our findings we suggest that wt p53 activity increases p53-p95 complex formation and that such interaction may play a central role in p53 mediated tumour suppression.

Key words: growth control/p53/transformation/tumour suppressor

Introduction

The nuclear DNA-binding phosphoprotein p53 is believed to be involved in the control of cell proliferation, differentiation and survival (Levine *et al.*, 1991; Weinberg, 1991; Shaulsky *et al.*, 1991; Yonish-Rouach *et al.*, 1991). Its inactivation by means of point mutations, rearrangements or gene losses is frequently observed in many types of human and rodent cancer (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Michalovitz *et al.*, 1991). Germ-line point mutations in the p53 gene constitute the genetic basis for the Li-Fraumeni cancer susceptibility syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). Wild-type (wt) p53 elicits a growth arrest when conditionally expressed in transformed cells (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990), promotes apoptosis (programmed cell death) in a myeloid leukaemic cell-line (Yonish-Rouach *et al.*, 1991) and is involved in pre-B cell differentiation *in vitro* (Shaulsky *et al.*, 1991). On the other hand, many naturally occurring p53 mutants exhibit transforming activities in addition to their failure to display the tumour suppressor activities of the wt allelle (Levine *et al.*, 1991; Michalovitz *et al.*, 1991).

The biochemical nature of p53 action is largely unresolved. It possesses DNA-binding properties (Lane and Gannon, 1983; Steinmeyer and Deppert, 1988; Kern *et al.*, 1991a) and has recently been shown to associate selectively with specific DNA sequences (Bargonetti *et al.*, 1991; Kern *et al.*, 1991b). Several studies have suggested that p53 has a role in the control of DNA replication (Braithwaite *et al.*, 1987; Gannon and Lane, 1987; Friedman *et al.*, 1990; Kern *et al.*, 1991b; Wilcock and Lane, 1991), whereas others indicate that it may be involved in either the activation (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990; Weintraub *et al.*, 1991) or the repression (Ginsberg *et al.*, 1991a; Mercer *et al.*, 1991; Santhanam *et al.*, 1991) of transcription. It is also still possible that p53 may regulate other, as yet undetermined processes.

p53 binds to gene products of at least three distinct DNA tumour viruses, namely the SV40 large T antigen, the 58 kDa E1B protein of adenovirus types 2 and 5 and the E6 proteins of papillomavirus types 16 and 18 (reviewed by Levine, 1990). This binding appears to be a hallmark of tumour suppressor gene products, as another tumour suppressor protein, pRB, binds to proteins encoded by the same viruses (Levine, 1990). These viruses may conceivably have taken advantage of a pre-existing property of the two tumour suppressor proteins, entailing the binding of cellular proteins in the course of performing their normal functions. This conjecture is supported by the recent identification of several cellular pRB-binding proteins which are competed by the viral proteins for binding (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Chellapan *et al.*, 1991; Chittenden *et al.*, 1991; Huang *et al.*, 1991; Kaelin *et al.*, 1991; Rustgi *et al.*, 1991). Clearly, identifying and characterizing cellular proteins to which p53 binds could deepen our understanding of its biochemistry.

In an attempt to identify cellular p53-binding proteins, we used cells expressing a temperature-sensitive (ts) murine p53 mutant, p53Val135. The ts protein behaves like other p53 mutants at 37.5°C, but assumes wt p53 activity at 32.5°C (Michalovitz *et al.*, 1990). In fibroblasts, this wt p53 activity leads to rapid growth arrest (Michalovitz *et al.*, 1990; Gannon and Lane, 1991; Martinez *et al.*, 1991). Consistent with the assumption that the conversion of p53 activity from wt to mutant is accompanied by the loss of binding to cellular factors, we could indeed demonstrate that three proteins co-precipitated with p53 from extracts of p53Val135-over-expressing fibroblasts growth-arrested at 32.5°C; the same proteins were barely detectable in p53 immunoprecipitates when cells were maintained in a proliferating state at 37.5°C. We demonstrate here that the more abundant co-precipitating protein, p95, is a p53-binding protein which exhibits only a marginal association with p53 in transformed cells overproducing non-suppressing p53 mutants. Fibroblasts subjected to the anti-proliferative activity of the ts p53 at 32.5°C contain a mixture of p53 populations in the mutant and wt conformations, respectively. In such growth-arrested cells, both wt and mutant p53 conformations are equally potent in binding p95. We propose that the p53-p95 interaction, which is enhanced specifically by wt p53 activity, may be central to the tumour suppressor activity of wt p53.

Results

Three polypeptides preferentially co-precipitate with p53Val135 in cells growth-arrested at 32.5°C

The identification of proteins that associate with wt p53 may provide crucial clues about the biochemical functions of p53. To this end, we took advantage of cell lines derived from rat embryo fibroblasts (REF) transformed by activated Ha-ras plus the ts murine p53 mutant, p53Val135 (Michalovitz *et al.*, 1990). At 37.5°C this p53 mutant exhibits pronounced transforming activities; the cells are highly transformed and proliferate vigorously. However, when the growth temperature is dropped to 32.5°C, p53 assumes a wt-like activity and the cells stop growing completely (Michalovitz *et al.*, 1990; Gannon and Lane, 1991; Martinez *et al.*, 1991). Several independent cell lines of this type were maintained at either 32.5°C or 37.5°C, labelled with [^{35}S]methionine and subjected to immunoprecipitation with the anti-p53 monoclonal antibody (mAb) PAb421. Figure 1 demonstrates the results in a representative cell line, clone 6 (Pinhasi-Kimhi *et al.*, 1986). Three co-precipitating polypeptides were clearly and reproducibly observable when cells were maintained at 32.5°C, but not at 37.5°C (Figure 1A, lane 2). These included a prominent 95 kDa protein, p95, as well as two minor proteins of ~80 kDa (long arrow) and ~58 kDa (short arrow). Labelling of the cells with [^{32}P]orthophosphate demonstrated that both p95 (Figure 1B) and the 58 kDa protein (short arrow, Figure 1B; pp58, Figure 1C) are phosphorylated; there was no indication that the 80 kDa protein was phosphorylated. In addition to hsc70, whose interaction with p53 has been characterized previously (Pinhasi-Kimhi *et al.*, 1986; Hinds *et al.*, 1987; Sturzbecher *et al.*, 1987), two additional co-precipitating polypeptides were preferentially observed in extracts from cells grown at 37.5°C, (Figure 1A, lane 4): (i) an ~110 kDa polypeptide (filled circle), which is probably identical to a protein previously characterized as a constituent of p53–hsc70 complexes (Clarke *et al.*, 1988) and (ii) an ~90 kDa polypeptide (open circle), which co-precipitated along with hsc70 (data not shown, see also Figure 5). None of the above polypeptides was precipitated by the control mAb PAb419, specific for SV40 large T antigen. Hence, at least three distinct proteins appear to co-precipitate specifically with p53Val135 when it exhibits wt anti-proliferative activity. Because of its abundance, we chose to concentrate on p95 in subsequent experiments.

In addition to clone 6, a battery of independent REF clones transformed by p53Val135 and Ha-ras exhibited an essentially similar co-precipitation pattern at 32.5°C versus 37.5°C (data not shown). This was also true of the REF-derived cell line Fi-XI-2-3 (Eizenberg and Oren, 1991), immortalized by p53Val135 alone (data not shown), as well as for lines transformed with the combination of p53Val135, ras and myc (data not shown, see also Figure 4B). These observations indicate that the nature of the additional oncogenes utilized in the process of cell transformation or immortalization is immaterial to the enhanced co-precipitation of p95 with p53, as long as the cells stop proliferating when shifted to 32.5°C.

The co-precipitation of p95 is not limited to rat fibroblasts; a comparable pattern was observed in the mouse fibrosarcoma line MCO-1-hcG9 # 23 (Figure 2A). This line

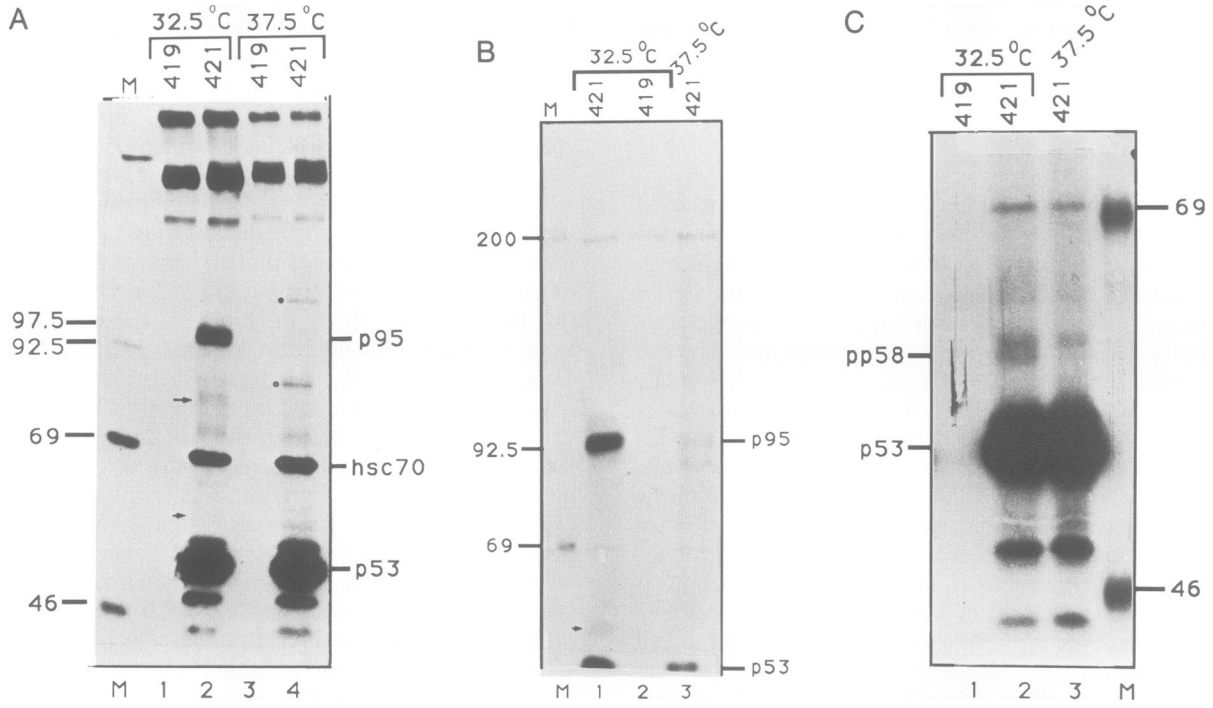

Fig. 1. Analysis of cellular proteins which co-precipitate with p53Val135. Clone 6 cells (Pinhasi-Kimhi *et al.*, 1986) were either continuously grown at 37.5°C or shifted to 32.5°C for 24 h and labelled with either [^{35}S]methionine (**A**) or [^{32}P]P_i (**B** and **C**) as described. Aliquots of cell extracts containing equal acid-insoluble radioactivity were immunoprecipitated with either the p53-specific mAb PAb421 (421) or with the anti SV40 large T antigen mAb PAb419 (419) as a control. Immunoprecipitates were resolved by 10% SDS–PAGE. Growth temperature and mAbs used are indicated above each lane. Positions of protein size markers (M) and of p53, hsc70, p95 and pp58 are also indicated. Additional indicators mark the 80 kDa co-precipitating protein (long arrow in A), pp58 (short arrows in A and B), the 110 kDa co-precipitating protein (filled circle in A) and the 90 kDa co-precipitating protein (open circle in A).

derives from the stable introduction of p53Val135 into the MCO 1 line, which is devoid of endogenous p53 protein owing to a splice site mutation (Halevy *et al.*, 1991; O.Halevy and M.Oren, unpublished data). As can be seen in Figure 2B, p95 from mouse cells migrates slightly more quickly than the corresponding rat species, suggesting that p95 represents an endogenous host cell protein rather than a product of the transfected DNA. Further evidence in support of this conclusion is presented in the following section.

p95 is a p53-binding protein

To rule out the possibility that p95 was precipitated as a result of coincidental cross-reactivity of PAb421, clone 6 extracts were reacted with several additional p53-specific monoclonal antibodies (mAbs). It was found that p95 could be precipitated by three additional distinct anti-p53 mAbs, PAb248, PAb242 (Figure 3) and RA3-2C2 (data not shown). Thus, the presence of p95 in the immune complexes was dependent on an authentic p53-specific reactivity.

p95 could conceivably be either a modified, more slowly migrating derivative of p53 or an unrelated protein forming a non-covalent complex with p53. These possibilities were examined using two independent strategies. First, a denaturation experiment was performed (Figure 4A). A ^{35}S-labelled extract of clone 6 cells maintained at 32.5°C was boiled for 10 min in the presence of 0.5% SDS and then diluted 25-fold and subjected to immunoprecipitation (lane 2). While this treatment caused a partial reduction in the amount of precipitable p53 as compared with untreated or mock-treated extracts (native proteins immunoprecipitated in the presence of 0.02% SDS), a substantial fraction of p53 could still be precipitated. On the other hand, denaturation led to an almost complete loss of p95, as well as of hsc70, which is also associated non-covalently with p53 in clone 6 (Pinhasi-Kimhi *et al.*, 1986).

In addition, the nature of p95 was probed using a smaller, functional p53 variant. In such a case, proteins that are derived directly from p53 would be expected to exhibit a corresponding size reduction. On the other hand, the size of a p53-associated protein should not be affected by the change in p53 itself. We therefore used the polymerase chain reaction (PCR) to generate a truncated p53Val135 derivative, p53Val135d360, that lacked the 30 C-terminal amino acid residues. Transformed cell lines were established following co-transfection of REF with p53Val135d360 together with myc and activated Ha-ras. One such line, RmVd360#1, which expressed the truncated p53 abundantly, was studied further. When RmVd360#1 cells were shifted to 32.5°C, they stopped growing completely (data not shown). Thus, the 30 C-terminal residues of p53 are dispensable for its antiproliferative activity. The p53 encoded by the truncated mutant clearly migrated more quickly than authentic p53 (Figure 4B, lanes 2 and 4). Nevertheless, p95 co-precipitated from ^{35}S-labelled extracts of either RmVd360#1 or clone 6 displayed identical mobilities (Figure 4B, lanes 3–5). These results unequivocally imply that p95 is indeed a distinct cellular protein that associates specifically with p53.

p95 – p53 interaction is enhanced by wt p53 activity

Since pronounced p95 binding was seen only in cells in which the ts p53 mutant was induced to display wt activity,

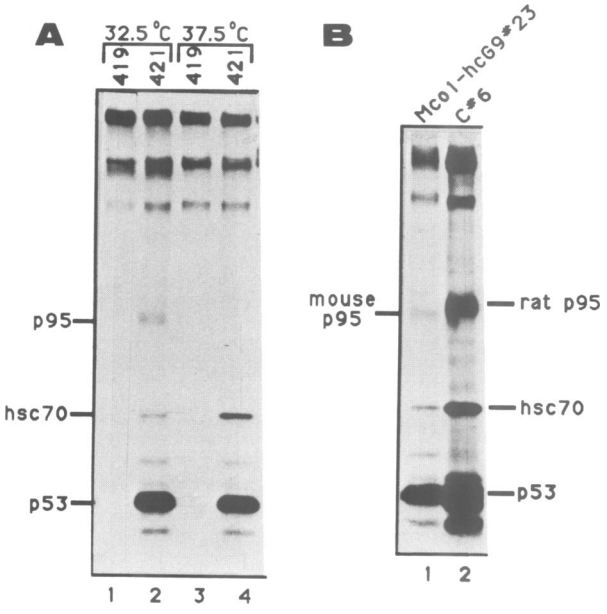

Fig. 2. p95 co-precipitates with p53Val135 from a mouse fibrosarcoma derived cell line. (**A**) Cell line Mco1-hcG9#23 was obtained by transfection of the mouse fibrosarcoma p53-negative line MCO 1 (Halevy *et al.*, 1991) with p53Val135 (O.Halevy and M.Oren, unpublished results). Cells were either maintained for 24 h at 32.5°C (lanes 1 and 2) or grown continuously at 37.5°C (lanes 3 and 4) and then labelled with [^{35}S]methionine. Aliquots of cell extract containing equal acid-insoluble radioactivity were immunoprecipitated with either PAb419 (lanes 1 and 3) or PAb421 (lanes 2 and 4) (**B**) Side-by-side electrophoresis of PAb421 immunoprecipitates from Mco1-hcG9#23 (lane 1) and clone 6 (C#6; lane 2) maintained for 24 h at 32.5°C.

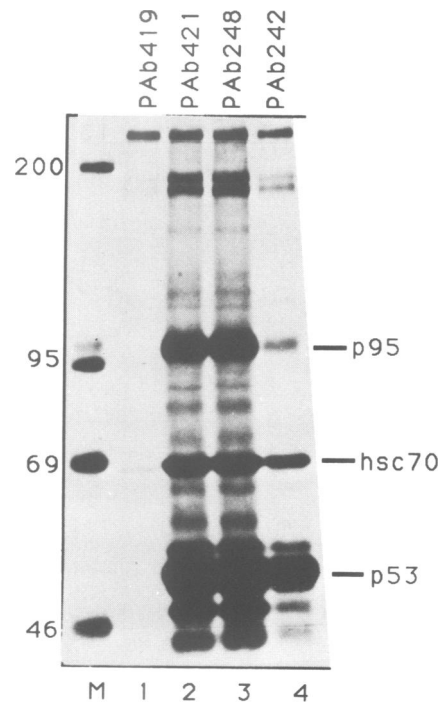

Fig. 3. p95 co-precipitates with different anti-p53 mAbs. Clone 6 cells maintained at 32.5°C for 24 h were labelled with [^{35}S]methionine as described. Cell extract aliquots containing equal acid-insoluble radioactivity were immunoprecipitated with the following mAbs: PAb419 (anti-SV40 large T, lane 1) and the anti-p53 mAbs PAb421 (lane 2), PAb248 (lane 3) and PAb242 (lane 4). Positions of molecular size markers (M), p53, hsc70 and p95 are indicated.

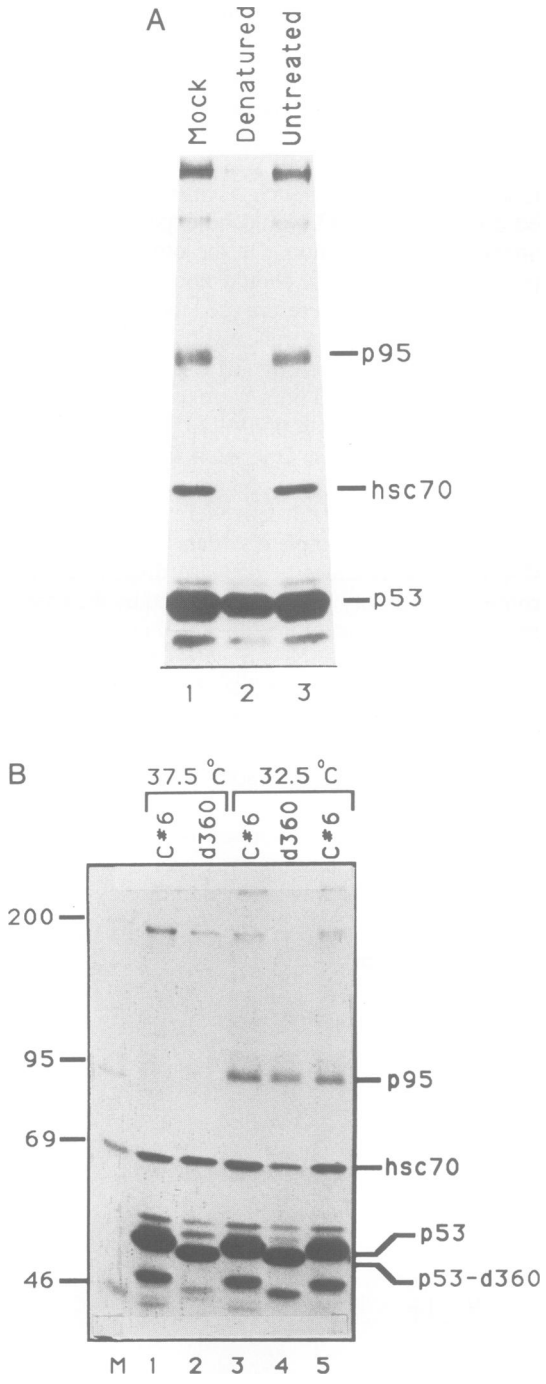

Fig. 4. p95 is a p53-binding protein. **(A)** p95 co-precipitation with p53 is sensitive to denaturation. ^{35}S-labelled extracts were made from clone 6 cells maintained for 24 h at 32.5°C. Aliquots containing equal acid-insoluble radioactivity were immunoprecipitated with PAb421 either in the presence of final SDS concentration of 0.02% (Mock, lane 1) or after boiling for 10 min in the presence of 0.5% SDS and then diluting 25-fold (Denatured, lane 2) or without further treatment (Untreated, lane 3). Positions of p53, hsc70 and p95 are indicated. **(B)** Size alteration of p53 does not affect the migration of p95 on SDS–PAGE. Cell line RmVd360#1 was selected among several cell lines generated by transformation of REF with Ha-ras, c-myc and p53Val135d360. The latter is a truncated derivative of p53Val135 lacking the 30 C-terminal amino acids. Clone 6 (odd lanes) and RmVd360#1 (even lanes) were either continuously grown at 37.5°C (lanes 1 and 2) or shifted to 32.5°C for 24 h (lanes 3–5). Cell extracts were prepared following labelling with [^{35}S]methionine. Aliquots of extracts were immunoprecipitated with mAb PAb248 and resolved by 10% SDS–PAGE. Positions of molecular size markers (M), p53, p53-d360, hsc70 and p95 are indicated.

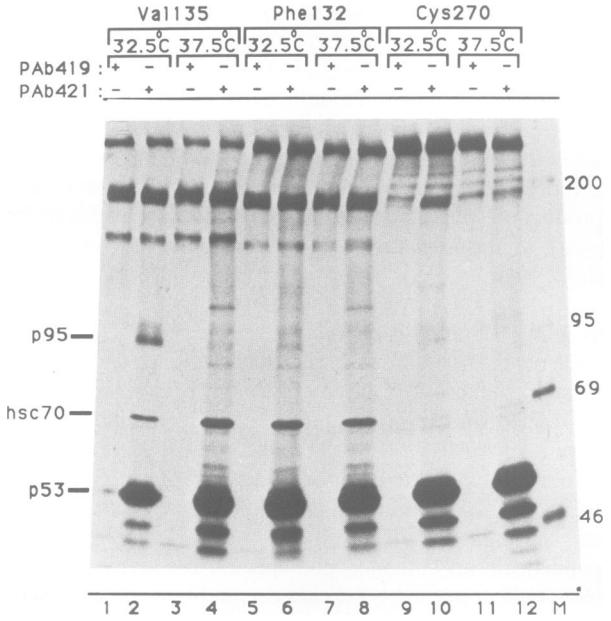

Fig. 5. Analysis of p95 co-precipitation in cells expressing various p53 mutants. Cells were either grown continuously at 37.5°C or shifted to 32.5°C for 24 h and then labelled with [^{35}S]methionine. Aliquots of cell extract containing equal acid-insoluble radioactivity were immunoprecipitated with either PAb421 or PAb419. Cell lines employed were clone 6 (lanes 1–4), R-Phe132#5, REF transformed with p53Phe132 + ras (Michalovitz *et al.*, 1990; lanes 5–8) and R-Cys270#2, REF transformed with p53Cys270 + ras (Halevy *et al.*, 1990; lanes 9–12). Growth temperatures and mAbs used in each immunoprecipitation reaction as well as mutation identities are indicated above the autoradiogram. Positions of molecular size markers (M), p53, hsc70 and p95 are indicated.

it appeared plausible that this property is abrogated by mutations that render p53 incapable of exerting antiproliferative effects. To confirm this conclusion further, we performed the same type of experiment with cells harbouring two non-ts p53 mutants, p53Phe132 (Michalovitz *et al.*, 1990) and p53Cys270 (Halevy *et al.*, 1990). Regardless of the temperature at which the cells were maintained, both mutant p53 species bound p95 to a much lesser extent than p53Val135 from cells growth-arrested at 32.5°C (Figure 5). It is noteworthy that while the two mutants differ in many respects, they are both completely devoid of antiproliferative effects (Halevy *et al.*, 1990). Thus, prominent p95 binding to p53 is detected only in the presence of wt p53 activity.

At 32.5°C the majority of the p53Val135 protein assumes a wt conformation, as detected by the specific mAb PAb246, while a considerable portion of it still retains the mutant conformation, specifically detected by mAb PAb240 (Martinez *et al.*, 1991). In light of the poor binding exhibited by other p53 mutants, as well as by p53Val135 at 37.5°C, we tested whether the binding of p95 by p53Val135 at 32.5°C was restricted to the specific subset of p53 molecules which assume the wild-type PAb246$^+$ conformation. Figure 6 shows clearly that this was not the case; both PAb246 (lane 1) and PAb240 (lane 2) co-precipitate p95 to comparable extents from extracts of clone 6 cells maintained at 32.5°C. In contrast, while both precipitated considerable amounts of p53 from cells maintained at 37.5°C, neither of them co-precipitated conspicuous amounts of p95 from such cells (lanes 3 and 4). In agreement with earlier reports

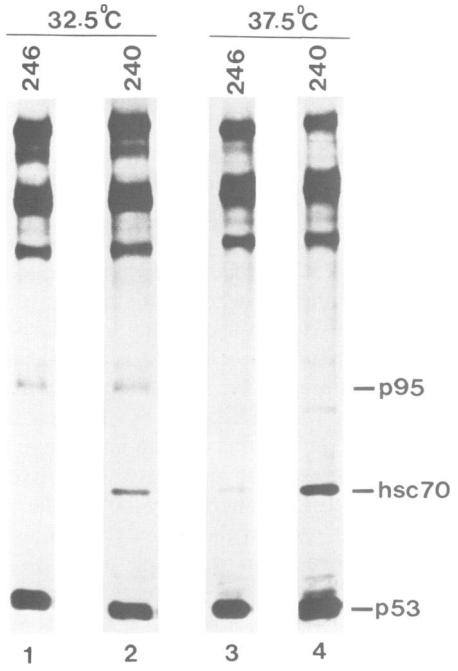

Fig. 6. p95 binds to both wt and mutant conformations of p53. Clone 6 cells were either maintained for 24 h at 32.5°C (Lanes 1–2) or continuously maintained at 37.5°C (lanes 3–4). [^{35}S]methionine-labelled cell extracts were made, which were subsequently immunoprecipitated with mAbs PAb246 (246, lanes 1 and 3) or PAb240 (240, lanes 2 and 4), and resolved by 10% SDS–PAGE. Positions of p53, hsc70 and p95 are indicated.

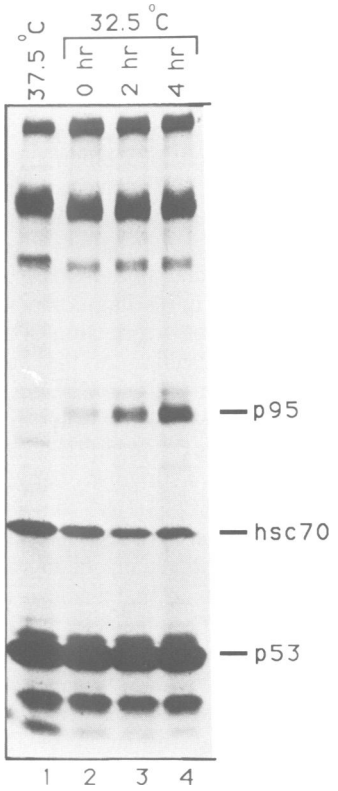

Fig. 7. Kinetics of appearance of p53–p95 complexes upon shifting to 32.5°C. Clone 6 cells were maintained at 37.5°C. 2 h of metabolic labelling with [^{35}S]methionine followed either no change in growth temperature (lane 1) or a shift to 32.5°C for the labelling period only (lane 2), for 2 h (lane 3) and for 4 h (lane 4) prior to labelling. Cell extracts were immunoprecipitated with PAb421 and resolved by 10% SDS–PAGE. Positions of p53, hsc70 and p95 are indicated.

(Sturzbecher *et al.*, 1988; Finlay *et al.*, 1988), the two antibodies could clearly distinguish between hsc70-bound (PAb240$^+$, mutant) and non-bound p53 (PAb246$^+$, wt). Therefore, it appears that p95 can bind to both mutant and wt p53, rather than binding specifically to wt p53. The parameter affected by the anti-proliferative activity of wt p53 is therefore, most probably, the availability of binding-competent p95 (see Discussion).

p95 levels in p53 immune complexes start to increase as early as within the first 2 h following the shift of the cells to 32.5°C (Figure 7, lane 2). This increase continues for at least another 4 h (Figure 7, lanes 3 and 4). While p53–p95 complex induction is relatively fast, it is still apparently slower than other transitions occurring upon shift of p53Val135 to 32.5°C, such as the nuclear translocation of p53, which is almost complete ~3 h after the temperature shift (Ginsberg *et al.*, 1991b), or the maximal reduction in hsc70 binding (Figure 7, lane 3), which reflects the maximal transition of p53 from the mutant to the wt conformation.

Discussion

The findings reported here demonstrate that several cellular proteins are specifically associated with p53 in cells subjected to wt p53-mediated growth arrest. Such associations are far less evident, if present at all, in cells overexpressing mutant p53 forms which fail to exert an anti-proliferative effect. It is therefore likely that at least some of these molecular interactions may underlie the biochemical processes responsible for the growth-inhibitory and tumour suppressor activities of p53.

In the present study, we concentrated on the analysis of the most abundant of these polypeptides, p95. This interaction of p95 with p53 is very tight and resists exposure to extreme conditions such as 8 M urea and 5 M guanidinium–HCl (data not shown). It is therefore possible that, once formed within the cell, this interaction may not be easily reversible.

The increase in the amount of p53–p95 complexes upon the induction of wt p53 activity in cells harbouring the ts mutant of p53 is rather striking. Yet, although very little p53–p95 complex formation is seen in cells carrying constitutively mutant p53 (Figure 5), p95 can associate equally well with mutant and wt p53 conformations in the growth-arrested cells (Figure 6). There are two alternative explanations for this surprising observation. One has to do with the criteria used for the definition of mutant and wt conformations of p53. When one compares the p53 populations present at 32.5°C versus 37.5°C in fibroblasts harbouring the p53Val135 ts mutant, the changes observed at the level of reactivity with conformation-specific monoclonal antibodies are rather moderate and incomplete (Martinez *et al.*, 1991; see also Figure 6). The same holds true for the change in the stability of p53 (Ginsberg *et al.*, 1991b), often considered as an additional indicator of the nature of a given p53 protein molecule (Halevy *et al.*, 1989; Reihsaus *et al.*, 1990). Yet the difference in the biological effects of the ts p53 at the two temperatures is most striking, changing from overtly transforming at 37.5°C (Halevy *et al.*, 1990; Michalovitz *et al.*, 1990) to strongly growth-inhibitory at 32.5°C (Michalovitz *et al.*, 1990; Gannon and Lane, 1991; Ginsberg *et al.*, 1991b; Martinez *et al.*, 1991).

It is thus plausible that the actual conformational change leading to regeneration of the wt-like biochemical activities of p53 is only partly reflected by PAb240/PAb246 reactivity. Accordingly, at 32.5°C even the PAb240$^+$ fraction of the protein may already possess some wt p53 features, which are sufficient to allow tight p95 binding.

Alternatively, p95 may truly have no preference for wt over mutant p53. In that case, one would have to assume that the hardly detectable levels of co-precipitating p95 in cells overexpressing mutant, non-inhibitory forms of p53 simply reflect the paucity of binding-competent p95 in such cells. This could merely be a reflection of actual total p95 levels, or else a manifestation of the balance between binding-competent and binding-incompetent subpopulations of p95. At the moment the only way whereby we can visualize p95 is through its co-precipitation with p53, which does not allow us to distinguish between these two alternatives. The kinetics of p53−p95 complex accumulation indicate that while this process is initiated almost concomitantly with the drop in temperature, it is nevertheless gradual, and thus appears to be enhanced by the ongoing presence of wt p53 activity in the cell. Such activity could, for instance, augment the synthesis of p95 at a transcriptional or post-transcriptional level, or induce a covalent modification in a pre-existing pool of binding-incompetent p95. In either case, the formation of the complex is apparently among the tightest molecular correlates of the cellular manifestations of wt p53 activity, tighter and more complete than the loss of association with hsc70 or the altered reactivity with conformation-specific monoclonal antibodies.

The molecular nature of p95 is not revealed by the present study. Several lines of evidence imply that p53 is a transcriptional regulator, acting both positively (Fields and Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990, 1991; Weintraub *et al.*, 1991) and negatively (Ginsberg *et al.*, 1991a; Mercer *et al.*, 1991; Santhanam *et al.*, 1991). Furthermore, mutations of the type failing to exhibit extensive p95-binding in our system are also deficient in the ability to exert such transcriptional effects (Ginsberg *et al.*, 1991a; Raycroft *et al.*, 1990, 1991; Weintraub *et al.*, 1991). It is thus possible that the interaction with p95 could be related to the transcription-regulatory activity of wt p53. For instance, p95 may be a transcription factor whose activity is altered by p53 binding. Such a possibility would be in line with the recent demonstration that two of the cellular proteins that associate with pRB (the product of the retinoblastoma susceptibility gene) are the transcription factors E2F and c-myc (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Chellapan *et al.*, 1991; Kaelin *et al.*, 1991; Rustgi *et al.*, 1991). In this respect, if the reason for the increased detection of p95 at 32.5°C is transcriptional activation of the p95 gene by wt p53, then this may reflect the existence of a regulatory loop linking the expression of the two proteins. In parallel to the proposed transcription-regulatory functions of p53, it has also been suggested that p53 may be involved in the regulation of DNA replication (Braithwaite *et al.*, 1987; Gannon and Lane, 1987; Friedman *et al.*, 1990; Wilcock and Lane, 1991; Kern *et al.*, 1991b). Hence, it is also possible that p95 may be a component of the cellular DNA replication machinery.

From our data it is not possible to conclude whether the effects of p53−p95 binding on the respective biochemical activities of each individual protein are positive or negative. It is thus possible that p95 is a functional partner of p53, acting in concert with p53 to inhibit cell proliferation. This would be consistent with the possibility that wt p53 acts to increase the absolute cellular levels of p95 in the course of implementing growth arrest. On the other hand, p95 may be a positive regulator of cell proliferation whose effects are antagonistic to those of wt p53. In such a case, it is possible that excess p53 may sequester p95 in an inactive or functionally impaired state, thus abolishing its growth-promoting function. The gradual increase in bound p95 seen in Figure 7 could thus be a reflection of this inactivation, in a way analogous to the accumulation of large T-bound p53 in SV40-transformed cells (Oren and Levine, 1981; Levine, 1990). Alternatively, it is possible that the complex is actually designed to restrict the biochemical activity of p53 rather than of p95. In this case an increase in p95 levels could be brought about, for instance, by negative feedback mechanisms reflecting an attempt by the cellular machinery to respond to and restrain the activity of excess wt p53. Obviously, the success in imposing a wt p53-mediated growth arrest through wt p53 overexpression implies that the maximum inducible levels of p95 are, in such cases, insufficient to block the effects of a large p53 excess.

The co-precipitation of an 85−90 kDa polypeptide (p90) with p53 from rat cells transfected with either mutant or wt human p53 has previously been reported by Hinds *et al.* (1990). On the basis of the observed interaction of p90 with transforming mutant p53, it has been suggested that it serves as a target for sequestration by mutant p53, in parallel with the titration of the endogenous wt p53 by the mutant in the transfected cells. It is possible that this p90 is identical to the 90 kDa polypeptide indicated in Figure 1A (lane 4), which co-precipitates preferentially with p53Val135 at 37.5°C. It is as likely, though, that it is in fact identical to the p95 characterized in the present study. The latter possibility is supported by the observation that the half-life of p95 in the complex with p53 is very short (data not shown), as has also been reported by Hinds *et al.* (1990) for p90. In such a case, however, our data would be more consistent with a role for p95 binding in growth inhibition and tumour suppression by wt p53, rather than in transformation by mutant p53.

Finally, while all the detailed experiments addressed only p95, it is equally possible that the main molecular targets of p53 are among the more minor p53-binding proteins. Such a possibility is quite likely in view of the very low steady-state levels of p53 in non-transformed cells. Two such potential candidates are pp58 and the 80 kDa protein, described in Figure 1. Further attempts should therefore also be directed towards the identification and functional characterization of these, as well as possibly additional, p53-binding proteins.

Materials and methods

Cell lines

Clone 6 (Pinhasi-Kimhi *et al.*, 1986) is a rat embryo fibroblast (REF) line generated by co-transformation with Ha-ras and p53Val135. R-Phe132 #5 are REF transformed by Ha-ras and p53Phe132 (Michalovitz *et al.*, 1990). R-Cys270 #2 are REF transformed by Ha-ras and p53Cys270 (Halevy *et al.*, 1990). Mco-1-hcG9 #23 is a derivative of the mouse fibrosarcoma cell line MCO 1 (Halevy *et al.*, 1991), stably transfected with p53Val135 (O.Halevy and M.Oren, unpublished data).

Cell growth, labelling and extraction

All cell lines were maintained at 37.5°C in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS). For temperature shift experiments, cells were seeded at ~25% confluence into several plates and incubated at 37.5°C for 24 h. Some of the culture dishes were then transferred to 32.5°C, while the others remained at 37.5°C. 24 h later, cells were metabolically labelled for 4 h (except for the experiments shown in Figure 7, in which the labelling was for 2 h) with either [^{35}S]methionine or [^{32}P]P_i in DMEM containing 2% dialysed FCS. Cells were subsequently harvested and extracted as described (Maltzman *et al.*, 1981).

Immunoprecipitation and gel electrophoresis

The mAbs used in this study are the anti-p53 mAbs PAb421 (Harlow *et al.*, 1981), PAb248, PAb242, PAb246 (Yewdell *et al.*, 1986), PAb240 (Gannon *et al.*, 1990) and the anti-SV40 large T antigen mAb PAb419 (Harlow *et al.*, 1981). Immunoprecipitation was carried out as described by Maltzman *et al.* (1981). In denaturation experiments, the extract was boiled for 10 min in the presence of 0.5% SDS, diluted 25-fold in cold immunoprecipitation buffer and immunoprecipitated. Control immunoprecipitation (mock) was carried out in this case with the addition of 0.02% SDS to the immunoprecipitation buffer. Immunoprecipitated polypeptides were resolved by 10% SDS–PAGE. Gels containing radiolabelled proteins were fixed and fluorographed in 1 M sodium salicylate.

Plasmid construction and transfection

The plasmid pLTR-p53Val135d360 is a derivative of pLTR-p53Val135 (Eliyahu *et al.*, 1985). It was constructed by PCR using a 3′ oligonucleotide which harbours a stop codon at a position corresponding to codon 361 of mouse p53. pLTR-p53Val135d360 was introduced into REF along with the plasmids pLTR-myc and pEJ6.6, expressing mutant human Ha-ras, as described before (Eliyahu *et al.*, 1984, 1989). Foci were trypsinized 9 days later and expanded into cell lines; from these the RmVd360 # 1 line was chosen for further work owing to its relatively high p53 content and temperature-sensitive growth.

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