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p53 Shares an Antigenic Determinant with Proteins of 92 and 150 Kilodaltons That May Be Involved in Senescence of Human Cells

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A panel of primary human cells and virus-transformed derivatives were tested for events that coincide with immortalization. In all primary and precrisis cells, two proteins of 92 and 150 kDa that shared an epitope with p53 were found; in most of their immortalized derivatives, however, they were absent. Expression of these proteins may be involved in senescence.

Human diploid cells, in contrast to rodent cells, are highly refractory to full transformation by viral and cellular oncogenes. Expression of the simian virus 40 (SV40) early region can lead to the induction of most characteristics of transformation in primary human cells (4). Although these cells show an extended lifespan, they are not immortalized and will irreversibly enter a G_1-arrested state followed by degeneration, called senescence or crisis phase. Adenoviruses (Ad) can transform human embryonic retinoblast (HER) cells with a reasonable efficiency, but other human cells are transformed with extremely low frequencies (6, 22). Ad-transformed foci that eventually appear generally become senescent after a limited number of cell divisions. Clonal immortalized cell lines have been isolated by the continuous passaging of large numbers of SV40- and Ad-transformed cells (12, 16, 22). The low frequency (approximately 10^{-8}) of immortalization suggests that this event requires the additional inactivation of a senescence gene (for a review, see reference 16). The extended lifespan after transformation with SV40, the dependence of immortalization on viral transformation, and the frequency of immortalization indicate that senescence can be partly suppressed by the SV40 T antigen and that in these cells inactivation of only one allele of a senescence gene may be sufficient for immortalization (12). Cell fusion studies have shown that immortalized transformed human cells can be divided into several groups that complement each other's loss of mortality (16). This indicates that more than one gene is involved in senescence. Senescence genes are assumed to belong to the class of tumor-suppressor genes. Recently, the product of the 11p13 Wilms' tumor locus, as well as the retinoblastoma protein (pRB), have been suggested to play a role in senescence (11, 17). The tumor-suppressor product p53 is another candidate for a senescence protein. p53 has been implicated in the immortalization of rodent cells (for reviews, see references 13–15 and references cited in reference 20). In primate cells, the interaction of SV40 large T and p53 is much weaker than in rodent cells, which might explain the reduced ability of SV40 to immortalize primate cells.

In our search of events leading to immortalization, we compared panels of primary human cells and their pre- and postcrisis transformed derivatives. In a first experiment, p53 from primary HER cells and an immortal Ad12 E1-transformed derivative was compared by immunoprecipitation with monoclonal antibody PAb122 (8) followed by one-dimensional (1-D) and 2-D electrophoresis. In agreement with earlier reports (5), p53 focused as a series of proteins with isoelectric points between 6 and 7. Its level was strongly increased in the Ad-transformed cells but no differences in isoelectric focusing were obvious (Fig. 1C and D). However, we observed a notable difference between the primary and the Ad-transformed HER cells: two proteins of 92 and 150 kDa coprecipitated with p53 in the primary cells but not in the transformed derivative (Fig. 1C and D and 2A). These proteins were not precipitated by more than 10 different monoclonal antibodies raised against nonrelated antigens that were used as negative controls (Fig. 1B; data not shown). Moreover, neither protein was detectable after 2-D electrophoresis of total-cell lysate (Fig. 1A). Thus, both the 92 and the 150 kDa proteins are minor cellular proteins and not generally contaminating polypeptides.

The 92-kDa protein was only sporadically detectable on 1-D gels (Fig. 2A). However, the signal of the 150-kDa protein was comparable to p53 on 1-D gels, while on 2-D gels it focused with difficulty and could not always be identified with certainty (Fig. 1C and 2A). Compared with the 92-kDa protein the 150-kDa protein was more critically dependent on the immunoprecipitation conditions used; increased stringency resulted in a strongly reduced signal of this protein (data not shown). Neither protein could be labeled with $^{32}PO_4$, in contrast to p53 (data not shown).

To test whether the 92- and 150-kDa proteins are associated with p53, several experiments were carried out. First, monoclonal antibodies PAb1620 (2) and PAb1801 (3), which both recognize epitopes on human p53 other than that recognized by PAb122, were compared in immunoprecipitation. Coprecipitation of the 92- and 150-kDa proteins appeared to be strictly limited to PAb122 (Fig. 1E). Therefore, either these proteins are not bound to p53 or association blocks the epitopes for the other two antibodies. This latter possibility is very unlikely, because PAb421, which recognizes an epitope distinct from but very close to the PAb122 epitope, also did not precipitate the 92- and 150-kDa proteins (21; data not shown). Second, we used PAb122 after three

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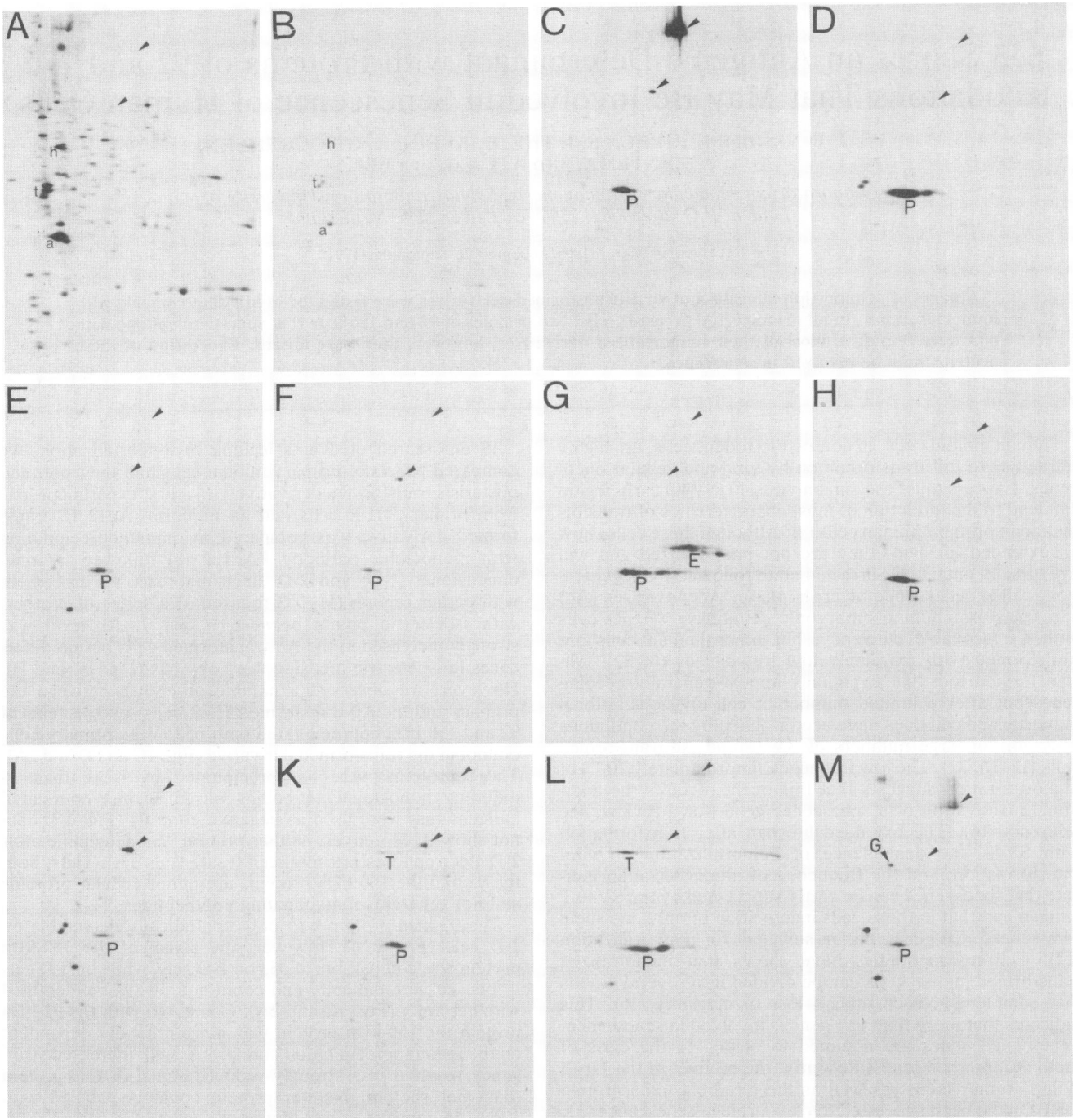

FIG. 1. 2-D gel analysis of [^{35}S]methionine-labeled proteins. The positions of the 92- and 150-kDa proteins are indicated by arrows. P, p53; T, SV40 large T; E, E1B-55kD; G, gelsolin. The positions of actin (a), tubulins (t), and the 73-kDa heat shock protein (h) are also indicated. Isoelectric focusing was performed with ampholines (LKB) of pH 3.5 to 10, 4 to 6, 5 to 8, and 9 to 10, in a 10:1:1:1 ratio, resulting in a pH range from 4.6 (left side; anode) to 8.4 (right side; cathode). (A) Total-cell lysate of VH10 (3.10^5 cpm); (B) immunoprecipitation from HER cells with a negative control monoclonal antibody, directed against blood-clotting factor VIII; (C) PAb122 precipitation from HER cells; (D) HER/Ad12 precipitated with PAb122; (E) VH10 precipitated with PAb1801; (F) Reprecipitation with PAb122. After immunoprecipitation with PAb122 from VH10 lysate and being washed 3 times in E1A buffer, the pellet was suspended in E1A buffer containing 1% SDS, boiled for 5 min, diluted 40 times in E1A buffer (9) without SDS, and reprecipitated with PAb122; (G) HEL/Ad5-7 cells precipitated with PAb122; (H) COLO 320 cells precipitated with PAb122; (I) PAb122 precipitation from primary VH10 cells; (K) VH10/SV precrisis cells precipitated with PAb122; (L) VH10/SV postcrisis cells precipitated with PAb122; (M) mixed immunoprecipitations from VH10 cells. A PAb122 precipitation from VH10 lysate (10 μCi) was mixed with an antigelsolin precipitation from VH10 lysate (2.5 μCi). Cells were labeled for 3 h, and immunoprecipitations were performed as previously described (20) in a buffer optimized for the coprecipitation of E1A-associated proteins (9) (A, C, E, F, G, H, and M) or in a buffer described to retain polymerase α complexes (18) (B, D, I, K, and L). The focusing dimension was followed by electrophoresis on 10% polyacrylamide gels.

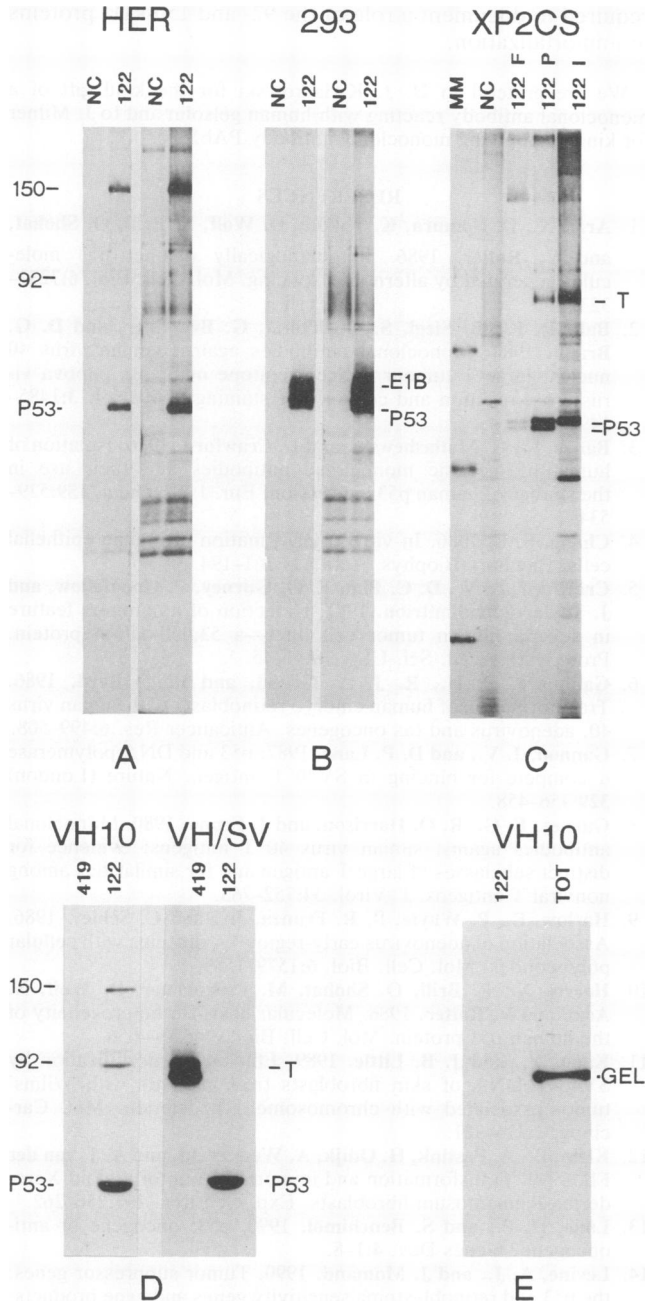

FIG. 2. 1-D analysis of p53 and coprecipitating proteins. Immunoprecipitations were performed with PAb122 (122), a negative control antibody (NC; anti-FVIII), or PAb419 (419; directed against SV40 large T antigen). (A and B) Two different exposures are shown: left lanes, 40 h; right lanes, 160 h. (A) Primary HER cells; (B) 293 cells; (C) XP2CS fibroblasts and SV40-transformed precrisis and immortal postcrisis derivatives, indicated by F, P, and I, respectively; (D) reprecipitations from primary VH10 cells and SV40-transformed postcrisis cells as described in the legend to Fig. 1. The first precipitations were carried out with PAb122, split in two, and reprecipitated with either PAb419 or PAb122; (E) Western blot from primary VH10 cells, performed as previously described (20). Cellular protein (1 mg) was precipitated with PAb122 and blotted subsequently (122), or 10 μg of total cellular protein was blotted directly (TOT). Blots were incubated with a monoclonal antibody directed against gelsolin (GEL). Positions of the 150- and 92-kDa proteins, p53, E1B-55kD (E1B), SV40 T antigen (T), and gelsolin (GEL) are indicated. All precipitations were done in E1A buffer (9).

Primary cells			Precrisis			Postcrisis		
	92kD	150kD		92kD	150kD		92kD	150kD
VH10	+	+	VH10/SV	+	+	VH10/SV	-	±
VH12	+	+						
HER	+	+	HER/SV	+	+			
						HER/Ad5	-	-
						HER/Ad12	-	-
HEL	+	+	HEL/Ad5-1	+	+	HEL/Ad5-7	-	-
			HEL/Ad5-5	+	+	HEL/Ad5-8	-	-
XP2CS	+	+	XP2CS/SV	+	+	XP2CS/SV	+	+
XP3MA	+	+						
XP2BI	+	+						
						293	-	±
						COLO 320	-	-
						T24	-	-
						HeLa	±	-

FIG. 3. Detection of 92- and 150-kDa proteins in primary human cells and pre- and postcrisis transformed cell lines.

sequential precipitations with PAb1801. After the PAb1801 precipitations, the 92- and 150-kDa proteins were still precipitated by PAb122 while p53 was no longer detectable (data not shown). Finally, we used an immunoprecipitation-dissociation-reprecipitation method. PAb122 precipitations were dissociated by boiling in 1% sodium dodecyl sulfate (SDS), which was followed by dilution and reprecipitation with the same antibody. The stable association between SV40 large T and p53 was completely dissociated by this procedure (Fig. 2D; VH/SV). However, the 92- and 150-kDa proteins were still precipitated by PAb122 after boiling the samples in 1% SDS (Fig. 1F and 2D; VH10), also indicating that their precipitation is not caused by association with p53. Subcloning of the PAb122-producing hybridoma by limiting dilution and the use of tissue culture supernatants from several independent clones showed that the precipitation of the 92- and 150-kDa proteins was not due to a contaminating antibody. We conclude that the 92- and 150-kDa proteins are not associated with p53 but are recognized by monoclonal antibody PAb122. Such specific cross-reactions of a monoclonal antibody can be accidental but can also point to a functional homology between proteins. The epitope of PAb122 is located at the extreme C terminus of p53 (21); it is conserved between mouse and man and is absent in the products of a subpopulation of alternatively spliced p53 mRNAs (1). The latter two facts may indicate that the epitope forms part of a functional domain.

The observed difference between primary and Ad12-transformed HER cells prompted us to screen a large panel of primary cells, virus-transformed cells, and human tumor cell lines by PAb122 precipitation followed by 2-D electrophoresis (Fig. 1 and 3). Seven isolates of primary human cells, including foreskin fibroblasts (Fig. 1I; VH10 and VH12), HER cells, human embryonic lung (HEL) cells, and three different xeroderma pigmentosum (XP) patient fibroblasts, all expressed the 92- and 150-kDa proteins. However, the 92-kDa protein was not detectable in any of five human Ad E1-transformed cell lines tested (Fig. 1G and 3), and only in 293 cells was a low amount of the 150-kD protein observed (Fig. 2B). Immortalized SV40(*ori*)-transformed VH10 cells did not express the 92-kD product (Fig. 1L), and 1-D analysis showed a reduction of 1 to 2 orders of magnitude in the expression of the 150-kD protein (Fig. 3). However, a protein of slightly higher molecular mass was detected in these cells (Fig. 1L). This might be the large subunit of DNA polymerase α (180 kDa), because of its location in 2-D gels (data not shown) and the existence of trimeric complexes between p53, SV40 large T, and DNA polymerase α (7). As

an exception, postcrisis SV40(*ori*)-transformed XP2CS cells expressed both the 92- and 150-kDa proteins (Fig. 2C) (see below). In addition, several human tumor cell lines were studied; the 150-kDa product was absent in the colon carcinoma cell line COLO 320 (Fig. 1H), the human bladder carcinoma T24, and cervical carcinoma HeLa cells. The 92-kDa protein was scarcely detectable in HeLa cells, at least at a 10-fold-lower level than in any of the primary cells. In contrast to the immortal transformed cell lines, the 92- and 150-kDa proteins were expressed in two precrisis Ad E1-transformed cell lines and three precrisis SV40-transformed cell lines (Fig. 1K and 3). Together, these data indicate that absence of the 92- and 150-kDa proteins is not a direct effect of expression of the viral oncogene products but correlates with immortalization of human cells. In most cases, the two proteins are detected simultaneously.

The immortal XP2CS/SV cells which exhibit normal expression of both proteins may represent another complementation group of immortalization. Interestingly, in the XP2CS fibroblasts the two alleles of p53 could be distinguished as a result of a variation in codon 72 (10). Whereas the immortal derivative of this cell line still expressed the 92- and 150-kDa proteins, it had lost expression of one allele of the p53 gene (Fig. 2C). This suggests that p53 can be involved in senescence.

It is clearly of importance to determine whether loss of the 92- and 150-kDa proteins is causally related to immortalization or represents an indirect effect. Direct involvement can only be demonstrated after cloning the gene(s) or identifying its products. The signal of the two proteins is very weak, particularly that of the 92-kDa protein. Therefore, our attempts to identify their nature have focused on comparison of their characteristics with known proteins. In a recent paper, Vandekerckhove et al. (19) described the 90-kDa protein gelsolin as the most prominently downregulated marker of transformed human cells. We compared gelsonin with the 92-kDa protein precipitated by PAb122. By using an anti-gelsolin monoclonal antibody, gelsolin could readily be detected on Western immunoblots containing 10 μg of cell lysate from human foreskin fibroblasts but not when a PAb122 immunoprecipitation from a 100-fold-greater amount of cell lysate was blotted (Fig. 2E). Moreover, gelsolin and the 92-kDa protein precipitated by PAb122 were found to be distinct when immunoprecipitations of the two proteins were mixed and compared by 2-D gel electrophoresis (Fig. 1M). In addition to gelsolin, we studied the DNA polymerase α protein complex, the E1A-associated 90-kDa protein, the pRB protein, the human glucocorticoid receptor, and the 80-, 90-, and 100-kDa heat shock proteins, none of which were found to colocalize on 2-D gels with the polypeptides precipitated by PAb122 (data not shown).

In conclusion, we detected two proteins of 92 and 150 kDa because of their cross-reactivity with an anti-p53 antibody. These proteins are absent in many immortalized human cells while viral transformation per se did not alter their level. We could not yet identify their nature nor could we verify a functional relationship with p53. In addition to the shared antigenic determinants, such a functional relation is also suggested by the loss of wild-type p53 in many human tumors and tumor cell lines, by the ability of mutant p53 to immortalize primary mouse and rat cells, and by the aberrant p53 expression in SV40- and Ad-transformed cells. Another interesting parallel is the absence of expression of one allele of p53 in the immortal XP2CS cells that still expressed the 92- and 150-kDa proteins. Obviously, additional studies are required to document a role of the 92- and 150-kDa proteins in immortalization.

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