

Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form

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Point mutations in the p53 gene are the most frequently identified genetic change in human cancer. They convert murine p53 from a tumour suppressor gene into a dominant transforming oncogene able to immortalize primary cells and bring about full transformation in combination with an activated *ras* gene. In both the human and murine systems the mutations lie in regions of p53 conserved from man to *Xenopus*. We have developed a monoclonal antibody to p53 designated PAb240 which does not immunoprecipitate wild type p53. A series of different p53 mutants all react more strongly with PAb240 than with PAb246. The PAb240 reactive form of p53 cannot bind to SV40 large T antigen but does bind to HSP70. In contrast, the PAb246 form binds to T antigen but not to HSP70. PAb240 recognizes all forms of p53 when they are denatured. It reacts with all mammalian p53 and chicken p53 in immunoblots. We propose that immunoprecipitation of p53 by PAb240 is diagnostic of mutation in both murine and human systems and suggest that the different point mutations which convert p53 from a recessive to a dominant oncogene exert a common conformational effect on the protein. This conformational change abolishes T antigen binding and promotes self-oligomerization. These results are consistent with a dominant negative model where mutant p53 protein binds to and neutralizes the activity of p53 in the wild type conformation.

Key words: antibody/conformation/mutation/p53/oncogene

Introduction

The p53 protein was first identified through its interaction with SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). It has been found in all vertebrate species examined (Soussi *et al.*, 1987). Soon after murine p53 was cloned it was shown to be a dominant transforming oncogene able to cooperate with activated *ras* in the transformation of early passage rodent cells (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). Subsequently, frequent inactivation of p53 was found in murine Friend virus induced erythroleukaemia cells, which led Ben-Chimol and his colleagues to propose that p53 may be a tumour suppressor gene (Ben-David *et al.*, 1988). In support of this, it has recently been shown in transfection experiments that the p53 clones which transform are all mutant (Hinds *et al.*,

1989) and that wild type p53 suppresses transformation (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989).

Interest in p53 was greatly stimulated by the finding that the protein is abnormally expressed in human colon (Van Den Berg *et al.*, 1989) and breast cancers (Cattoretti *et al.*, 1988). The gene for human p53 is on the short arm of chromosome 17, a frequent site of allele loss in common human cancers (Yokota *et al.*, 1987; Mackay *et al.*, 1988; Baker *et al.*, 1989; James *et al.*, 1989). Direct sequencing of p53 from tumours and tumour cell lines is beginning to suggest that p53 mutation is a very common event in human cancer (Nigro *et al.*, 1989; Takahashi *et al.*, 1989). The mutations which are detected in human and murine tumours and cell lines are mostly missense point mutations in highly conserved amino acids (Finlay *et al.*, 1988; Nigro *et al.*, 1989; Takahashi *et al.*, 1989).

Neither the biochemical function of p53 nor the precise effects of mutation are understood. One promising avenue resulted from the discovery that normal p53 can block the binding of DNA polymerase α to SV40 large T antigen (Gannon and Lane, 1987) and inhibit SV40 DNA replication (Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988b; Wang *et al.*, 1989), in contrast with mutant p53 which is less effective in both assays. Using a new monoclonal antibody we have found that mutations which activate p53 as an oncogene and inactivate it as a tumour suppressor exert a common conformational effect on the protein. Mutant murine p53 genes lead to the synthesis of a form of p53 which displays the PAb240 epitope, fails to bind to SV40 large T antigen and associates with HSP70. We show using two site immunoassays that the mutant form is able to bind to normal p53. This may explain how transfected mutant genes can inactivate the tumour suppressor activity of endogenous wild type genes, and provides the basis for a single hit model for inactivation of wild type p53 in common cancers.

Results

PAb 240, a new monoclonal antibody to p53, recognizes p53 in a wide range of species by cell staining and immunoblotting

Most monoclonal antibodies to p53 are species specific (Thomas *et al.*, 1983; Banks *et al.*, 1986; Yewdell *et al.*, 1986). Those which are not (PAb421, PAb122 and PAb410) bind to a small region near the C terminus of the protein (Harlow *et al.*, 1981; Wade-Evans and Jenkins, 1985). Since the amino acid sequence of p53 has been well conserved in evolution it is likely that antibodies to conserved epitopes will bind to functionally important sites. To prepare antibodies to conserved epitopes we generated monoclonal hybridomas from a mouse immunized with a β -galactosidase-p53 fusion protein and screened the secreted antibodies by cell staining on mouse and human cell lines (Figure 1). We rejected antibodies which reacted in an

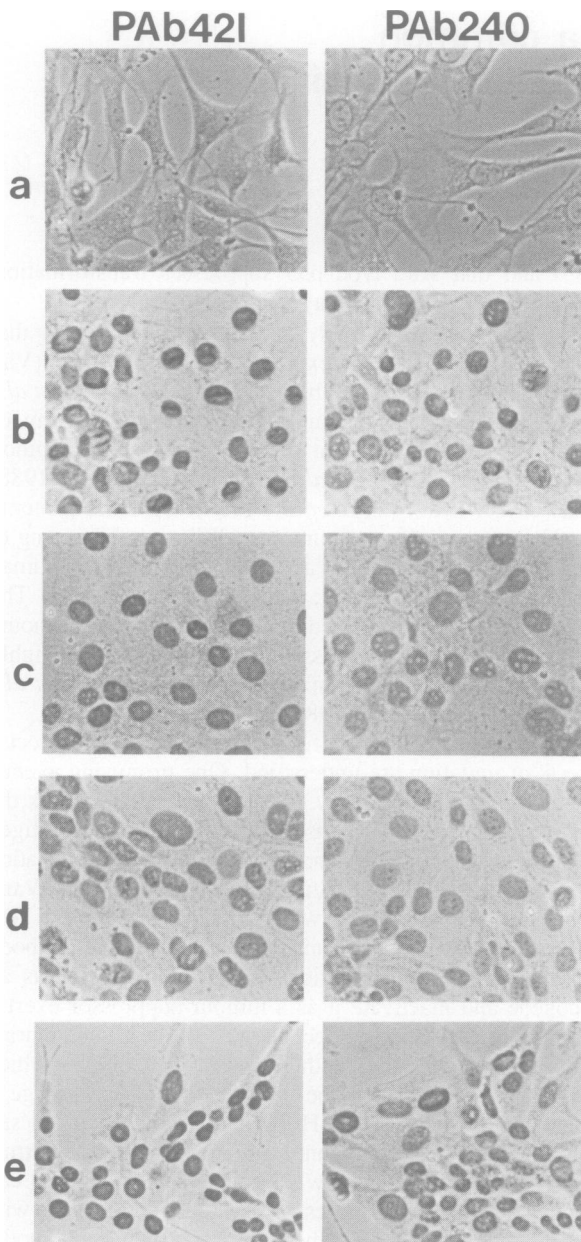


Fig. 1. Immunoperoxidase staining of various human and murine cell lines with PAb421 and PAb240. 3T3 cells, which contain very little p53, were used in the initial hybridoma screen to eliminate antibodies which reacted with irrelevant antigens. a, 3T3; b, T3T3; c, Cos; d, SVK14; e, SVA31-E7 cells.

ELISA with a synthetic peptide corresponding to the PAb421 epitope. Three hybridomas (PAb240, PAb241 and PAb243) were isolated and cloned. PAb240 was selected for further study because it reacts with human, mouse, rat, hamster, bovine and chicken p53 in immunoblotting experiments (Greaves, 1988).

PAb240 defines a novel, highly conserved, denaturation resistant epitope on p53

PAb240 binds to bacterially expressed fragments of murine p53 encoding amino acids 156–389 or 14–335 but fails to react with a stable fragment encoding amino acids 214–389 (Greaves, 1988). Thus, the epitope probably lies

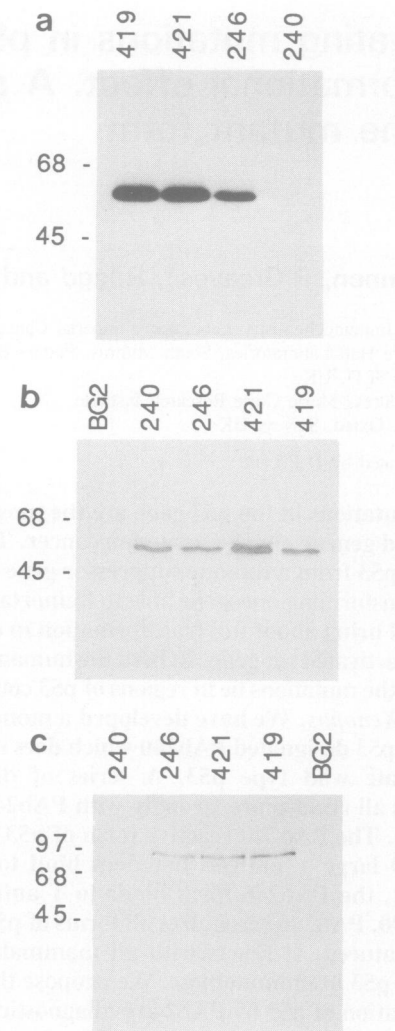


Fig. 2. Immunoblot of immunoprecipitates. Antibodies listed above the lanes were used for immunoprecipitation. a, SVA31-E7 cell immunoprecipitates probed with rabbit anti-p53 serum; b, SV40 transformed C3H T101/2 cell immunoprecipitates probed with rabbit anti-p53 serum; c, immunoprecipitates as in (b) probed with rabbit anti-T antigen serum. a, ¹²⁵I-labelled primary antibody; b and c, indirect peroxidase technique. M_r is shown in kd.

between amino acids 156 and 214 on murine p53. Neither a synthetic peptide encoding amino acids 161–179 (including conserved region III, Soussi *et al.*, 1987) nor any of the existing anti-p53 antibodies tested (200.47; RA32C2; PAb242, 246, 248 and 607) inhibit binding of PAb240 to murine p53 in two site ELISA assays using PAb421 coated microtitre plates. PAb240 thus defines a new epitope on p53 both in terms of its location in the linear sequence and in steric competition assays.

PAb240 cannot bind to murine p53 when it is bound to T antigen

Two SV40 transformed mouse cell lines were studied by immunoblotting of PAb240 immunoprecipitates (Figure 2). Bound proteins were analysed by immunoblotting with anti-T and anti-p53 monoclonal antibodies. PAb240 failed to precipitate p53 from SVA31 E7 cells (Figure 2a), although control anti-p53 and anti-T antibodies readily demonstrated the presence of p53 and the T–p53 complex in these cells.

Since PAb240 stains the nuclei and detects p53 after immunoblotting of whole cell extract, the PAb240 epitope must be in a cryptic form on the p53 protein in these cells. The simplest explanation is that PAb240 only recognizes denatured p53. Examination of a second cell line (SV40 transformed C3H T101/2 cells) showed that this was not the case. Clear immunoprecipitation of p53 with PAb240 was seen when extracts of this cell line were analysed. PAb240 and PAb246 each immunoprecipitated about half as much p53 as PAb421 (Figure 2b). Remarkably, no T antigen was found in the PAb240 immunoprecipitate, but roughly equal amounts of T antigen were detected in the PAb421, PAb246 and anti-T immunoprecipitates (Figure 2c). This suggests that SV40 transformed C3H T101/2 cells contain two populations of p53, a PAb246 reactive form that is entirely complexed to T antigen and a PAb240 reactive form that is completely free of T antigen. As described below, *in vitro* T-p53 association assays support this idea since PAb246 recognizes *in vitro* assembled complex, but PAb240 does not.

PAb240 immunoprecipitates mutant p53

p53 mutation frequently results in loss of the PAb246 epitope (Finlay *et al.*, 1988; Sturzbecher *et al.*, 1988a). Since the experiments above imply that the PAb240 epitope may be present on p53 molecules lacking the PAb246 epitope we decided to test whether PAb240 is specific for mutant p53. We performed immunoprecipitations on a range of cell lines containing well characterized wild type and mutant p53 molecules (see Materials and methods for details). T3T3 and C6 cells were examined by immunoprecipitation from [³⁵S]methionine labelled cell extracts (Figure 3). T3T3 cells contain large amounts of p53 which is competent to bind T antigen and is efficiently immunoprecipitated by PAb246 (Yewdell *et al.*, 1986). All previously available anti-p53 antibodies readily immunoprecipitate p53 from T3T3 cells, whereas PAb240 clearly does not (Figure 3a), despite being able to detect p53 by immunoblotting (not shown). No trace of HSP70 is seen in the immune precipitate. C6 cells, rat embryo fibroblasts transformed by cotransfection of a mutant murine p53 gene and an activated *ras* gene, contain separate PAb246 binding and HSP70 binding populations of p53 molecules (Finlay *et al.*, 1988). Both PAb246 and PAb240 are able to immunoprecipitate p53 from C6 cells, although neither antibody precipitates as much p53 as the other anti-p53 antibodies shown (PAb421, 242 and 248). PAb240 precipitates p53 bound to HSP70, whereas PAb246 does not (Figure 3b). Immunoblotting of immunoprecipitates (Figure 4) allowed us to divide cell lines into three groups: those which contain only PAb246 positive, T antigen binding p53 (F9 and T3T3 cells); those which contain only PAb240 positive, HSP70 binding p53 (RKT101-Y and MethA cells); and those which contain a mixture of both (C6 and p53A1 cells). The presence of HSP70 was confirmed by reprobating the blots with polyclonal mouse anti-HSP70 ascites (not shown). Since the sequence of the p53 gene in F9 cells is known to be wild type (Finlay *et al.*, 1988) it appears that the PAb240 epitope is only displayed on p53 proteins which are mutant. In contrast, the PAb246 epitope is displayed on wild type p53 and on a fraction of the p53 present in cells expressing mutant p53. For clarity we refer to the PAb240 positive form as the mutant conformation and the PAb246 form as the wild type or pseudo-wild type conformation.

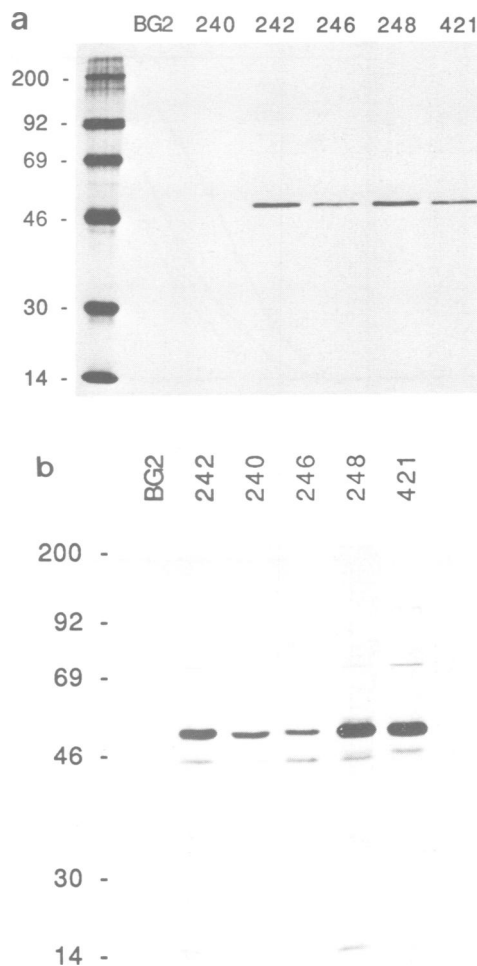


Fig. 3. Immunoprecipitation from [³⁵S]methionine labelled cell extracts. Antibodies listed above the lanes were used for immunoprecipitation. a, T3T3 cells; b, C6 cells.

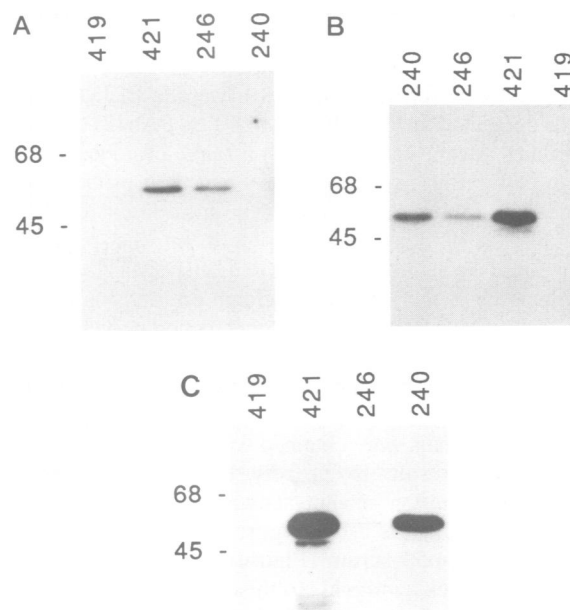


Fig. 4. Immunoblot of immunoprecipitates. Blots were probed with [¹²⁵I]-labelled rabbit anti-p53 serum. a, F9 cells; b, p53A1 cells; c, RKT101-Y cells.

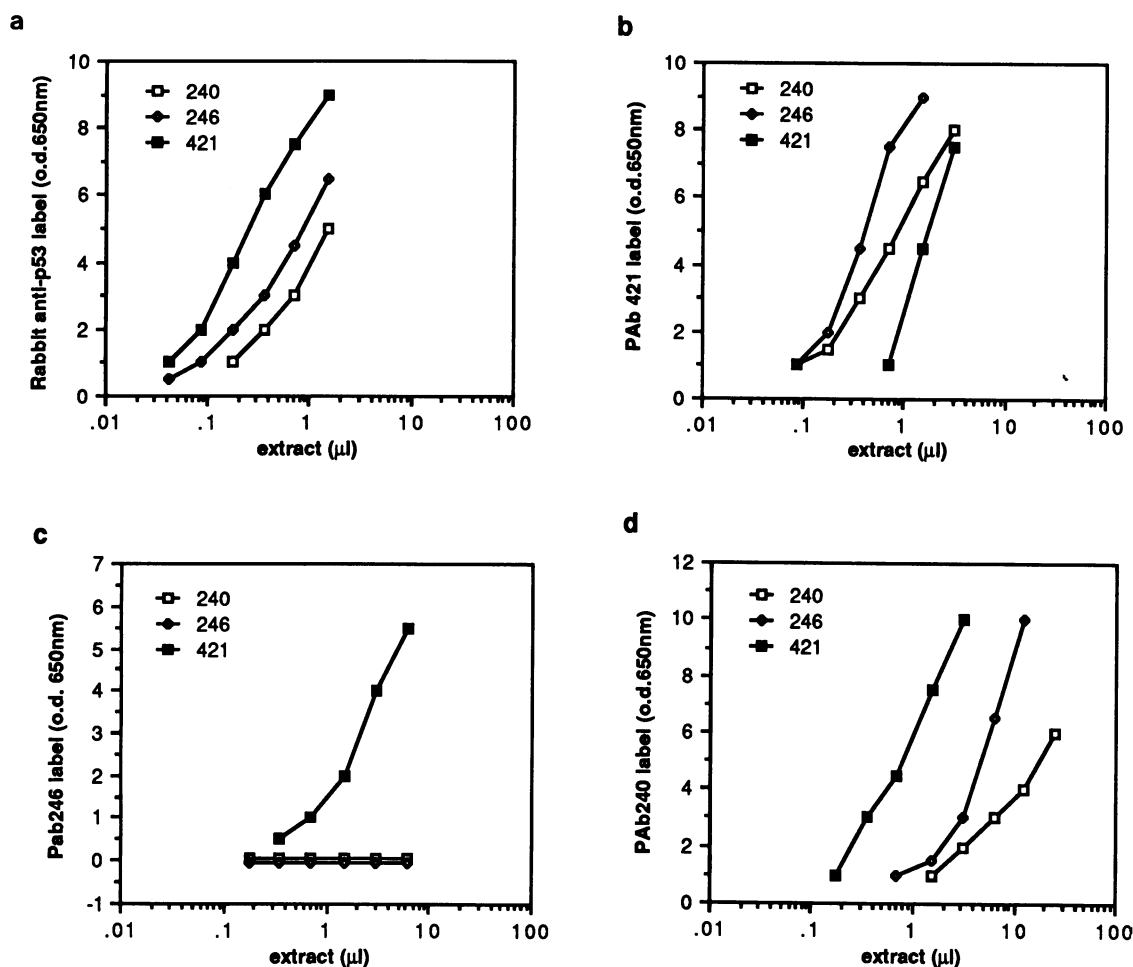


Fig. 5. Two site immunoassays using C6 cell extract. Each graph shows assays performed with PAb240, 246 and 421 plates as indicated and probed with biotin labelled antibody. a, rabbit anti-p53 serum; b, PAb421; c, PAb246; d, PAb240. See text for details.

Mutant p53 can form heterodimers with wild type p53

To explore further the nature of the two forms of p53 in C6 cells we performed a series of two site ELISA assays. Plates coated with PAb246, PAb240 or PAb421 ('capture antibodies') were incubated with a range of concentrations of C6 cell extract, washed briefly and then probed with a range of biotinylated anti-p53 antibodies ('labelled antibodies') to detect p53 captured on the plate. p53 homodimers or higher oligomers can be specifically detected in this assay if the same monoclonal antibody is used for both capture and detection. When a labelled polyclonal anti-p53 antibody was used for detection it was clear that all three capture antibodies worked well with C6 cell extract (Figure 5a). The strongest signal was obtained with the PAb421 plate while lower signals were obtained with PAb246 and PAb240 plates. This result is broadly in keeping with the immunoprecipitation results shown in Figure 3b. When labelled PAb421 was used (Figure 5a) instead of labelled polyclonal anti-p53 serum (Figure 5a) the PAb421 plates gave a much weaker signal. In this situation the polyclonal detects all p53 whereas the PAb421 only detects molecules displaying two copies of the PAb421 epitope (one for capture and one for detection), i.e. the polyclonal detects monomers and oligomers but the PAb421 only detects oligomers. Therefore, comparison of Figure 5a and b gives an

approximate estimate of the amount of p53 in the oligomeric form. About 5–10% of the p53 is oligomeric on this basis.

PAb246 was very effective as a capture antibody in Figure 5a, b and d but only worked as a label on PAb421 plates (Figure 5c). This lack of reciprocity with PAb246 has been noted before (Yewdell *et al.*, 1986). It was striking that no binding of labelled PAb246 could be detected on PAb246 plates even at very high concentrations of cell extract. This implies that no oligomers display multiple copies of the PAb246 epitope. Finally, labelled PAb240 (Figure 5d) gave a strong signal with PAb421 plates and weak but still significant signals with PAb246 and PAb240 plates. The result with the PAb240 label and the PAb246 plate is especially important since it implies that either the two epitopes can be simultaneously present on a fraction of p53 molecules, which is unlikely in view of the immunoprecipitation results, or that p53 in the mutant conformation can bind to p53 in the pseudo-wild type conformation.

PAb240 cannot bind to p53 that is complexed to T antigen *in vitro*

We have used plate assays to look at the ability of different antibodies to recognize the T–p53 complex formed *in vitro* on mixing a fixed amount of SV40 T antigen with a range of dilutions of C6 cell extract (Figure 6). The complex is readily detected on PAb246 and PAb421 plates with a

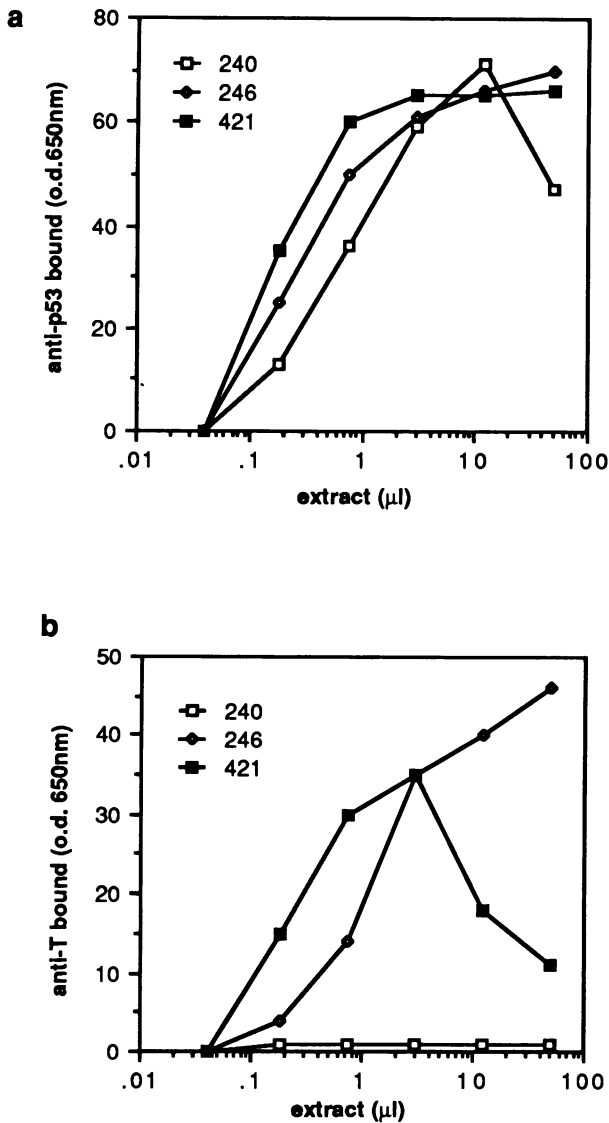


Fig. 6. Two site immunoassays to study T-p53 complexes formed *in vitro* in C6 cell extract. ELISA plates as in Figure 5 probed with biotin labelled antibody. a, rabbit anti-p53 serum; b, anti-T antibody (PAb419).

labelled anti-T antibody (Figure 6b) but undetectable on PAb240 plates, despite clear evidence using a labelled anti-p53 antibody that p53 itself is present on PAb240 plates (Figure 6a). Thus only p53 molecules in the pseudo-wild type conformation can bind T. The complete absence of a T antigen signal from the PAb240 plate suggests that the oligomers which display both the PAb246 and PAb240 epitope (Figure 5d) cannot bind to T. This is in agreement with the immunoprecipitation results from the SV40 transformed C3H T101/2 cells (Figure 2). At high concentrations of cell extract less T antigen is captured by PAb421 plates than at intermediate concentrations of cell extract. This is in contrast with the results on PAb246 plates where the amount of T antigen increases throughout the range of concentrations tested.

Immunohistochemical observations on mutant and wild type p53

It is clear from Figure 1 that PAb240 is able to stain the nuclei of cells from which it is unable to immunoprecipitate

p53. This presumably results from denaturation which occurs on fixation with acetone/methanol. Nevertheless, the ability of PAb240 and PAb246 to distinguish different populations of p53 is not lost altogether. In C6 and p53A1 cells PAb240 stains both the nucleus and the cytoplasm, whereas PAb246 only stains the nuclei strongly (compare Figure 7a and b). Nuclear staining was particularly weak in RKT101-Y cells, which contain very little p53 in the wild type conformation. This suggests that p53 in the wild type conformation is preferentially located in the nucleus and p53 in the mutant conformation is preferentially located in the cytoplasm. We have noticed additional variations in the staining of C6 and p53A1 cells. Some cells fail to stain in the nucleus with any of the anti-p53 antibodies although the same cells stain strongly in the cytoplasm. Hinds *et al.* (1987) have presented immunoprecipitation data suggesting that the ratio of PAb246 positive to HSP70 positive p53 in cells transfected with the C6 p53 plasmid (pLTRp53cG) is quite variable. The variation in C6 nuclear staining may reflect differences in this ratio. To investigate the expression of wild type p53 we stained F9 teratocarcinoma cells with five different anti-p53 antibodies (PAb240, 242, 246, 248, and 421). In all cases ~30% of the cells showed nuclear staining (Figure 7d), possibly reflecting cell cycle variation in p53 level. Even among the positive cells the strength of staining was quite variable.

Discussion

A model for the effect of mutation on p53

The principal conclusion from the data presented here is that different activating mutations exert a common conformational effect which results in expression of the PAb240 epitope on mutant p53 molecules. These molecules can adopt either a pseudo-wild type (PAb246 positive) or an overtly mutant (PAb240 positive) form, with the proportion of molecules in each state dependent on the precise mutation. Levine's group found that PAb246 positive molecules had a much shorter half-life than HSP70 bound (i.e. PAb240 positive) molecules (Finlay *et al.*, 1988). This implies that there is not free exchange between the two states. We do not know what prevents this from occurring, although it is possible that incorrect folding immediately after synthesis produces an abnormal structure so stable that HSP70 cannot correct it. Alternatively, overtly mutant molecules may undergo abnormal post-translational modification. We have preliminary data suggesting that, at least in bacterially expressed p53, inappropriate disulphide bond formation prevents expression of the PAb246 epitope (Midgely and Lane, in preparation). In unsynchronized cultures PAb240 positive molecules show apparently normal levels of phosphorylation (unpublished data). It is plausible that p53 normally switches between functional states during the cell cycle, for example, in levels of phosphorylation, but there is no reason to suppose that these are the same as the two states we describe here.

Wang *et al.* (1989) found that *in vitro* SV40 DNA replication was inhibited by low concentrations of p53 purified from C6 cells but not by larger amounts. There is a striking correlation between their result and our data (Figure 6b) which suggests that there is less wild type p53 available at high than at intermediate concentrations of C6 cell extract. T antigen is apparently not limiting in our assay

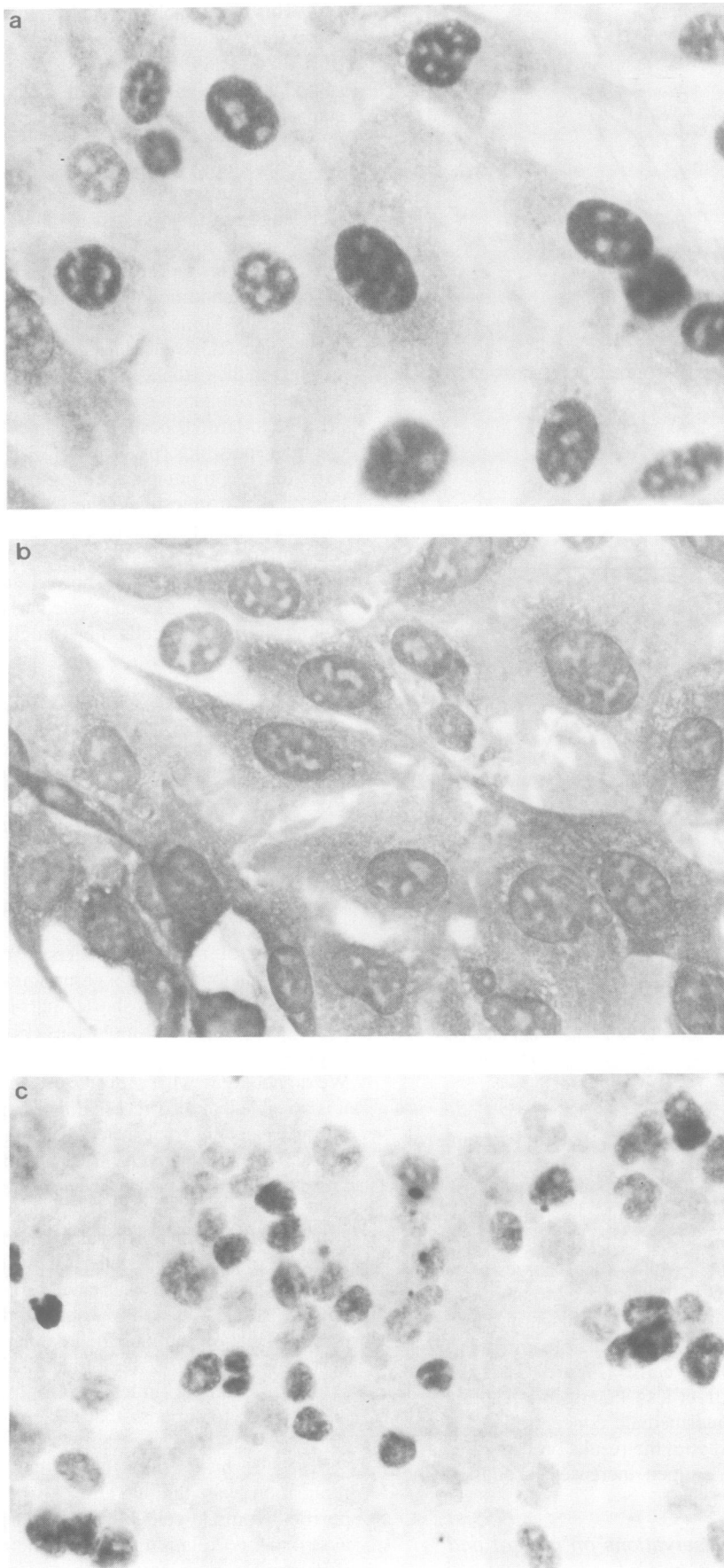


Fig. 7. Anti-p53 cell staining showing variation in the distribution and intensity of staining of p53A1 cells (a and b) and F9 cells (c). a, PAb246; b and c, PAb240.

because there is no drop off in signal with PAb246 plates. Our finding that hetero-oligomers exist between pseudo-wild type and overtly mutant p53 in these cells (Figure 5d) may provide an explanation for this result. At low concentrations we expect p53 to be mainly monomeric, in other words free wild type p53 is available to inhibit T antigen. At higher p53 concentrations inactive heterodimers form which chelate wild type p53 and relieve the block to replication. Similar behaviour may underlie the *in vivo* transition from tumour suppression to transformation which accompanies p53 mutation. This model predicts that mutations which cause more of the p53 protein to adopt the overtly mutant conformation should be more potent oncogenes and this does indeed appear to be the case (Finlay *et al.*, 1988). The model is also consistent with the finding that p53 mutants which are stable but in the pseudo-wild type state are not transforming (Rovinski and Benchimol, 1988). According to this model SV40 T antigen and adenovirus E1b behave as perfect p53 mutants because they bind stably to both wild type and pseudo-wild type p53 molecules.

Materials and methods

Cell lines

T3T3 cells, a spontaneously transformed 3T3 cell line, were isolated in our laboratory. C6 cells and p53A1 cells are rat embryo fibroblasts transformed by the pLTRp53cG plasmid and activated *ras*. C6 cells were provided by M.Oren. RKT101-Y cells are rat embryo fibroblasts transformed by the MSVKH215cG plasmid and activated *ras*. RKT101-Y and p53A1 cells were provided by A.Levine. SVA31-E7 cells, an SV40 transformed mouse fibroblast cell line, were originally provided by Y.Ito. SV40 transformed C3H T101/2 cells were provided by J.Pipas. MethA cells, a methylcholanthrene transformed cell line, were provided by L.Crawford. SVK14 cells, an SV40 transformed human keratinocyte cell line, were provided by B.Lane. F9 teratocarcinoma cells were provided by P.Rigby. Cos cells were provided by Y.Gluzman. Mutations identified in these cell lines are described in Finlay *et al.* (1988).

Antibodies

Polyclonal anti-T and anti-p53 antigen sera were prepared by immunizing rabbits with pure T antigen (Simanis and Lane, 1985) and bacterially expressed full length p53 respectively. Polyclonal anti-HSP70 ascites was prepared by the method of Lacy and Voss (1986) in mice immunized with HSP70 purified as described in Welch and Feramisco (1985). Monoclonal antibodies PAb242, 246 and 248 were developed in this laboratory (Yewdell *et al.*, 1986). PAb421 and 419 (Harlow *et al.*, 1981) were provided by E.Harlow.

Hybridoma fusion

A p53- β -galactosidase fusion protein containing p53 sequence from amino acids 14–389 (derived from the pSV53C p53 cDNA clone) was gel purified and injected into BALB/c mice. Spleen cells from a hyperimmune animal were fused to SP2 cells as described in Harlow and Lane (1988). PAb240 is an IgG1 monoclonal antibody with kappa light chains.

Immunoprecipitation

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). For metabolic labelling 70% confluent cells were grown for 8 h in 20 μ Ci/ml [³⁵S]methionine (Amersham). Cells were lysed in 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP40, 1 mM PMSF for 30 min on ice. The cell extract was centrifuged at 100 000 g for 30 min and the pellet discarded. The extract was preabsorbed with Protein G sepharose (Pharmacia), monoclonal antibody was added and the mixture was left overnight at 4°C on a rotating wheel. Protein G beads were added and the incubation was continued for 1 h. The beads were then washed four times in lysis buffer. Denaturing PAGE and immunoblotting were performed as described in Harlow and Lane (1988) using BioRad minigel apparatus.

Immunoassays

96 well microtitre plates were incubated overnight at room temperature with 50 μ l per well of 30 μ g/ml pure antibody, rinsed in phosphate buffered saline

(PBS) and blocked for 2 h in 20% dried milk in PBS. Cell extract was prepared as above. 50 μ l of extract or serial dilutions in lysis buffer were added to each well and incubated overnight at room temperature. The plates were washed in 0.1% NP40 in PBS, biotinylated second antibody was added and the incubation continued for 3 h. The plates were washed again and streptavidin conjugated horseradish peroxidase (HRP) was added for 30 min. HRP was visualized with tetramethylbenzidine and the enzymatic reaction monitored in a Molecular Devices ELISA plate reader at 650 nm. For details see Harlow and Lane (1988). For T-p53 complex assays 100 ng pure T antigen (Simanis and Lane, 1985) was mixed with each 50 μ l dilution of extract in a separate well, incubated for 1 h and then transferred to the appropriate antibody coated wells.

Immunohistochemistry

Subconfluent monolayers of cells were fixed for 2 min in 50% acetone in methanol and air dried. Staining was performed as described in Harlow and Lane (1989) using hybridoma supernatant followed by HRP conjugated rabbit anti-mouse immunoglobulin (Dako) and diaminodenzidine hydrogen peroxide substrate.

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