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13. ABSTRACT (Maximum 200 words) p53 is a cellular guardian of the genome integrity and a key player in preventing tumor formation; p53 mutation has been implicated in several types of cancer such as breast cancer. A very important fact in its functioning is how p53 selects which one of its cognate sites to bind and when. We think that the differential association of p53 with cellular proteins is a key biochemical event in p53 functioning. By DNA affinity chromatography using the p53 binding site present in the promoter 2 of the MDM2 gene (MDM2 P2-affinity chromatography) and nuclear extract from 3-4 cells, we showed the recruitment of the TATA Binding Protein (TBP) and the transcription factor SP1 to the MDM2 P2. The recruitment of TBP and SP1 to MDM2 P2 seems to be p53 dependent because we could not TBP or SP1 in the MDM2 P2 elution fractions when we used nuclear extract from 10-1 cells, which do not have p53.				
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FOREWORD

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PREFACE.

The Department of Defense, Breast Cancer Research grant has been determinant in our training and development as breast cancer researchers. Thanks to its support and by facing the challenges presented by our daily work, we have been greatly enriched; our comprehension of the breast cancer pathology has matured and broaden and our technical skills have grown.

We are immensely grateful to the DOD Breast Cancer Research Program for giving us this opportunity and look forward to making our liaison stronger.

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INTRODUCTION.

p53 is a tumor suppressor protein (Baker, S.J. et al. 1990; Levine, A.J. et al. 1991) involved in the control of cell division (Michalovitz, D. et al. 1990; Martinez, J. et al. 1991; Hartwell, L. et al. 1992; Livingstone, L. et al. 1992). When the cell suffers damage in its DNA, the p53 gene product responds depending on the extension and nature of the damage as well as on the cell type. p53 promotes either growth arrest (G1, G2/M, or postmitotic) (Kastan, M. et al. 1991; Kastan, M. et al. 1992; Kuerbitz, S.J. et al. 1992; Agarwal, M.L. et al. 1995, Lanni J.S. and Jacks T. 1998) or apoptosis, programmed cell death, (Clarke, A.R et al.; Lowe et al., 1993). These events prevent the cell from giving rise to a genetically altered progeny, a happening that can lead to tumor formation and cancer.

The purpose of this project is to gain more understanding about the molecular mechanisms by which p53 exerts its function(s). Some of these mechanisms are already known. p53 is a sequence-specific DNA binding protein (El-Deiry, W. S., et al. 1992; Kern, S.E. et al. 1991; Funk, W.D. et al. 1992; Bargonetti, J. et al. 1991; Bargonetti, J. 1993; Pavletich, N.P. et al. 1993), known to act as a transcription factor (O'Rourke, R.W et al 1990; Farmer, G. et al. 1992; Kern, S.E. et al. 1992). *In vitro* and *in vivo* experiments have shown that p53 can activate transcription of some genes such as GADD45, (Kastan, M. et al. 1992; Hollander, M.C. et al. 1993; Zhan, Q. et al 1993), MDM2 (Perry, M.E. et al 1993; Juven, T. et al. 1993; Fornace, A.J. et al. 1993; Barak, Y. et al. 1993), Waf1/p21 (El-Deiry, W. et al. 1993; Friedman, P. et al. 1992) as well as suppress transcription for other DNA elements (Subler, M.A. et al. 1992; Ginsberg, D. et al. 1991; Ragimov, N. et al. 1993; Deb, S. et al 1992). In addition, p53 can bind to DNA elements for which no function has yet been identified, for example, The Ribosomal Gene Cluster (RGC) (Kern, S.E. et al. 1991).

To further understand the mechanisms underlying p53 function(s), our goal is to identify cellular proteins that interact with the wild type p53 present in the normal breast cell line MCF10A (Muller, F. R. et al. 1994), with the wild type p53 present in the breast cancer cell line ZR75-1 (Engel, W.L., et al. 1978), and with the mutant form of p53 Hist 273 present in the breast cancer cell line MDA-MB-468 (Bartek, et al. 1990; Nigro, J.M., et al 1989; Chen, J.Y. et al. 1993). This analysis will be done by DNA affinity purification using the p53 specific binding sites present in the Ribosomal Gene Cluster (RGC) (Kern, S.E. et al. 1991) and in the Promoter 2 (P2) of the MDM2 gene (Juven, T. et al. 1993). The binding of p53 to its cognate site present in the MDM2 (P2) promoter activates transcription of the MDM2 gene (Barak, Y. et al. 1993; Barak, Y. et al. 1994), while the function of the p53 binding site present in the RGC is not known.

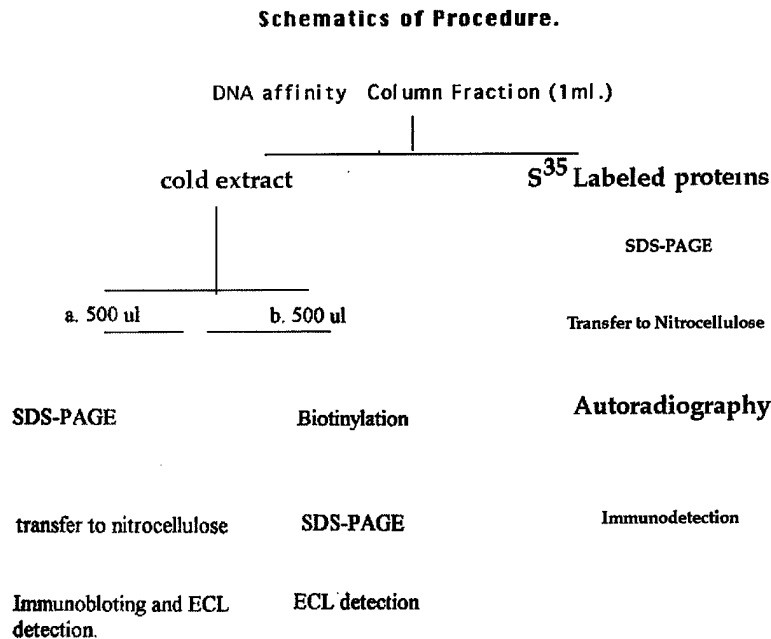
The p53 cellular level, under normal circumstances, is very low but upon DNA damage it is increased due to decreased degradation of the protein (Hall, P. A., et al. 1993). Because of this, and as a first control, we have set up the experimental conditions using the Ts. mutant p53 Val.135 present in the mouse

fibroblast cell line 3-4. The Ts. mutant p53 Val. 135 provides a well documented system for wild-type p53 dependent growth arrest, when the cells are grown at 32 °C (Michalovitz, D. et al. 1990; Martinez, J. et al. 1991).

There is evidence to suggest that p53 is part of a transcription complex (es) and that a differential association with various proteins might be part of the molecular mechanism by which p53 selectively activates or represses the genes under its control. *In vitro* and *in vivo* experiments have shown that p53 has the ability to associate with other proteins and that this association modulates p53 function. For example, the well-known transcription factor SP1 associates with p53 and the Sp1-p53 complex binds DNA (Borellini et al. 1993; Bargonetti et al. 1997). Even more, there is an Sp1-p53 binding motif present in some regulatory DNA regions (Borellini et al.; Macleod, M.C. et al. 1993) and Sp1 binding to DNA changes in the presence of a mutant p53 which is not able to bind DNA on its own (Bargonetti et al. 1997). Other proteins, with a relevant role in transcription, associate with p53; among them, the TATA binding Protein (TBP) and its associated factors Taf II 40 and Taf II 60 (Seto et al. 1992; Liu, X. et al 1993; Truant, R. et al. 1995). Besides the above mentioned, there have been other reports of p53 associated proteins; among them: **TFIIH** (Xiao, H. et al. 1994); **TAFII40** and **TAFII60** (Truant, R. et al. 1995); **p300/CBP** (Lill, N. L., et al 1997); **DP-1** (Sorensen, T. S., et al. 1996); **WT1** (Maheswaran, S. et al 1995); **Mdm2** (Momand, J. et al. 1992); **RPA** (Abramova, N. A. et al. 1997; Dutta, A. et al. 1993); and others (Sepehrnia, B., et al. 1996; Chen, Y. et al 1994). Most of these p53-associated proteins have been identified by co-immunoprecipitation experiments and using either in vitro translated proteins or overexpressing systems. We think that the presence of the p53-binding site, makes a difference regarding p53 association with other proteins and also that cellular posttranslational modification to p53 also influence this association. To the best of our knowledge, this is the first time that p53-associated proteins are being studied by DNA affinity chromatography.

MATERIALS AND METHODS.

Diagram 1.



Cells.

The 10-1 cell line is a mouse fibroblast cell line that does not have p53 because the p53 gene is deleted (Martinez et al., 1991). The 3-4 cell line is a stable p53 expressing cell line derived from the 10-1 cell line by cotransfection with the temperature-sensitive (Ts) mutant p53-Va1135 plasmid (ppLTRp53cGva1135) (Michelovitz et al., 1990) and a Neomycin resistant plasmid. In order to have a nuclear preparation with high amounts of p53 to use as a control, we infected *Spodoptera frugiperda* (Sf21) cells with a recombinant baculovirus expressing the wild-type human p53.

Nuclear Extracts.

We grew 3-4 and 10-1 cells at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) supplemented with 10% of heat inactivated Fetal Bovine Serum (FBS). When the plates were 80% confluent, we shifted the cells to 32 °C for 4 hours. We harvested the cells with 500 microliters, per plate, of lysis buffer, 8.8 milliliters of stock buffer (Hepes pH 7.5, 20mM; Glycerol, 20%; NaCl, 10mM; MgCl₂, 1.5 mM; EDTA, 0.2 mM; Triton X-100; DTT, 1mM; PMSF, 1mM; Aprotinin, 50 micrograms/ ml.; Leupeptin, 50 micromolar.) and 6.2 milliliters of water. Then we centrifuged the cell suspension at

2,000 rpm for 15 minutes to pellet the nuclei. We resuspended the pellet in 500 microliters, per plate, of nuclear lysis buffer (stock buffer 8.8 ml., NaCl 5M 1.47 ml. water 4.7 ml.) and rocked 1 hr. at 4 °C. Then, we centrifuged the preparation in microfuge for 10 minutes, to pellet the debris, recovered supernatant, aliquoted and kept it at -80 °C for further use.

We grew *Spodoptera frugiperda* (Sf 21) cells at 27 °C in TC-100 medium (GIBCO), supplemented with 10% of heat inactivated Fetal Bovine Serum. We infected them with recombinant baculovirus containing the human p53 gene and, 48 hours post-infection, we harvested the cells by centrifugation at 2,000 rpm for 15 minutes. We resuspended the pellet in 1.6 milliliter of Cowi lysis buffer / plate. We left the pellet suspension on ice for 30 minutes, spun at 2,000 rpm for 15 minutes and removed supernatant. Then, we spun the supernatant in a ss34 rotor at 20,000 rpm for 30 minutes, aliquoted the supernatant and kept at -80 °C.

Sephacryl S 300 gel filtration. Our goal experiment is a DNA affinity chromatography. In order to protect the DNA from being destroyed by nucleases, it is necessary to rid the crude nuclear extract of such nucleases. To achieve this purpose, we passed the crude nuclear extract through a column made of Sephacryl S 300 (Pharmacia) and perform the gel filtration procedure according to the manufacturer's specifications. We made a column 25 centimeters in length, 2 centimeters in width, used Tris buffer 0.1 molar KCL (TM⁺) to run the column and collected 25 fractions, 1 milliliter each. We discarded the first 10 ml. as void volume, pooled the rest of the fractions and stored the fraction pool at -80 °C.

Quantification. We normalized the experiments either for total protein or for amount of p53. For this purpose, we used the Bradford method to determine the amount of total protein contained in the nuclear extracts as well as in the Sephacryl fraction pool. We determined the amount of p53 by densitometry.

Electrophoretic Mobility Shift. Before using the Sephacryl fraction pool for the DNA affinity Chromatography, we tested its specific binding ability by Electrophoretic Mobility Shift Assays (EMSAs). We incubated 2.4 ng. of p53 contained in the crude nuclear extract and Sephacryl fraction pool preparation with ³²P labeled synthetic deoxyoligonucleotides (Operon), under DNA binding conditions (Hepes pH 7.8, 20mMolar; KCl, 100mMolar; EDTA, 1mMolar; Glycerol, 10%; DTT, 1mMolar; salmon sperm DNA, 1ug. per 30 microliters reaction). We included antibody against p53 (Pab 421) in some of the reactions. The incubation time was 20 minutes. Then, we resolved the DNA-protein complexes in a 4% nondenaturing acrylamide gel and visualized them by autoradiography. For the DNA affinity elution fractions, we incubated 5% of each fraction with SCS oligo (Superconsensus cognate site)(Operon) to detect p53, TFIID consensus oligonucleotide (Santa Cruz), to detect TBP and HIV LTR oligo (Operon), for SP1.

DNA affinity chromatography. Our goal is to identify cellular proteins that associate with wild-type p53 when p53 is bound to two of its cognate sites (RGC and MDM2 P2).

We decided to do it by identifying the proteins that co-purify with p53 using DNA affinity purification. For this purpose, we constructed two DNA affinity columns. One containing the p53 binding site present in the Ribosomal Gene Cluster (RGC) and the other one with the p53 binding site present in the Promoter 2 of the MDM2 gene (MDM2 P2). We constructed them by crosslinking the respective DNA deoxyoligonucleotides to CNBr activated Sepharose, according to the method of Kadonaga (Kadonaga, J. T. et al.). We loaded 10 micrograms of p53, contained in the Sephacryl S 300-fraction pool from 3-4 cells or the corresponding amount of total protein from the 10-1 cells, we passed the sample, over the column, 10 times at gravity flow, over each column. Then, we washed each column, 4 times, with 2 milliliters of buffer Z 0.1 molar KCl and eluted with 1 milliliter fractions of buffer Z with increasing salt concentration (KCl, 0.2 to 1.0 molar) (Kadonaga, J. T. et al). Then we processed the elution fractions as described in diagram 1. The synthetic deoxyoligonucleotides used to construct the DNA affinity columns were for RGC top, 5' TCGAGTTGCCTGGACTTGCCTGGCCTTGCCTTTTC3' and MDM2 P2 top, 5'GATCCCTGGTCAAGTTGGGACACGTCCGGCGTCGGCTGTCTCGGAGGAGCTAA GTCCTGACATGTCTCCG3'.

ECL detection of biotinilated proteins. When we performed the DNA affinity Chromatography experiments with cold nuclear extract, we split each elution fraction from the DNA affinity column in two parts, one for immunodetection by western blot and another half for detection of all the proteins present in such fractions by biotinylation, diag. 1. After the biotinylation step, we resolved the proteins by electrophoresis in a 10% SDS- Polyacrylamide gel, transferred them to a nitrocellulose membrane and incubated with Streptavidin-Horseradish Peroxidase. Finally, we visualized the protein-Biotin-Streptavidin-Horseradish Peroxidase complex (es) by the addition of Enhanced Chemoluminescence (ECL) kit reagents, which have the ability to react with the Peroxidase enzyme and produce a luminic signal that was captured on a film.

Metabolic labeling with S³⁵. We decided that biotinylation was not the best approach to detect all the proteins in the DNA affinity elution fractions. We made this decision based on the fact that not all the proteins biotinilate at the same extent and because it gives a high background (we will discuss this fact when we present the results). As an alternative, we metabolically labeled the cells by growing them in Dulbecco's Modified Eagle Medium (95 % Methionine free and 5% complete) supplemented with S³⁵ Methionine (20 uCi/ml.) for 5 hours. The proteins were resolved by SDS-PAGE electrophoresis and visualized by autoradiography.

Western Blot. To determine the presence of p53 in the elution fractions from the DNA affinity columns and to identify the proteins that co-purify with it, we resolved the DNA affinity elution fractions by SDS-PAGE in a 10% acrylamide gel. After resolving them, we transferred the proteins to a nitrocellulose membrane, over night at 0.3 amperes, and then probed with the corresponding antibodies.

Immunoprecipitation. Most of the proteins reported to associate with p53 have been identified by co-immunoprecipitation. We wanted to compare the proteins that co-purify

with p53 from the DNA affinity columns with those that co-immunoprecipitate with it. We made immunobeads by crosslinking PAb 421(anti p53) to Sepharose A beads. We incubated 15 microliters of anti p53 immunobeads with the protein preparation (nuclear extract or Sephacryl S300 fraction pool) in Buffer Z, 0.1 molar KCl, for 3 hours. After the incubation period, we washed the beads, 5 times, with 500 microliters of buffer Z 0.1 molar KCl and resolved the immunoprecipitated proteins by SDS-PAGE.

Cloning of MDM2 P2 into pGem3Z. We digested a PGL2 basic plasmid containing the MDM2 gene, with the restriction enzymes NsiI (Gibco) and ApaI (Gibco), which gave a 400-bp fragment . We then digested this 400 -bp fragment with Hin PIII (Gibco) to obtain our target fragment, which was 279 basepairs and had AccI cohesive ends. Next, we linearized pGem3Z(-) (Promega) by digestion with the restriction enzyme AccI. After this, we set up a ligation reaction having the excised MDM2 P2 fragment (279 bp), linearized pGem (3199bp) and T4 DNA ligase (Gibco), to obtain the cloned plasmid pGem-mdm2 that has 3478 bp. Finally, we obtained a piece of DNA having the Lac operator next to the mdm2-binding site by digesting pGem-mdm2 with the restriction enzyme EaeI. This digestion produced 3 fragments of sizes 1,442, 1,443 and 593 basepairs; the 593 basepairs one is our target DNA fragment. All the reactions were performed according to the manufacturer's specifications and as described in Molecular Biology Protocols. Ed.

STATEMENT OF WORK.

Task 1. Month 1-3: Run RGC affinity column with nuclear extracts from 3-4, TR9-7, ZR75-1, MDA10A, MDAMB468 and MDAMB157 cells; resolution and detection of these fractions, as described in methods.

Task 2. Month 4-5: **a.** Characterization, by molecular, weight of the proteins found in task 1, and partial identification by immunodetection. **b.** Cloning of human MDM2 P2 fragment in pGem plasmid, for GRAB system.

Task 3. Month 6-7: **a)** Construction of MDM2 affinity column and testing with extracts from insect cells infected with p53 containing baculovirus. **b)** Establishing the conditions for GRAB system using MDM2 fragment cloned in task 2

Task 4. Month 8-10: Run MDM2 affinity column with nuclear extracts from 3-4, TR9-7, ZR75-1, MDA10A, MDAMB468 and MDAMB157 cells; resolution and detection of these fractions as described in methods.

Task 5. Month 11: Characterization of the proteins found in task 4., by molecular weight and partial identification by immunodetection.

Task 6. Month 12-14: Run RGC affinity column with nuclear extracts from ZR75-1, MDA10A, MDAMB468 and MDAMB157 cells after treatment with Actinomycin D a chemotherapeutic agent known to induce G1 growth arrest in normal cells; resolution and

detection of these fractions as described in methods.

Task 7. Month 15-17: Same as task 6 but with MDM2 affinity column.

Task 8. Month 18: Characterization of proteins found in task 6 and 7 by molecular weight and partial identification by immunodetection.

Task 9. Month 19-21: Run RGC and MDM2 affinity columns with nuclear extracts from ZR751, MDA10A, MDAMB468 and MDAMB157 cells at the end of S phase, obtained by elutriation as explained in methods or drug treatment; resolution and detection of proteins as described in methods.

Task 10. Month 22-24: Same as task 9 but with nuclear extracts from cells at G1 phase of the cell cycle.

Task 11. Month 25: Characterization of the proteins found in tasks 9 and 10, by molecular weight and identification by immunodetection.

Task 12. Month 26-27: a) Run MDM2 GRAB system with nuclear extract from ZR75-1, MDA10A, MDAMB468 and MDAMB157 cells; resolution and detection of protein as described in methods.

b) Cloning of RGC fragment in pGem plasmid, for the GRAB system.

Task 13. Month 28: Same as task 12 a. but with RGC GRAB system.

Task 14. Month 29: Characterization and identification of the proteins found in tasks 12 a. and 13.

Task 15. Month 30-31: Run RGC GRAB system for nuclear extract from ZR75-1, MDA10A, MDAMB468 and MDAMB157 cell treated with Actinomycin D; resolution and detection of proteins as described in methods.

Task 16. Month 32-33: Same as task 15 but with MDM2 GRAB system.

Task 17. Month 34: Characterization of the proteins found in tasks 15 and 16, by molecular weight and identification by immunodetection.

Task 18. Month 35-36: a. Run RGC and MDM2 GRAB systems with nuclear extracts from ZR75-1, MDAMB468 and MDAMB157 cell at the end of G1 phase of the cell cycle.

b. Characterization of the proteins found in task 19, by molecular weight and identification by immunodetection.

RESULTS AND DISCUSSION.

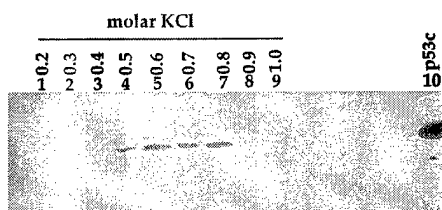
Our goal is to identify cellular proteins that associate with wild-type 53 when p53 is bound to 2 of its naturally occurring cognate sites, the binding site present in the MDM2 gene promoter 2 (MDM2 P2) and the other one present in the Ribosomal Gene Cluster (RGC). We chose to do it by DNA affinity Chromatography.

Our original plan was to perform the RGC affinity Chromatography experiments first and then, to perform the MDM2 affinity Chromatography ones; this idea is expressed in our Statement of Work, tasks 1 to 5. Now we think that it is more convenient to run both columns simultaneously. This is why, we are going to present our results according to their relevance to our goal and not in the order presented in the Statement of Work; although we will specify which task they belong to.

Task 3a. Construction of MDM2 affinity column and testing with extract from insect cells infected with p53 containing baculovirus.

We made an MDM2 P2 column as described in methods,. In order to check the accuracy of the column, before using it for the experiments, we performed a chromatographic experiment with the MDM2 P2 affinity column using control extract from insect cells infected with recombinant baculovirus having the human p53 gene; fig. 1 shows this result.

FIGURE 1.



Site specific isolation of wild-type p53 by MDM2 affinity chromatography.

10ug of p53, contained in the Sephacryl S300 fraction pool prepared from Insect Cell extract, were loaded onto the MDM2 affinity column; the sample was recycled 10 times through the column at gravity flow. The column was washed, 4 times, with 2 ml. of buffer Z 0.1 M KCl. The column was eluted with buffer Z having different KCl concentrations (0.2M to 1.0M) and 1ml fractions corresponding to the elution steps, were collected, resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with a mix of anti p53 monoclonal antibodies 1801, 421 and 240. Lane 1 to 9, fractions 0.2 to 1.0 molar KCl; lane 10, p53 control.

A portion of the loaded p53 remained specifically bound to the MDM2 column after the washes, and eluted with higher salt concentration, 0.5 to 0.8 molar KCl (lanes 4 to 7),

indicating that wild-type p53 can be isolated by MDM2 affinity chromatography. It is noteworthy that only a portion (in the order of nanograms) of the p53 loaded (10 micrograms) binds to the MDM2 column. This fact might indicate that the MDM2 P2 p53-binding site selects for a specific type of p53.

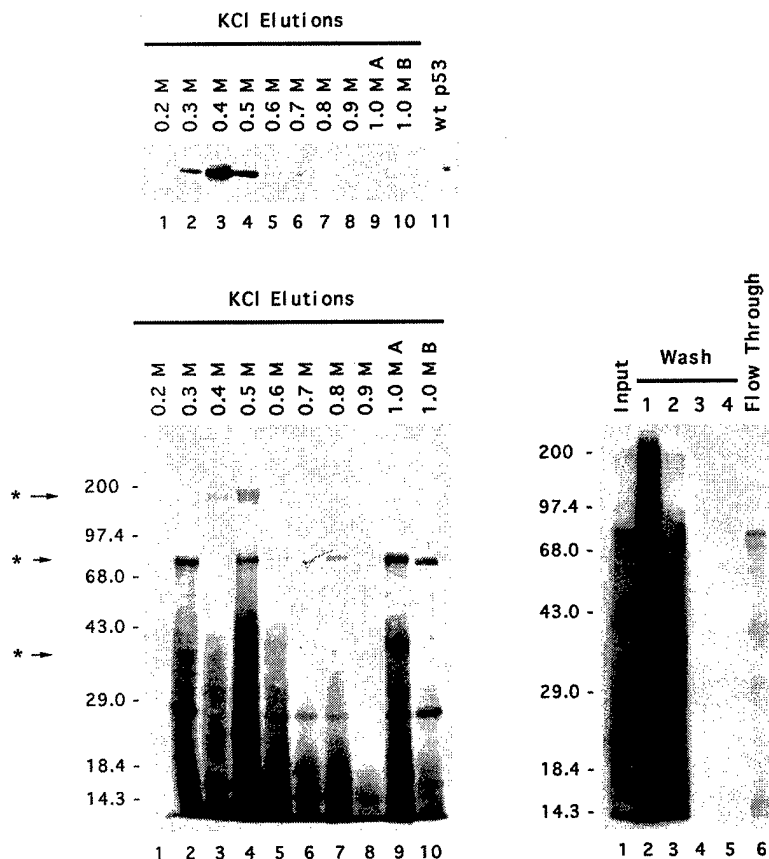
We had previously constructed and checked the RGC column, in the same way we did for the MDM2 column.

Task 4 and Task 1. MDM2 and RGC affinity chromatography with nuclear extracts from 3-4, TR 9-7, ZR75-1, MDA10A, MDAMB468 and MDAMB157 cells; resolution and detection of these fractions.

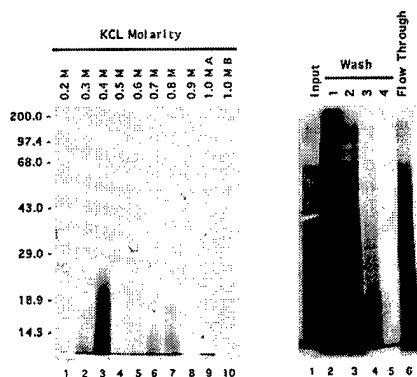
In order to look for mammalian p53- associated proteins and, to set up a control for the breast cell lines MDA10A, MDA MB 468, MDA MB 157 and ZR75-1, we performed MDM2 and RGC affinity chromatography experiments using nuclear extract from 3-4 cells (wild-type p53 conformation when grown at 32 °C). Fig. 2.

FIGURE 2.

a. 3-4 cells.



c. 10-1 cells.



Isolation of p53 and p53-associated proteins from 3-4 cell line by MDM2 affinity

chromatography. 10ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cell extract or the equivalent 5 mg. of total protein from the 10-1 cells, were loaded onto the MDM2 affinity column; the sample was recycled 10 times through the column at gravity flow. The column was washed, 4 times, with 2 ml. of buffer Z 0.1 M KCl. The column was eluted with buffer Z having different KCl concentrations (0.2M to 1.0M) and 1ml fractions corresponding to the elution steps were collected. Each elution fraction was split in two parts: a. The elution fractions were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with a mix of anti p53 antibodies (421, 1801, 240). Lane 1 to 10, elution fractions 0.2 to 1.0 molar KCl; lane 11, p53 control. b. The elution fractions were biotinylated, resolved by SDS-PAGE, transferred to a nitrocellulose membrane and visualized by chemoluminescence. Left panel, lane 1 to 10, elution fractions 0.2 to 1.0 molar KCl. Right panel, lane 1, 1% of the input, lanes 2 to 5, washes 1 to 4; lane 6, 1% of flowthrough. c. The elution fractions from 10-1 cells, were biotinylated, resolved by SDS-PAGE, transferred to a nitrocellulose membrane and visualized by chemoluminescence. Left panel, lane 1 to 10, elution fractions 0.2 to 1.0 molar KCl. Right panel, lane 1, 1% of the input, lanes 2 to 5, washes 1 to 4; lane 6, 1% of flowthrough.

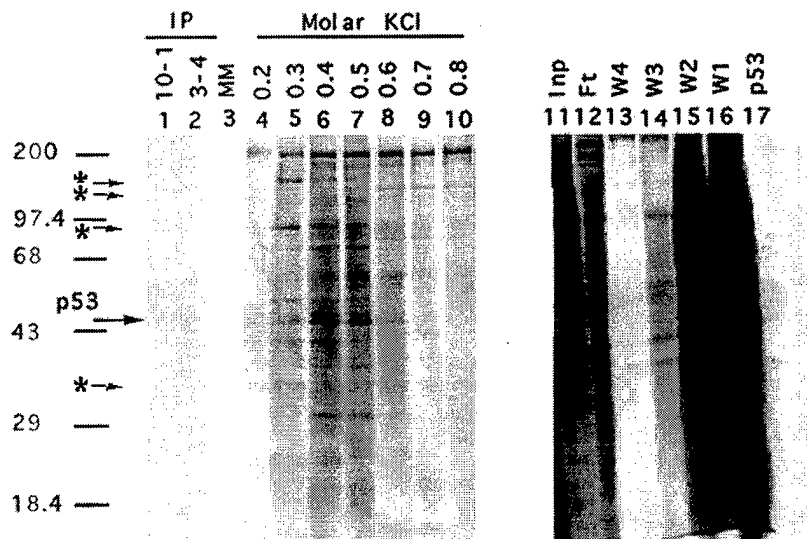
We successfully isolated p53 from the 3-4 cells by MDM2 affinity chromatography. From the MDM2 affinity column, p53 from 3-4 cells, elutes at 0.3, 0.4 and 0.5 molar KCl, fig. 2a. lanes 2, 3 and 4. From the biotinylated fractions, Fig. 2b. left panel, we can see a group of proteins that co-purify with p53 (arrows). We think that these associated proteins bind the MDM2 site in a p53 dependent manner because we could not detect such proteins when we performed the MDM2 affinity chromatography with cell extract from 10-1 cells which, do not have p53, compare fig. 2b. left panel with fig. 2c, left panel. Of special interest are the bands marked with * in fig. 2b, left panel which, might be related to SP1, TBP and TBP associated factors II (Taf IIs), we will discuss their relevance in our next set of results. It is important to notice that, we could detect p53 by immunodetection, but we could not see it when the proteins were biotinylated, compare fig. 2a. and fig. 2b left panel. So although we could detect some putative p53 associated proteins by biotinylation, there are some other proteins (for example p53) that do not biotinylate well. Because of this fact, and because of its high background, we concluded

that biotinylation was not a good approach to detect all the proteins present in the DNA affinity fractions.

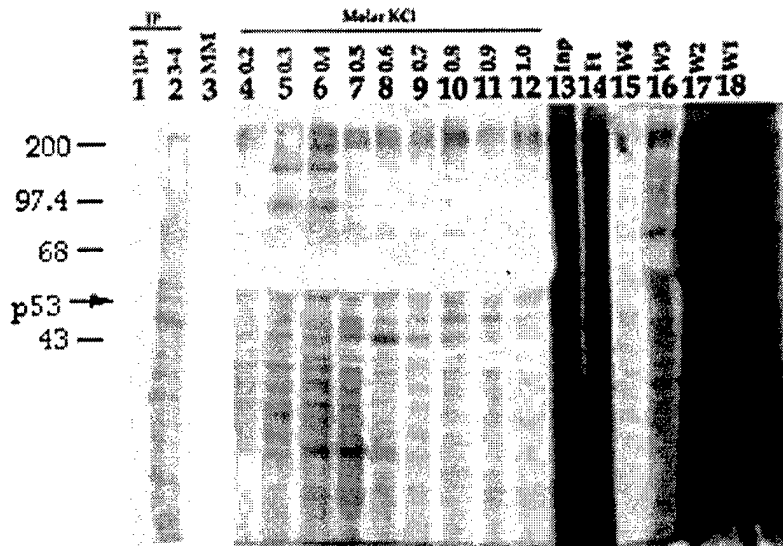
In order to identify p53-associated proteins, it is important to detect the proteins that co-elute with p53 from the RGC and the MDM2 affinity columns. We had chosen the biotinylation of such proteins to accomplish this goal but, as we discussed before, this technique was not appropriate. As an alternative to the biotinylation, we chose to label the cellular proteins with ^{35}S . We grew the 3-4 cells in DMEM containing ^{35}S Methionine, prepared the nuclear extract and Sephacryl S300 fraction pool, as described in methods. Then, we used this Sephacryl S300 fraction pool preparation to perform MDM2 and RGC affinity chromatography experiments. We included an immunoprecipitation from 10-1 (no p53) and 3-4 (ts.Val 135 p53) cells as a control for those proteins that coimmunoprecipitate with p53 in the absence of the DNA binding site. Fig. 3.

FIGURE. 3

a. Mdm2 affinity column.



b. RGC affinity column.



Autoradiographic detection of p53-associated proteins isolated by MDM2 and RGC affinity chromatography. 10ug of p53, contained in the Sephacryl S300 fraction pool prepared from S^{35} labeled 3-4 cell extract, were loaded onto each one of the MDM2 and RGC affinity columns; the sample was recycled 10 times through the column at gravity flow. The column was washed, 4 times, with 2 ml. of buffer Z 0.1 M KCl. Then, the column was eluted with buffer Z having different KCl concentrations (0.2M to 1.0M) and 1 ml. Fractions corresponding to the elution steps were collected. The elution fractions were resolved by SDS PAGE, transferred to a nitrocellulose membrane and visualized by autoradiography. As a comparison for proteins that associate with p53 in an MDM2 or RGC binding- site independent manner, 100 ng. of p53 contained in the same Sephacryl S300 fraction pool preparation used for the affinity chromatography, were incubated with 421 anti p53 immunobeads and immunoprecipitation was performed as specified in methods; immunoprecipitation for 10-1 cells was normalized for total protein. 3a. Lane 1 and 2, immunoprecipitation, IP, from 10-1 and 3-4 cells; lane 3 molecular markers; lane 4 to 10, MDM2 elution fractions 0.2 to 0.8 molar KCL; lanes 11 and 12, 1% of input (Inp) and flowthrough (Ft); lanes 13 to 16, washes 4 to 1; lane 17, p53 control. 3b. Lanes 1 and 2, immunoprecipitation, IP, from 10-1 and 3-4 cells; lane 3 molecular markers; lanes 4 to 12, RGC elution fractions 0.2 to 1.0 molar KCl; lanes 13 and 14, 1% of input (Inp) and flowthrough (Ft); lanes 15 to 18, washes 4 to 1.

We obtained a profile of the cellular proteins that elute from the MDM2, fig. 3a. and RGC, fig. 3b affinity columns. In the case of the MDM2 affinity column, we observed prominent bands at about 190, 105, 95 and 38 kilodaltons, fig. 3a. *. Based on the reports in the literature, we think these bands might be related to SP1 (190,105 and 95kda. and TBP (about 38 kda.) we will discuss them in the next task. In fig. 3b. we show the S^{35} elution profile for the RGC affinity column. In this profile, we also see some proteins that

elute from the column but we can not offer any discussion about their relevance because we could not clearly immunodetect detect p53 in this experiment.

Because establishing the conditions took longer than we expected, we have not performed the experiments with the nuclear extracts from the other cell lines, besides 3-4. Now, we have overcome these difficulties, we have constructed new RGC and MDM2 affinity columns and we are in the process of performing these experiments.

Task 2a. and Task 5. Characterization, by molecular, weight of the proteins found in task 1 and task 4. Partial identification of those proteins, by immunodetection.

Several proteins have been reported to bind to p53; and the interaction between p53 and such proteins has been analyzed by co-immunoprecipitation experiments, even more, most of those experiments have been performed with in vitro translated proteins or with overexpressing systems. From these reported proteins, we chose a subset whose interaction with p53 makes sense from a physiological point of view and whose molecular weight corresponds to the bands that we observed in our results from the DNA affinity chromatography.

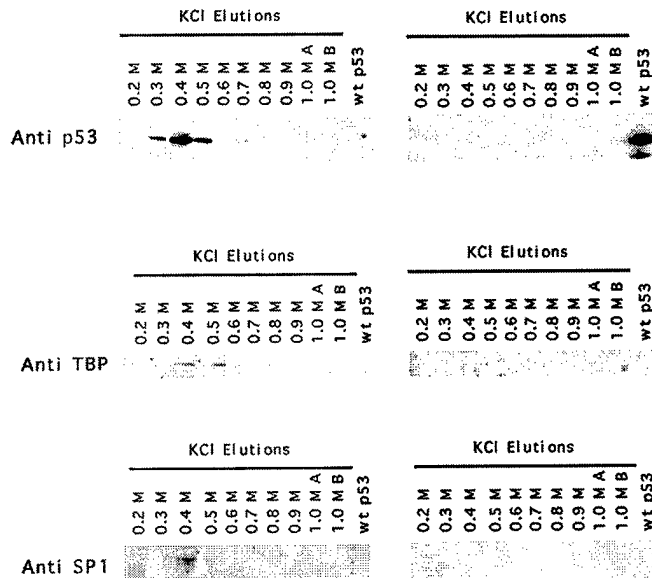
Our results from the MDM2 affinity chromatography with 3-4 cells nuclear extract made us suspect the presence of SP1 (bands in the range from about 95 to about 190) and TBP (band about 38) in the elution fractions, fig. 2b. left panel and fig 3a. To investigate the identity of these bands, we probed the same membrane shown in fig. 2a. with antibodies against the putative proteins. Fig. 4.

FIGURE 4.

Mdm2 affinity chromatography of 3-4 and 10-1 cells

a. 3-4 Cells

b. 10-1 Cells



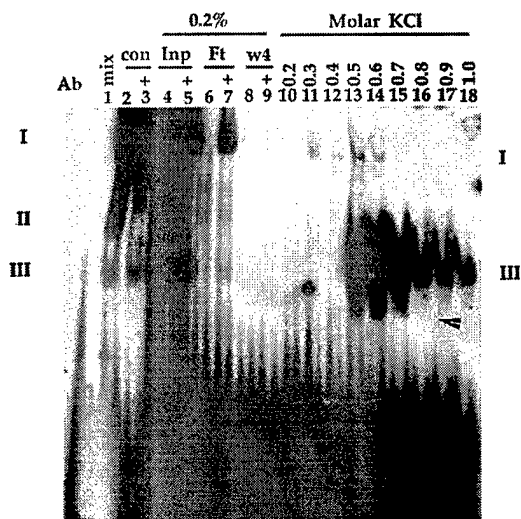
Recruitment of SP1 and TBP to the MDM2 P2 promoter. a. 10 μ g of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cell extract, were loaded onto the MDM2 affinity columns; the sample was recycled 10 times through the column at gravity flow. The column was washed, 4 times, with 2 ml. of buffer Z 0.1 M KCl. Then, the column was eluted with buffer Z having different KCl concentrations (0.2M to 1.0M) and 1ml fractions corresponding to the elution steps were collected. The elution fractions were resolved by SDS PAGE, transferred to a nitrocellulose membrane and probed with a mixture of anti p53 421, 1801 and 240 monoclonal antibodies, for p53; anti TBP (N-12, Santa Cruz) for TBP and anti SP1 (Pep 2, Santa Cruz) for SP1. Panel b shows the results when we carried out the experiment with 5 mg. of total protein from 10-1 cell extract. Lanes 1 to 10, elution fractions 0.2 to 1.0 molar KC (KCl elution); lane 11, p53 control (wt p53).

We observed proteins of the right sizes corresponding to TBP and SP1, that reacted with the specific antibodies. TBP eluted at fractions 0.4 and 0.5 molar KCl, while SP1 eluted with fraction 0.4 molar KCl; their elution profile correlates with the one for p53, elution fractions 0.3, 0.4 0.5 molar KCl, fig. 4a. We think that the binding of TBP and SP1 to the MDM2 site is p53 dependent because we could not see either TBP or SP1 when we ran the MDM2 affinity column with nuclear extract from 10-1 cells (no p53). Fig. 4a Vs fig. 4b. The amount of TBP and SP1 that we detected in the elution fractions from the MDM2 affinity column, is very low. This might reflect a real stoichiometry of their

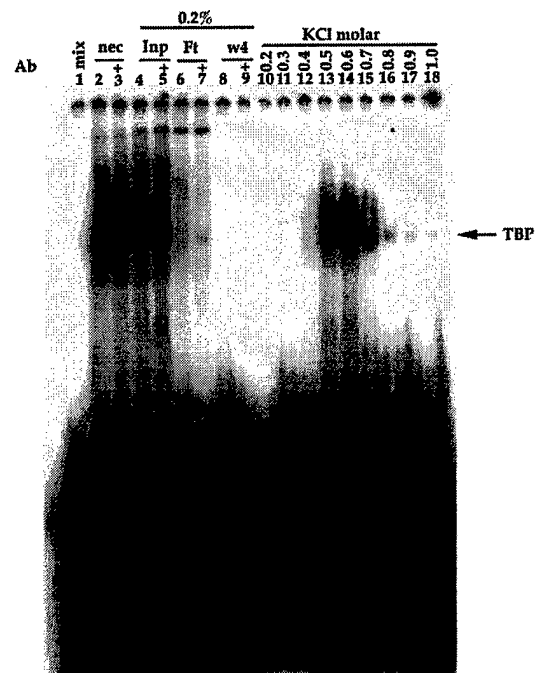
association or might also be due to the fact that we stripped the membranes before reprobing with the anti TBP and anti SP1 antibodies, leading to a loss of protein bound to the nitrocellulose membrane. We are in the process of repeating this experiment for the third time.

In order to confirm the presence of TBP and SP1 in the elution fractions from the MDM2 affinity column, we performed another MDM2 affinity chromatography and we used 5% of each elution fraction to perform EMSA experiments with oligonucleotides specific for p53 fig. 4c; for TBP, fig. 4d.

4c. p53



4d. TBP



Recruitment of SP1 and TBP to the MDM2 P2 promoter. 10ug of p53, contained in the Sephacryl S300 fraction pool prepared from cold nuclear extract from 3-4 cells, were loaded onto the MDM2 affinity column; the sample was recycled 10 times through the column at gravity flow. The column was washed, 4 times, with 2 ml. of buffer Z 0.1 M KCl. Then, the column was eluted with buffer Z having different KCl concentrations (0.2M to 1.0M) and 1ml fractions corresponding to the elution steps were collected. 5% of each elution fraction (0.2 to 1.0 molar KCl) and 0.2% of the input (Inp), flowthrough (Ft) and wash 4 (W4) were incubated with P³² labeled oligonucleotides. SCS oligo (Superconsensus Sequence), a synthetic idealized cognate binding site for p53 (4c), TFIID Consensus Oligonucleotide (Santa Cruz) for TBP (4d) and HIV oligo (Santa Cruz) that has three binding sites for SP1 (4e). The protein-DNA complexes were resolved in a non-denaturing 4% acrylamide gel and visualized by autoradiography. **4 c. p53 binding to SCS oligonucleotide.** Lane 1, mix without protein; lanes 2 and 3, p53 control (con); lanes 4 and 5 0.2 % of input (Inp); lanes 6 and 7, 0.2 % of flowthrough (Ft); lanes 8 and 9, 0.2 % of wash four (W4); lanes 10 to 18, elution fractions 0.2 to 1.0 molar KCl (Molar KCl). Anti p53 421 antibody was included in some of the reactions because it activates p53 DNA binding and allows better visualization of the p53-DNA complex, lanes 3, 5, 7, 9 and 10 to 18. **4d. TBP binding to TFIID Consensus Oligonucleotide.** Lanes 1, mix without protein; lane 2 and 3 nuclear extract for control (nec); lanes 4 and 5 0.2 % of input (Inp); lanes 6 and 7, 0.2 % of flowthrough (Ft); lanes 8 and 9, 0.2 % of wash four (W4); lanes 10 to 18, elution fractions 0.2 to 1.0 molar KCl (Molar KCl). Anti TBP antibody (Santa Cruz) was included in some of the reactions, lanes 3, 5, 7 and 9.

We detected p53 supershift in the elution fractions from the MDM2 affinity column; p53 eluted with 0.3 to 0.6 molar KCl, fig. 4c. band I, lane 11 to 14. In this elution profile we also detected a very prominent complex, fig. 4c, band III, lanes 13 to 18. This complex migrated faster than the p53 supershift one, fig. 4c. band III Vs band I. The complex detected in band III might correspond to a different oligomeric form of p53, to a different p53 conformation or might also correspond to a non-specific DNA binding protein. We are in the process of performing competition experiments to determine if the complex represented by band III in fig. 4c., corresponds to a specific DNA binding protein(s) and if p53 is present in this complex.

TBP was recruited to the MDM2 affinity column and eluted with 0.5 to 0.8 molar KCl, fig. 4d, lanes 13 to 16. It is noteworthy that TBP starts to elute from the MDM2 affinity column with 0.5 molar KCl, the same salt concentration at which p53 elution peaks, compare fig. 4c. lane 13, band I with fig. 4d., lane 13. We included anti TBP N-12 antibody (Santa Cruz) to produce a supershift that would confirm the identity of the band as TBP but we found out later that this particular antibody does not produce a supershift.

Sp1 also seems to be recruited to the MDM2 affinity column. In the EMSA experiments with the SP1 binding oligonucleotide, we observed two species present in the elution fractions. One of these species migrated as the control and the other one migrated faster than the control (data not shown) Our results were not conclusive and we are in the process of performing competition experiments to elucidate this matter.

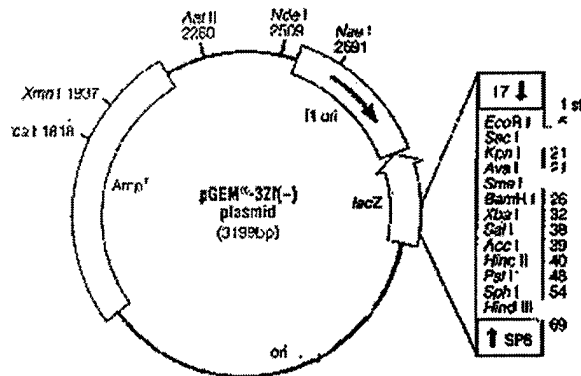
Because establishing the conditions took longer than expected, we have not performed the experiments using nuclear extract from the other cell lines (TR9-7, MDA10, MDAMB468, MDAMB157 and ZR75-1), as proposed.

Task 2b. Cloning of the MDM2 P2 fragment in pGem, for GRAB system and Task 3b. Establishing the conditions for GRAB system using the MDM2 fragment cloned in task 2.

Our goal is to identify proteins that associate with p53 when p53 is bound to its cognate site in the P2 of the MDM2 gene and in the RGC; we are doing it by DNA affinity chromatography using the mentioned DNA sites crosslinked to CNBr activated Sepharose beads as described in methods. This method has a size limitation for the piece of DNA that can be crosslinked to the beads, approximately 100 base pairs. The GRAB method gives more flexibility regarding the size of the DNA fragment to be used, allowing us to use not only the p53 binding site but also flanking regions which might be important for the binding of associated proteins. Comparison of GRAB results with those from the DNA-Sepharose affinity columns would show if the p53 binding sequence is enough for the assembly of such complexes.

The GRAB system, as described by Levens et al. 1985, requires a DNA fragment having the MDM2 P2 binding site next to the Lac operator. The p53-binding site - Lac operator fragment is then incubated with a Lac repressor-beta-galactosidase fusion protein. The fusion protein binds to immobilized antibody raised against Beta galactosidase. All this assembly is used as a solid matrix that is then incubated with the different cell extracts mentioned before. As opposed to a salt elution gradient, the DNA bound protein(s) complex is eluted by the addition of IPTG that binds to Lac repressor with high affinity. Our strategy was to excise the MDM2 P2 site from a PGL2 basic plasmid that has the MDM2 gene and to clone this site into pGem 3Z(-) plasmid (Promega), which has the Lac Operator next to a cloning site, diag. 2.

DIAGRAM 2.

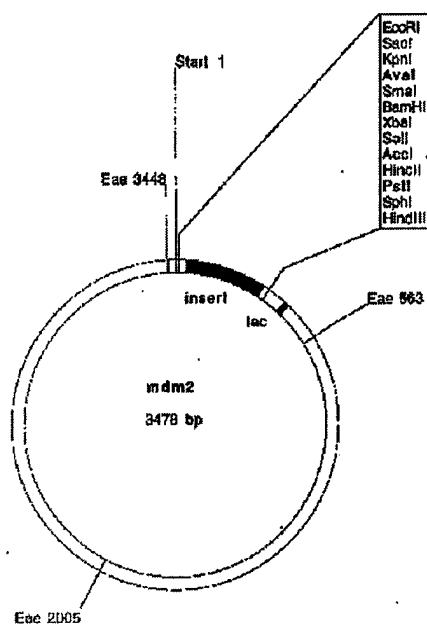


pGem 3Z(-) map.

We obtained the MDM2 P2 binding digesting the PGL2 basic plasmid with NsiI and ApaI, which gave a 400 bp . We then digested this 400 bp. fragment with Hin PIII to obtain our target fragment, which was 279bp and had AccI cohesive ends. After cloning this 279-bp fragment into linearized pGem (3199bp), we obtained the cloned plasmid pGem-mdm2 that has 3478 bp, diagram 3. In order to check the ligation, we run a small aliquot of the pGem-mdm2 DNA on an agarose gel, to compare its size with the size of pGem before ligation, fig. 5a.

In order to obtain the desired DNA fragment , mdm2-grab, which is 593 bp. and contains the MDM2 p2 site next to the Lac operator, we digested pGem-mdm2 plasmid with the restriction enzyme Eae. Mdm2-grab is the piece of DNA necessary to perform the GRAB experiments. Figure 5 b.

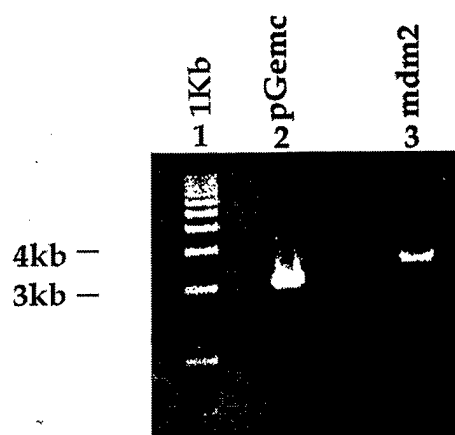
DIAGRAM 3.



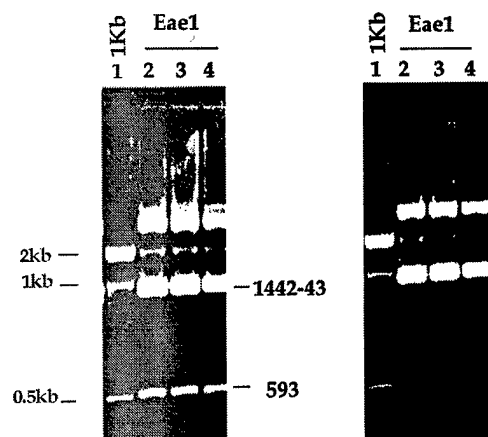
pGem-mdm2 map. Diagram 3 represents the plasmid obtained after cloning the MDM2 P2 site into pGem3Z (-) plasmid (Promega). The diagram shows the size of pGem-mdm2, the position of the MDM2 P2 insert (long dark region), the position of the Lac operator(short black region) and the restriction sites for the enzyme Eae.

FIGURE 5.

a.



b.



a. Cloning of MDM2 P2 fragment into pGem3Z (-). Aliquots of pGem3Z(-) and pGem-mdm2 were resolved in an agarose gel and visualized by Ethidium Bromide fluorescence. Lane 1, 1 Kb.

Ladder; lane 2, pGem before ligation (pGemc); lane 3, pGem after ligation of the MDM2 P2 fragment (mdm2).b. mdm2-grab fragment. digestion reactions from 3 pGem-mdm2 clone preparations were resolved in a preparative agarose gel, visualized by Ethidium Bromide fluorescence and the mdm2-grab fragment excised for electroelution. Lane 1, Kb ladder; lanes 2 to 4, digestion of three pGem-mdm2 clones (EaeI). Right panel, before cutting mdm2-grab fragment out; left panel, after cutting it out for electroelution.

We successfully cloned the 279-bp fragment containing the MDM2 P2 site into the pGem 3Z(-) plasmid as evidenced by the slower migration of the pGem-mdm2 plasmid compared with pGem plasmid before ligation, fig. 5a. By digestion of pGem-mdm2 with EaeI, we obtained our target DNA fragment (mdm2-grab, 593 bp.), fig. 5b left panel. Using an agarose preparative gel, we purified mdm2-grab and electroeluted it for further use.

Our goal, with GRAB system, was to have more DNA of varied sequence flanking the p53 binding site and compare these results with the ones obtained from the CNBr-Sepharose-MDM2 affinity chromatography in which, the fragment of DNA used has only the p53 binding site without flanking DNA. As we can see in fig.3a. lanes 4 to 10, many proteins bind to the MDM2 site on its own so, by using a bigger piece of DNA (MDM2 P2 binding site plus flanking sequences) with GRAB, we would allow more proteins to bind to this site making it very difficult to know which ones are relevant and specific. Because of this fact, we decided that GRAB system was not a good alternative to accomplish our goal and did not proceed further with it.

CONCLUSIONS.

p53 is a cellular guardian of the genome integrity and a key player in preventing tumor formation and p53 mutation has been implicated in several types of cancer such as breast cancer. To understand p53 functioning, will enable the scientific community to develop better-targeted therapeutical measures to fight this disease. A very important fact in its functioning is how p53 is prompted to bind to its cognate sites; and how to select which sites to bind and when. As a contribution to answering this question, we are in the process of identifying proteins that associate with p53 when p53 is bound to its cognate sites in the MDM2 gene and in the Ribosomal Gene Cluster, because we think that, the differential association of p53 with cellular proteins is a key biochemical event in p53 functioning.

By DNA affinity chromatography using the p53 binding site present in MDM2 P2 (MDM2 P2- affinity chromatography), we showed the recruitment of the TATA Binding Protein (TBP) and the transcription factor SP1 to the promoter 2 on the MDM2 gene (MDM2 P2) as detected by Western blot fig. 4a, left panel and by Electrophoretic Mobility Shift Assay (EMSA) fig. 4d and 4e. The recruitment of TBP and SP1 seem to be p53 dependent because we could not detect either one in the MDM2 P2 elution fractions, when we used cell extract from 10-1 cells (no p53), fig. 4a. left panel (3-4 cells) Vs. fig. 4a. right panel (10-1 cells). Using MDM2 P2- affinity chromatography, we successfully

purified p53 from 3-4 cells, fig. 2a. and 4c. as well as from recombinant-baculovirus infected Sf9 cells, fig. 1.; to the best of our knowledge, this is the first time that p53 has been isolated using this technique. It is noteworthy that p53 from 3-4 cells seems to have a different affinity for MDM2 P2 than p53 from Sf9 cells, fig. 4a, right panel, elution fractions 0.3 to 0.5 molar KCl and fig. 4c. elution fractions 0.3 to 0.6 molar KCl; Vs. fig. 1., elution fractions 0.5 to 0.8 molar KCl. This different affinity might be due to the fact that p53 in 3-4 cells is modified, after translation, in a different way than p53 in the Sf9 cells is. The difference in the affinity mentioned before and the fact that not all the p53 loaded onto the MDM2 P2 affinity column (10 micrograms) binds to it (eluted p53 is in the order of nanograms) suggest that the MDM2 P2 affinity column is selecting for a subset of p53 molecules. If this is the case, MDM2 P2 affinity chromatography and in general, p53-binding site affinity chromatography, would be a very useful technique to isolate different p53 conformations and to perform biochemical analysis with them.

Under our experimental conditions, the GRAB system was not a good alternative to identify p53 associated proteins because it would increase the number of non-specific proteins bound to the MDM2 P2 p53-binding site making more difficult their identification. Biotinilation of the proteins in the MDM2 P2 elution fractions did not give us acceptable results because it increased the backgrounds and because it did not mark all the proteins at the same extent, making the detection of p53 associated proteins more difficult and unreliable.

REVISED STATEMENT OF WORK.

This first year of work has been very intense and very interesting, bringing us a whole myriad of challenges and difficulties that we did not expect. It is true that these unaccounted for challenges and difficulties caused us to fall behind schedule. But, it is also very true that facing these challenges and surmounting these difficulties has given us a great deal of experience and, precisely because of them we now have a better understanding of the project and realize, even more, the relevance of its results in the context of fighting breast cancer.

Because of the reasons above mentioned, we ask you, to kindly consider our new Statement of Work, which we consider to be not only more accurate in terms of timing but also more meaningful in terms of the way the results are produced.

Task 1. Define the experimental conditions for MDM2 affinity chromatography and for MDM2-GRAB as well as establish positive and negative controls for the MDM2-p53 associated proteins using nuclear extract from 3-4 and 10-1 cells respectively. Month 1-12.

Task 2. Define the experimental conditions for RGC affinity chromatography as well as establish positive and negative control for RGC-p53 associated protein using nuclear extract from 3-4 and 10-1 cells respectively. Month 13-15.

Task 3. Perform MDM2 and RGC affinity chromatography with nuclear extract from the

normal breast cell line MDA10A, before and after treatment with the DNA damaging agents Actinomycin, Comptothecin and Zeocin. Compare these results with the ones from tasks 1 and 2. Month 16-19.

Task 4. Perform MDM2 and RGC affinity chromatography with nuclear extract from the breast cancer cell line ZR75-1 that has wild type p53, before and after treatment with the DNA damaging agents Actinomycin, Comptothecin and Zeocin. Compare these results with the ones from tasks 3. Month 21-23.

Task 5. Perform MDM2 and RGC affinity chromatography with nuclear extract from the breast cancer cell line MDAMB468 that has the mutant p53 His 273, before and after treatment with the DNA damaging agents Actinomycin, Comptothecin and Zeocin. Compare these results with the ones from tasks 3 and 4. Month 24-28.

Task 6. Perform MDM2 and RGC affinity chromatography with nuclear extract from the breast cancer cell line MDAMB157 that does not have p53, before and after treatment with the DNA damaging agents Actinomycin, Comptothecin and Zeocin. Compare these results with the ones from previous tasks. Month 29-32.

Task 7. Perform MDM2 and RGC affinity chromatography with nuclear extract from elutriated ML1 cells at different stages of the cell cycle. Compare these results with the ones from previous tasks. Month 33-36.

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