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A 53-kilodalton protein common to chemically and virally transformed cells shows extensive sequence similarities between species

(nuclear antigens/Epstein–Barr virus/amino acid sequence comparisons)

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ABSTRACT A heat-stable DNA-binding protein with subunits of about 53 kilodaltons (kDal) was purified from two virally transformed human cell lines (Epstein–Barr virus-positive Raji and Namalwa) and two mouse tumor cell lines (methylcholanthrene-induced Meth A sarcoma and TA3 mammary carcinoma). All four 53-kDal proteins showed closely related total amino acid compositions, similar peptide maps, and identical NH_2-terminal amino acid sequences for 20 residues. These 53-kDal proteins are therefore evolutionarily highly conserved, independent of whether they originate from virally or chemically transformed cells. The NH_2-terminal sequence and the protein chain as a whole are not hydrophobic; however, some unexpected residue distributions were observed. Comparisons with other proteins reveal no clear sequence similarity with known tumor antigen structures, homologous immunoglobulins, or some other proteins of known sequence. Epstein–Barr virus-determined nuclear antigen also appears to have a different NH_2-terminal sequence. Thus, the results show that the 53-kDal proteins represent a unique protein type with little species variation; this finding suggests that these proteins must perform an important common function in different transformation systems.

Cellular proteins with subunits about 53 kilodaltons (kDal) have been described in several transformed cell systems and in other cells in active growth. A protein of this size occurs in cells transformed by simian virus 40 (SV40) (1–11), associated with the large tumor (T) antigen, and in cells transformed by other papovaviruses. A 53-kDal protein has also been detected in Epstein–Barr virus-transformed cells, where it may be complexed with the Epstein–Barr virus-determined nuclear antigen (EBNA) (12), and in cells transformed with Abelson murine leukemia virus (13). In nonviral systems, proteins of this size have been reported from methylcholanthrene-induced sarcomas (14, 15), concanavalin A-stimulated cells (16), embryonic cells (17) [including embryonal carcinoma cells (3)], and normal thymus (15). A 53-kDal protein is also a common feature in several human tumor cell lines (11, 18). Thus, this class of proteins appears ubiquitous in rapidly proliferating cell populations.

53-kDal proteins are coded for by cellular DNA. The fact that they may be associated with T antigen and are found in a variety of different tumor systems and embryonic tissues suggests that 53-kDal proteins may be of general importance in transformation or growth regulation. It also appears possible that such a protein may form a link between viral, chemical, and other mechanisms of cellular transformation.

Immunological studies suggest that some 53-kDal proteins from several sources are similar, regardless of the etiological agent involved (18, 19). These findings are also consistent with peptide mapping experiments (15), although peptide differences have also been noticed (3). As yet, there is no direct proof that all these studies concern the same protein; even the molecular mass values reported have been slightly different, and various forms of 53-kDal protein have been found to occur in a single cell (12). To date, direct chemical characterization has been lacking, and no sequence data have been reported for any of the 53-kDal proteins.

In the present work, 53-kDal proteins were purified from two different mouse ascites tumor cell lines and from two human lymphoblastoid cell lines. In addition, EBNA was partly purified from one of these cell lines. All four preparations of 53-kDal proteins were analyzed by NH_2-terminal sequence determinations, two types of peptide mapping, and amino acid analysis.

The sequence data for the purified 53-kDal proteins allow direct comparisons between strains and species. The sequence data also form a basis for future comparisons with other independently isolated 53-kDal proteins. Comparisons of the 53-kDal protein sequences with those of other proteins of special interest revealed no clear similarities, although a few regions of questionable resemblance were noticed.

MATERIALS AND METHODS

Cell Lines. Meth A ascites sarcoma, a methylcholanthrene-induced mouse sarcoma (14) and TA3 mouse mammary carcinoma (20) were the starting material for the murine 53-kDal protein purification. The human 53-kDal proteins were obtained from Raji and Namalwa Burkitt lymphoma lines (12), grown as stationary suspension cultures. EBNA was prepared from Raji cells.

Protein Purifications. The 53-kDal proteins from all four cell lines were purified essentially by the method described for the Raji and Ramos cell line proteins (12). The purification scheme is summarized in Fig. 1. Complete extraction, efficient purification (by heat treatment and by DNA-cellulose chromatography), and inhibition of proteolysis (by use of a protease inhibitor and of rapid steps), are essential. Therefore, freezing of the cells before extraction, rapid heating and cooling during heat treatment, use of a large DNA-cellulose column, and addition of phenylmethylsulfonyl fluoride throughout are the critical steps, as judged by final yield and purity. Yields were about 300 μg of purified 53-kDal protein from 50 g of cells for all four cell lines. Once acceptable purity had been ascertained by $NaDodSO_4$/polyacrylamide gel electrophoresis and by analysis

Abbreviations: kDal, kilodalton(s); 53-kDal protein, heat-stable DNA-binding protein with subunits about 53 kDal in molecular mass, purified from transformed cells; EBNA, Epstein–Barr virus-determined nuclear antigen; SV40, simian virus 40; T antigen, tumor antigen.

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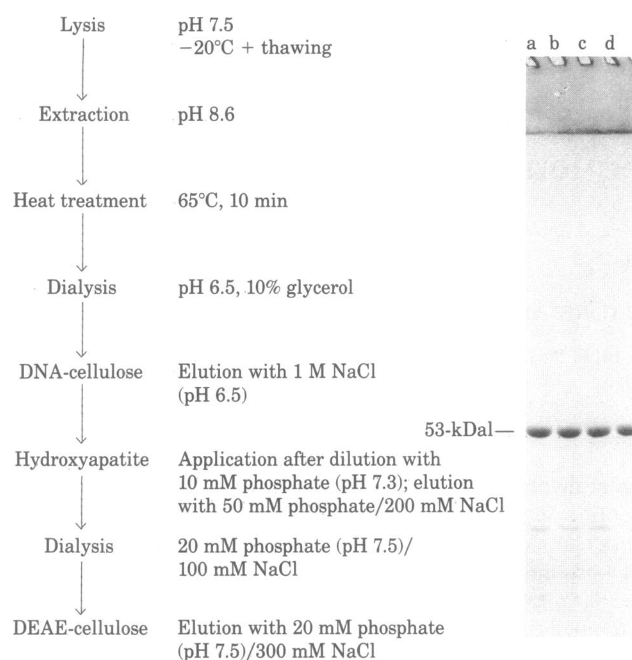

FIG 1. Purification scheme of the 53-kDal proteins (*Left*) and NaDodSO$_4$/polyacrylamide gel patterns of final products (*Right*) from the Raji (lane a), Namalwa (lane b), TA3 (lane c), and Meth A (lane d) cells. The gel was stained with Coomassie blue. The conditions for elution in the chromatography steps apply to batchwise preparations in large scale from cells lacking EBNA. For separation of EBNA and the 53-kDal protein, additional NaCl elutions or a NaCl-gradient elution is required as further detailed in ref. 12.

of total composition, the purified proteins were precipitated with trichloroacetic acid (15% final concentration), washed with acetone, and stored frozen.

Analytical HPLC. The purified material was dissolved in NaDodSO$_4$ (0.5% in water) and analyzed by HPLC on reverse-phase chromatography (μBondapack C$_{18}$, Waters Associates), in 0.05% trifluoroacetic acid, using a linear gradient of acetonitrile (from 0% to 60% in 30 min) for elution (21).

Peptide Mapping and Total Compositions. Samples were treated with staphylococcal extracellular protease, or with CNBr, and peptides obtained were resolved by NaDodSO$_4$/polyacrylamide gel electrophoresis (12). Total amino acid compositions were analyzed on a Beckman 121M or a Durrum D500 amino acid analyzer, after hydrolysis for 24 hr with 6 M HCl in evacuated tubes at 110°C.

Sequence Analysis. The proteins were applied in 0.5% NaDodSO$_4$ to a Beckman 890 liquid-phase sequencer, equipped with a Beckman cold-trap and a Sequemat P-6 auto-converter. Degradations were usually performed in the absence of Polybrene, using a 0.1 M Quadrol protein program. Some samples were also degraded in the presence of precycled Polybrene (22) and applied in formic acid to an unmodified liquid-phase sequencer. Phenylthiohydantoin derivatives were identified by HPLC, using gradients of acetonitrile in sodium acetate (23) on either a Hewlett–Packard instrument equipped with an Ultrasphere-cyano column (Beckman) or a Waters Instrument, equipped with a Zorbax octadecylsilica column (Du Pont) and with simultaneous recording of absorbance at 254 and 313 nm.

Carboxymethylation. The protein (20 μmol) was dissolved in 0.5% NaDodSO$_4$ (500 μl) and mixed with concentrated Tris buffer to give 0.67 M Tris and 0.42% NaDodSO$_4$, pH 8.3 (600 μl). It was reduced with dithiothreitol (6 μmol) for 3 hr at 37°C and carboxymethylated in the dark under nitrogen with neutralized iodo[2-^{14}C]acetic acid (18 μmol) from Amersham (specific radioactivity of 1.6 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) (24). Reagents were removed by chromatography on Sephacryl S-300 Superfine (Pharmacia), 0.5 × 110 cm, in 0.1% NaDodSO$_4$/0.1 M ammonium bicarbonate, and the eluted protein was concentrated by evaporation under a stream of nitrogen.

RESULTS

The 53-kDal Protein. The purified preparations gave essentially one band on NaDodSO$_4$/polyacrylamide gel electrophoresis, corresponding to a molecular weight of 53,000, with only trace amounts of bands corresponding to lower molecular weight contaminants. The pattern obtained on HPLC is shown in Fig. 2. In agreement with the results from electrophoresis, two peaks of protein were recovered, the first peak being the major entity.

Upon electrophoresis of material from each of the two HPLC peaks, the first peak eluted was found to correspond to the 53-kDal protein, whereas the second peak corresponded to three minor bands with lower molecular weights, as also shown in Fig. 2.

Amino Acid Compositions. The 53-kDal protein preparations from all cell lines had amino acid compositions highly similar to those reported for 53-kDal proteins from human Raji and Ramos cells (12). This was also true for the main component (in the first peak) after HPLC as shown in Table 1. The previously unknown cysteine/half-cystine content of the protein was estimated in one preparation after reduction and [^{14}C]carboxymethylation. The results on amino acid analysis for carboxymethylcysteine and on ^{14}C measurements are consistent; they show cysteine/half-cystine to be an uncommon residue in the 53-kDal protein (Table 1).

The closely related compositions of the murine and human 53-kDal components suggest that the proteins from the two species are highly similar.

Peptide Mapping. Different samples of the four preparations were treated with CNBr or a staphylococcal protease specific

Table 1. Amino acid compositions of the 53-kDal proteins from different sources.

Amino acid residue	Composition, mol %			
	Raji	Namalwa	Meth A	TA3
Cys				0.9
Asx	12.1	11.3	11.4	11.2
Thr	3.1	3.5	3.4	3.1
Ser	5.6	6.0	6.4	7.1
Glx	15.5	14.3	14.3	14.7
Pro	8.2	7.4	7.0	5.8
Gly	13.5	13.1	13.6	15.9
Ala	6.7	7.9	6.9	7.0
Val	4.7	4.9	4.7	4.5
Met	0.4	1.0	1.1	1.2
Ile	2.6	2.9	2.7	2.5
Leu	4.9	5.6	5.6	4.6
Tyr	0.6	0.6	0.9	0.9
Phe	3.1	3.1	3.3	3.4
Lys	8.3	8.6	8.4	8.2
His	2.6	2.5	2.4	2.3
Arg	8.0	7.3	7.9	6.8

The protein samples were from the first HPLC peak (see Fig. 2). Tryptophan was not determined, and cysteine/half-cystine was determined only in TA3. Mean standard deviation for all values is 0.6 (with a maximum of 1.4 and a minimum of 0.2 in values for individual residues). Within these limitations, all values for the 53-kDal protein agree with those obtained before (12). Calculations are based on HCl hydrolyses for 24 hr.

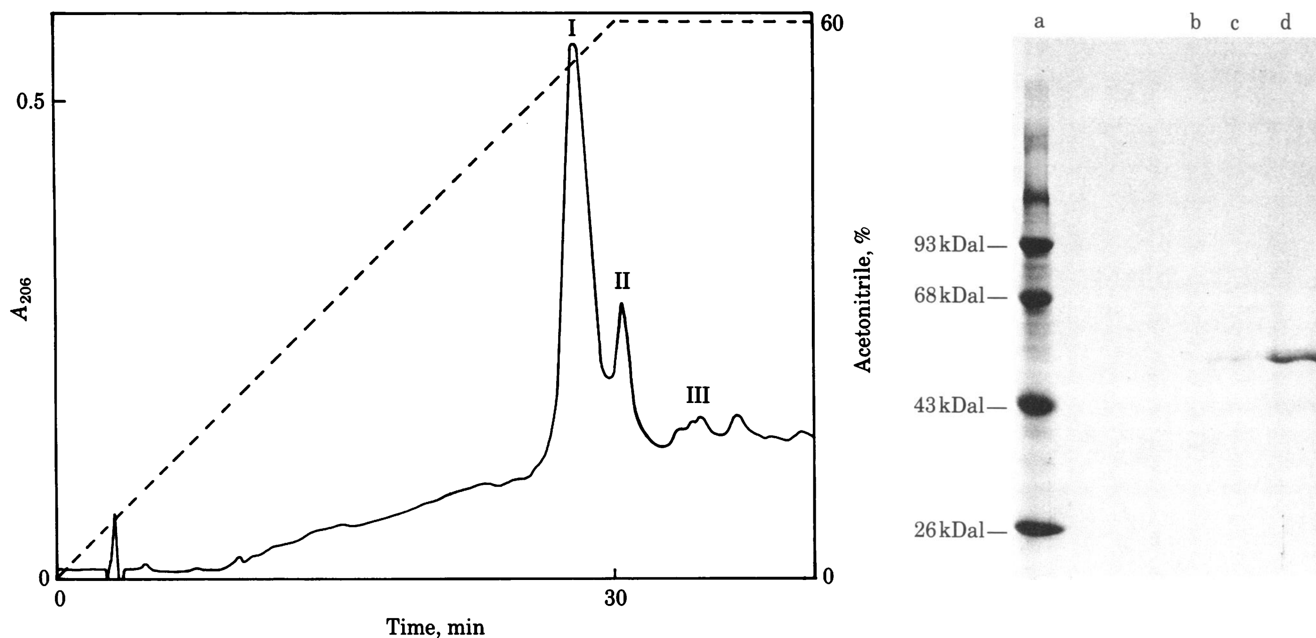

FIG. 2. Analysis of the 53-kDal protein preparation on HPLC. (*Left*) The sample was injected into a μBondapak C_{18} column as a 0.5% $NaDodSO_4$ solution in 0.05% trifluoroacetic acid; elution was with a gradient of acetonitrile, applied as shown by the broken line. (*Right*) $NaDodSO_4$/polyacrylamide gel electrophoresis of the material from each peak: lane a, marker proteins with sizes shown; lane b, peak II; lanes c and d, peak I, diluted 1:10 and undiluted, respectively.

for glutamic acid (25, 26). Subsequent analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis gave the results shown in Fig. 3. The proteins from the two human strains gave largely indistinguishable patterns after treatment with either CNBr or protease. The same was true for the pair of the two murine proteins. On comparison of the 53-kDal proteins from the two species, great similarities were also noticed, but minor deviations in a few of the bands were evident.

It may be concluded that the mouse and human 53-kDal components are very similar in primary structure, in agreement with other peptide comparisons (15) and immunological studies (18, 19).

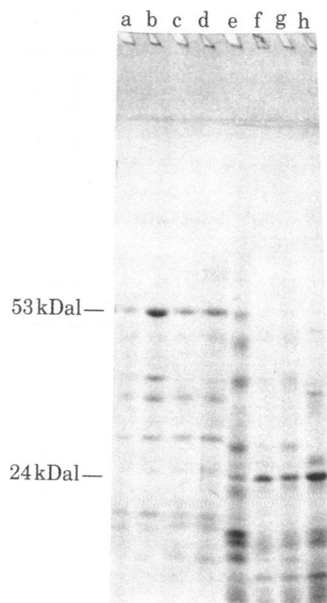

FIG. 3. Peptide mapping in $NaDodSO_4$/polyacrylamide gel of samples from each of the four 53-kDal proteins analyzed (Raji, lanes a and e; Namalwa, lanes b and f; TA3, lanes c and g; Meth A, lanes d and h). Conditions for digestions with staphylococcal protease (lanes a–d) and for cleavage with CNBr (lanes e–h) were as given in ref. 12.

Sequence Analysis. The results of NH_2-terminal sequence analysis of the four 53-kDal protein preparations are shown in Table 2. The human protein from Raji cells was degraded three times with identical results, using different preparations, conditions (with and without Polybrene), and sequencers (an ordinary instrument and one equipped with a cold trap and autoconverter).

All positions with clearly identifiable residues gave the same result, independent of protein source (Table 2). The initial coupling yield of up to 70% is high enough to establish that the sequence corresponds to the major 53-kDal component in the preparations. Even in mixtures with a considerable amount of the second component on HPLC, no other sequence was observed, suggesting either that contaminants are NH_2-terminally blocked or that they correspond, at least to some extent, to NH_2-terminal fragments of the major component.

Assignments were made for the first 20 residues except for position 10. This position was therefore suspected to correspond to half-cystine/cysteine, which is nondetectable on analysis of native proteins. Therefore, the TA3 53-kDal protein was $[^{14}C]$carboxymethylated in $NaDodSO_4$ after reduction with dithiothreitol. The total composition of the modified protein showed recovery of carboxymethylcysteine (Table 1) in an amount consistent with the degree of ^{14}C incorporation. As expected, sequence analysis of this preparation showed the presence of ^{14}C at position 10 (Table 2) in an amount consistent with the repetitive yield of the degradation.

Consequently, amino acids have been ascribed to the first 20 residues (Table 2), and in no case was any difference discovered among the NH_2-terminal sequences of the four proteins.

EBNA. EBNA prepared from the Raji cell line (12) was also submitted to HPLC and to sequence analysis. However, the results show that the preparations were not homogeneous (data not shown). Two major peaks were seen on HPLC and two se-

Table 2. Results of NH_2-terminal sequence analysis of four 53-kDal protein preparations

Degradation step	Residue	Raji	Namalwa	Meth A	TA3
1	Pro	11.3	4.5	4.0	(+)
2	Gly	5.5	+	0.9	(+)
3	His	+	(+)	(+)	
4	Leu	7.4	4.0	5.3	1.9
5	Gln	2.8	1.2	1.0	0.5
6	Glu	+	+	0.9	+
7	Gly	4.7	+	0.9	+
8	Phe	5.9	3.1	2.8	1.1
9	Gly	3.2	+	0.8	+
10	Cys				+
11	Val	5.8	2.0	2.1	+
12	Val	5.0	1.9	2.4	+
13	Thr	+	+	0.7	+
14	Asn	1.1	+	0.6	+
15	Arg	2.6	(+)	0.5	
16	Phe	3.6	+	2.8	0.7
17	Asp	+	+	0.8	+
18	Gln	1.4		0.5	+
19	Leu	2.2		2.1	+
20	Phe	3.5		2.8	+
Amount degraded, nmol		15	10	12	6
Repetitive yield, %		95	96	97	94
Initial coupling, %		70	50	50	30

Degradations were performed with a modified liquid-phase sequencer in 0.1 M Quadrol without Polybrene; the samples were applied in 0.5% $NaDodSO_4$. + indicates identification by HPLC; numerical values show nmol recovered; and (+) indicates low yield (His, Arg) or the presence of contaminants (TA3). Cys in position 10 was not detectable in the native proteins, but was identified after [^{14}C]carboxymethylation of the TA3 protein (also indicated by +). Residues 18–20 in Namalwa 53-kDal protein were not analyzed.

quences were obtained. Neither of these appeared to be highly similar to the NH_2-terminal structure of the 53-kDal protein.

These results, therefore, show that EBNA is structurally different from the 53-kDal protein, in agreement with previous observations from amino acid composition and peptide mapping (12).

DISCUSSION

Our results establish that the 53-kDal proteins found in virally and chemically transformed cells of mouse and human origin are very similar. These proteins have been shown to be identical in the NH_2-terminal sequence (Table 2) of the first 20 residues, to have highly similar amino acid compositions (Table 1), and to have closely related peptide maps (Fig. 3). In addition, the 53-kDal protein purified from Meth A cells crossreacts with a rat monoclonal antibody made by hyperimmunization with rAW 112 cells (13) and a mouse monoclonal antibody raised against a methylcholanthrene-induced sarcoma, cMS4 (18) (data not shown). Therefore, these proteins may be expected to have evolutionarily highly conserved structures in murine and human tissues. This fact further suggests that the 53-kDal protein has an important and constant function in these two species. The finding of conserved structures is also compatible with previously reported peptide comparisons (15) and antigenic cross-reactivities (18, 19).

Another characteristic of the 53-kDal proteins is their binding to DNA, as shown by the present purification scheme and the fact that these proteins are primarily associated with nuclei. This association is of great interest in view of the possibility that this interaction may relate to the role of large T antigen in promoting DNA synthesis.

The NH_2-terminal amino acid found for each purified 53-kDal protein is proline. A protease cleaving X-Pro would be required to form such a chain if an acid cleavage has not occurred during the purification. However, this NH_2-terminal residue is reproducibly recovered in high yield (Table 2), and is ascribed to the main component, which can be shown to have the original 53-kDal size (Fig. 2). The finding of an NH_2-terminal proline residue, therefore, suggests that the enzymes processing nascent protein chains need not have the restrictions in substrate specificity against peptide bonds involving proline that is frequently noticed with other proteases. This conclusion is also compatible with the finding of NH_2-terminal proline in some other proteins (27).

Now that sequence data for a 53-kDal protein have become available, the primary structure was screened for similarities with that of small and large T antigens from SV40 (28), and that of the middle T antigen of polyoma virus (29). Also examined were the sequences of two structurally known immunoglobulin γ chains from mouse and one such μ chain from man (27), because lymphoblastoid cell lines are one source of the 53-kDal protein. Finally, all proteins with known sequences (27) identical to those found in the 53-kDal protein around proline (Pro-Gly-His) and cysteine (Gly-Cys-Val-Val) residues were analyzed, which included other immunoglobulin chains. All possible 20-residue segments of 13 different proteins were compared with the 53-kDal protein NH_2-terminal structures in a total of 4478 alignments (30). The results are given in Table 3; they show that in no case was a clearly significant alignment discovered. The three most similar pairs between the 53-kDal protein sequence and any other protein (see Table 3) involved internal segments of the SV40 large T antigen (one sequence with seven identities, starting at position 268), the polyoma middle T antigen (six identities, starting at position 191), and human myoglobin (six identities, starting at position 22). If one considers the remaining, nonidentical, residues, the myoglobin

Table 3. Comparisons of the 53-kDal protein sequence with the structures of T antigens, immunoglobulins, and proteins with similar sequences around proline or cysteine

Protein compared with 53-kDal protein	Number of alignments	Number of 20-residue alignments		
		Seven identities	Six identities	Five identities
SV40 small T	187	—	—	—
SV40 large T	639	1	—	3
Polyoma middle T	445	—	1	3
Human Ig μ	581	—	—	4
Mouse Ig γ_1	453	—	—	—
Mouse Ig γ_2	460	—	—	—
Human Ig ε	560	—	—	2
Human Ig λ_v	122	—	—	—
Dihydrofolate reductase	199	—	—	1
Myoglobin	166	—	1	—
Flavodoxin	151	—	—	1
Aldolase	374	—	—	1
Azurin	141	—	—	—
Total	4478	1	2	15

All possible 20-residue fragments from each protein were aligned with the NH_2-terminal structure of the 53-kDal protein and the number of identities was recorded in each alignment.

alignment actually appears to be the best, although all proteins examined lack any statistically significant identity. It is concluded from this comparison that the 53-kDal proteins bear no clear relationship to transformation-related proteins with known structures, to proteins of similar cellular origins, such as the immunoglobulins, or to proteins with similar structures around proline or cysteine residues.

Protein kinase activites appear to be important in transformation (31–36) and have been found to be associated with the 53-kDal protein of Meth A (19). Because the 53-kDal type of protein is associated with EBNA in Epstein–Barr virus-transformed cells and with large T antigen in SV40-transformed cells, it might be possible that the viral proteins serve as phosphoacceptors for a 53-kDal kinase or else influence such a kinase. It is of interest to note in this context that dose factors may be important in transformations (37, 38). Independent of these and other possible functional roles, the present results demonstrate close homology between different 53-kDal proteins and give the structural data for such proteins.

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