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13. ABSTRACT (Maximum 200) Mutation of the p53 gene is very frequent in breast cancer. In normal cells induction of wild-type p53 function leads to either cell cycle arrest or cell death. Loss of this function can contribute to oncogenic cell transformation. Additionally the presence of mutant forms of p53 in breast tumor cells may actually facilitate the process of tumorigenesis. The properties of mutant p53 proteins in vitro and in breast tumor cell lines will be studied, experiments will focus on analysis of the structure and modification of mutant p53 proteins as well as the effect of cellular signalling on p53 function. Additionally it is planned to establish breast cell lines expressing inducible mutant p53 to determine the effect of such mutants on parameters of cell cycle, growth and death. We have discovered that all mutant forms of p53 tested are capable of binding specifically to p53 response elements present in p53 target genes at lower but not at physiological temperatures. Furthermore, we have identified a means by which such binding is stabilized at the higher temperature. This will allow us to explore means to develop molecules that might have the outcome of converting p53 in breast tumor cells from mutant to wild-type in function. One approach will be to develop a yeast-based screen for mutant p53 modifying genes. Such reagents would have clear therapeutic advantages.			
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DAMD-17-94-J-4275

The goal of the research funded by DAMD-17-94-J-4275 is to characterize the role of mutant p53 in breast cancer progression and to develop means to counteract the tumorigenic potential of mutant p53.

INTRODUCTION:

The p53 tumor suppressor protein plays a pivotal role in transmitting a signal from agents that induce genotoxic stress to genes that control the cell-cycle and apoptosis (1,2). p53 is a DNA binding dependent transcriptional activator which binds specifically to sites in genomic DNA that contain two or more copies of the consensus sequence: 5' R R R C A/T T/A G Y Y Y 3' (3). Such sites are identified as p53 response elements in a number of genes. Thus when cells are stressed by processes such as DNA damage or hypoxia the p53 protein normally present in low quantities in cells and in a latent, inert form, is activated both quantitatively and qualitatively to induce several target genes. Among these are included the genes expressing GADD45 (4), WAF1/p21/CIP1 (5), mdm2(6), cyclin G (7), bax (8) and IGFBP3 (9). Each of these genes contains a p53 response element and is therefore a likely target for p53 as a transcriptional activator to induce their expression. Each thus is likely to play a role in the p53 pathway in which, as a result of DNA damage, normal cells either undergo cell cycle arrest or cell death. When p53 is mutated and cannot respond thus to DNA damage, cells display the loss of growth control that is characteristic of tumorigenesis.

Mutation of the p53 tumor suppressor gene is among the most frequent events in breast cancer. Such mutation is frequently manifested as loss of one allele coupled with missense mutation of the other allele. Strikingly the location of the missense mutations are within the central region of the molecule (10) and this region contains the DNA binding domain (11). This highlights the likelihood that specific DNA binding and sequence specific transactivation is essential for the tumor suppressor function of p53 and that DNA binding is absent from mutant forms of p53. The missense mutant p53 proteins are frequently expressed at very high levels in breast tumor cells (12), and the p53 status in breast cancer has been linked closely to detection of p53 protein by immunostaining (13). Therefore understanding the function of the wild-type p53 protein and how it is altered when p53 is mutated will be critical to evaluating the prognosis of breast cancer. Importantly, the study of the properties of mutant p53 in breast cancer will hopefully lead to the development of ways to convert mutant protein to wild-type in function. The original aims of this proposal are as follows:

Specific Aims:

- (1) Analysis of the structural properties of mutant forms of p53 that are found in breast cancer and how they differ from that of the wild-type form as well as among themselves.
- (2) Analysis of the DNA binding properties of mutant forms of p53 in breast cell lines with

the aim of (a) finding cellular genes that are targets of mutant p53 activation and (b) conversion to or stabilization of the wild-type DNA binding activity of mutant forms of p53.

(3) Identification of cellular proteins from mammary cell lines that might be involved in mutant p53 gain-of-function in breast cancer.

BODY

Since submitting the most recent revised progress report (12/08/96) there have been no publications from this laboratory that were supported by DAMD-17-94-4275. However, progress is ongoing with respect to the statement of work in the original proposal, as outlined below.

(1) The role of the N-terminus in regulation of DNA binding of wild-type and mutant forms of p53.

Wild-type p53, by means of sequence-specific transcriptional activation (SST), induces target genes that regulate cell cycle and cell death. Tumor-derived mutant forms of p53, however, are defective in SST and cannot induce cell arrest or apoptosis. However, we discovered that many tumor mutant forms of p53 are conditionally defective and can bind to DNA in temperature sensitive fashion (14). We observed that several hot-spot mutant forms of p53 can bind to and even activate transcription from p53 response elements at sub-physiological temperatures, but not at 37°C.

The hypothesis to be tested is that mutant p53 proteins can be regulated such that they can assume a wild-type p53 conformation ideally leading to tumor cell death. Our goal is to determine ways in which mutant forms of p53 can be stabilized to bind productively to DNA under physiological temperatures.

Three general approaches are planned:

(1) We previously identified a region within the N-terminus of p53 approximately encompassing residues 45-55 that when bound by antibody will stabilize mutant p53 binding at 37°C (14). To extend this finding the following projects are planned:

(a) we have further characterized the role of the N-terminus in regulating DNA binding by the central core domain of p53:

We found that antibodies which bind to the N-terminus such as PAb 1801 (and other proteins that can interact with the N-terminus (specifically TAFs 42 and 62, TBP and mdm2) affecting the rate of disassociation of p53 from DNA. Quantitative competitive gel mobility shift assays and DNaseI footprinting were being used to reach these conclusions. This project is well underway and it is expected that we will be able to submit a manuscript within a few months.

(2) Using a yeast-based assay we have initiated a project in which we will screen for genes that can enable mutant p53 to activate transcription from wild-type p53 responsive promoters. The budding yeast *Saccharomyces cerevisiae* does not contain a p53 homologue, despite the conservation of the cell cycle machinery from yeast to man. Constitutive expression of human wild-type p53, but not tumor-derived mutant p53, is

able to activate transcription of reporter genes under the control of a p53-binding site (15).

Based on the observations of Ishioka et al. (16) and with constructs kindly provided by Dr. Richard Iggo, we have developed a genetic screen that should allow the isolation of p53 interacting proteins. Budding yeast were transformed with reporter plasmids containing various p53-binding sites driving the expression of *HIS3*. These include the p53-responsive elements from the *RGC*, *p21*, *mdm2*, *cyclinG*, *GADD45*, *Bax* and *IGF1* genes. Additionally, an idealized p53 binding site SCS (17) was cloned into the reporter construct as well. Ishioka et al. (16) demonstrated that the *RGC-ΔUAS1:pGAL1:HIS3* on a *TRP1/CEN* reporter is responsive to wild-type p53, by virtue of wild-type p53 binding to the RGC p53-binding sites, and allowing growth on histidine lacking medium (His⁻). We have confirmed and extended their observations with the other sites mentioned above (Tables 1 and 2). These reporter strains were transformed with 20 different tumor-derived and, in some cases, "hot-spot" p53 mutant constructs (e.g.: *pADH:p53^{R273H}* on *LEU2/CEN*) or control vector. We have also confirmed the observation of Ishioka et al. (16) who demonstrated that none of the tumor-derived p53 mutants tested were able to bind to and activate the RGC reporter construct, hence, no growth on His⁻ medium (see Tables 1 and 2). However, interestingly, we have observed that a subset of the mutants can activate transcription from a subset of the p53-responsive elements. In many, but not all, cases the transcriptional activation exhibited by mutant p53 proteins (eg.: p53^{Y220C}, and p53^{I254F}) is temperature sensitive (Table 1). moreover, p53 mutants were identified which retain wild-type-like function with certain p53 responsive elements (eg.: p53^{P177L}, p53^{R267W}, and p53^{C277Y}), even at physiological temperature, i.e. 37°C (Table 2). Conclusion: wild-type p53 displays transcriptional activation properties in budding yeast that are similar to those in mammalian tumor (including breast tumor) cells. No tumor derived mutant form of p53 displays the ability to activate the full spectrum of p53 responsive elements. However, some mutants are capable of activating transcription from some p53-responsive promoters.

For the "transactivation assay", once the initial characterization is completed, select strains will be transformed with either a human or mouse cDNA expression library (*URA3* marked) which has been constructed from a cell line where the status of p53 is known. Preferentially, p53 should be either mutated or deleted to reduce the amount of false positives obtained when screening (wild-type p53 would bind to and activate the reporter). Additionally, we will use a high copy number yeast genomic library (*URA3* marked). Budding yeast may contain a protein(s) capable of binding to and restoring wild-type function to mutant p53. Regardless of the library used, transformants will be replica plated onto His⁻ medium and scored for those colonies which grow at 30C, as well as 37C. The assumption is that only those library plasmids which encode for a protein(s) which can interact with or modify mutant p53 and confer wild-type DNA-binding function will grow in the absence of histidine. However, the possibility exists that the library plasmid may encode a protein which binds to and activates the reporter construct on its own. To test this, all library plasmids isolated will be retransformed back into the reporter strain and assayed for their ability to grow on His⁻ medium. We are interested

only in those library plasmids which require mutant p53 to grow on His⁻ medium. Additionally, the library plasmids isolated will be assayed for the ability to allow growth on His⁻ medium when other tumor derived mutants are present in the assay (e.g.: p53^{R175H}, p53^{R248W}, and p53^{Y220C}). It is important to show that the library plasmid is not specific for one tumor derived mutant but for a family of tumor derived mutants. Based on the results of the initial analysis described above, should genes be identified that confer wild-type activity on mutants it will be necessary to examine a number of different p53 response elements in order to determine the effectiveness of such gain-of function modifying genes.

(3) Role of p53 in response to antineoplastic agents.

As described in the previous progress report and in Chen et al. (18) we have established a number of human tumor cell lines which contain inducible p53 genes. Most of the work since the last report has utilized H1299 cells containing either wild-type p53, tumor derived mutants (his175, ser249, trp248), p53 (22/23), p53 Δ 30 (amino acids 1-363) and p53 Δ 62-91 (amino acids 1-61 fused to 92-393). The question posed addresses whether wild-type p53 can cooperate with anti-neoplastic agents to induce cell death and if so, which aspect of p53 is involved in this cooperation. We have observed that tumor derived mutants are incapable of inducing apoptosis, as measured by quantitation of cells with sub-G1 content of DNA, or by Annexin staining, under any conditions tested to date. However, wild-type p53 cooperates synergistically to induce cell death by the same experimental criteria, with a number of anti-neoplastic agents, including etoposide, daunorubicin, 5-fluoro uracil, camptothecin and hydroxyurea. p53, however, fails to cooperate with the spindle poison colchicine. Importantly, by Western blot analysis there are no significant changes in the level of p53 protein detected before or after treatment with any of the above agents.

Progress made with respect to the Statement of Work

As I explained in my last report, much of what we set out to accomplish has changed due to discoveries made in ours' and others' laboratories. Overall, however, the goal of our grant is to study and modulate mutant p53 function in order to eventually develop p53-based therapeutics for treatment of breast cancer remains the same. Thus the description of the tasks and our progress therein is very similar to that of the last report. Where additional progress has been made, the text is italicized.

Task 1: Examination of the properties of mutant p53 proteins:

(a) biochemical analyses of mutant p53 immunopurified proteins expressed from currently available baculoviruses: phosphate mapping, proteolysis protocols, hsp binding and oligomerization analyses

(a) We have examined the ability of mutant p53 proteins to be phosphorylated by cyclin dependent kinase, have examined thier state of oligomerization by sucrose gradient sedimentation analysis and non-denaturing gels. Neither of these tasks are ready for publication at present because the initial results did not provide any new insight into why mutants differ from wild-type protein with respect to DNA binding. With respect to examination of the effect of proteolysis, we did have some success, the results of which were pulbished in late 1993, i.e. very soon after the incipiation of the grant. Much of our work, as mentioned in the original report was predicated on our discovery of the temperature sensitive phenotype of mutant p53 proteins and so we have tried to further understand this property in biochemical terms. We have discoverd that wild-type p53 is also temperature sensitive such that it fails to resolve on non-denaturing gels at 37°C in contrast to experiments performed at 25°C. We observed that creatine phosphate will stabilize wild-type p53 at 37°C both for DNA binding and gel resolution. However, creatine phosphate does not stabiliz mutant p53 at 37°C.

(b) Cultured breast cell lines: develop purification procedures for mutant p53 from cells.

Since the last report we have focussed on examination of the DNA binding properties of p53 from extracts of yeast and mammalian cells. We have established conditions whereby p53 DNA binding can be detected in the absence of the "activating antibody" PAb 421.

(c) comparative analyses of breast cell derived p53 proteins.

(b) We did not develop procedures for the isolation of mutant p53 from breast cell lines

because our preliminary experiments did not warrant this at the time. Thus, having not documented any significant differences with purified proteins it did not seem effective to proceed with this line of research.

(d) construction of baculoviruses expressing additional mutant p53s found in breast cells with aim of continuing comparative analysis of different mutant forms of p53..

(c) When we started our research on mutant p53 supported by DAMD17-94-J-4275 we had generated baculoviruses expressing four of the mutant forms of p53 as baculovirus expression vectors. Since then we have successfully constructed a fifth, ser 149, and are in the process of making the sixth (and final) "hotspot" mutant, ie mutated at codon 282. We have actually had a number of cloning difficulties with this, but are optimistic that we will solve our problem. *Still no progress at the present time has been made in generating this additional baculovirus. However, we have initiated a project in which p53 is expressed in and purified from bacteria and are hopeful that this will provide an excellent and abundant source of wild-type and mutant p53 protein. During the final stage of this grant support period it is planned to examine many of the properties outlined in Task 1 using this source of p53 protein.*

Task 2: Analysis of the DNA binding properties of mutant p53 proteins:

(a) Continue studies on the temperature sensitive phenotype of mutant p53 proteins performing gel-shift, DNase I footprinting and methylation interference assays. Examine exiting p53 response elements, adding more as they become available from the literature. As new mutants become available, determine the generality of the ts phenotype.

These studies have been completed and were published: Friedlander et al. (14) We went on to examine the temperature sensitive phenotype *in vivo* as well and these results were included in the above paper. However, the results mandated a more careful analysis of the effects of transfected mutant p53 in tumor cells and these data yielded the interesting and important observation that one mutant, ala143, is defective in inducing apoptosis at 32°C in contrast to wild-type p53, but discriminates between p53 responsive genes. This was a collaborative study with M. Oren (Weizmann Institute) and was also recently published (19).

(c) Expand observations that PAb 1801 stabilizes DNA binding by mutant proteins: (i) generate mutants within the 1801 epitope region (ii) expression of N-terminal region of p53 with the aim of generating additional antibodies to further characterize the stabilization effect (iii) isolation of peptides from a phage display library that bind to the 1801 epitope region of p53 and testing whether they can stabilize mutant p53 DNA binding at 37°C.

We have recently initiated experiments designed to characterize the PAb 1801 epitope. As described above we have made the very interesting observation that, in contrast to antibodies directed against other epitopes in p53, this antibody has a marked effect on the dissociation rate constant of p53 bound to its cognate DNA. We attempted repeatedly to generate a baculovirus expressing the N-terminus of p53 without success. This may be due to the observation that the N-terminus has very little structure and is very protease sensitive. However, we have made a construct expressing the N-terminus fused to GST and can cleave the N-terminus (amino acids 1-83) from the GST. We will attempt to generate antibodies to this N-terminal peptide.

(b) Search for consensus sites for mutant p53 proteins operating selection assays at both 25°C and 37°C. Once identified, use sites as response elements in reporter assays in insect cells (grown at 27°C) or mammalian cells at 37°C.

A graduate student, T. Zhang, spent one year trying to isolate by using an established PCR based selection protocol DNA sequences that are bound by mutant forms of p53. She was unable to get the protocol to work, and, based on the experience of other members of our laboratory, it may be that our PCR machine is not optimal for this type of procedure. We are hoping to purchase a newer and better PCR machine shortly (funds permitting) and are anxious to make a new stab at this. This becomes particularly important given our exciting observation that some mutant p53 proteins display altered promoter specificity.

Task 3. Identification of proteins or peptides that interact with mutant p53.

Attempts to identify proteins that interact with mutant p53 are ongoing. In addition to using columns and immunoprecipitations from tumor cell lines, we have initiated a yeast screening program to identify factors that interact *functionally* with mutant p53 to restore its transcription function which is our penultimate goal. Progress towards that aim is described above and in Tables 1 and 2. Even though these experiments do not conform strictly to what we originally planned, they certainly conform in the best spirit of our intended goal which is to identify ways to convert mutant forms of p53 to the wild-type form in function. While we intend to pursue the other goals in parallel, i.e. identifying small peptides that can function in a similar matter, we need to find another collaborator or source of phage display libraries since our original collaborator, C. Siegal, is no longer working in this area. On a related note however, we had tried the effects of two peptides that had been isolated by screening a phage display library with our wild-type and mutant p53 proteins, and failed to see any effect of these peptides on either wild-type and mutant forms of p53 protein. We have been offered assistance by a colleague with experience in this area, and if necessary we will purchase a phage display library for these experiments. It should be noted that we have made what I think is an important

discovery regarding p53 in 1996, namely that there are a class of promoters that are activated by wild-type but not by mutant, apoptosis-defective forms of p53 (19). Furthermore, our data in yeast imply that the situation is far more complicated. Based on these observations, we must modify our original aim such that we ascertain which sites that mutants can bind to are relevant to induction of apoptosis. Thus, one good bet would be to require that a mutant be converted to a form that can bind to and activate the ba promoter. Although it is not clear whether ba induction is necessary and sufficient for apoptosis in breast cancer cells, this would still give an idea if we could convert mutant p53 to the most functional version of wild-type p53 as well.

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Table 1. Activation at sub-physiological temperatures

Cis-acting Element		β					
		Cont.	W.T.	M160I A161T	H193R	β Y220C	β I254F
none	24°	-	-	-	-	-	-
RGC	24°	-	+	-	-	-	-
SCS	24°	-	+++	+++	₁ +	+++	+++
	30°	-	+++	+++	-	+++	+++
p21	24°	-	+++	+++	₁ +	+++	+++
	30°	-	+++	+++	-	₂ +++	+++
mdm2	24°	-	+++	₁ +	-	₃ +	₁ +
	30°	-	+++	-	-	-	-
Cyclin G	24°	-	+++	+++	-	₁ +	₁ +
	30°	-	+++	+++	-	-	-
GADD45	24°	-	++	₁ +	-	₃ +	₁ +
	30°	-	+++	-	-	-	-
Bax	24°	-	-	-	-	-	-
	30°	-	+	-	-	-	-
IGF(A)	24°	-	-	-	-	-	-
IGF(B)	24°	-	-	-	-	-	-

"-"= no growth "+"= growth "++"= moderate growth
 "+++ "= wild-type growth "β"= β-scaffold maintenance

1: "+" growth after 2 days @ 24°C, no growth, 3 days, 30°C
 2: "+" growth by streak-out @ 30°C
 3: "+" growth after 3 days @ 24°C, no growth, 4 days, 30°C

Table 1. The ability of wild-type and tumor-derived p53 mutants to transactivate p53-responsive reporters at sub-physiological temperatures. Strains expressing wild-type or mutant p53 (from the *pADH1* promoter on a *LEU2/CEN* plasmid) and containing one of the following p53-responsive (*SCS:HIS3*, *RGC:HIS3*, *p21:HIS3*, *mdm2:HIS3*, *GADD45:HIS3*, *cyclinG:HIS3*, *Bax:HIS3*, *IGFBP3-BoxA:HIS3*, *IGFBP3-BoxB:HIS3* on *TRP1/CEN*) or control (Δ *UASpGAL1:HIS3* on *TRP1/CEN*) reporters were: 1) streaked out onto SC minus leucine minus tryptophan minus histidine plates and grown for 3 days at 24°C, 2 days at 30°C, and 2 days at 37°C; and 2) replica plated from SC minus leucine minus tryptophan plates grown at 30°C to SC minus leucine minus tryptophan minus histidine plates and grown for 2 to 3 days at 24°C and 1 day at 30°C, unless otherwise noted (see Table legend). Growth of the mutant p53-containing strains on histidine-deficient media was scored against the strain expressing wild-type p53 and containing the *p21:HIS3* reporter (+++). "Cont." is a *LEU2/CEN* vector expressing no p53, "W.T." is the wild-type p53 expressing plasmid pCUB7.

Table 2. Activation of reporters at the physiological temperature

Cis-acting Element		L22E							
		Cont.	W.T.	W23S	V143A	P177L	R267W	C277Y	R283H
none	30°	-	-	-	-	-	-	-	-
RGC	30°	-	++	-	-	-	-	-	-
SCS	30°	-	+++	+++	+++	+++	+++	-	+++
	37°	-	+++	+++	-	+++	+++	-	+++
p21	30°	-	+++	+++	¹ ++	+++	+++	² +++	+++
	37°	-	+++	+++	¹ +	++	+++	² +++	+++
mdm2	30°	-	+++	⁴ -	-	-	-	-	³ +
	37°	-	+++	-	-	-	-	-	+
Cyclin G	30°	-	+++	⁵ +++	-	-	-	-	+++
	37°	-	+++	⁵ +++	-	-	-	-	++
GADD45	30°	-	+++	⁴ ++	-	-	-	-	+
	37°	-	+++	⁴ ++	-	-	-	-	+
Bax	30°	-	+	-	-	-	-	-	-
	37°	-	+	-	-	-	-	-	-
IGF(A)	30°	-	-	-	-	-	-	-	-
IGF(B)	30°	-	-	-	-	-	-	-	-

"-" = no growth "+" = growth "++" = moderate growth "+++ " = W.T. growth
 "*" = contact mutant "•" = required for Mdm2 and TAF binding

- 1: "+++ " growth @ 24°C, heterogenous colony size @ 24°, 30°, and 37°C
- 2: "+" growth by streak-out @ 30° and 37°C
- 3: growth scored after 3 days
- 4: "+" growth by streak-out @30°C
- 5: "++" growth by streak-out @30° and 37°C

Table 2. The ability of wild-type and tumor-derived p53 mutants to transactivate p53-responsive reporters at physiological temperature.

Strains expressing wild-type or mutant p53 (from the *pADH1* promoter on a *LEU2/CEN* plasmid) and containing one of the following p53-responsive (*SCS:HIS3*, *RGC:HIS3*, *p21:HIS3*, *mdm2:HIS3*, *GADD45:HIS3*, *cyclinG:HIS3*, *Bax:HIS3*, *IGFBP3-BoxA:HIS3*, *IGFBP3-BoxB:HIS3* on *TRP1/CEN*) or control (Δ *UASpGAL1:HIS3* on *TRP1/CEN*) reporters were: 1) streaked out onto SC minus leucine minus tryptophan minus histidine plates and grown for 3 days at 24°C, 2 days at 30°C, and 2 days at 37°C; and 2) replica plated from SC minus leucine minus tryptophan plates grown at 30°C to SC minus leucine minus tryptophan minus histidine plates and grown for 2 to 3 days at 24°C and 1 day at 30°C, unless otherwise noted (see Table legend). Growth of the mutant p53-containing strains on histidine-deficient media was scored against the strain expressing wild-type p53 and containing the *p21:HIS3* reporter (+++). "Cont." is a *LEU2/CEN* vector expressing no p53, "W.T." is the wild-type p53 expressing plasmid pCUB7.