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Tumour Suppressor Genes and Molecular Chaperones [and Discussion]

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Tumour suppressor genes and molecular chaperones

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SUMMARY

The two tumour suppressor genes that are most commonly inactivated in human cancer are the p53 gene on chromosome 17 and the retinoblastoma (Rb) gene on chromosome 11. Recent studies of both gene products suggest that they are able to act as powerful negative regulators of cell division. The Rb gene seems to exert this activity by physically complexing to a variety of specific transcription factors and inactivating their function. The capacity of Rb protein to bind these factors is regulated by phosphorylation. The Rb protein can therefore be seen to act as a chaperone for these factors. The p53 protein also may act in part by regulating transcription but may also interact directly with the DNA replication apparatus. The growth suppressive function of p53 is induced by DNA damage leading to an attractive model of p53 as an essential checkpoint control. The p53 protein interacts with members of the hsp70 chaperone family which we now show can regulate its function.

1. INTRODUCTION

(*a*) *The genetic basis of cancer*

The development of malignant cancer in man involves multiple genetic changes. These changes occur sequentially over a prolonged period of time and are associated with the progressive evolution of clones of altered cells. The last decade has seen a revolution in our understanding of the process through the determination of many of the specific genes involved in this process. Whilst research in the early part of the 1980s concentrated on the importance of activating mutations in the proto-oncogenes such as *ras*, the last few years have seen an increasing emphasis on understanding the function of the tumour suppressor genes. The activity of these genes protects individuals from developing neoplasia. Their crucial role is apparent from the devastating affect of germ-line transmission of inactivating mutations in these genes. Affected individuals inheriting one defective copy of the tumour suppressor gene show very high incidences of neoplasia with a characteristic early onset. In accordance with Knudson's hypothesis (Knudson 1971), examination of tumours from these individuals shows that a second somatic mutational 'hit' has taken place that inactivates the wild-type allele, thus leading to complete loss of function in the tumour tissue. In individuals who inherit two wild-type alleles of the tumour suppressor gene, tumours occur less frequently, but somatic inactivation of both alleles of certain suppressor genes may commonly be found in their tumours.

(*b*) *The p53 and Retinoblastoma suppressor genes*

Two of the best-characterized tumour suppressor genes are the retinoblastoma gene, Rb, germ-line mutations in which are responsible for hereditary retinoblastoma (Levine & Momand 1990) and the p53 gene (Lane & Benchimol 1990), germ-line mutation in which is the cause of a generalized cancer susceptibility syndrome, the Li-Fraumeni syndrome (Malkin *et al.* 1990; Srivastava *et al.* 1990). The products of the Rb gene and the p53 gene both have a powerful capacity to negatively regulate cell growth. The retinoblastoma protein has many of the properties of a molecular chaperone, whereas the p53 protein's activity may be regulated by members of the hsp 70 family of chaperones (Pinhasi-Kimhi *et al.* 1986; Sturzbecher *et al.* 1987; Hainaut & Milner 1992; Hupp *et al.* 1992; Gannon & Lane 1991) and itself play a key role in the cellular response to geneotoxic stress (Kastan *et al.* 1991; Kuerbitz *et al.* 1992; Lane 1992). Understanding the activities of both gene products may have profound implications for the diagnosis and treatment of cancer because inactivation of their function is very common in a wide range of different tumour types.

2. MATERIALS AND METHODS

(*a*) *Production of p53*

The production and purification of mutant and wild-type p53 proteins by using recombinant *E. coli* systems has recently been described in detail (Hupp *et al.* 1992; Midgley *et al.* 1992). In brief, p53 was purified by heparin agarose and gel filtration chromatography from lysates of *E. coli* cells that overproduce p53 protein. These cells were developed by using a T7 polymerase-based expression plasmid system into which the full-length human wild-type p53 has been cloned.

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(*b*) *p53 DNA binding assay*

A gel mobility shift assay to measure p53 sequence-specific DNA binding activity was developed by using a ^{32}P-labelled double-stranded oligonucleotide probe containing the p53 consensus binding site defined by El-Deiry *et al.* (1992).

3. RESULTS AND DISCUSSION

(*a*) *Rb as a chaperone for transcription factors*

The Rb protein is a 110 000 Da molecular mass nuclear phosphoprotein (Lee *et al.* 1987). It physically interacts with the dominant oncogene products of three different DNA tumour viruses, as well as specific cellular transcription factors and components of the cell cycle network, including members of the cyclin family and the cdc-2 protein kinase family of proteins (Hu *et al.* 1992). As such it is attractive to imagine the Rb protein as playing an essential coordinating role linking the regulation of the cell cycle to the regulation of transcription. The ability of Rb to bind to its target proteins is profoundly regulated by its state of phosphorylation, as only the hypophosphorylated forms are able to bind to SV40 large T (Ludlow *et al.* 1989) and the E2F transcription factor (Chittenden *et al.* 1991; Chellappan *et al.* 1991). This phosphorylation of Rb is itself subject to cell cycle control, and growing evidence suggests that the hypophosphorylated form of Rb may be in a highly oligomeric state. This may form an insoluble anchor in the nucleus that would act to sequester transcription factors from their targets, effectively holding them in a latent state (Templeton *et al.* 1991). When cells respond to growth signals that encourage them to enter the cycle, specific kinases of the cdc-2 family are activated which in turn phosphorylate Rb. This leads to the release of Rb from its insoluble state and the coordinated release of transcription factors into an active state. The loss of this negative control loop caused by inactivation of Rb may predispose towards the development of neoplasia by removing a critical control on cell division.

(*b*) *Regulation of p53 function by the hsp70 chaperones*

Mutation of the p53 protein is one of the most common events in the development of human tumours (Hollstein *et al.* 1991). Usually these mutations involve the complete loss of one allele accompanied by point mutation of the other allele. Most of these point mutations are mis-sense mutations so that mutant protein is expressed in many tumours. Although the normal p53 protein normally has a very short half-life, the mutant protein produced in the environment of the tumour cell is very stable and accumulates to high levels. This accumulation of p53 can be readily detected by immunohistochemistry and quantitative enzyme linked immunosorbant assay (ELISA) techniques. The accumulation of p53 is emerging as a very interesting marker of poor prognosis in breast (Thor *et al.* 1992) and gastric cancer (Martin *et al.* 1992). The mutant p53 proteins in transformed cells are often found in a tight physical complex with hsc 70 protein (Pinhasi-Kimhi *et al.* 1986; Sturzbecher *et al.* 1987; Hupp *et al.* 1992). This complex may be of functional significance because a temperature-sensitive mutant of p53 that acts as a tumour suppressor gene at 32°C but not at 37°C is only found complexed to hsc 70 at the higher temperature, whereas a series of constitutive mutants in p53 are complexed to hsc 70 at both temperatures (Gannon & Lane 1991). This suggests that the formation of a stable complex with hsc 70 may inactivate p53 function.

(*c*) *Two conformations of p53 protein, only one of which is bound to hsc 70*

A detailed immunochemical analysis of mutant p53 proteins has allowed us to identify monoclonal antibodies that react exclusively with two different conformational forms of the protein (Bartek *et al.* 1990; Gannon *et al.* 1990). Interestingly, many of the different point mutations in p53 found in tumour cells affect the conformation of p53 so that an epitope that is normally cryptic on the wild-type protein (but can be exposed by denaturation) is now exposed at the protein surface, and the protein can be immunoprecipitated from solution by antibody (PAb240) directed to this epitope. This mutant conformation of the protein also loses its ability to bind to certain other monoclonal antibodies (PAb246 and PAb1620) that recognize conformationally sensitive sites on the protein. This conformational change may be an important regulator of p53 function, as only protein in the 'wild-type' conformation is targeted by SV40 T antigen, whereas only protein in the 'mutant' conformation binds to hsc 70 (Bartek *et al.* 1990; Gannon *et al.* 1990; Hainaut & Milner 1992). The 'mutant' conformation of p53 may well be inactive in growth suppression. In certain transformed rat cell lines, this change in conformation is also associated with changes in protein stability, the 'mutant' conformation having a longer half-life. The different conformations may also differ in their interaction with the nuclear transport mechanism, as the mutant conformer tends to accumulate in the cytoplasm whereas the wild-type conformer is predominantly nuclear. Both of these effects may be mediated by the interaction with hsc 70. Certainly there is a precedent for the effect of hsc 70 on the nuclear transport from studies of the transport of 'cytoplasmic' mutants of SV40 large T antigen.

(*d*) *Using phage display libraries to define the conformational change in p53*

To try and understand this common conformational change in more detail, it seemed sensible to try and define precisely the cryptic epitope recognized by the PAb240 antibody. To do this, phage that bind the antibody were isolated from a random hexapeptide phage display library (Scott & Smith 1990). By using a high-stringency selection using very low concentrations of antibody in the final rounds of selection, four phage were isolated that were all bound with high

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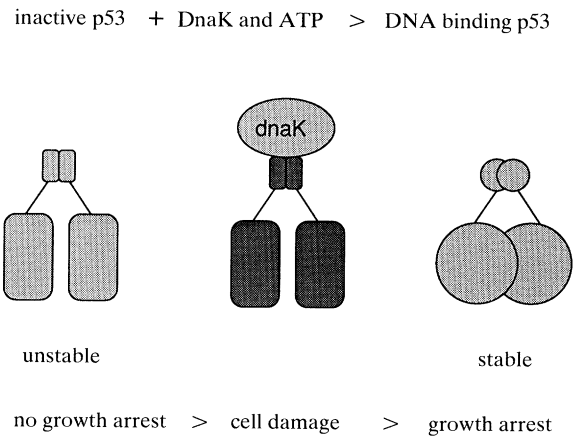

Figure 1. A model of activation of the DNA binding function of p53 by DnaK.

affinity by the PAb240 antibody. It was striking that, on sequencing, all four phage were found to encode highly related hexapeptides. All of the inserts encoded a contiguous four amino acid sequence arginine, serine, histidine, valine (RHSV) that is present in p53. Further work confirmed the central importance of these amino acids in the PAb240 epitope (Stephen & Lane 1992). The location of the epitope in the centre of the p53 molecule fits well with current ideas of p53 structure and may provide a target for novel therapeutics. As current work suggests that the C terminus of p53 may bind hsc 70, the result also suggests that conformational changes in the central region of the protein may also affect the C terminus to alter its display of an hsc 70 binding site.

(*e*) *The function of p53*

The precise function of the p53 protein has not yet been determined. However, recent work has shown that it can act as a transcriptional regulator and has a sequence-specific DNA binding activity (see Lane (1992); Vogelstein & Kinzler (1992) for review). This capacity to bind to DNA seems to be essential for the growth suppression function of p53 as all mutant p53s tested have lost this activity, and mutants of p53 that are temperature sensitive for DNA binding are also temperature sensitive for growth suppression. Two models for this relation can be suggested. Either p53 acts as a transcription factor to promote the transcription of growth arrest genes or repress the transcription of growth promoting genes, or it is possible that p53 may interrupt DNA replication directly, as it has been shown to do in viral models (Braithwaite *et al.* 1987; Sturzbecher *et al.* 1988), by interacting with cellular initiator proteins or blocking cellular origins of replication. In either case the regulation of this function and its loss in p53 mutant tumour cells becomes a matter of great interest. We have recently shown that the DNA binding activity of p53 is normally cryptic but can be constitutively activated by removal of the carboxy terminal 30 amino acids of the protein. Antibodies to this C terminal region can also act as potent activators (Hupp *et al.* 1992).

(*f*) *Regulation of p53 function by DnaK*

We attempted to find cellular proteins that could activate the cryptic DNA binding activity of p53. By using gel mobility shift assays we found that DnaK purified from *E. coli* was an efficient activator of DNA binding *in vitro*. The reaction was ATP dependent and required a small molar excess of DnaK to p53. This activation reaction is extremely intriguing and may have physiological relevance, as current models of p53 function suggest that it may act as part of a damage control pathway. It would them make sense that activation of p53 may be done by inducible members of the heat shock family of chaperones. The activity probably reflects a subtle conformational reorganization of the p53 molecule because both the active and inactive forms of the protein are oligomeric (figure 1).

(*g*) *The p53 checkpoint pathway*

Treatment of cells with a wide range of DNA damaging agents blocks the degradation of p53 (Maltzman & Czyzyk 1984; Lu *et al.* 1992; Kastan *et al.* 1991) leading to accumulation of the active protein. The high levels of p53 then seem to play a critical role in arresting DNA synthesis in the responsive cell (Kuerbitz *et al.* 1992). The p53 protein can then play a key role in maintaining genetic stability, acting as an inducible growth arrest signal that allows the cell to respond to DNA damage and blocks the appearance of clones of cells with an altered genetic complement. Such a model can explain the central role that inactivation of p53 might play in the evolution of a malignant tumour. Inactivation of p53 allows the more rapid evolution of variant clones within the tumour. This then creates a larger pool of variants from which more malignant variants will be selected. The induction of the DNA binding function of p53 by an hsc 70-like chaperone shows that a chaperone may play a key role in regulating this vital protein's tumour suppressor function.

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Discussion

W. J. Welch (*Department of Medicine and Physiology, University of California, San Francisco, U.S.A.*). Would the authors like to comment on why p53 is so labile with respect to mutation relative to some other oncogenes like *ras*, where the mutations are very specific around the GTP site?

D. P. Lane. I think that the simplest explanation is that the principal effect of a mutation is to inactivate a function. However, this is not a satisfactory explanation since there is increasing evidence that the mutant proteins have activity. One way to rationalize the data is to assume that all these mutations occur in a regulatory domain, and the amino and carboxytermini which might contain functional sites are preserved. So although there is a broad spectrum of mutation they may all share the same effect, i.e. the alteration of the folding pathway in the central part of the molecule, leaving the two ends intact and potentially functional. The mutant proteins retain the ability to form oligomers, and so can form oligomers with wild-type p53 and hence inactivate its function in a dominant-negative way.

P. Viitanen (*Du Pont de Nemours, Wilmington, U.S.A.*). Does either the carboxyterminal truncation or the

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antiserum against the carboxyterminus that activates p53 prevent the binding of hsp 70?

D. P. Lane. No, but this has not been tried in a reconstitution experiment, so we do not know whether the two compete for binding. We do know that the antiserum is able to efficiently immunoprecipitate the p53–hsp 70 complex. We are trying to determine the exact site of hsp 70 binding.

G. H. Lorimer (*Du Pont de Nemours, Wilmington, U.S.A.*). Does the protein isolated from p53 mutant cells grown at permissive temperature have the same *in vitro* properties as the wild-type protein? This might be expected if the mutant affects folding such that the protein cannot fold properly at the restrictive temperature, but does fold properly at the permissive temperature.

D. P. Lane. The mutant protein isolated from cells grown at the permissive temperature shows the same sequence-specific binding to DNA as the wild-type protein. However, the correlation between the functional shift, the growth arrest and the antibody change is not linear, because growth arrest is seen with a very small change in the proportion of molecules in one conformation or the other.

G. H. Lorimer. You will see only those molecules that remain in a monomeric state: any aggregated molecules will be lost in the precipitate during purification.

M. Zylicz (*University of Gdansk, Poland*). Have the authors determined whether there is any phosphorylation or dephosphorylation of the carboxyterminal domain of p53 in the presence of DnaK?

D. P. Lane. No.

M. F. Perutz (*MRC Laboratory of Molecular Biology, Cambridge, U.K.*). Is p53 a dimer or a larger oligomer? I ask because most DNA-binding proteins are dimers, and their activity is often regulated by the distance between the two DNA binding sites. Mutations that occur in the centre of the protein could alter this distance and so alter the affinity for DNA. The distance between these sites is critical since it determines whether the protein can or cannot bind to two successive major grooves in the DNA. Alternatively, p53 could be an allosteric protein and the mutation could alter the equilibrium between the two conformations.

D. P. Lane. There is very good evidence that p53 is at least a dimer, and consensus says that it is a tetramer. From our own experiments it certainly binds to DNA as at least a dimer, but the nature of native gels means that we cannot be certain that it is not binding as a tetramer.

W. J. Welch. I think we should remember that DNA binding does not necessarily imply transcription. Has anyone determined which genes may be bound by p53?

D. P. Lane. I agree. There is no progress at identifying which genes are bound by p53. There may be a more direct mechanism by which p53 inhibits DNA replication than via the transcription of particular genes. A more direct mechanism is attractive as it is rapid, and avoids the problem of transcribing possibly damaged genes.

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