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Purification and partial amino acid sequence analysis of the cellular tumour antigen, p53, from mouse SV40-transformed cells

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The cellular tumour antigen p53 is implicated in the transformation process. To compare p53 from transformed cells and their normal counterparts in detail, and so to identify any structural differences that might alter p53 function, requires information on the primary structure of the protein. By making use of immunochemical techniques we have been able to purify nanomole amounts of p53. This was sufficient, using high sensitivity automated gas-phase sequencing techniques to determine the amino acid sequence of two tryptic peptides from p53. Their sequences agree completely with the predicted polypeptide sequence derived from a cloned cDNA for p53 mRNA and provide the first data on the amino acid sequence of p53. A combination of the high sensitivity amino acid sequencing procedures used here and cDNA sequence analysis should provide the complete amino acid sequence of p53.

Key words: p53/protein purification/protein sequencing

Introduction

Studies on a variety of transformed cells have revealed cellular phosphoproteins of 48 000 – 54 000 daltons which may vary in amount, or state of modification in the transformed cell as compared with non-transformed controls (for reviews, see Klein, 1982). These proteins have by general agreement been termed p53, although they show variation in their apparent mol. wts. Such proteins have been found in association with the SV40 large T antigen in SV40-transformed mouse 3T3 cells (Lane and Crawford, 1979; Linzer and Levine, 1979; McCormick and Harlow, 1980), in various mouse transformed and tumour cell lines (DeLeo *et al.*, 1979; Rotter, 1983), in a number of human tumour cell lines (Crawford *et al.*, 1981), and associated with the adenovirus E1b 58 K protein in adenovirus-transformed cells (Sarnow *et al.*, 1982a, 1982b). All these proteins appear to be very similar in structure on the basis firstly of comparisons of their respective *Staphylococcus aureus* V8 protease or tryptic digestion products (Jay *et al.*, 1979; Simmons *et al.*, 1980) and secondly of their shared reactivity with a variety of monoclonal antibodies (Gurney *et al.*, 1980; Harlow *et al.*, 1981; Rotter *et al.*, 1980; Dippold *et al.*, 1981). These p53 proteins are, therefore, likely to be the products of homologous genes in the different cell types studied. However, a protein of 53 000 daltons, found in association with the Epstein-Barr virus antigen, EBNA (Luka *et al.*, 1980) now appears to be unrelated to p53 (Luka *et al.*, 1983; D.Lane, personal communication).

Experiments by two groups (Milner and Milner, 1981; Mercer *et al.*, 1982) suggest that p53 has an essential role in the control of replication and cell division, but as yet the precise function of p53 in either normal or transformed cells is not known. Analysis of p53 by two-dimensional gel electrophoresis shows a marked heterogeneity for this protein in both its isoelectric point and apparent size (Crawford *et al.*, 1981). The extent to which p53 is phosphorylated also varies markedly between different cell types, being generally lower in nontransformed cells (Crawford *et al.*, 1981). It is possible, therefore, that it is the distribution of p53 between different molecular forms (either differing in the extent of post-translational modification, or being the products of differentially regulated p53 genes) that is important. An understanding of the primary structure of the protein would provide the essential basis for investigation of the relationship between its structure and function. Here we report the purification of nanomole quantities of p53 from an SV40-transformed mouse 3T3 cell line and the determination of a partial amino acid sequence from this material using a high-sensitivity automated gas-phase sequencing technique. These data, in conjunction with nucleotide sequence information, should allow the complete amino acid sequence of p53 to be defined.

Results

Protein purification

p53 was purified using a suitably scaled-up immunoprecipitation and SDS-gel electrophoresis protocol, adapted to minimise the risk of impurities contaminating the final preparation, as described in Materials and methods. The mouse SV40-transformed 3T3 cell line SVA31E7 (E7) was chosen to provide the starting material for this purification. Of those tested by Benchimol *et al.* (1982), this cell line produces the most p53. A typical batch procedure used extract prepared from ~1.6 x 10^9 cells by homogenisation in detergent-containing buffer. A small amount of ^{32}P-labelled E7 extract was added to the bulk extract to provide a labelled tracer for p53. The extract was immunoprecipitated with PAb421 and a 1% sample run on an SDS-polyacrylamide gel (Figure 1). Lane a shows the Coomassie blue stained proteins and lane b the ^{32}P-labelled proteins in this sample. ^{32}P-Labelled species characteristic of p53 and large T from this cell line co-migrate with heavily stained protein bands. A super-T antigen, similar to that described by Kress *et al.* (1979), produced by these cells is also present in the immunoprecipitate. Polypeptides with mol. wts. of 25 000 and 110 000 (Figure 1, lane a), correspond to immunoglobulin light and heavy chains, respectively. The latter migrate as dimers, some of which retain bound light chains, because the sample was prepared without heating and as a result the dissociation of IgG into subunits is minimal (McCormick and Harlow, 1980). To check that there was no significant con-

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The contribution of J.Jenkins to the work described in this paper is restricted to the isolation, in collaboration with Sam Benchimol and others, of the cDNA clone for p53, limited data on which we present in our Discussion.

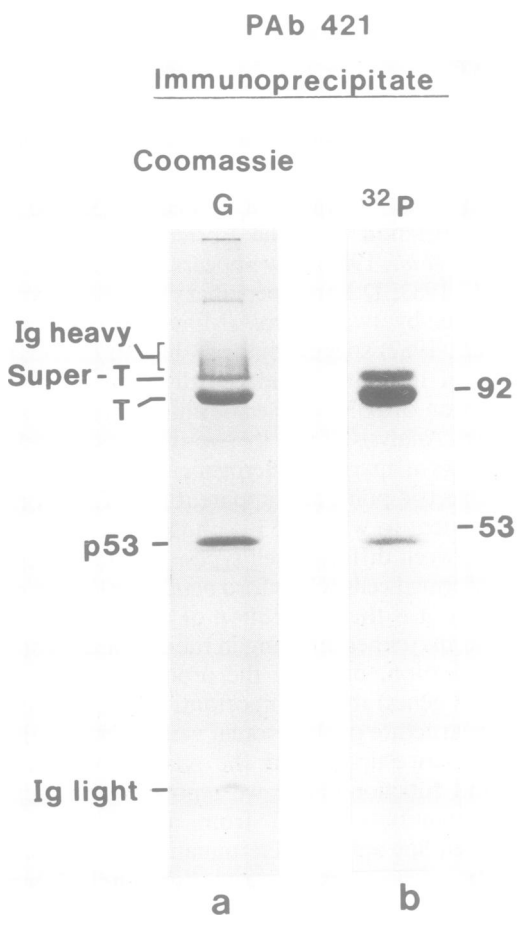

Fig. 1. The large T/p53 complex was immunoprecipitated from an extract of ~1.6 x 10^9 SVA31E7 mouse cells (of which a small proportion had been metabolically labelled with [^{32}P]phosphate) using anti-p53 monoclonal antibody PAb421, and eluted into 2.5 ml of SDS gel sample buffer at 0°C. 25 μl of this sample was analysed on a 10% SDS-polyacrylamide gel, which was then stained with Coomassie blue stain **(lane a)** before being dried and autoradiographed **(lane b)**. The positions of components of the immunoprecipitate and mol. wt. marker proteins are shown on the left and right, respectively.

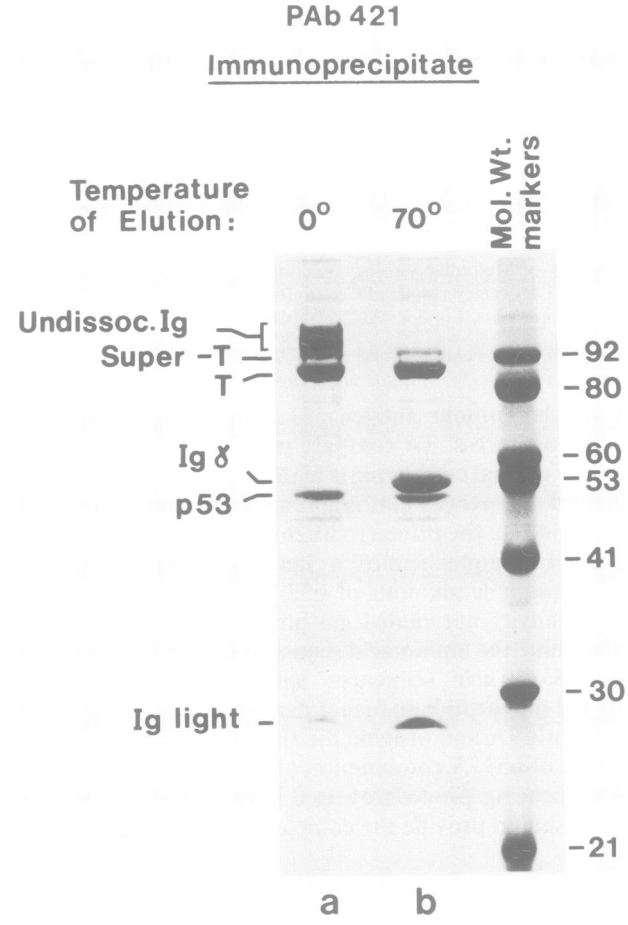

Fig. 2. Samples of immunoprecipitated proteins in SDS-gel sample buffer as in Figure 1 were either loaded directly **(lane a)**, or heated to 70°C for 5 min before loading **(lane b)**. The components of the immunoprecipitates are identified on the left, and the sizes of mol. wt. marker proteins are shown on the right of the figure.

tamination of p53 with γ-chain monomer, aliquots of immunoprecipitate were, on a separate occasion, either kept at 0°C or heated to 70°C for 5 min before loading. The pattern of Coomassie-stained proteins derived from these samples is shown in Figure 2. A comparison of lanes a and b shows that on heating, the γ-chains run as a heavily stained band of ~50 K, clearly distinguishable from the p53 band present in both lanes. No monomer heavy chain was detected in the unheated sample, and the light chain band was of much reduced intensity. A set of protein species of 100–120 K are seen only in the unheated sample. These represent the various possible partially dissociated IgG molecules.

After elution of the p53 protein from the appropriate slices of the preparative gels used to separate the bulk of the sample analysed in Figure 1, the purity and yield were tested by rerunning a 1.25% aliquot on an analytical gel, as shown in Figure 3. Single bands of both stained (lane a) and labelled (lane b) protein are apparent which precisely co-migrate with an apparent mol. wt. of ~48 000, estimated from the mobility of marker proteins. Based on the amount of bound Coomassie stain, the final yield of p53 protein from 1.6 x 10^9 cells was, in this case, ~75 μg, 50% of the p53 initially immunoprecipitated.

p53 is defined by its reactivity with antibodies such as PAb421 and by this definition the ^{32}P-labelled material of 48 000 mol. wt. that had been derived from the original immunoprecipitation with PAb421 was p53. To ensure that all of the Coomassie-stainable protein that had been purified shared the immunological and chemical properties of p53, we tested its ability to rebind either to PAb421 or to the control anti-large T antibody, PAb419. PAb421 rebound ~50% of the input of purified p53, whereas PAb419 showed no specific precipitation of this material (data not shown). Since PAb421 only rebinds ~50% of [^{35}S]methionine-labelled p53, purified in this way, in a direct binding assay (E.Harlow, unpublished data), we conclude that the Coomassie-stainable protein in the purified preparation represents the same population of molecules as the labelled antigen, p53.

Amino acid composition of p53

p53, prepared as described, was further concentrated, and the level of SDS in the sample reduced, either by an electrophoretic procedure or by precipitating with trichloroacetic acid (TCA) and redissolving in 98% formic acid. Samples prepared by either method, or protein further subjected to performic acid oxidation (Hirs, 1967; Smith *et al.*, 1978), were then hydrolysed and their amino acid content determined (Darbre and Waterfield, 1983). Data from an experiment using between 0.1 and 0.2 nmol of TCA-precipitated, unoxi-

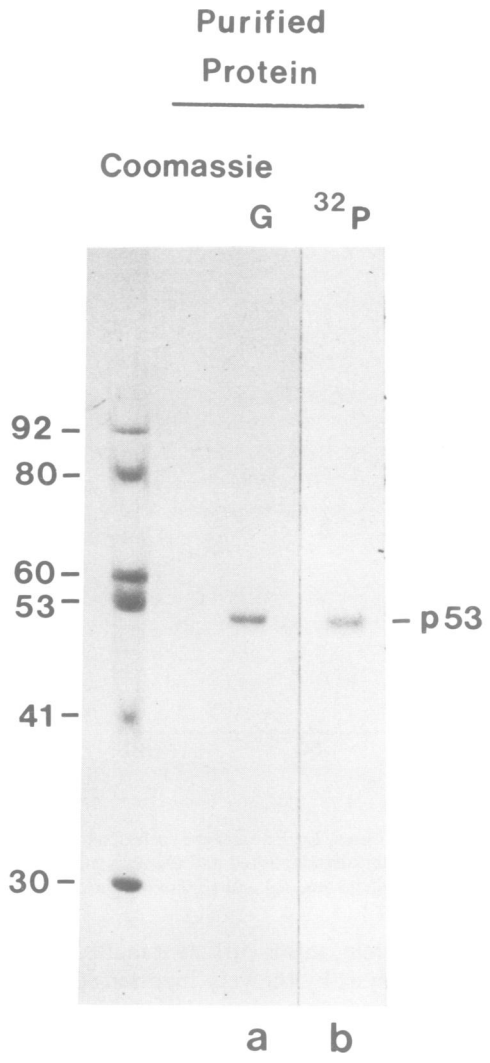

Fig. 3. p53 protein from E7 cells, some of which had been labelled *in vivo* with [^{32}P]orthophosphate, was purified by electrophoretic separation of components of an anti-p53 antibody immunoprecipitate and subsequent electroelution from the appropriate slice of polyacrylamide gel. An aliquot of this material was then re-electrophoresed through an SDS-polyacrylamide gel alongside proteins of known mol. wt. The gel was firstly stained with Coomassie blue **(lane a)** and then dried and autoradiographed **(lane b)**. The sizes of the marker proteins are indicated on the left of the figure.

dised p53 protein are shown in Table I. Since proline, cysteine and tryptophan could not be determined in our protocol, these amino acids were assumed to contribute 12.5 mol% of the protein as a basis for calculation of the contributions of the other 17 amino acids. The composition data reveal that p53 contains no excess of negatively charged residues; its pI of between 5.5 and 6.5 is therefore probably due to the observed phosphorylation of several serine and threonine residues along the length of the polypeptide chain (van Roy *et al.*, 1981). One third of the residues (excluding proline) are hydrophobic and one eighth are hydroxy-amino acids. The estimated methionine content of 2.9% would imply 11 or 12 methionines per 400 residues (the predicted length of p53 based on its apparent mol. wt. and a typical mean residue mol. wt. of 120).

Amino acid sequence of p53

Sequence analysis of intact p53 at a sensitivity of 10 pmol

Table I. Amino acid composition of p53

Amino acid	Description	mol%
D/N	charged/polar	5.9
E/Q	charged/polar	10.6
S	polar	8.1
T	polar	3.7
G	polar	6.2
Y	polar	5.0
F	hydrophobic	3.7
V	hydrophobic	5.5
I	hydrophobic	2.8
L	hydrophobic	10.1
A	hydrophobic	7.7
M	hydrophobic	2.9
R	charged	6.9
K	charged	6.9
H	charged	1.4
Total*		87.4

Approximately 0.1 nmol of p53 was hydrolysed in 50 μl 6 N HCl with 0.5 μl 1,4-butanedithiol for 22 h at 110°C under N_2, and the products analysed by h.p.l.c. Data here and subsequently are presented using the single letter amino acid code (A - Ala, C - Cys, D - Asp, E - Glu, F- Phe, G - Gly, H - His, I - Ile, K - Lys, L - Leu, M - Met, N - Asn, P - Pro, Q - Gln, R - Arg, S - Ser, T - Thr, V - Val, W - Trp, Y - Tyr).
*P, C and W were not detectable by our procedures. These amino acids were assumed to contribute 12.5% of the residues of p53 for calculation of mol% figures for the other 17 amino acids (see text).

failed to detect any derivative from 0.5 nmol of protein, indicating that the amino terminus of the protein was blocked. We therefore digested the protein with trypsin to isolate one or more internal peptides which could then be sequenced. Tryptic peptides were generated from ~1 nmol of performic acid-oxidised p53, and separated by h.p.l.c. as described. The absorbance of the column eluate at 206 nm was monitored continuously and the resulting profile is shown in Figure 4. A well separated peak eluting at 32% acetonitrile, and designated K9, was selected for further analysis. The composition of this peptide was determined from a 50 μl aliquot of the appropriate column fraction (0.5 ml) and is shown in Table II. After allowing for column background, the material had an approximately integral composition, but appeared to contain two arginine residues, which suggested that it was the product of incomplete tryptic digestion.

250 μl of the fraction containing K9 was used for amino acid sequence determination by the method described. Assuming that the composition data referred to a single peptide, this corresponded to a loading of ~60 pmol of peptide. Each degradation cycle yielded a single phenylthiohydantoin-derivatised (PTH-) amino acid as detected by h.p.l.c., confirming the purity of the peptide. The repetitive yield of derivative at each cycle is shown in Figure 5, together with the deduced amino acid sequence. It was not possible to quantitate the recovery of serine and threonine derivatives due to their instability. No derivative was identified at cycle 14, and the last residue detected was leucine at cycle 17. The repetitive yield at this point had fallen considerably from that achieved in cycles 1 – 10, suggesting that peptide K9 did not extend much beyond this 17th residue. The peptide designated K2 in Figure 4 was similarly analysed and gave an identifiable amino acid derivative at seven of eight positions (see Figure 6; Discussion), with recoveries similar to those for K9.

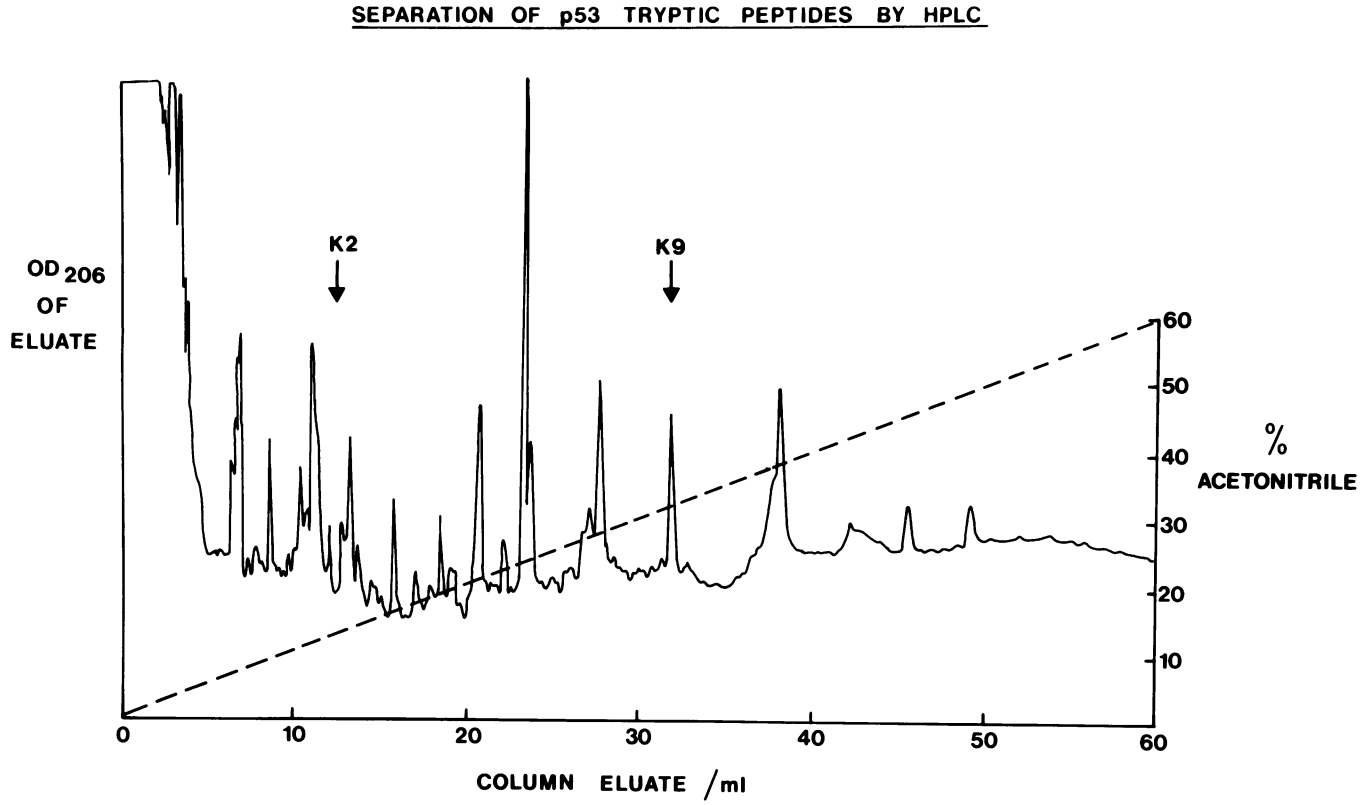

Fig. 4. Tryptic peptides derived from 1 nmol of purified p53 were redissolved in 50 μl of 0.1% TFA, 6 M urea, loaded onto a Synchropak RP-P column and eluted with a linear 0–60% acetonitrile gradient in 0.1% TFA. The optical density at 206 nm of the eluate was monitored and plotted against the cumulative eluate volume. Full scale on the ordinate represents an optical density of 0.2. The positions of the peaks designated K2 and K9 are indicated.

Table II. Amino acid composition of p53 peptide K9

Amino acid	Peptide K9	
	Amount/pmol	Composition (Arg = 2)
D/N	24	2.0
E/Q	17	1.4
S	27	2.4
H	–	–
G	24	2.0
T	12	1.0
R	23	2.0
A	3	0.3
Y	1	0.1
V	–	–
F	–	–
I	9	0.8
L	31	2.8
K	–	–
P,C,W,M	ND	ND

A 50 μl aliquot (10%) of the K9 peptide fraction was lyophilised, and the peptide hydrolysed with 50 μl of 6 N HCl at 110°C under N_2 for 22 h. Determination of the amino acid content of the hydrolysate was as described in the text. For key to the single-letter amino acid code see Table I.

Discussion

We have described the purification of the cellular tumour antigen p53. The material obtained by the immunoprecipitation protocol adopted is considered to be homogeneous p53 by the criterion of its specific rebinding to the anti-p53 antibody, PAb421. Whilst this protocol yields only one or two nanomoles of protein, this is sufficient material to allow protein chemical analysis by the very high sensitivity techniques now available.

This material was used initially to determine the amino acid composition of p53 although, with the procedures adopted, it was not possible to measure its proline, cysteine or tryptophan content. To calculate the mol% contributions of the other 17 amino acids, we assumed that one eighth of the p53 polypeptide is contributed by proline, cysteine and tryptophan. This assumption is based on the observed frequency of these amino acids in the predicted translation product of a 277 codon open reading frame in a recently derived cDNA clone (see below). There are several major differences between the composition of p53 determined here and the published composition of the 53 000 dalton EBNA-associated protein (Luka *et al.*, 1980; Jörnvall *et al.*, 1982). Most notable are the differences between the figures for aspartic acid plus asparagine (5.9% p53; 11.4% methA 53kD), glycine (6.2%; 13.6%), leucine (10.1%; 5.6%) and tyrosine (5.0%; 0.9%). These data are therefore in good agreement with the suggestion (Luka *et al.*, 1983) that the 53 000 dalton EBNA-associated protein is not related to the SV40 large T-associated cellular protein, p53.

We have prepared and purified tryptic peptides from p53, and selected one for sequence analysis. This peptide, designated K9, gave an unambiguous sequence for 17 cycles, except that at cycle 14 no residue was positively identified. When the sequence of peptide K9 was compared with the polypeptide translation product predicted from an open reading frame in a cDNA clone (Benchimol *et al.*, in preparation), a precise match was found (Figure 6). This allowed us both to confirm that the cDNA clone was derived from an

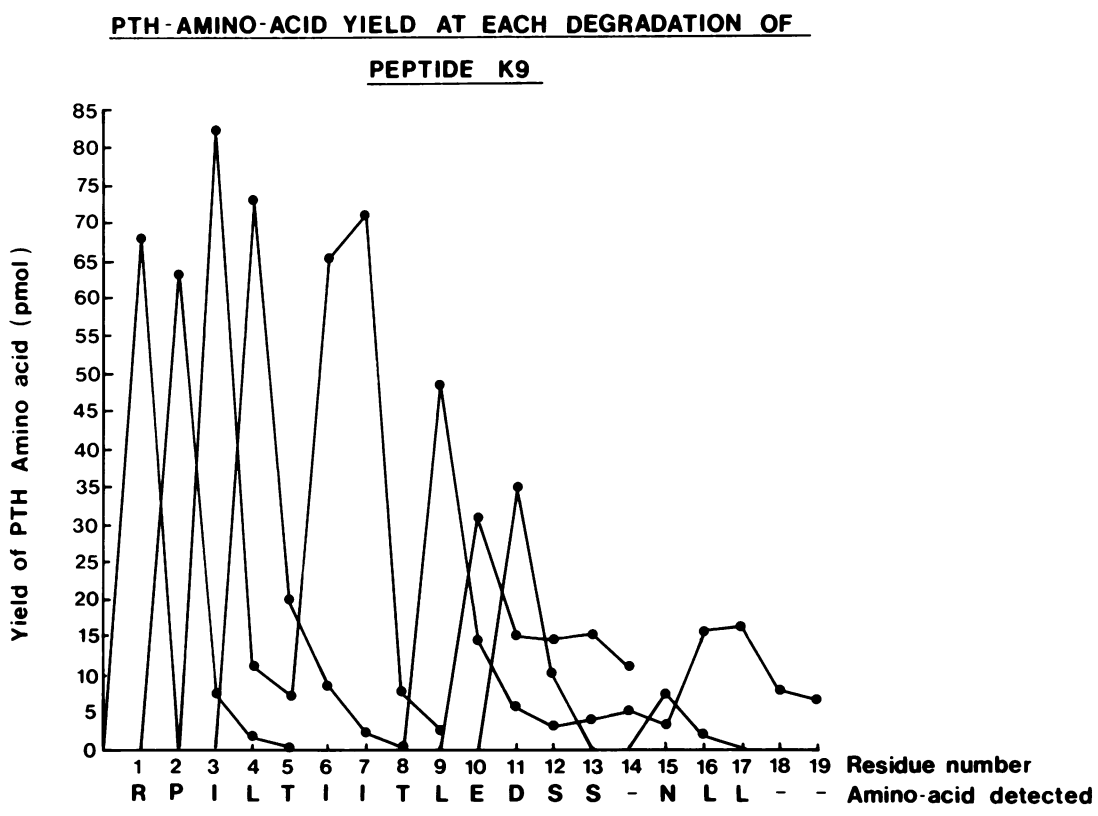

Fig. 5. Peptide K9 was subjected to automated sequence analysis as described. The input estimated from composition determination was 60 pmol, without allowance for any losses during the hydrolysis. The recovery of PTH-amino acids after each cycle was calculated and these recoveries plotted against the degradation cycle number. The residue identified at each cycle is indicated beneath the appropriate cycle number. PTH-ser and PTH-thr derivatives could not be quantitated but, where indicated, were unambiguously identified on the basis of their characteristic elution times from the h.p.l.c. column. For a description of the single letter amino acid code see Table I.

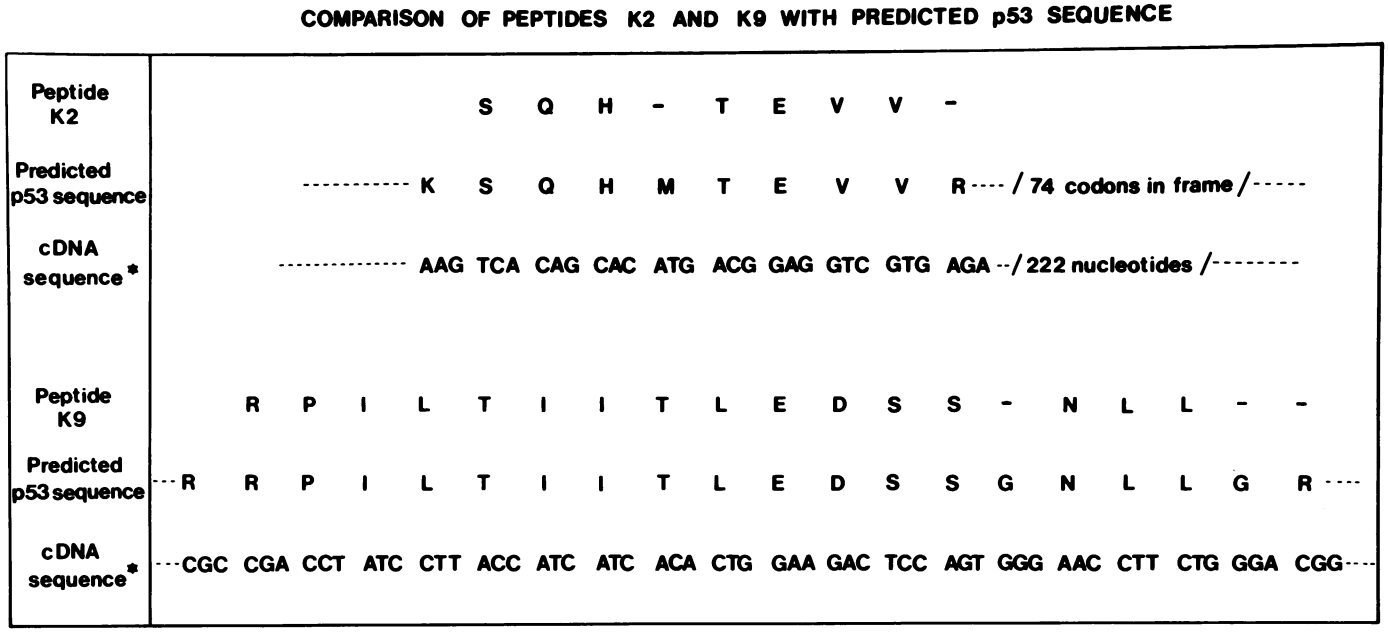

Fig. 6. Alignment of p53 peptide sequences K2 and K9 with the sequence of a polypeptide predicted from the DNA sequence. *The cDNA sequence is taken from Benchimol *et al.* (in preparation). For a description of the single letter amino acid code see Table I.

mRNA coding for p53 protein, and to derive a predicted sequence for peptide K9 in which residues 14 and 18 are glycine, and residue 19, the C-terminal residue, arginine. Our inability to detect these residues is attributable in part to the very small quantities of material used, and also to a particular difficulty with glycine detection at this level. Glycylglycine, added to the sequencer to remove potential N-terminal blocking agents that contaminate the reagents used, gives rise to a variable background release of glycine at each cycle. Immediately upstream of the initial arginine codon in the cDNA clone is a

second arginine codon. This may explain why K9, which was produced from p53 by tryptic digestion, has arginine as its first residue. When two arginine residues lie adjacent in the polypeptide chain, trypsin frequently cuts exclusively between these two target residues.

There are some discrepancies between the determined composition of K9 and its composition predicted from its sequence. Principally, there are two isoleucine residues, one leucine and one threonine residue that cannot be reconciled with the determined composition. Peptide bonds between hydrophobic residues are known to be relatively resistant to acid hydrolysis. Presumably, the peptide bonds between positions 3 and 9 in the peptide failed to hydrolyse completely, giving low figures for the residues involved.

A second peptide, K2, was also sequenced using the same techniques. Its sequence is shown in Figure 6, aligned with the predicted translation product of the same cDNA clone. Methionine at position 4 was not detected because this residue had been converted, by performic acid oxidation, to methionine sulphone, which does not form an analysable PTH-derivative, and the C-terminal arginine was, again, not detected. Peptides K2 and K9 are separated in the predicted sequence of p53 by 74 amino acids.

The techniques employed here have allowed us to establish the sequence of tryptic peptides from p53, and will be used to sequence further peptides. The data presented are the first protein sequence data to be derived from p53. With this information, the correct reading frame within a series of p53 cDNA clones (to be described elsewhere: Benchimol *et al.*, in preparation) has been established. A combination of protein and cDNA sequence analysis will allow the complete amino acid sequence of p53 to be defined. The availability of such a sequence should allow significant advances in the study of p53 function. For example, synthetic peptides derived from the p53 sequence could be used to raise antisera, which could then be used to purify p53 from cell extracts. Such antisera would be particularly valuable for this purpose since the antibody-antigen interaction can be readily broken by the addition of excess peptide once the immunoselection has been achieved, allowing elution of purified material without the use of harsh denaturing conditions (Walter *et al.*, 1982). Ultimately, it is studies of native p53 that will lead to an understanding of how p53 functions in normal and transformed cells.

Materials and methods

Reagents

All reagents were analytical grade except as follows. SDS was from Serva, and was recrystallised twice from 80% ethanol. Glycerol was Aldrich Chemicals, Gold Label grade. Spectrapor membrane dialysis tubing was prepared for use by successively heating to 80°C in 10 mM EDTA, 5% sodium bicarbonate, then ddH_2O and then 0.05% SDS in which it was subsequently stored at room temperature. Bovine TPCK-treated trypsin was from Worthington. Reagents used for analysis of amino acid composition and amino acid sequence were obtained from Rathburn (Walkerburn, Scotland) or from BDH (Poole, UK).

Cells and antibodies

SVA31E7 is an SV40-transformed mouse 3T3 cell line and was kindly provided by Y.Ito. The cells were cultured either on 90 mm NUNC dishes or on NUNC bioassay trays in Dulbecco's modified Eagles medium (E4) supplemented with 5% fetal calf serum. Monoclonal antibody PAb421 has activity towards both rodent and human p53 proteins, and PAb419 has anti-SV40 large T antigen activity (Harlow *et al.*, 1981).

Cell labelling and preparation of extracts

Just confluent monolayers of E7 cells ($1-2 \times 10^7$ cells per 90 mm dish) were labelled with 1.0 mCi of [^{32}P]orthophosphate (carrier-free, Amersham International) for 3 h in 2.5 ml E4 medium lacking phosphate. They were then drained, rinsed in ice-cold Tris-buffered saline and lysed on ice with 0.5 ml 1% Nonidet P-40 (NP-40), 150 mM NaCl, 50 mM Tris pH 8.0 (lysis buffer) per dish. Lysate was collected by tilting the dish after 20 – 30 min and pipetting off the supernatant.

Extracts of unlabelled cells were prepared by homogenisation in hypotonic buffer containing detergent. Briefly, washed cells were broken in ~4 ml/10^8 cells of 25 mM Tris pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, (PB) containing 2% Triton X-100, 1 mg/ml heparin, 1 μg/ml cycloheximide. The supernatant from a 5 min, 15 000 r.p.m. spin in a Sorvall SS34 rotor was made 0.1 M in $MgCl_2$ and left at 0°C for 1 h before layering over an equal volume of PB containing 0.5% Triton X-100, 0.5 mg/ml heparin, 1 μg/ml cycloheximide, 0.5 M sucrose and spinning at 15 000 r.p.m. for 15 min as above. The supernatant from this spin was taken as the large T/p53-containing cell extract. Before use, extracts were precleared to remove any material capable of being non-specifically precipitated in the subsequent immunoprecipitation by incubation with 0.1 ml of 10% *S. aureus* strain Cowan 1 (SAC) per ml of lysate for 30 min on ice, and clearance by centrifugation firstly at 10 000 r.p.m. for 10 min in a Sorvall SS34 rotor and then either at 28 000 r.p.m. for 2 h in a Beckman SW28 rotor or at 40 000 r.p.m. for 1 h in an SW40 rotor depending on the total volume to clear any remaining denatured protein aggregates. Smaller scale preparations based on the standard NP-40 lysis technique were equally successful.

Immunoprecipitation

Preparative immunoprecipitation was as follows. Precleared extract was diluted at 4°C with 2 volumes of 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 8.0 (NET) per volume of supernatant and immunoprecipitated overnight with 0.1 volumes of tissue culture fluid from hybridoma PAb421 (containing ~30 μg/ml anti-p53 antibody). Immune complexes were collected with 200 μl 10% SAC per ml of antibody fluid (Kessler, 1975) for 30 min at 4°C, washed twice in 10 ml NET buffer per 100 μl 10% SAC and finally eluted by resuspending in 100 μl sample buffer [2% SDS, 100 mM dithiothreitol (DTT), 10% glycerol, 25 mM Tris pH 6.8] per 100 μl 10% SAC at 4°C and holding at that temperature for 1 h. Bacteria were then removed by centrifugation and the supernatant either used directly or stored at −20°C.

Purified protein was rebound to antibody in the presence of background protein. 1 – 5 μg purified protein in 10 – 25 μl 20 mM ammonium bicarbonate, 0.02% SDS was diluted with 1.0 ml NET/gel buffer (1 x NET buffer containing 0.05% NP-40, 0.02% NaN_3 and 0.25% gelatin) and immunoprecipitated at room temperature for 30 min with 1.5 μg purified antibody. Immune complexes were collected on 20 μl 10% SAC, washed twice in NET/gel buffer and eluted into 25 μl sample buffer.

Gel electrophoresis and electroelution

Analytical gels were as described by Laemmli (1970). Protein mol. wt. markers were phosphorylase b (92 K), catalase (80 K), glutamate dehydrogenase (60 K), alcohol dehydrogenase (41 K), carbonic anhydrase (30 K) and soybean trypsin inhibitor (21 K). Gels were stained in 50% TCA containing Coomassie blue dye and destained in 7% acetic acid, 25% methanol for ~4 h with several changes before drying if required. Preparative gels were as described by Neville (1971). Large T and p53 were located in preparative gels by overnight exposure of the wet gel under Saran Wrap to Kodak SB-5 film and the appropriate slices cut from the gel. These were mashed by passage through 5 ml syringes into 1 ml of 0.2 M Tris/acetate pH 7.4, 1.0% SDS, 0.1% DDT per 0.1 ml polyacrylamide and confined in a small piece of precleaned dialysis tubing. This was then placed in a flat-bed electrophoresis tank in 50 mM Tris/acetate pH 7.4, 0.1% SDS, 0.5 mM sodium thioglycolate and subjected to a 100 V, 100 mA electric field for 3 h, with current reversed for the final 30 s. The material was then extensively dialysed against 20 mM ammonium bicarbonate, 0.02% SDS, before filtration through GF-C filters to remove gel fragments.

Preparation of tryptic peptides

Protein for digestion was precipitated with TCA and oxidised with performic acid (Hirs, 1967) as described by Smith *et al.* (1978). After drying the protein several times from dH_2O, it was then redissolved in 100 μl of fresh 1% ammonium bicarbonate, digested with bovine trypsin at a ratio of 1:50 w/w with p53 for 4 h at 37°C, and peptides then lyophilised three times from dH_2O. Peptides were then separated by h.p.l.c. (Bennett *et al.*, 1980). The mixture was taken up in 50 μl of 6 M urea, 0.1% trifluoracetic acid (TFA) and loaded onto a 75 x 4.5 mm Synchropak RP-P column (Synchron Inc., Linden, IN). This was then developed with a 0 – 60% linear gradient of acetonitrile in 0.1% TFA over a 1 h period. The eluate was monitored for absorbance at 206 nm and collected in 0.5 ml fractions.

Amino acid composition analysis

Lyophilised protein (~0.1 nmol) was taken up in 50 μl of 6 N HCl and

0.5 μl of 1,4-butanedithiol added. Samples were then deaerated, sealed under vacuum, and hydrolysed at 110° for 22 h. After removing the acid under vacuum, amino acids in the hydrolysate were analysed by automated pre-column derivatisation with *o*-phthalaldehyde using a C-8 'Short One' reverse phase column (Rainni Insts., Woburn, MA) as described by Darbre and Waterfield (1983).

Protein sequence analysis

Amino acid sequences were determined using a gas phase sequencer built and operated as described by Hewick *et al.* (1981), using high sensitivity detection methods for PTH-amino acid analysis employing a C-8 Zorbax (Dupont) reverse phase column as described by Waterfield (1983).

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