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Analysis of the most representative tumour-derived p53 mutants reveals that changes in protein conformation are not correlated with loss of transactivation or inhibition of cell proliferation

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In an effort to correlate the biological activity of the p53 protein with its conformation, we analysed 14 p53 mutants representative of the most frequently observed protein alterations in human cancers, at codons 175, 248 and 273 (22% of all mutations thus far reported), all three of which contained a CpG dinucleotide. Strikingly, most of the mutants at codons 248 and 273 did not display any change in their conformation, as probed by monoclonal antibodies PAb240 and PAb1620 or by binding to hsp70 protein. For all 14 mutants tested, we found a strict correlation between the transactivation properties of p53, tested either on RGC sequences or using the WAF-1 promoter, and inhibition of cell proliferation. All these mutants showed nuclear localization. Several mutants, present at a low incidence in human tumours, displayed wild-type activity in all our assays, suggesting that the presence of a mutation is not strictly correlated with p53 protein inactivation in tumours. Further analysis of nine thus far undescribed p53 mutants at codon 175 revealed a wild-type or mutant behaviour. All these results suggest that the occurrence of a mutation is dependent on two criteria: (i) the mutability of a given codon, such as those containing a CpG dinucleotide; (ii) the resulting amino acids, eventually leading to synthesis of a p53 conferring a growth advantage on the cell.

Key words: cell proliferation/monoclonal antibodies/p53 mutants/transactivation

Introduction

Human cancer is a multi-step process resulting from damage to genes involved in regulation of cell growth, cell differentiation and cell apoptosis (Weinberg, 1989; Vogelstein and Kinzler, 1993). Examination of progressive stages of neoplasia has identified the accumulation of a multitude of genetic changes (mutation, gene rearrangement or amplification) in tumorigenic cells. One current model postulates that 5–10 mutations are required for a cancer to form (Renan, 1993). According to this multi-hit concept, gene alterations occur through a sequential process in a single cell and its progeny (Nowell, 1976). Among the genes which are frequently mutated in human cancers, the *p53* gene is the most common target for abnormalities (Hollstein *et al.*, 1991; Caron de Fromentel and Soussi, 1992). The spectrum of cancers in which *p53* mutations has been found includes cancers of the breast, colon, bladder, pancreas and lung, as well as brain tumours, leukaemias, lymphomas, gastric carcinomas, hepatocarcinomas (HCC) and osteosarcomas. Furthermore, germline *p53* mutations have been reported to be the basis for the high frequency of tumours arising in Li–Fraumeni families (Malkin *et al.*, 1990; Srivastava *et al.*, 1990).

Two recent reports have analysed the significance of all *p53* point mutations in relation to tumour type (Hollstein *et al.*, 1991; Caron de Fromentel and Soussi, 1992). Several important points emerged from this work: First, >90% of these mutations were clustered in the central part of p53, and mainly affected amino acids conserved in all p53 species. This observation was made on 380 mutations, and it was noted that a bias could have arisen from the fact that many sequencing studies were performed only on exons 5–8. Nevertheless, more recent analysis of the whole gene shows that <5% of such mutations lie outside the central region. A second point concerns the nature of point mutations impairing the *p53* gene. A high incidence of G→T transversion was observed in cancers such as lung carcinomas and HCC. These two cancers are known to be associated with exogenous mutagens such as benzo[*a*]pyrene (Takahashi *et al.*, 1989; Chiba *et al.*, 1990; Iggo *et al.*, 1990) or aflatoxin B1 (Bressac *et al.*, 1991; Hsu *et al.*, 1991). The association of a *p53* alteration with exogenous carcinogens has recently been highlighted by the work of Brash *et al.*, who showed that *p53* mutations in squamous cell carcinomas of the skin mainly affect pyrimidine dimers (Brash *et al.*, 1991), a phenomenon known to be associated with a UV effect. The third important point concerns amino acids which are designated as mutation hotspots. Three amino acids (Arg175, Arg248 and Arg273) account for 22% of all mutations. These three codons (CGN) contain a CpG dinucleotide, and the high frequency of C→T transitions at this position is consistent with lack of DNA repair which occurs after deamination of the 5-methylcytosine.

Several investigators have postulated that generation of the multiple mutations found in cancer cells is the result of a decrease in the genetic stability of the genome of a normal cell (Nowell, 1982; Livingstone *et al.*, 1992; Yin *et al.*, 1992). It is thus important to distinguish between those neutral mutations that are acquired by the malignant cell and those that truly contribute to the malignant phenotype. Several clues indicate that p53 alterations are involved in the transforming process. (i) They are mainly localized in the highly conserved region of the p53 protein, which has recently been shown to be involved in the specific DNA binding activity of wild-type p53 (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993). (ii) When available, the analysis of normal tissue from a patient indicates that the mutation is not present, ruling

out the polymorphism hypothesis. (iii) Analysis of several p53 mutants has shown that they have lost their normal properties, such as transactivation of the reporter gene or the capacity to inhibit cell growth. Nevertheless, such studies on p53 mutants have focused either on specific p53 mutants, such as Ala143, His175 or His273 (Hinds *et al.*, 1990; Kern *et al.*, 1991b; Slingerland *et al.*, 1993), or on less common mutants involved in specific neoplasias, such as Li–Fraumeni syndrome (Frebourg *et al.*, 1992), Burkitt's lymphoma (Vousden *et al.*, 1993) or cervical carcinoma (Crook and Vousden, 1992).

Examination of the frequency of the p53 mutants at the three hotspot codons (Arg175, Arg248 and Arg273) clearly shows that some mutational events are found at high frequency compared with others, which are either rare or absent. The present study was undertaken to analyse the behaviour of a large number of p53 mutants found at these three hotspot codons in order to answer the following questions. (i) What feature(s) determines the unequal frequency of a mutational event at a given codon: the DNA sequence, the resulting amino acid or both? (ii) Are all these mutants really altered in each of their biological functions? (iii) Are all these p53 mutants equivalent in their loss of activity or conformation? (iv) Which biological activity should be tested to infer p53 alteration?

To address these questions, the behaviour of wild-type and mutant p53 was tested either through the conformation of the protein or its biological activity. Conformation was tested by probing with conformational monoclonal antibodies (mAbs), such as PAb240 and PAb1620, or by interaction with heat shock protein 70 (hsp70). Biological activities were assessed by transactivational assays or the cell growth inhibitory effect.

Our results show that: (i) loss of biological activities in some p53 mutants does not correlate with a change in the conformation of the protein; (ii) loss of cell proliferation inhibition is always linked to loss of transactivating properties; (iii) some mutants found in human cancers behave like wild-type p53.

Results

Frequency of p53 alterations at codons Arg175, Arg248 and Arg273

Strikingly, the three hotspots described for p53 mutations are located in arginine codons, with a CpG dinucleotide as the two contributing bases of the codon. This dinucleotide is known to be frequently methylated in the vertebrate genome. Deamination of the 5-methylcytosine can generate a C→T transition after DNA replication. The symmetry of dinucleotide CpG suggests that the 5-methylcytosine residue on each strand of the DNA could be the target of deamination. If we consider the mutational event on a given strand of the DNA (usually the coding strand), mutational events can be depicted as C→T or G→A transitions occurring at an equal rate.

Table I summarizes mutational event data reported in the literature at the three hotspot codons. It corresponds to a compilation of 2305 mutations, including 322 insertions, deletions or splice mutations. These p53 mutants at the three hotspots correspond respectively to 5.3%, 8.6% and 7.7% of the total number of point mutations found in various human cancers.

Table I. Summary of mutational events found at codons 175, 248 and 273

Transition/ transversion	CGC175 (Arg)	CGG248 (Arg)	CGT273 (Arg)
Transition			
G→A	92 (His)	69 (Gln)	73 (His)
C→T	2 (Cys)	79 (Trp)	63 (Cys)
Transversion			
C→A	2 (Ser)	0	0
C→G	1 (Gly)	2 (Gly)	2 (Gly)
G→T	3 (Leu)	16 (Leu)	11 (Leu)
G→C	2 (Pro)	1 (Pro)	3 (Pro)

A database of all p53 mutations found in human cancers was established from data published up to February 1994 (270 publications and 2305 mutations). Only point mutations at the three hotspot codons are described.

They correspond mainly to transitions (G→A or C→T): 94/102 for codon 175, 148/167 for codon 248 and 136/152 for codon 273. For codons 248 and 273, two types of mutational event are equally represented, e.g. 69 G→A and 79 C→T for codon 248 and 73 G→A and 63 C→T for codon 273. In contrast, there is a striking imbalance for codon 175, with 92 G→A (Arg→His) and only two C→T (Arg→Cys). This low incidence of Cys175 mutants could be due to differential accessibility of the DNA sequences to repair enzymes or to a differential intrinsic spontaneous mutability or lack of oncogenicity of this mutant p53. With regard to the latter point, two human tumours have been found to harbour such a mutation, raising the question of the significance of this mutation in the neoplastic process. Does a p53 with a C→T transition at codon Arg175 have transforming properties? In general, considering all the various mutation types seen at these hotspot codons, are they really involved in abnormal behaviour of the p53 protein?

In order to address the fundamental question of the role of p53 in neoplastic transformation, we have produced a library of p53 mutants at these three hotspot positions and have tested their properties.

Construction of a p53 mutant library and assessment of p53 activity

Using *in vitro* mutagenesis, we constructed a library of 23 p53 mutants at positions Arg175, Arg248 and Arg273 (Tables II and III). We focussed our attention on codon 175, since it is the position of the bias described above. p53 mutants in the library could be classified into two categories: those occurring in human cancers (14 mutants) and artificial mutants not detected in human cancers (nine mutants). All p53 mutants were constructed by oligonucleotide-directed mutagenesis and were controlled by sequencing.

Among the tests aimed at assessing the wild-type or mutant phenotype of the p53 protein, we selected those most relevant to the transformation process: those related to the conformation of the protein, such as its interaction with hsp70 (Pinhasi-Kimhi *et al.*, 1986; Stürzbecher *et al.*, 1987) or its reaction with two conformational antibodies, PAb240 (Gannon *et al.*, 1990) and PAb1620 (Gamble and Milner, 1988); and those related to the biological activity

Table II. p53 mutants at codon 175

	Conformation			Transactivation[a]	Interference (%)[b]	Inhibition		Codon[e]
	PAb1620	PAb240	hsp70			Ratio[c]	ICC (%)[d]	
pCMVneoBam	NA	NA	NA	NA	NA	5.3;39	0	
pC53SN3 (Arg)	+	−	−	+	7	1.0	0;1.6	CGC
pC53CX3 Ala143	−	+	+	−	83	2.7;8.9	38;78	
His	−	+	+	−	74	8.2;12.2	60;70	**CAC**
Cys	+	−	−	+	11	1.4;1.8	0;2.2	**TGC**
Leu	+	−	−	+	30	0.2;1.24	0;6	**CTC**
Pro	+	−	−	+	26	1.2;2.0	0;12	**CCC**
Ser	+	−	−	+	13	1.2;2.6	0;16	**AGC**
Gly	NA	NA	NA	−	112	3.0;8.2	0;0.5	**GGC**
Lys(f)	+	−	−	+	12	1.0	0;1.0	**AAG**
Asn(f)	+	−	−	+	27	0.4;1.7	0;13	**AAC**
Gln(f)	+	−	−	+	24	1.8;4.4	10;54	**CAA**
Ile(f)	+	−	−	+	11	0.5;6.0	0.5;4	**ATC**
Thr(f)	+	−	−	+	8	0.8;1.6	0;0.6	**ACC**
Asp(f)	−	+	+	−	72	5.2;6.0	57;72	**GAC**
Phe(f)	−	+	+	−	71	4.2;7.1	40;75	**TTC**
Trp(f)	−	+	+	−	92	2.1;21.8	34;60	**TGG**
Tyr(f)	−	+	+	−	68	6.4;13.6	45;63	**TAC**

[a]p53 mutants negative for transactivation always have an activity of 0–5% compared with wild-type p53. All co-transfections were performed at least three times.
[b]100% corresponds to c-*jun* promoter activity cotransfected with the pCMVneoBam vector. The value for the p53 mutant corresponds to the mean of at least three transfections.
[c]This value corresponds to the ratio of clones obtained between the p53 mutant and the wild-type p53 (pC53SN3). All these experiments were performed three to five times. The two values correspond to the minimal and maximal values obtained in the various experiments.
[d]This corresponds to the frequency of G418 clones which express p53 as tested by ICC analysis using HR231 monoclonal antibody. The two values correspond to the minimal and maximal values obtained in the various experiments.
[e]Substituted nucleotides are shown in bold.
[f]Artificial mutants.
NA, not applicable.

Table III. p53 mutants at codons 248 and 273

	Conformation			Transactivation	Interference (%)[b]	Inhibition		Codon
	Pab1620	Pab240	hsp70			Ratio	IC (%)[d]	
Arg (wt)	+	−	−	+	7	1.0	0;1.6	CGG
Leu	+	−	−	−	48	1.7;17.4	27;46	**CTG**
Pro	+	−	−	−	74	0.9;8.1	12;61	**CCG**
Gln	+	−	−	−	51	1.6;11.7	36;40	**CAG**
Trp	+	−	−	−	53	3.9;24.6	52;57	**TGG**
Arg (wt)	+	−	−	+	7	1.0	0;1.6	CGT
Cys	+	−	−	−	41	8.3;18	9;37	**TGT**
Leu	+	−	−	−	47	3;8	14;44	**CTT**
His	+	−	−	−	40	8;15.3	33;44	**CAT**
Pro	−	+	+	−	114	3.0;15	31;59	**CCT**

See footnotes to Table II.

of p53, such as the transactivating properties (Kern *et al.*, 1991b; El-Deiry *et al.*, 1992) or the proliferation activity (Baker *et al.*, 1990; Diller *et al.*, 1990). p53 transactivation was tested using two complementary assays. The first used a reporter gene linked to a p53 DNA binding response element. Wild-type p53 can transactivate the reporter gene through DNA binding, whereas mutant p53 is devoid of this activity. Several p53 sequence-specific DNA elements have been characterized and two main consensus sequences were derived from these studies: the RGC sequence, which has been found within the human ribosomal gene cluster or in the muscle-specific creatine phosphokinase promoter (Kern *et al.*, 1991b; Zambetti *et al.*, 1992) and the p53 CON consensus found either in the gadd45 or the WAF-1 gene (El-Deiry *et al.*, 1992, 1993; Kastan *et al.*, 1992). Both sequences can confer wild-type p53 responsiveness when cloned upstream of a minimal promoter. Nevertheless, Chen *et al.* have described heterogeneity of the transactivational activity which is dependent of the DNA target sequences (Chen *et al.*, 1993a). Consequently, we have monitored the transactivational activity of the p53 using reporter plasmids

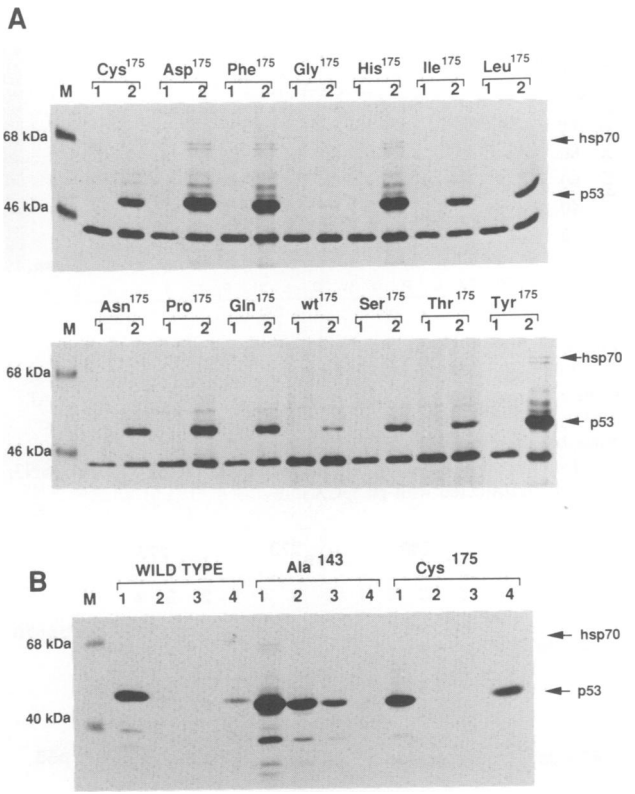

Fig. 1. Conformation analysis of p53 mutants at codon 175. Saos-2 cells were transfected with 10 μg various p53 mutant plasmids. After transfection (48–72 h), cells were metabolically labelled for 2 h as described in Materials and methods. Extracts were prepared and used for immunoprecipitation. (**A**) Extracts were immunoprecipitated with the control mAb PAb419 (lane 1) or the p53 mAb B17 (lane 2). (**B**) Extracts were immunoprecipitated with HR231 (lane 1), PAb240 (lane 2), HP64 (lane 3) or PAb1620 (lane 4).

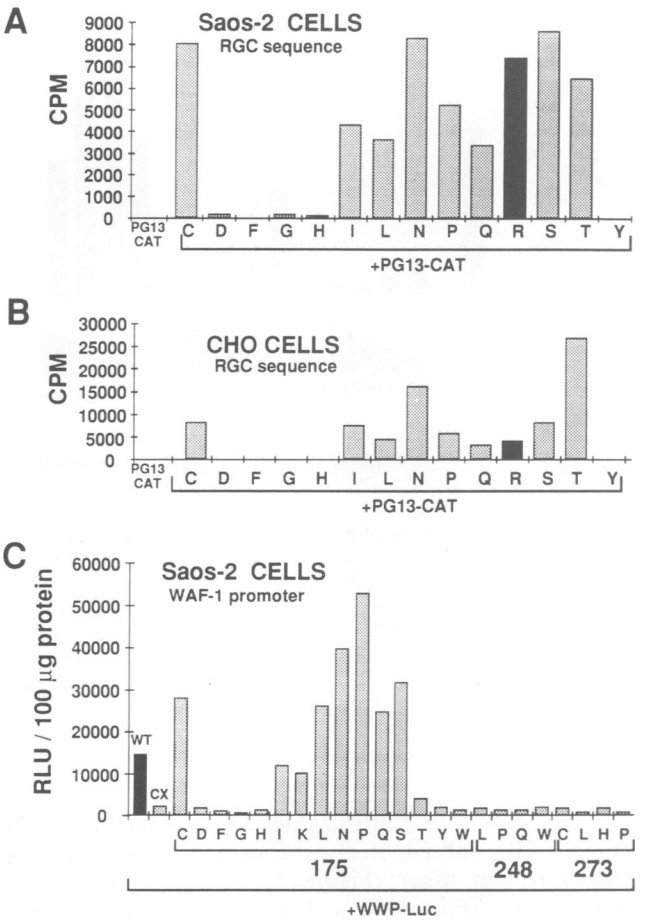

Fig. 2. Transactivation properties of p53 mutants. Saos-2 cells or CHO cells were co-transfected with 2 μg reporter plasmid (PG13-CAT or WWP-Luc) and 3 μg p53-expressing vector; 48 h after transfection, the activity of the reporter enzyme was measured as described in Materials and methods. The black bar corresponds to the wild-type p53. The reporter plasmids were PG13-CAT in experiments **A** and **B** and WWP-Luc in experiment **C**. WT, cells transfected with pC53SN3; CX, cells transfected with pC53CX3.

containing the two different p53 target sequences, PG13-CAT with the RGC sequence and WWP-Luc with the promoter of the WAF-1 gene for the CON sequence.

The second transcriptional assay is based on the finding of Oren *et al.*, who showed that wild-type p53 down-regulates the activity of c-*fos*, c-*jun* or c-*myc* promoters (Ginsberg *et al.*, 1991; Ragimov *et al.*, 1993). Recently, it has been demonstrated that this down-regulation occurs via interference with the binding of wild-type p53 to transcription factors such as the TATA binding protein (Seto *et al.*, 1992). Mutant p53 is devoid of such activity. We chose to test these two transactivating properties, since they are totally complementary.

The p53 mutants at codon 175

Preliminary experiments were performed using the well-defined p53 mutant expressed by pC53CX3 (Ala143). Immunoprecipitation with PAb1620 and PAb240 makes it possible to distinguish between the wild-type conformation ($PAb1620^+/PAb240^-$) and the altered conformation ($PAb1620^-/PAb240^+$). Wild-type p53 is never immunoprecipitated by PAb240 (Figure 1B), whereas the control mutant, Ala143, is no longer PAb1620 reactive and has acquired the PAb240 epitope. Furthermore, the mutant p53 is able to interact with hsp70 (Figure 1B). This mutant p53 has lost the transactivating properties of a reporter gene through binding of the RGC or WAF-1 sequence and does not interfere with transcription from the c-*jun* promoter (Table II and data not shown). Transfection of the Ala143 mutant into Saos-2 cells does not lead to growth arrest. Immunocytochemical (ICC) analysis shows that >50% of clones transfected with mutant p53 expressed high amounts of the protein, whereas clones obtained by transfection with wild-type did not express any p53 (Table II).

All the results obtained for the p53 mutants at codon 175 are summarized in Table II. The mutant His175, which is found in numerous human cancers, is clearly mutant on the basis of its conformation ($PAb1620^-/PAb240^+$ and $hsp70^+$) (Figure 1A), as well as on the basis of its biological activity. This mutant has lost its transactivation activity on either the RGC or the CON sequences (Figure 2) and does not interfere with c-*jun* promoter activity (Figure 3). Saos-2 cells transfected with this mutant are tolerant to p53 expression, as they have lost the ability to inhibit cell proliferation. Of the clones obtained upon G418 selection, 60–70% express high levels of p53, as tested by immunocytochemical (ICC) analysis (Table II).

The mutant Cys175 exhibits wild-type properties. It is

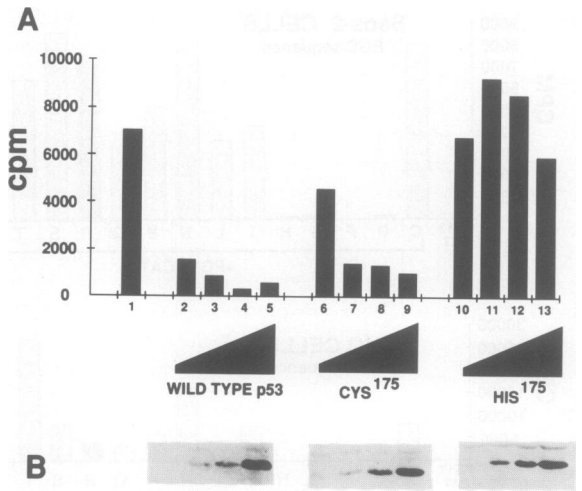

Fig. 3. Interference activity of p53 mutants on the c-*jun* promoter. Two micrograms of c-junCAT plasmid were co-transfected with p53-expressing vectors; 72 h after transfection, CAT activity was measured as described in Materials and methods. (**A**) Lane 1, control experiment with c-junCAT and 3 μg pCMVneoBam which does not express p53; lanes 2–5, co-transfection with 0.05, 0.25, 0.5 and 1 μg wild-type p53-expressing vector; lanes 6–9 and 10–12 with 0.05, 0.25, 0.5 and 1 μg either Cys175- or His175-expressing vector. (**B**) Western blot experiment showing the increasing amounts of p53 in the transfection assay.

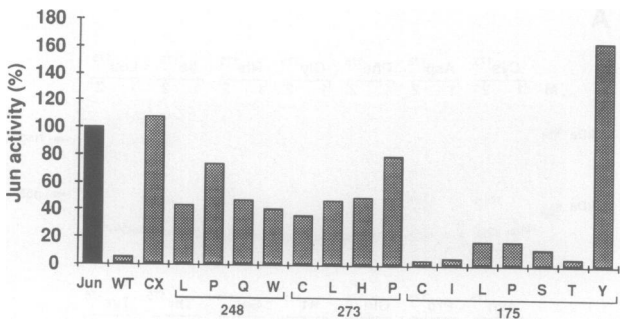

Fig. 4. Interference activity of p53 mutants on the c-*jun* promoter. Two micrograms of c-junCAT plasmid were co-transfected with p53-expressing vector (1 μg). Seventy-two hours after transfection, CAT activity was measured as described in Materials and methods. The black bar corresponds to the activity of the c-*jun* promoter co-transfected with pCMVneoBam. WT, cells transfected with pC53SN3; CX, cells transfected with pC53CX3.

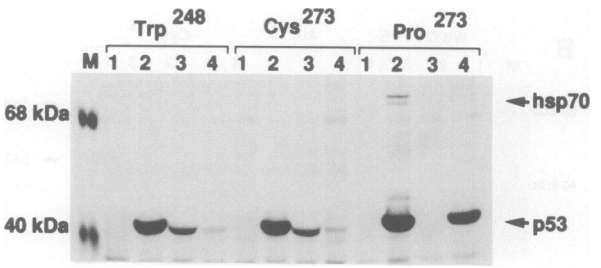

Fig. 5. Conformation analysis of p53 mutants at codon 248 or 273. Saos-2 cells were transfected with 10 μg various p53 mutant plasmids; 48 h after transfection, cells were metabolically labelled for 2 h as described in Materials and methods. Extracts were prepared and used for immunoprecipitation. Lane 1, control with normal mouse sera; lane 2, HR231; lane 3, PAb1620; lane 4, PAb240.

only recognized by PAb1620 and does not bind to hsp70 (Figure 1B). Indeed, it also behaves as a wild-type in transactivation assay (Figure 2), interferes with c-*jun* transcription (Figure 3) and inhibits cell proliferation (Table II). None of the clones obtained after G418 selection express p53 protein (tested by ICC analysis).

Unexpectedly, three other mutants, Pro175, Ser175 and Leu175, which have been found in human tumours, also have wild-type behaviour (Table II). They have a wild-type conformation using either mAbs or hsp70 binding criteria (Figure 2A and Table II). These three mutants also have wild-type biological activity, as they are able to transactivate, to repress expression of the c-*jun* promoter and to inhibit cell proliferation (Table II). Thus, with all criteria tested, these p53 mutants behaved like the wild-type.

Mutant Gly175, which was found once in a human cancer, also shows unexpected behaviour in the sense that it does not transactivate, does not interfere with c-*jun* expression and does not inhibit cell proliferation (Table II). ICC analysis shows that clones obtained after G418 selection did not express detectable p53 protein, whereas other mutants yielded p53-expressing clones (Table II). Short term expression of the Gly175 mutant was difficult to achieve and required greater amounts of DNA in transfection experiments in order to detect very weak expression of p53, either by immunoprecipitation (Figure 2) or by ICC analysis (Figure 6A). These results have been observed for several independent Gly175 mutant clones. The entire ORF of this mutant has been sequenced to verify that no second mutation had occurred during the mutagenesis process (data not shown). Northern blot analysis of RNA expressed by this mutant compared with that of other mutant or wild-type p53, indicated that such expression was similar (data not shown). All these data suggest that this Gly175 protein is rapidly degraded, leading to very weak expression of the p53 protein. Conformation assays using the specific mAbs have not been possible due to the low levels of p53 protein expressed in transfection experiments.

Several artificial mutants (not found in human cancers) were also tested. Such studies had not been previously performed. We raised the question of p53 mutant selection during neoplastic transformation, because certain amino acid substitutions have never been found at position 175. Either they do not encode mutant p53 and cannot be selected during cell transformation or the mutational event leading to such a mutant is not possible, leading to under-representation of such a mutant.

Some of these mutants, such as Lys175, Asn175, Thr175, Gln175 and Ile175, have wild-type properties, whereas others (Tyr175, Trp175, Asp175 and Phe175) show mutant behaviour (Table II and Figures 1 and 2). Again, there is a close correlation between alterations in the p53 protein visualized by monoclonal antibodies or specific interaction with hsp70 and the loss of biological properties of the wild-type p53 protein (Tables I and II).

p53 mutants at codons 248 and 273

Only those mutants most frequently found in human cancers at these two positions were constructed. The results obtained differed from those with mutants involving codon 175. In terms of biological properties, none of these

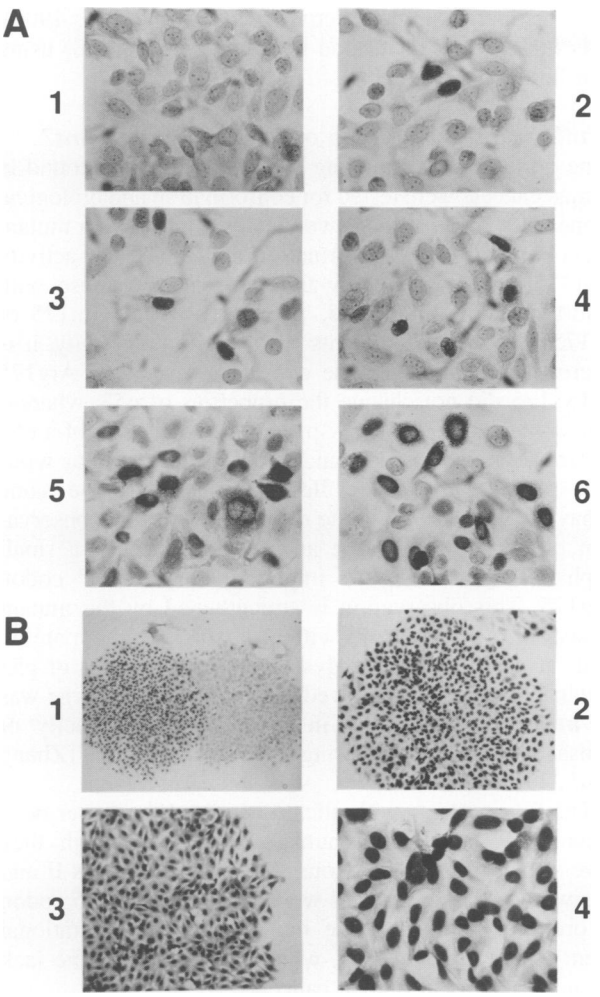

Fig. 6. Localization of p53 mutants. ICC was performed either on Saos-2 cells 72 h after transfection with 10 μg p53-expressing plasmids (**A**) or on stable Saos-2 clones obtained after G418 selection (**B**). Cells were transfected with: (**A**)—(1) mock transfection; (2) pC53SN; (3) p53 Cys175; (4) p53 Gly175; (5) p53 His175; (6) p53 Trp248 (×400); (**B**)—(1) p53 Trp248 (×50); (2) p53 His175 (×100); (3) p53 Cys273 (×100); (4) p53 Cys273 (×400).

mutants was able to transactivate a reporter gene (Table III) or to interfere with c-*jun* activity (Table III and Figure 5). They also failed to inhibit cell proliferation, and clones obtained after G418 selection expressed p53 protein (Table III and Figure 6). On the other hand, all but one mutant, Pro273, had a wild-type conformation (Figure 4 and Table II). They possessed the PAb1620 epitope, were not immunoprecipitated with PAb240 mAb and did not bind to hsp70 (Figure 4). These experiments were performed several times with similar results. Pro273 had an altered conformation, since it bound to hsp70, had lost the PAb1620 epitope and possessed the PAb240 epitope (Figure 4).

Localization of p53 mutants in Saos-2 cells

Using transient transfection experiments followed by ICC staining with HR231, we analysed the localization of the p53 protein inside the cells (Figure 6A). Whatever the position of the mutation, all mutants expressed a nuclear p53 protein (Figure 6A). Nevertheless, we repeatedly observed a difference in the staining pattern of p53 protein with wild-type behaviour and that of mutant protein with loss of activity. Over-expressed wild-type protein had a strict nuclear localization, whereas mutant protein could also be found in the cytoplasm (Figure 6A). This observation was consistent with the notion that mutant p53 is more stable and cannot be entirely translocated within the nucleus. Furthermore, the frequency of stained cells was always higher with mutant p53 than with the wild-type. This could be explained by a growth arrest induced by the overexpression of wild-type p53 in the cell (Diller *et al.*, 1990). Analysis of clones obtained after stable transfection also showed a nuclear localization (Figure 6B).

Discussion

The present work represents the first systematic study of a large panel of p53 mutants using various *in vivo* assays capable of discriminating between the molecular conformation of p53 and its biological activity. Other studies have focused either on a small number of mutants (Hinds *et al.*, 1990; Dittmer *et al.*, 1993; Slingerland *et al.*, 1993), on specific properties of p53 (Kern *et al.*, 1991a), on specific mutants found in given cancers such as Li–Fraumeni syndrome, lung cancer, cervical carcinoma, HCC or Burkitt's lymphoma (Crook and Vousden, 1992; Frebourg *et al.*, 1992; Medcalf *et al.*, 1992; Chen *et al.*, 1993a,b; Hao *et al.*, 1993; Puisieux *et al.*, 1993; Vousden *et al.*, 1993), on properties of p53 not directly related to the transformation process (Bartek *et al.*, 1993), on artificial mutants (Schmieg and Simmons, 1993) or on murine p53 (Halevy *et al.*, 1990). The panel of mutants analysed in the present study corresponded to 22% of the mutants observed in human cancers and was not restricted to a specific type of neoplasia.

p53 mutants found in human cancers: diversity of their behaviour

Three codons (Arg175, Arg248 and Arg273) were shown to be hotspots for mutations in human cancers. This high rate of mutation can be partially explained by the observation that these Arg codons (CGN) contain a CpG dinucleotide, two of which (175 and 273) have been described as being methylated *in vivo* (Rideout *et al.*, 1990). This methylation increases the probability of mutations in such sites because of the ability of 5-methylcytosine to undergo deamination, resulting in a thymine. Examination of mutational events at the three codons has revealed a bias at codon 175 leading to underrepresentation of the Cys175 mutant. A detailed study of the conformation or the biological properties of this mutant demonstrated that it clearly has a wild-type behaviour. This is in striking contrast to His175 p53, the mutant behaviour of which is in accordance with its high frequency in human cancer. Nevertheless, Cys175 was found twice in human cancers. The first case was a colorectal carcinoma in which three different mutations were found in the same p53 allele (Ishioka *et al.*, 1991). The other two mutations were a C→T transition at codon 273 (Arg273→Cys273) and a neutral T→C change at codon 173 (Val173→Val173). The finding of a second mutation at codon 273, which is known to produce a p53 protein with altered properties, suggested that the transition

at codon 175 was not necessary for neoplastic transformation and could occur as a neutral mutational event in a manner similar to that at codon 173. In the second tumour case, the C→T transition at position Arg175 was found in a uterine carcinoma (Enomoto *et al.*, 1993). No other mutation was found, but only exons 5–8 (aa 126–306) were examined in this work. Taken together, all these observations strongly suggest that the transition C→T at position 175 (Arg→Cys) does not lead to a p53 protein with altered properties, providing an explanation for the under-representation of this mutational event at that position compared with the other two hotspots.

A similar observation was made in the study of the Ser175 mutant. It has been twice described in human cancers, whereas the present study indicated that it had wild-type behaviour. Like the Cys175 mutant, it was found once in an HCC associated with a second mutation at position 272 (Murakami *et al.*, 1991). Mutations at codon 272 have been described in various cancers. It was also found in an HCC without another mutation, but in that case only exons 5–8 were sequenced (Tanaka *et al.*, 1993).

The other two mutants, Leu175 and Pro175, exhibit wild-type activity in spite of their description in human cancers as single point mutations. Although we cannot totally exclude the possibility that these mutants have an altered activity not covered by our assays, it is tempting to conclude that they correspond to a wild-type phenotype, thereby suggesting that their role in cell transformation is doubtful. This assumption is strengthened by the low frequency of occurrence of these mutants.

The phenotype of mutant Gly175 warrants our interest. Its biological properties are clearly those of a mutant p53, but appear to be a consequence of a high degradation rate of the protein by an unknown mechanism. A similar situation is observed in cells infected with human papilloma virus (Scheffner *et al.*, 1990; Crook *et al.*, 1991). In these cells, the interaction of wild-type p53 with the E6 protein led to degradation of the p53 protein. Pulse chase analyses were not performed on this p53 mutant, as it is very difficult to detect its expression in a transient transfection assay. That observation indicated that such mutants cannot be detected in neoplastic cells by immunohistochemical analysis and suggested a new mechanism for p53 inactivation.

p53 mutants at position 248 and 273 had clearly lost their biological properties, although most of them (seven out of eight) retained a wild-type conformation. These observations were noteworthy, as it is generally assumed that all p53 mutants have lost the PAb1620 and gained the PAb240 epitope. That view is subject to caution, however, as we demonstrated here that most of the p53 mutants found at positions 248 and 273 had a wild-type conformation. Compared with position 175, positions 248 and 273 were less susceptible to conformation change.

The p53 tertiary structure is presently unknown, but it is clear that the various p53 regions containing the three hotspot codons are not alike. Milner and Medcalf have shown that mutants at position 248 are less susceptible to a co-dominance effect (Milner and Medcalf, 1991). This position is frequently found as a germline mutation in cancer-prone families. Using a murine system, Halevy *et al.* showed that various tumour-derived mutants could have distinct biological activities (Halevy *et al.*, 1990). This finding was confirmed by Hinds *et al.* (1990) using four human p53 mutants.

Artificial mutants: to be or not to be a mutant?

Nine mutants corresponding to substitutions not found in human cancers were tested for conformation and biological properties. They fell into two classes, those with a mutant phenotype for both conformation and biological activity (Tyr175, Trp175, Asp175 and Phe175) and those with wild-type activity (Lys175, Asn175, Thr175, Gln175 or Ile175). Among the mutants with wild-type activity, it is interesting to note that the conservative change Arg175 to Lys175 did not change the properties of p53, whereas conversion of Arg175 to Asp175 led to synthesis of a p53 mutant. Mutants Asn175 and Gln175 had a similar wild-type phenotype and the Ile175 mutant had the same behaviour as Leu175. These results suggest that conservation of a charged residue and the presence of a small aliphatic side chain are important features of codon Arg175. This observation is strengthened by the mutant behaviour of p53 mutants with aromatic side chain amino acids at position 175. Analysing a different series of p53 mutants, Zhang *et al.* showed that a change in charge was an important component in DNA binding capacity or transactivation activity using the CON element (Zhang *et al.*, 1993).

The four mutants with altered activity have never been previously described in human cancers, although they have strong mutant behaviour. As shown in Tables II and III, two nucleotide changes were needed in the 175 codon in order to generate these mutants. Such a mutational event is very rare *in vivo*, which could explain the lack of such mutants in human cancers.

Assessment of p53 behaviour

We have demonstrated that a change in p53 conformation, as tested with PAb1620 and PAb240, is always correlated with binding to hsp70. Nevertheless, this change in conformation is not an absolute prerequisite for p53 inactivation, as several inactive p53 mutants present a wild-type conformation, and it should not be taken as a criterion for p53 status. Descriptions of p53 mutants, especially those at infrequent mutation positions, should be viewed with caution as long as their biological properties have not been tested. This is critical for assessment of p53 germline mutation. To avoid these problems, a yeast functional assay has been developed based on the transcriptional activity of p53 (Ishioka *et al.*, 1993).

p53 mutations are usually scattered in a region of 180 amino acids [highly conserved domains II–IV (Soussi *et al.*, 1990)]. Modifications in amino acids at various positions in this region do not necessarily lead to a completely identical phenotype. In fact, previous works have already shown that several mutations encode p53 proteins which are dissimilar in their properties. This has led to the proposal of various classes of p53 mutants according to their behaviour (Michalovitz *et al.*, 1991).

A higher degree of complexity was reached in the work of Unger *et al.*, showing that p53 transactivating properties can vary from one cell type to another (Unger *et al.*, 1992). In the present study, we did not detect any differences in

the behaviour of the two consensus DNA elements with any of the p53 mutants, suggesting that more studies are needed to gain a full understanding of p53 mutant behaviour.

Analysis of the p53 database revealed another unexpected bias. In lung cancer, transitions or transversions can frequently be found at codons 248 and 273, whereas codon 175 is always spared in this neoplasia. Transfection of a lung cancer cell line (Calu-I, no p53) with several p53 mutants, including His175, showed no transcriptional activity of the reporter plasmid bearing the RGC motif (K.Ory and T.Soussi, unpublished results). Several questions remain unresolved concerning p53 alterations and their exact relationship with the structure and function of the p53 protein in a given cell environment.

Materials and methods

Cell culture and DNA transfection

Saos-2 and CHO cell lines were obtained from the American Type Culture Collection. They were maintained in Dulbecco's modified Eagle medium (DMEM) with glucose (4.5 g/l), L-glutamine and sodium pyruvate. The medium was supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were grown at 37°C in a humidified 5% atmosphere.

For transient transfection, 10^6 cells were plated on 60 mm dishes and transfected on the following day by the calcium phosphate procedure (Graham and van der Eb, 1973). The amount of DNA used for transfection varied according to the type of analysis performed (labelling or transactivation assay) and is shown in the figure legends. The precipitate was left on the cells for 15 h (4 h for long term transfection) before washing twice with DMEM and feeding with 5 ml complete medium. Assays were usually performed 48–72 h after transfection. Long term transfection for cell growth arrest assay was performed as described by Baker *et al.* (1990): 5×10^5 cells were plated into 25 cm^2 flasks and transfected on the following day by the calcium phosphate procedure using 0.5 μg plasmid. Two days after transfection, the cells were split into two flasks in medium containing 0.5 mg/ml geneticin (G418, Gibco-BRL). After 4 weeks, one flask was stained using Giemsa while the other was assessed for p53 protein expression by immunocytochemistry using HR231 mAb, as described by Legros *et al.* (1993).

Plasmids and mutant construction

pCMVneoBam is an expression vector containing the cytomegalovirus constitutive promoter and the neomycin resistance gene under the control of the simian virus 40 promoter. pC53SN3 is derived from pCMVneoBam by insertion of a wild-type *p53* cDNA, whereas pC53CX3 contains a single point mutation in the *p53* cDNA at codon 143 (substitution of valine by alanine). The PG13-CAT reporter plasmid contains the RGC p53 DNA binding sequence linked to a minimal polyoma early promoter (Kern *et al.*, 1991b). In WWP-Luc, the luciferase reporter gene is under the control of the WAF-1 gene promoter (El-Deiry *et al.*, 1993). All these plasmids were kindly provided by B.Vogelstein. BH11 was constructed by subcloning the full length cDNA of human *p53* (clone H8, a generous gift from V.Rotter) into the *Eco*RI site of the phagemid Bluescribe+ (Stratagene) close to the T7 promoter. A reporter plasmid with the human c-*jun* promoter linked to the CAT reporter gene was provided by M.Yaniv.

For mutagenesis at codon 175, we used oligonucleotide-directed mutagenesis of the wild-type *p53* cDNA on the single-stranded form of phagemid BH11, obtained via induction of bacteria with a helper phage. The method of Kunkel was used to preferentially select mutant clones (Kunkel, 1985). All cDNA were checked by DNA sequencing and mutant *p53* cDNAs were subcloned in pCMVneoBam. All constructs were checked again by DNA sequencing.

For the mutations at codons 248 and 273, we used the method described by Deng and Nickoloff using the USE Kit (Pharmacia) (Deng and Nickoloff, 1992). This method allowed oligonucleotide-directed mutagenesis to be performed on double-stranded DNA. pC53SN3 was used as a target for this mutagenesis. All the clones were checked by DNA sequencing.

Direct transactivation assay of p53 activity was performed with either PG13-CAT or WWP-Luc. For the interference assay, the c-*jun*–CAT reporter plasmid was used as described by Ginsberg *et al.* (1991).

Monoclonal antibodies

Monoclonal antibodies HR231 and B17 recognized both wild-type and mutant mammalian p53 via an epitope localized in the C-terminus (HR231) and N-terminus of p53 (B17) (Legros *et al.*, 1993, 1994). PAb240 (Yewdell *et al.*, 1986) (specific for p53 with altered conformation) and PAb1620 (Ball *et al.*, 1984) (specific for a wild-type like conformation) were kindly provided by D.Lane and E.May, respectively. HP64 is a new monoclonal antibody produced in our laboratories (Y.Legros, A.Meyer and T.Soussi, manuscript submitted). It is specific for the mutant conformation of p53. The epitope recognized by HP64 is clearly distinct from that recognized by PAb240. PAb419, a monoclonal antibody specific for SV40 large T antigen, was used as a negative control. All monoclonal antibodies were used as purified immunoglobulins.

Metabolic labelling and immunoprecipitation

Cells were metabolically labelled for 2 h with 200 μCi [^{35}S]methionine and cysteine (Expre^{35}S^{35}S, NEN Dupont). Proteins were extracted with 0.5 ml modified RIPA buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP 40, 0.5% sodium deoxycholic acid) and protease inhibitors. After 30 min on ice, extracts were cleared for 30 min at 15 000 r.p.m. at 4°C. Cell lysates were precleared twice by adsorption with *Staphylococcus aureus* (Immunoprecipitin, GIBCO-BRL) and non-immune serum. Equal amounts of *in vivo* radiolabelled proteins were immunoprecipitated with specific antibodies for 2 h on ice. Antigen–antibody complexes were collected by using immunoprecipitin and washed three times in lysis buffer. Immunoprecipitates were analysed by SDS–PAGE.

CAT assay

Cells were harvested 40–48 h after transfection and lysed by five successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for the reporter enzyme activity. CAT activity was measured by the method of Neumann *et al.* and luciferase activity as described by N'Guyen *et al.*, using a Berthold LB9501 luminometer (Neumann *et al.*, 1987; N'Guyen *et al.*, 1988).

Transfection assays for transactivation were repeated at least three times. All extracts were also tested for p53 expression by Western blot (using HR231) in order to ensure that results of the transactivation assay were not due to variations in p53 expression.

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