The EMBO Journal vol.12 no.3 pp.1029–1037, 1993

The transforming and suppressor functions of p53 alleles: effects of mutations that disrupt phosphorylation, oligomerization and nuclear translocation

Joyce M.Slingerland, John R.Jenkins[1] and Sam Benchimol

The Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 500 Sherbourne St, Toronto, Ontario, Canada M4X 1K9, and [2]The Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, UK

Communicated by D.P.Lane

Mutant p53 alleles that have a recessive phenotype in human tumors can, in cooperation with an activated H-*ras* gene, transform rat embryo fibroblasts (REFs). Mutant p53 proteins differ from wild type, and from each other in conformation, localization and transforming potential. Missense mutations in codons 143, 175 and 275 confer strong transforming potential. A serine 135 p53 mutant has an intermediate transforming potential, while the histidine codon 273 allele transforms weakly, if at all. In contrast to the wild type p53 gene, mutant p53 alleles with strong transforming ability cannot suppress the transformation of REFs by other oncogenes. The His273 allele retains partial suppressor function in this assay. The relevance of p53 oligomerization, phosphorylation and nuclear translocation to the transforming potential of mutant p53 and to wild type p53 suppressor function were examined. The inability of mutant p53 polypeptides to form homodimers correlates with loss of transforming function. Monomeric variants of wild type p53 protein, however, retain the ability to suppress focus formation. Phosphorylation of serine residues 315 and 392 is not required for the transforming function of mutant p53, nor is serine 315 required for suppressor function when these alleles are constitutively expressed in REF assays. Nuclear translocation-defective mutant and wild type p53 proteins retain transforming and suppressor function in REF assays.

Key words: nuclear translocation/oligomerization/p53/suppression/transformation

Introduction

The cellular p53 protein was implicated in oncogenesis initially by its high level of expression in tumors and in chemically and virus transformed cells (Crawford, 1983). Evidence for the direct involvement of p53 in neoplasia was provided when it was shown that the p53 gene could immortalize normal, early passage cells in culture and could cooperate with a *ras* oncogene to induce transformation of REFs (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984; Hinds *et al.*, 1987; Rovinski and Benchimol, 1988). The observation that transforming potential is restricted to mutant p53 alleles (Hinds *et al.*, 1989) and that cotransfected wild type p53 alleles could suppress focus formation by other oncogenes supported a shift in the paradigm surrounding the function of p53 (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). Genetic analysis of the p53 gene in murine and human tumors revealed that this gene frequently undergoes mutation and loss of heterozygosity during neoplastic progression (Mowat *et al.*, 1985; Baker *et al.*, 1989; Nigro *et al.*, 1989; Takahashi *et al.*, 1989). The selection against wild type p53 expression in tumors led to the hypothesis that the p53 gene encodes a negative growth regulator (Lane and Benchimol, 1990). Furthermore, reconstitution of constitutive wild type p53 expression has proven incompatible with continued proliferation in certain cell lines (Baker *et al.*, 1990b; Diller *et al.*, 1990; Johnson *et al.*, 1991). In a number of studies, interruption of cell cycle progression at the G_1/S transition was observed following reintroduction of wild type p53 alleles (Diller *et al.*, 1990; Mercer *et al.*, 1990a). In other cell lines, wild type p53 expression slowed growth, promoted apoptosis or cellular differentiation and resulted in a less neoplastic phenotype (Chen *et al.*, 1990; Mercer *et al.*, 1990b; Shaulsky *et al.*, 1991a; Yonish-Rouach *et al.*, 1991; Cheng *et al.*, 1992).

To reconcile the observed transforming potential of mutant p53 alleles with the selection against wild type p53 in tumors, it was postulated that in REFs, the protein encoded by transfected mutant p53 alleles acts in a dominant negative manner to bind and functionally inactivate the endogenous rat p53 protein (Hinds *et al.*, 1989; Munroe *et al.*, 1990). In REF clones transformed by mutant p53 and *ras*, or immortalized by p53 alone, coprecipitation of the transfected mutant polypeptide and endogenous rat p53 protein has been observed (Rovinski and Benchimol, 1988; Hinds *et al.*, 1990) in support of this model. Although this hypothesis has been widely accepted, coexistence of mutant and wild type p53 alleles is rarely seen in human tumors, suggesting that these mutations do not confer a fully dominant negative phenotype *in vivo* (Slingerland *et al.*, 1991). In spite of the apparent recessive nature of p53 mutations in tumors, certain human mutant p53 alleles do have transforming potential in cellular assays (Hinds *et al.*, 1990; Farrell *et al.*, 1991; Slingerland and Benchimol, 1991).

The present studies were undertaken to explore the transforming potential of a series of human tumor derived human p53 alleles and to assess the conformation and location of the encoded p53 proteins in REF clones derived from transformed foci. The relevance of p53 oligomerization, phosphorylation and nuclear translocation to the transforming function of mutant p53 and to the suppressor function of wild type p53 was assessed.

The C-terminal portion of p53 is largely unaffected by mutational change in human tumors; however, it contains several functionally significant regions. A major nuclear localization signal (NLSI) has been identified between codons 316 and 325 as have two putative minor translocation sites lying further downstream (Dang and Lee, 1989; Addison *et al.*, 1990; Shaulsky *et al.*, 1990b). The intracellular

localization of p53 appears to change during the cell cycle. In synchronous NIH 3T3 cells, p53 protein is cytoplasmic in G_1, shifts into the nucleus at the G_1/S transition, and remains there until the completion of the G_2/M phases (Shaulsky *et al.*, 1990a). To test the relevance of nuclear translocation of p53 to its transforming and suppressor functions, transforming point mutant p53 alleles were rendered NLS defective as were wild type p53 alleles and these were used in REF assays.

Two C-terminal sites of serine phosphorylation at codons 315 and 392 have been identified *in vivo* (Samad *et al.*, 1986; Meek and Eckhart, 1988). The serine 315 lies within a consensus recognition site for the cell cycle regulatory $p34^{cdc2}$ kinase (McVey *et al.*, 1989; Shenoy *et al.*, 1989) and can be phosphorylated by the cdc2 kinase *in vitro* (Bischoff *et al.*, 1990; Sturzbecher *et al.*, 1990), raising the possibility that the action of p53 protein is modulated by interaction with the cdc2 kinase or with other cdk homologues. The serine at codon 392 is also a site of potential physiologic significance, since it is phosphorylated *in vitro* by casein kinase II (Meek *et al.*, 1990). Moreover, serine 392 is also the site for covalent attachment to an RNA oligonucleotide, the significance of which is not known (Samad and Carroll, 1991). REF assays were performed to ascertain whether phosphorylation at either codons 315 or 392 is required for transforming mutant p53 alleles to cooperate with *ras*. Further studies addressed whether serine phosphorylation at codon 315 is required for suppression of transformation by wild type p53.

The domains of p53 responsible for p53 homo-oligomerization have recently been defined (Sturzbecher *et al.*, 1992). In the C-terminus of p53 protein an amphipathic α-helix is followed by a basic region. The hydrophobic face of the α-helix mediates p53 homodimerization. The basic region plays a role in the formation of tetramers and higher order p53−p53 complexes. To test directly the requirements for p53−p53 homo-oligomerization in REF transformation, mutant p53 alleles with transforming ability were rendered oligomerization defective by mutating the regions encoding the α-helical or the basic region motifs. To address whether p53 self-association correlated with wild type p53 suppressor function, vectors encoding oligomerization-defective p53 proteins were assayed for their ability to suppress REF transformation by other oncogenes.

Results

Transformation and suppressor assays

Human tumor derived missense mutant p53 alleles were assayed for their ability to cooperate with an activated human H-*ras* gene to transform early passage REFs. Mutations at codons 143, 175 and 275 conferred transforming potential with numbers of foci 6- to 30-fold greater than the background produced by *ras* alone (mean values are shown in Table IA). p53ser135 had weaker transforming activity with numbers of foci 3- to 6-fold greater than *ras* alone (Table IC). The p53his273 mutant failed to transform in cooperation with *ras* when it was controlled by the SV40 promoter (Slingerland and Benchimol, 1991) and transformed weakly when it was expressed by the human β-actin promoter (Table IB).

Human wild type p53 has been shown to suppress transformation by human papillomavirus E7 and *ras* genes (Crook *et al.*, 1991). The transforming allele βhup53ala143 has lost suppressor function in this assay. The weakly transforming mutant βhup53his273 showed an incomplete suppressor phenotype producing a consistent reduction in the number of foci formed but to a lesser extent than that seen with wild type p53 (Table IIA).

Transforming activity of p53 oligomerization-defective mutants

The C-terminal region of p53 was first implicated in p53−p53 oligomerization in studies of the sedimentation profiles of a truncation mutant lacking amino acids 343−393 (Milner *et al.*, 1991). The C-terminus of p53 contains an amphipathic α-helical region (amino acids 334−356)

Table I. Transformation of rat embryo fibroblasts by p53 and *ras*

	Plasmid DNA	Mean no. of foci	SEM	No. of expts.
A.	*ras*	2	1	9
	CMVhup53wt + *ras*	4	2	4
	CMVhup53his175 + *ras*	17	3	4
	CMVhup53tyr275 + *ras*	15	6	6
	CMVhup53ala143 + *ras*	16	5	6
	CMVhup53ala392 + *ras*	2	1	3
	CMVhup53ala143ala392 + *ras*	18	3	3
B.	βhup53wt + *ras*	2	1	5
	βhup53his273 + *ras*	8	1	4
	βhup341K344E348E355K + *ras*	1	0	4
	βhup365A372L379A386L + *ras*	3	2	4
	βhup53ala143 + *ras*	15	1	4
	βhup53ala143KEEK + *ras*	2	0	3
	βhup53ala143ALAL + *ras*	14	3	3
C.	pECH53ser135 + *ras*	10	2	6
	pECH53ser135KEEK + *ras*	4	1	6
	pECH53ser135ALAL + *ras*	12	1	6

Table II. p53 mediated suppression of REF transformation by HPV E7 and *ras*

Plasmid DNA	Mean no. of foci	SEM	No. of expts.
ras	3	2	6
E7 + *ras*	47	6	7
βhup53wt + E7 + *ras*	4	1	9
A. Point mutation			
βhup53ala143 + E7 + *ras*	37	4	5
βhup53his273 + E7 + *ras*	21	2	3
B. Oligomerization			
βhup341K344E348E355K + E7 + *ras*	2	1	3
βhup365A372L379A386L + E7 + *ras*	4	1	3
C. Phosphorylation of serine 315			
βhup53ala315 + E7 + *ras*	4	3	2
βhup53asp315 + E7 + *ras*	4	1	2
D. Nuclear localization			
βhup53Δ366 + E7 + *ras*	7	1	3
βhup53NLSI + E7 + *ras*	8	4	3
βhup53NLSIΔ366 + E7 + *ras*	2	1	3
βhup53ala143NLSI + E7 + *ras*	49	12	3
βhup53his273NLSI + E7 + *ras*	51	7	3

followed by a basic region (amino acids 363–386). Alteration of multiple hydrophobic amino acids within the α-helix completely abolishes p53 self-association, while alteration of residues in the basic region allows the formation of p53 homodimers but not tetramers and higher order oligomers (Sturzbecher *et al.*, 1992). The protein encoded by a p53 mutant construct with lysine at codon 341, glutamic acid at 344, glutamic acid at 348 and lysine at codon 355 designated hup341K344E348E355K, sediments as a monomer on sucrose gradients. A construct designated hup365A372L379A386L encodes a p53 protein with alanine at codon 365, leucine at codon 372, alanine at 379 and leucine at codon 386. This variant p53 polypeptide is competent to form only homodimers.

To test the effect of oligomerization defects on transformation, expression vectors were constructed bearing p53 alleles with transforming mutations (either serine 135 or alanine 143) and in which the hydrophobic face of the C-terminal α-helix was altered by the four amino acid changes lysine (K) 341, glutamic acid (E) 344, glutamic acid (E) 348 and lysine (K) 355. These alleles were designated pECH53ser135KEEK and βhup53ala143KEEK. The transforming abilities of both p53ser135 and p53ala143 were abolished when the mutant proteins were rendered monomeric (Table I). The competence of βhup53ala143KEEK to express p53 protein was demonstrated by p53 immunoprecipitation following transient transfection into the p53 protein-negative cell line SKOV-3 (data not shown). Moreover, a C-terminal p53 peptide fragment that is capable of binding to p53 proteins having intact α-helix and basic regions was unable to bind the p53ala143KEEK protein expressed transiently whereas it could bind to wild type p53 (data not shown). This is consistent with the idea that the p53ala143KEEK protein is defective for self-oligomerization. Mutant vectors capable of dimerization but deficient in the formation of higher order complexes were constructed by introducing four codon changes in the basic region, alanine (A) 365, leucine (L) 372, alanine (A) 379 and leucine (L) 386 into the transforming point mutant alleles. These were designated pECH53ser135ALAL and βhup53ala143ALAL. The dimerization competent proteins encoded by pECH53ser135ALAL and βhup53ala143ALAL retained transforming function. Neither the monomeric p53 protein encoded by βhup341K344E348E355K nor the dimeric p53 product of βhup365A372L379A386L could cooperate with *ras* to transform REFs (Table I).

Suppressor activity of oligomerization-defective p53 proteins

Both the construct encoding monomeric p53, βhup341K344E348E355K and that encoding dimeric p53, βhup365A372L379A386L suppressed E7 and *ras* transformation to the same extent as that seen with the fully wild type construct βhup53wt (Table IIB). Thus, while the transforming effects of mutant p53 required a dimerization competent molecule, both monomeric and dimeric forms of p53 retained wild type suppressor function in this type of assay.

The effect of serine phosphorylation on transforming and suppressor functions of p53

p53 mutations converting serine 315 to either alanine or aspartic acid both failed to confer transforming potential. Conversion of codon 315 to alanine or aspartic acid in the transforming mutant vector βhup53ala143 did not abolish its transforming function (Table IIIA). Loss of serine phosphorylation at codon 315 did not abrogate wild type suppressor function on cotransfection of this p53 variant with E7 and *ras* (Table IIC). An alanine 392 mutant allele also failed to cooperate with *ras*. Conversion of codon 392 to alanine in CMVhup53ala143ala392 also failed to affect its transforming ability (Table IA).

Transforming and suppressor functions of nuclear translocation-defective p53 mutants

Mutation of the major nuclear localization signal (NLSI), in which lysine residues at codons 319, 320 and 321 were converted to alanine, did not confer transforming ability on wild type p53. In addition, inclusion of this NLSI mutation on a vector encoding hup53ala143 did not change its transforming activity (Table IIIB).

While the hup53his273 allele was weakly or non-transforming, and the hup53NLSI allele failed to cooperate with *ras*, the hup53his273NLSI mutant allele was strongly transforming in cooperation with *ras* (Table III).

Mutation of NLSI did not alter the suppressor function of p53 protein (Table IID). p53ala143 vectors with mutation of NLSI, remained unable to suppress REF transformation mediated by E7 and *ras*. While the hup53his273 allele was weakly transforming, it retained a partial suppressor phenotype on cotransfection with E7 and *ras*. However, the NLSI-defective allele, hup53his273NLSI, had not only acquired strong transforming potential on cotransfection with *ras*, it also failed to mediate suppression of focus formation by E7 and *ras* (Table IID).

The requirement for the two minor nuclear translocation signals NLSII and NLSIII (Shaulsky *et al.*, 1990b) for wild type p53 function was addressed by constructing a 3′ deletion mutant hup53Δ366 that removed codons 366–393 from the p53 cDNA. This region includes NLSII and NLSIII. The encoded truncated protein behaved similarly to wild type p53 protein in its ability to suppress transformation. The mutant p53 allele with the NLSI mutation and in which the minor nuclear translocation signals NLSII and NLSIII had been

Table III. Effects of phosphorylation and nuclear localization on transformation

Plasmid DNA	Mean no. of foci	SEM	No. of expts.
A. Phosphorylation of serine 315			
ras	5	2	4
βhup53ala315 + *ras*	2	1	2
βhup53asp315 + *ras*	1	1	2
βhup53ala143 + *ras*	27	4	6
βhup53ala143ala315 + *ras*	25	6	6
βhup53ala143asp315 + *ras*	30	10	6
B. Nuclear localization			
βhup53Δ366 + *ras*	1	1	3
βhup53NLSI + *ras*	4	3	3
βhup53NLSIΔ366 + *ras*	1	1	3
βhup53ala143NLSI + *ras*	24	5	3
βhup53his273 + *ras*	9	1	3
βhup53his273NLSI + *ras*	29	3	3

Table IV. p53 and *ras* transformed REF clones

Mutant p53 allele	Transforming potential[a]	Cloning efficiency	Epitope map PAb421	PAb240	PAb1620	PAb1801	Hsc70 binding
p53ser135	++	7/9	+	+	+	+	−
p53ala143	+++	8/10	+	+	−	+	+
p53his175	+++	5/5	+	+	−	+	+
p53tyr275	+++	4/4	+	−	−	+	+
p53his273	+	5/5	+	−	+	+	−
p53wt	−		+	−	+	+	−

[a] +++ mean number of foci >6-fold over *ras* alone; ++ mean number of foci 3- to 6-fold over *ras* alone; + mean number of foci <3-fold over *ras* alone.

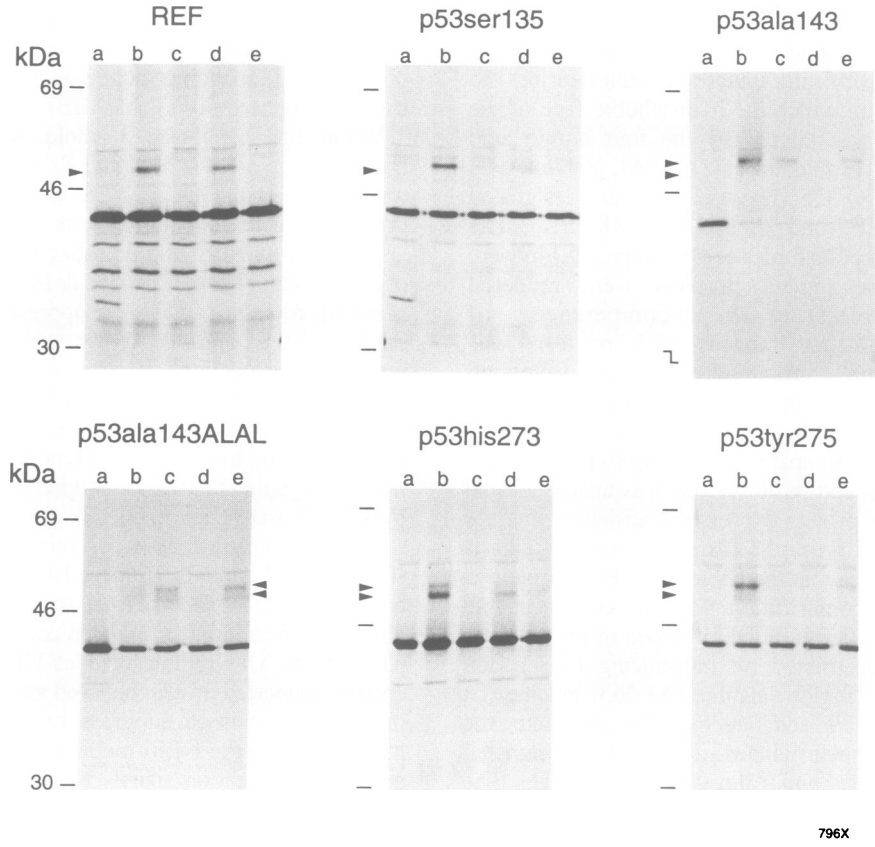

Fig. 1. Expression of p53 protein in transformed rat embryo fibroblast clones. Cells were labelled metabolically with [^{35}S]methionine for 1 h. Extracts were prepared and immunoprecipitated with (**a**) control antibody PAb419, and the following p53 antibodies: (**b**) PAb421, (**c**) PAb240, (**d**) PAb1620, (**e**) PAb1801. The panels represent labelling of normal REFs, and clones transformed by an activated H-*ras* gene together with the indicated human mutant p53 alleles. Where two arrows are shown, the upper arrow indicates human p53 protein and the bottom arrow points to the endogenous rat p53 protein.

deleted, hup53NLSIΔ366, also suppressed focus formation on cotransfection with E7 and *ras* (Table IID).

p53 protein expression in transformed REF clones

To verify the expression of the transfected human p53 cDNA in transformed clones, and to assess the conformational state of the encoded p53 proteins, several of the foci generated with the *ras* oncogene and mutant p53 cDNAs were isolated, expanded in culture and metabolically labelled with [^{35}S]methionine. Clones were established in culture with high efficiency (Table IV) and all clones tested grew in methylcellulose culture (data not shown). The epitope maps of the p53 variants encoded by the mutant alleles are presented in Figure 1 and summarized in Table IV. Alleles bearing mutations at codons 143, 175, 273 and 275 all had the proline polymorphism at amino acid residue 72 and encoded proteins with slower mobility on gel electrophoresis than the codon 135 mutant with arginine at codon 72 (Matlashewski *et al.*, 1987). Rat p53 protein migrated with a mobility identical to the human p53 arginine 72 polymorph (Figure 1).

The Ala143 and the His175 mutants encoded proteins that reacted with the mutant specific anti-p53 antibody PAb240 and failed to react with PAb1620. The codon 275 mutant encoded a PAb240-negative, PAb1620-negative protein. The weakly transforming p53his273 allele encoded a PAb240-negative, PAb1620-positive protein that was distinguishable from rat wild type p53 protein by its slower

mobility and PAb1801 positivity. The Ser135 mutant was previously shown, on transient transfection into SKOV-3, a human cell line lacking endogenous p53 protein, to react with PAb240 but not PAb1620 (Slingerland and Benchimol, 1991). Reactivity of p53 protein with PAb1620 in the p53ser135 transformed REF clone (Figure 1) may represent an alternative conformation of the mutant protein in a different cellular context, or reflect binding of the mutant protein to the endogenous rat wild-type p53 protein with the latter reacting with PAb1620. Although PAb1801 reacted weakly with some of the human mutant p53 polypeptides in immunoprecipitation assays (Figure 1), longer film exposures and immunofluorescence staining indicated that all the mutant p53 proteins listed in Table IV were recognized by this antibody. p53ala143, p53his175 and p53tyr275 proteins co-immunoprecipitated with a protein having the same mobility on polyacrylamide gels as the heat shock 70 (hsc70) protein, while p53ser135 and p53his273 proteins failed to bind to the putative hsc70 protein.

Immunoprecipitation of labelled p53 protein expressed in one βhup53ala143ALAL transformed clone was seen with PAb240 and PAb1801; however, reactivity with PAb421 was reduced, reflecting the changes in the PAb421 epitope in the C-terminus.

The ability of the primate p53-specific antibody PAb1801 to co-immunoprecipitate a protein having the mobility of rat p53 along with hup53ala143ALAL protein indicates that these peptides can form a complex (Figure 1).

Localization of mutant p53 protein in transformed REF clones

Independent clones transformed by *ras* and either p53ser135, p53ala143, p53his175 or p53tyr275 were examined by indirect immunofluorescence staining with the human p53-specific monoclonal antibody PAb1801. In all transformants expressing high levels of human p53 protein, strong nuclear and variable levels of cytoplasmic staining were observed. Immunofluorescence of p53 protein in a REF clone transformed by CMVhup53ala143 and activated *ras* is shown in Figure 2. Nuclear cytoplasmic dissynchrony was a common feature of all clones analysed, with the frequent presence of multinucleate cells bearing up to six nuclei (Figure 2c). Within each clone analysed, the pattern of reactivity of p53 protein with monoclonal antibodies PAb1801, PAb421 and PAb240 was identical. All three recognize both cytoplasmic and nuclear forms of p53 protein. While each of these three antibodies failed to stain the nucleoli, prominent nucleolar staining was detected with PAb1620 in all clones tested (Figure 2d). No nucleolar staining, indeed no reactivity with any p53 antibody was detected in the p53-negative cell line SKOV-3. The human p53 specificity of antibody PAb1801 was confirmed by its failure to react with either the rat p53 protein in untransformed REFs or with the abundant murine mutant p53 protein detected by PAb421 in a REF clone transformed by *ras* and murine p53pro193 (data not shown).

In cell lines established from the few foci derived from *ras* and p53his273 transfections, the distribution of p53 immunofluorescence differed from that seen in clones expressing the strongly transforming p53 alleles. p53his273 protein stained strongly in the nucleus with levels of cytoplasmic fluorescence indistinguishable from background (Figure 3b).

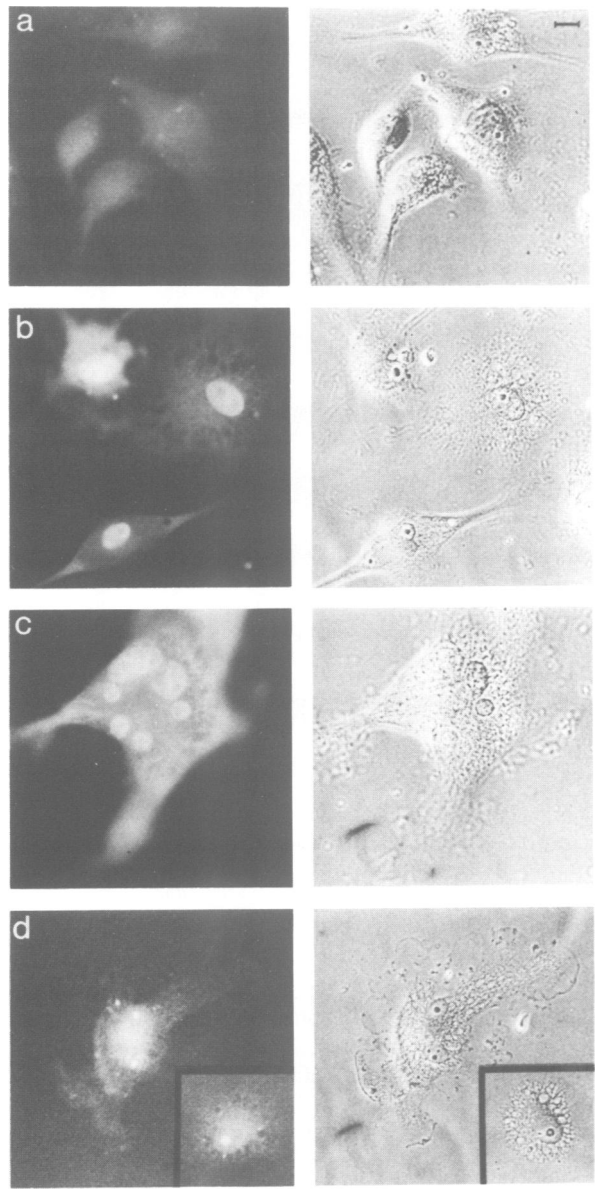

Fig. 2. Intracellular localization of mutant p53 protein in a REF clone transformed by CMVhup53ala143 and activated *ras*. Fluorescence micrographs of cells reacted with (**a**) control antibody IgG2a, and p53 antibodies (**b**) PAb240, (**c**) PAb421 and (**d**) PAb1620 are shown on the left. Photomicrographs on the right represent the same cells as on the left seen under phase contrast. Bar indicates 20 μm.

Effect of NLS defects on p53 protein localization

To verify that mutations in the major nuclear localization signal, NLSI, converting codons 319, 320 and 321 from lysine to alanine, produce p53 proteins with a cytoplasmic localization, *ras* transformed REF clones in which the non-transforming hup53NLSI allele was co-transfected were isolated. One of these was established in culture and p53 protein visualized by immunofluorescence. The hup53NLSI allele encoded a cytoplasmic, PAb1801-reactive protein (Figure 3e). Three E7 and *ras* transformed clones expressing the hup53Δ366 allele were also examined by immunofluorescence and showed exclusively nuclear p53 staining of low intensity with PAb1801 (Figure 3d). Thus the putative minor nuclear translocation signals at codons 369–375 and 379–384 (Shaulsky *et al.*, 1990b), that are

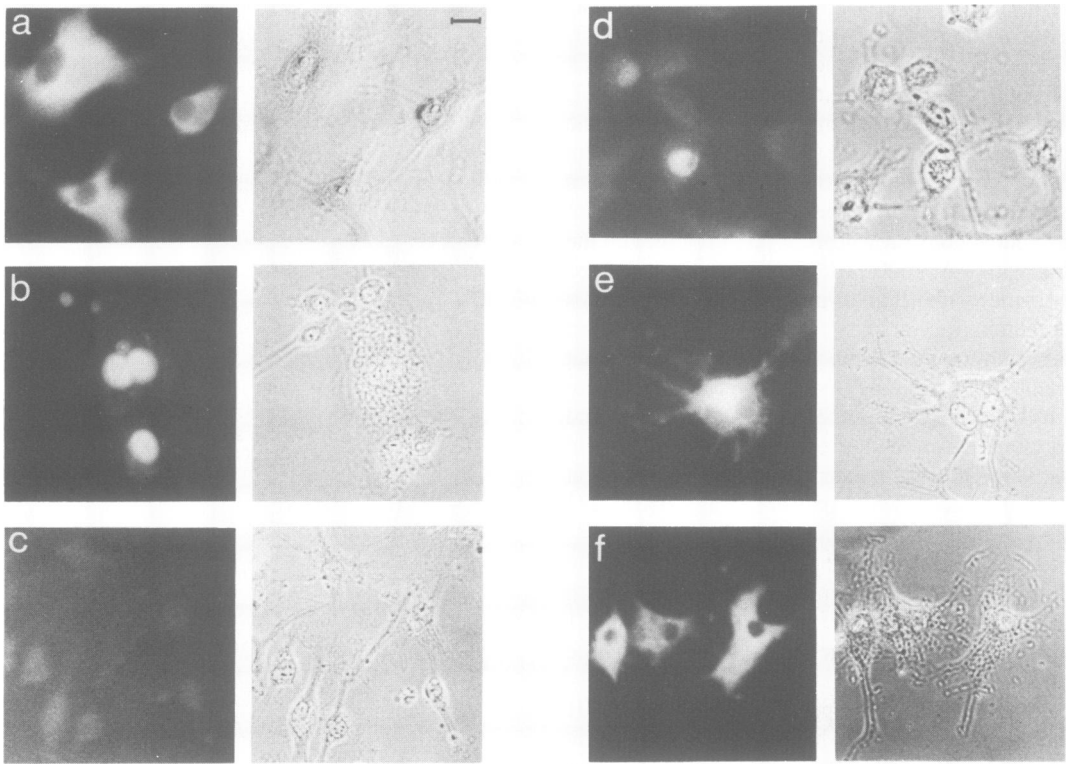

Fig. 3. Intracellular localization of mutant p53 protein in transformed REF cell lines. Immunofluorescence micrographs of cells reacted with p53 antibody PAb1801 (panels **a**, **b**, **d**, **e** and **f**) and control antibody IgG1 (panel **c**). Photomicrographs on the right show the same cells as on the left seen under phase contrast. (**a**) A REF clone transformed by *ras* and βhup53his273NLSI, showing intense cytoplasmic p53 staining; (**b**) a REF clone transformed by *ras* and βhup53his273; (**c**) the same cell line as in (b) reacted with control antibody IgG1; (**d**) a REF clone transformed by *ras*, HPV E7 and βhup53Δ366; (**e**) a REF clone transformed by *ras* and expressing the cytoplasmic protein product of the nuclear translocation mutant βhup53NLSI; (**f**) a REF clone transformed by *ras* and βhup53ala143NLSI. Bar indicates 20 μm.

deleted in the hup53Δ366 construct, do not appear to affect the localization of p53 protein.

The protein encoded by hup53his273 stained strongly in the nucleus in REF clones (Figure 3b). However, in all of five independent lines that were established from foci transformed by *ras* and the hup53his273NLSI allele, strong cytoplasmic p53 staining was seen (Figure 3a). The protein encoded by hup53ala143NLSI was also cytoplasmic in each of three independent transformed clones (Figure 3f), while the p53 protein was localized in both nucleus and cytoplasm of the hup53ala143 transformed clones (Figure 2).

Discussion

Mutation of the tumor suppressor gene p53 is the most commonly reported genetic change in a wide variety of human malignancies (Hollstein *et al.*, 1991; Caron de Fromentel and Soussi, 1992). In almost all cases, where p53 mutations have been identified *in vivo*, both alleles have been affected, either by point mutation with loss of the wild type homologue, or by the presence of compound heterozygous mutations (Nigro et al., 1989; Baker *et al.*, 1990a; Slingerland *et al.*, 1991). Thus, p53 mutations appear to act recessively in human tumors. Each of the point mutations in human p53 shown here and elsewhere (Hinds *et al.*, 1990; Slingerland and Benchimol, 1991) to confer transforming potential in REF assays arose in tumors having undergone bi-allelic change at the p53 locus. Thus, while point mutations at codons 135, 143, 175 and 275 confer dominant transforming ability in REF assays, and the mutant proteins may interfere with wild type p53 function, these mutations are unlikely to behave as complete dominant negatives *in vivo*. At some point in tumor progression, there is a selection for complete loss of wild type p53 expression. The ability of human mutant p53 alleles to override the effect of endogenous rat wild type p53 in REF assays results from the constitutive overexpression of multiply integrated mutant p53 alleles and may not entirely reflect pathologic processes involving mutant gene products during tumorigenesis and cancer progression.

The missense point mutations of p53 arising in human tumors give rise to p53 proteins that differ in their conformation, their transforming potential, and in their intracellular distribution in transformed REF clones. The conformational differences are detected by the patterns of reactivity with p53 antibodies. These observations support the notion that p53 mutations, arising *in vivo*, differ one from another in the degree to which they alter wild type p53 function. The proteins encoded by p53ala143 and p53his175 are PAb240 positive and PAb1620 negative; the p53tyr275-encoded protein did not react with either PAb240 or PAb1620; and in the transformed REF clone producing p53ser135, reactivity with both PAb240 and PAb1620 was noted. While mutant alleles p53ala143, p53his175, and p53tyr275 are strongly transforming, p53ser135 is intermediate in this effect and p53his273 transforms weakly or not at all.

The p53 mutation converting codon 273 arginine to

histidine is frequently detected in human tumors (Hollstein *et al.*, 1991). This mutant protein has a wild type epitope map, PAb240-negative and PAb1620-positive. The p53his273 allele is weakly transforming in REF assays, even when it is highly expressed from the strong human β-actin promoter and may, therefore, lack the ability to interfere with wild type p53 protein in a dominant negative manner. This is supported by the observation that when single copies of wild type human p53 and p53his273 alleles were coexpressed in SAOS-2, an osteosarcoma cell line lacking endogenous p53 protein expression, the wild type p53 phenotype of slowed growth, decreased saturation density, and reduced tumorigenicity was dominant (Chen *et al.*, 1990). The p53his273 allele, however, is not simply a negative allele. p53his273 protein appears to have acquired a novel function since its introduction into SAOS-2 cells, in the absence of wild type p53, promoted cell growth to a higher saturation density than that of parental cells (Chen *et al.*, 1990). The contribution, if any, of this putative novel function of p53his273 protein towards REF transformation is unknown. The transforming potential of different mutant p53 alleles in REF assays may be determined, not only by the degree to which the encoded proteins can inactivate wild type p53 protein function, but also by the acquisition of novel properties that promote cellular transformation (Wolf *et al.*, 1984).

Recent work suggests that intracellular partitioning of p53 protein between nucleus and cytoplasm may represent one of the cellular mechanisms controlling the duration and level of activity of p53 protein at growth regulatory sites in the nucleus (Shaulsky *et al.*, 1990a,b; Gannon and Lane, 1991). In the present studies, REF clones established from foci produced by *ras* and transforming mutant p53 alleles demonstrated abundant immunofluorescence staining of p53 protein with PAb421, PAb240 and PAb1801 in both the nucleus and cytoplasm. In none of the REF clones analysed was the p53 encoded by tumor derived point mutant alleles entirely cytoplasmic as was reported for lines derived from transformation with the murine temperature sensitive mutant Val135 (Michalovitz *et al.*, 1990; Martinez *et al.*, 1991). Expression of the weakly transforming p53his273 mutant allele was restricted to the nucleus.

The discrete nucleolar staining seen only with PAb1620 may represent binding to a conformationally distinct species of p53 that migrates to the nucleolus. It may also result from crossreactivity with another protein, although nucleolar staining with PAb1620 was not detected in non-transformed REFs. The functional significance of conformational changes in p53 protein and of nucleolar staining with PAb1620 bear further study.

In experiments designed to investigate the importance of nuclear translocation on the transforming activity of mutant p53 and on the transformation suppressing activity of wild type p53, we found that an intact NLSI site was not required for either activity. p53ala143NLSI cDNA containing mutation of the three lysine residues which constitute the core of NLSI, behaved similarly to p53ala143 in its ability to complement H-*ras* in REF transformation assays even though the encoded protein was localized to the cytoplasm. Moreover, the weakly transforming p53his273 allele became strongly transforming when the intact NLSI site was replaced with the mutated NLSI site. This type of synergism between mutations involving two regions of the p53 gene has not been reported previously. It is possible that the increased transforming potential of this double mutant was due to the ability of the encoded protein to retain the endogenous rat p53 protein in the cytoplasm. However, the same NLSI mutation did not accentuate the transforming activity of the p53ala143 allele. Alternatively, introduction of the NLSI mutation into the p53his273 allele may produce a conformational change in the encoded protein that renders it transformation competent and abolishes its negative growth regulatory activity as reflected by its inability to suppress transformation mediated by E7 and *ras*. A cDNA clone (hup53NLSIΔ366) with a mutated NLSI site in which NLSII and NLSIII were deleted retained the ability of wild type p53 to suppress transformation of REFs by E7 and *ras*.

These data differ from those previously reported by Shaulsky *et al.* (1991b) using mouse p53 cDNA clones where mutation of NLSI decreased the ability of normal p53 to suppress transformation mediated by E1A plus H-*ras*, and mutation of both NLSI and NLSII completely abolished suppressor activity. Shaulsky *et al.* (1990b, 1991b) have also reported that a transformation competent murine p53 allele that is truncated at the 3′ end and, hence, is missing NLSII and NLSIII is rendered transformation incompetent by a single mutation that disrupts NLSI. Although we cannot readily explain these differences, it is pertinent to note that mouse and human p53 proteins are being compared and that these may have different structural properties. Moreover, different assays are used in both studies and the mutations that target the NLSI site are not the same.

Loss of the putative cdc2 phosphorylation site at serine codon 315 had no effect on transformation by the Ala143 mutant p53 allele or on suppression of E7 and *ras* transformation by wild type p53. Similarly the loss of the casein kinase II phosphorylation site at serine 392 failed to affect transformation by hup53ala143. It is conceivable that, as with the retinoblastoma gene product, cell cycle dependent phosphorylation of p53 protein may control its association with other cellular proteins and its growth regulatory function(s) (reviewed in Cooper and Whyte, 1989). However, the modulation of p53 function by phosphorylation of serine residues 315 and 392 may be irrelevant to the phenotypes displayed by both transforming mutant p53 alleles and suppressing forms of wild type p53 when these alleles are constitutively overexpressed in REF assays.

We have demonstrated that changes in the hydrophobicity of the C-terminal α-helical motif of p53 abolishes the transforming potential of human tumor derived mutant p53 alleles. Since these changes also prevent p53 homodimerization, one possibility is that dimerization is required for the transforming activity of mutant p53. Mutant alleles encoding p53 polypeptides that can form dimers but not tetramers, however, retain the ability to cooperate with *ras* in REF transformation.

Monomeric variants of wild type p53 suppress REF transformation by E7 and *ras* as effectively as native p53. The monomeric product of hup341K344E348E355K can also inhibit DNA replication at the SV40 origin (Sturzbecher *et al.*, 1992). It is of considerable interest, therefore, to determine if the oligomerization-defective variants of p53 retain or lose activity in the various assays that have been described for p53 (reviewed in Levine *et al.*, 1991). Studies addressing the ability of native, monomeric and dimeric variants of p53 to bind DNA, to regulate DNA replication,

to modulate the transcriptional activity of various genes, and to suppress the growth of transformed cells may identify those biochemical and functional properties of p53 that are most relevant to its transformation and tumor suppressor activity.

We observed that loss of p53 homodimerization correlates with loss of transforming ability. The transforming function of mutant p53 alleles may be mediated in part by p53 homodimers that have acquired novel transactivation function due to mutation-related conformational changes. The observation that alleles encoding NLSI-defective mutant p53 retain transforming potential, and that NLSI-defective wild type p53 variants can suppress transformation is consistent with p53 protein acting indirectly with other proteins to modify gene expression. Constitutively expressed wild type p53 proteins may bind and sequester strong coactivators of cell cycle regulatory genes, thereby inhibiting their effect and causing cell cycle arrest. Alternatively, constitutive expression of wild type p53 would accentuate the repression of genes whose expression is normally down-regulated by p53 during cell growth. Both of these indirect effects on gene transcription could be mediated effectively by nuclear translocation-defective p53 molecules interacting with cofactors in the cytoplasm.

Materials and methods

Recombinant vectors

The pEJ6.6 vector is a pBR322 derivative containing an activated *ras* gene from the human bladder carcinoma cell line EJ (Shih and Weinberg, 1982). pJ4Ω16-E7 is a derivative of pJ4Ω containing an intact E7 gene and part of the E1 gene of the human papillomavirus type 16, under the control of the Moloney murine leukemia virus LTR (Storey *et al.*, 1988). pECH53 contains the wild type human p53 cDNA (Matlashewski *et al.*, 1987) in the SV40 derived expression vector pECE (Ellis *et al.*, 1986). pECH53ser135 is identical to pED-1 (Johnson *et al.*, 1991) and bears the mutant human p53 cDNA derived from primary blasts of a patient with acute myelogenous leukemia in which a point mutation converts cysteine at codon 135 to serine (Slingerland *et al.*, 1991). A series of human p53 cDNA expression vectors was constructed in the pBC12CMV vector described by Cullen (1986). The wild type human cDNA in the CMV-promoted vector was reconstructed from clone pR4-2 (Harlow *et al.*, 1985) by PCR directed mutagenesis converting histidine codon 273 to the wild type arginine. Subsequently, oligomerization-defective mutants hup341K344E348E355K and hup365A372L379A386L were created by site-directed mutagenesis of the wild type human p53 cDNA (Sturzbecher *et al.*, 1992). Mutants of serine codon 315 and serine 392 were created in a similar manner to produce hup53ala315 and hup53asp315 and hup53ala392. Hup53ala143 and hup53tyr275 were cloned from PCR amplified cDNA derived from two different human colon carcinoma samples. In both tumors, the p53 gene had undergone loss of heterozygosity and expressed only mutant p53. Hup53his175 was created by site directed mutagenesis and is identical to the human mutant p53 allele detected in a number of human tumors (Baker *et al.*, 1989).

The p53 cDNA cassettes were excised from either the pECH53 (SV40)-promoted vector or the CMV-promoted constructs with *Hin*dIII and *Bam*HI and religated into pHβApr-1-neo, a vector bearing the human β-actin promoter (Gunning *et al.*, 1987). βhup53wt was derived from pECH53 in this manner. All other p53 constructs bearing the β-actin promoter are designated βhup53. βhup53his273 was derived from pR4-2 (Harlow *et al.*, 1985).

Tumor derived, mutant alleles βhup53ala143 and pECH53ser135 were rendered oligomerization defective by excision of the cDNA encoding codons 224−stop TGA with *Bsu*36I and *Bam*HI and replacement of this fragment with the *Bsu*36I−*Bam*HI cassettes from either hup341K344E348E355K or hup365A372L379A386L.

Tumor derived point mutants lacking the phosphorylation site at serine codon 315 were created by swapping the *Bsu*36I−*Bam*HI fragment from the hup53ala315 or hup53asp315 into βhup53ala143. These vectors are designated βhup53ala143ala315 and βhup53ala143asp315. The same method was used to construct CMVhup53ala143ala392.

The p53 protein encoded by pR4-2 was rendered defective for nuclear translocation by the conversion of the three lysine residues at codons 319−321, to alanine (Addison *et al.*, 1990). This NLSI-defective p53 cDNA was transferred into the β-actin vector to produce βhup53his273NLSI. The region containing the NLSI mutations was excised from βhup53his273NLSI with *Bsu*36I and *Bam*HI. This was used to replace the corresponding region of the hup53ala143 cDNA. A wild type p53 derivative bearing the NLSI mutations Ala319, Ala320 and Ala321 was constructed using the two 5′ cDNA fragments generated by *Nfs*I restriction of the hup53wt cDNA and the 3′-most fragment of the *Nfs*I restricted hup53his273NLSI cDNA insert, all ligated together into the pHβApr-1-neo vector. This was designated βhup53NLSI.

The nuclear translocation domains NLSII and NLSIII (Shaulsky *et al.*, 1990b), located between codons 369 and 375, and codons 379−385, respectively, were removed by a truncation of the p53 cDNA inserts at codon 366. Truncated p53 cDNA vectors βhup53Δ366, and βhup53NLSIΔ366 were derived from βhup53wt, and βhup53NLSI, using the 5′sense PCR primer JS-6 (exon 7, CCCATCCTCACCATCATCACACTGGAAG) and the 3′antisense primer, Δ366 (exon 10, GTCAGGATCCTCAGGAGTG-AGCCCTGCTCCC). The Δ366 primer was designed to contain two stop codons followed by a *Bam*HI restriction site to facilitate religation of PCR amplified fragments back into the β-actin driven parent vector.

Transformation assays

Rat embryo fibroblasts (REFs) were prepared from 14 day old Fisher rat embryos as described (Rovinski and Benchimol, 1988). 3×10^5 tertiary passage REFs were plated on 60 mm diameter dishes in 5 ml of α minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS) and transfected on the following day. The calcium phosphate precipitate (Graham and Van der Eb, 1973) contained 10 μg of NIH3T3 carrier DNA and 2 μg of pEJ6.6, with or without 2 μg of SV40- or CMV-promoted p53 vector or 5 μg of β-actin-promoted p53 vector. In assays of E7 and *ras* suppression, 1.25 μg of pJ4Ω16-E7 was used in the transfection cocktail. Sixteen hours post-transfection, cells were washed with phosphate-buffered saline and fresh medium applied. Approximately 36 h post-transfection, cells were trypsinized and replated on to three 60 mm dishes. Foci were scored 10−14 days post-transfection. To assess the transient expression of transfected vectors, the transfection protocol was modified as described (Slingerland and Benchimol, 1991).

Antibodies

PAb421 is a pan-species specific anti-p53 monoclonal antibody recognizing the C-terminal p53 epitope between codons 373 and 381 (Harlow *et al.*, 1981). PAb240 binds an epitope displayed on mutant p53 polypeptides from several species (Gannon *et al.*, 1990). PAb1620 recognizes wild type rat and human p53 and a subset of mutant p53 proteins (Milner and Cook, 1987) and PAb1801 recognizes an epitope in exon 4 unique to human p53 (Banks *et al.*, 1986).

Metabolic labelling and immunoprecipitation

Cells were metabolically labelled with [^{35}S]methionine and p53 protein was immunoprecipitated from cell lysates as previously described (Slingerland *et al.*, 1991). The lysis buffer contained 25 mM Tris pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin.

Immunofluorescence studies

Transformed REF clones, normal tertiary passage REFs and a p53-non-producer, human ovarian adenocarcinoma cell line SKOV-3 (Johnson *et al.*, 1991) were grown on sterile glass coverslips in 35 mm culture dishes to semi-confluence. Cells were then washed twice with PBS containing 1% bovine serum albumin (BSA), fixed on ice with −20°C methanol for 30 min and then stained with 50 μl of anti-p53 antibody hybridoma supernatant (~1 μg of anti-p53 antibody). IgG2a or IgG1 murine polyclonal antibody (1 μg) was used as negative primary antibody control. Following two washes with PBS containing 1% BSA, cells were incubated with a 1:40 diluted solution of fluorescein-conjugated goat anti-mouse antibody (FITC) for 1 h at room temperature, then rinsed twice, blotted dry and the glass coverslips mounted on glass slides using an 80% glycerol solution. p53 fluorescence was visualized with a Zeis Axioscope microscope and cells were photographed.

Acknowledgements

We thank Penny Johnson for help with the immunofluorescence assay and Stephen Chung for excellent technical assistance. This work was supported by the National Cancer Institute of Canada and the Medical Research Council

of Canada. J.M.S. is supported by a Fellowship from the National Cancer Institute of Canada.

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Received on August 17, 1992; revised on November 19, 1992