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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Proliferating cell nuclear antigen (PCNA) is a multifunctional enzyme involved in multiple cellular processes including DNA replication and repair. During DNA replication, PCNA functions as an accessory factor for the DNA polymerases □ and □ and are part of a multiprotein DNA replication complex termed the DNA synthesome. Isolation and analysis of the of the DNA synthesomes from non-malignant and malignant breast cells has previously shown that replication fidelity is significantly reduced in malignant cells as compared to non-malignant cells. This reduction in replication fidelity in malignant cells is accompanied by a structural alteration to PCNA. In attempts to explain how this structural alteration to PCNA present in malignant cells could result in lowered replication fidelity, the ability PCNA present in malignant cells to interact with $p21^{WAF1}$ was examined. Initially identified as a cyclin-dependent kinase inhibitor, $p21^{WAF1}$'s ability to inhibit DNA replication in response to DNA damage has been well characterized. Interestingly, $p21^{WAF1}$ inhibits DNA replication by interacting with PCNA, and an inability of $p21^{WAF1}$ to interact with the structurally altered PCNA present in malignant cells could have tremendous mutagenic potential. However, research soon proved that the effects of this structural change to PCNA would not be so simple. Examination of the interaction of $p21^{WAF1}$ with PCNA revealed a third form of PCNA present in malignant cells that preferentially bound $p21^{WAF1}$. Elucidation of three isoforms of PCNA present in malignant breast cells may therefore represent signaling events that link DNA replication to DNA repair through structural alterations to PCNA.

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INTRODUCTION

DNA replication occurs through the coordinated action of multiple proteins, which together form a discrete multiprotein complex in breast cells termed the DNA synthesome. Studies performed with the DNA synthesome isolated from non-malignant and malignant breast cells have demonstrated that the fidelity of DNA replication was significantly reduced in malignant cells when compared to their non-malignant counterparts. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the proteins that comprise the DNA synthesome revealed that proliferating cell nuclear antigen (PCNA), a processivity factor for the DNA polymerases δ and ε and an essential component of the DNA synthesome, was altered in the malignant cells when compared to the non-malignant cells. DNA sequence analysis of the *PCNA* gene uncovered coding regions for *PCNA* that were identical in malignant cell and non-malignant cells, and because the *PCNA* sequence is not different in malignant cells, it was concluded that the altered 2D-PAGE migration pattern of the PCNA protein present in malignant cells must be the due to the presence of one or more modifications added to the protein post-translationally. The presence of post-translational modifications on PCNA is intriguing because PCNA is essential not only for DNA replication, but it is also required during DNA repair. One of the ways cells limit the mutagenic potential of a DNA damaging event is by inhibiting DNA replication. This not only prevents the duplication of errors in the DNA, but also theoretically allows the cell time to repair the damage before DNA replication is re-commenced. Paradoxically, the inhibition of DNA replication occurs as a result of the induction of $p21^{WAF1}$. Once induced, $p21^{WAF1}$ binds to PCNA and blocks PCNA's ability to interact with polymerase δ and other proteins involved in the DNA replication process. How then does $p21^{WAF1}$ signaling inhibit PCNA's function in DNA replication while unaffecting its role in DNA repair? The answers to these questions have remained elusive, and the $p21^{WAF1}$ signaling events that take place as a result of DNA damage still poorly understood. Interestingly, we have recently found that, the interaction of PCNA and $p21^{WAF1}$ was dependent upon the post-translational state of PCNA. The data also suggest that $p21^{WAF1}$ has the ability to differentially interact with isoforms of PCNA, and this ability may represent a signaling pathway that coordinates the functions of PCNA during DNA replication and repair. These findings may therefore clarify the molecular mechanisms by which cells are able to inhibit DNA replication while facilitating DNA repair. Therefore, with the insight gained from the elucidation of these signaling mechanisms, we may not only further our understanding of the biochemical events that are occurring in breast cancer, but by applying this knowledge, we may ultimately develop better and more practical approaches towards diagnosis and treatment of the disease.

PROPOSAL BODY

I. RESULTS.

A. The csPCNA has an Acidic pI and the nmPCNA has a Basic pI, and It is Not the Result of a Genetic Mutation.

To better characterize the two forms of PCNA found in MCF7 cells, the pI values for the two forms were assigned. First, partially purified PCNA samples from MCF7 cells were resolved by 2D-PAGE in the presence of 2D-PAGE protein standards. The resolved gels were subsequently silver stained and the gels were then imaged using a scanning densitometer. Melanie II 2D-PAGE analysis software was then used to assign pI values (Figure 1). The pI for the csPCNA was determined to be 4.74, very close to the calculated pI for PCNA, 4.56. The pI for the nmPCNA, on the other hand, was much more basic, 6.96. We then sought to explain this shift in the pI of PCNA found in malignant cells. We postulated that the shift in pI of PCNA could result from changes in the gene coding for PCNA or changes to the protein after it has been translated. First, the shift could result from either a mutation in the DNA of the coding sequence for PCNA or alternative splicing of the RNA. PCNA cDNA was therefore isolated from MCF7 and MCF10A cells and sequenced. After isolating and sequencing the PCNA cDNA from these cells numerous times, it was concluded that the coding sequence for PCNA was unaltered in malignant MCF7 cells as compared to non-malignant MCF10A cells, and identical to the previously published sequence for PCNA (Bechtel *et al.*, 1998). These data suggest that the shift in pI of the PCNA identified in malignant cells must not be the result of either a mutation in the coding region of PCNA or an alternative splicing. The change in 2D-PAGE migration pattern for PCNA must therefore be due to post-translational modification and/or conformational change of the protein. The post-translational modification could add charges to the protein causing it to focus at a different pI. Additionally, a change in conformation of PCNA could expose charged amino acid residues present in the primary structure of PCNA and cause a pI shift.

B. The csPCNA and nmPCNA have Two Different Molecular Masses.

We first decided to analyze the structure of PCNA present in MCF7 cells using mass spectrometry. Analysis of PCNA was accomplished using Surface Enhanced Laser Desorbtion/Ionization (SELDI) mass spectrometry. The surface chemistry of the SELDI sample plate enables the capture of specific molecules on its surface, and this allows for the isolation and purification of target proteins and peptides prior to mass spectral analysis. Therefore, we immobilized anti-PCNA antibodies on the surface of a SELDI plate to specifically capture PCNA. MCF7 P4 protein fraction was then added to the surface of the SELDI plate and non-specifically bound proteins were washed away. The full-length PCNA polypeptide immobilized on the surface of the plate was then analyzed using a SELDI mass spectrometer. The resulting mass spectrum showed the presence of two peaks (Figure 2) resolving at 29,080 and 29,982 mass over charge (m/z) ratio. Because of the acidity of the matrix, proteins analyzed by MALDI generally have a +1 charge and therefore have a mass (in Da) identical to their m/z value. We therefore concluded that these two peaks correspond to the two forms of PCNA found in the MCF7 cells. Because the pI value assigned to the csPCNA was so close to the pI calculated from PCNA's primary sequence, csPCNA most likely represents a non-modified form. And not having a modification would cause PCNA to have a mass near its theoretical mass of 28,768 Da Indeed, the lower molecular weight peak (29,080 Da) differs by only 1% from that of the theoretical mass for PCNA and most likely represents a non-modified csPCNA. The peak at

29,982 Da would then be the nmPCNA, suggesting that the nmPCNA harbors a post-translational modification, which causes a basic shift in pI and has a molecular mass of ~900 Da.

Two of the most characterized post-translational modifications are phosphorylation and poly (ADP) ribosylation. Phosphorylation adds 80 Da to the protein mass per phosphate molecule and nmPCNA could represent PCNA with the addition of 11 to 12 phosphates. However, there are major reasons why we believe nmPCNA is not modified by phosphorylation. First, the shift in pI of nmPCNA is towards the basic side of the gel suggesting that it has a higher abundance of positively charged chemical groups, and phosphate groups are negatively charged. However, the addition of phosphate groups to PCNA could cause conformational changes in the secondary structures of PCNA and this change could expose positively charged amino acid residues resulting in the basic charge of nmPCNA. Also, if this were true, we would expect to see multiple forms of PCNA with multiple pIs. The same problems are encountered when poly (ADP) ribose is considered. Poly (ADP) ribose, much like phosphorylation, contains negative charged phosphate groups, but poly (ADP) ribose has a molecular mass closer to 300 Da, so nmPCNA would contain three poly (ADP) ribose moieties. Additionally, Western blotting using anti-poly (ADP) ribose moiety antibody identified nmPCNA as harboring poly (ADP) ribosylation (Bechtel *et al.*, 1998). However, until the exact location and structure of the moiety is identified alternative types of post-translational modifications must be considered.

Some other modifications that must be considered are mono (ADP) ribose and polyamine conjugation. First, the presence of mono (ADP) ribose could explain the results that demonstrate recombinant PCNA as having the same pI as nmPCNA because bacteria contain mono (ADP) ribosylases. Additionally, polyamines, unlike most other modifications, contain a positive charge, and this would explain the shift of nmPCNA to the to the basic side of the gel. Interestingly, the PCNA protein sequence contains consensus sites for both mono (ADP) ribose and polyamine conjugation, suggesting that these modifications may be present on PCNA. This also begs another question. Are there multiple types of modifications? Clearly, this question is not a simple one to solve. If there are multiple modifications present on PCNA, there are most probably associated changes in secondary structures, which accompany these modifications, and multiple modification and changes in secondary structures most likely contribute to the pI change of nmPCNA. Because the exact nature of the modification(s) present on the PCNA molecule are currently not known

D. Recombinant PCNA is Indistinguishable from nmPCNA (rPCNA=nmPCNA).

The PCNA cDNA isolated from MCF7 cells was sub cloned into an expression plasmid and expressed in *E. coli* BL21(DE3) cells fused to a calmodulin binding protein (CBP) affinity tag. Recombinant PCNA (rPCNA) was then purified by affinity chromatography using calmodulin affinity resin. The purified CBP-tagged PCNA was then analyzed by SDS-PAGE followed by staining with Coomassie blue and Western blotting (Figure 3A). The CBP tag present on rPCNA was then cleaved with thrombin protease, and the resulting rPCNA was resolved by 2D-PAGE and Western blotted (Figure 4B). The Western blot demonstrated that rPCNA resolves at the basic side of the gel at the same position as the nmPCNA, and this was confirmed by the addition of rPCNA to MCF7 P4 protein fraction followed 2D-PAGE and Western blotting. The results showed that the spot corresponding to the nmPCNA gets larger with the amount of rPCNA added, and no new spots corresponding to PCNA appear (data not shown). This discovery was interesting because, as described earlier, the nmPCNA was thought to contain the post-translational modification, and these results suggested that *E. coli* might be capable of modifying PCNA. In addition, these data also suggest that there may be some factor

other than PCNA's DNA or amino acid sequence that is leading to the development of csPCNA in MCF7 cells.

E. Mass Spectrometric Analysis of Recombinant PCNA Resolved by SDS-PAGE Does Not Identify a Post-Translational Modification.

To investigate whether rPCNA harbors a post-translational modification, the CBP affinity tag was removed from rPCNA with thrombin protease, and it was resolved by 10% SDS-PAGE and stained with Coomassie brilliant blue protein stain. Recombinant PCNA was then excised from the gel and sent to Harvard's Microchemistry Facility for analysis. At the facility, the band was subjected to proteolytic cleavage with trypsin and the resulting peptide were extracted and analyzed by ion trap mass spectrometry. To our surprise, analysis of rPCNA by tandem mass spectrometry showed a complete absence of post-translational modification. The results of amino acid sequencing of rPCNA suggested that PCNA is not post-translationally modified as originally suspected, or it is losing the modification at some step prior to the sequencing step.

This result is consistent with the stability of (ADP) ribose found on proteins. If the bacteria were adding mono (ADP) ribose to the rPCNA, the moiety would be very labile similar to that observed with poly (ADP) ribosylation. In fact, numerous freeze-thawing events and extensive lengths of time in the freezer have been shown to cause the rPCNA to revert to the acidic side of the gel and focus as csPCNA.

F. Attempts to Electroelute nmPCNA from Two-Dimensional Gels Results in its Conversion to csPCNA

In order to analyze the differences between csPCNA and nmPCNA, it was essential that the two forms be isolated from one another. An initial attempt to separate the two forms of PCNA was done by resolving PCNA from MCF7 cells by 2D-PAGE and electroeluting csPCNA and nmPCNA from the gel into two separate fractions. Although exposure of the two forms of PCNA to harsh denaturing conditions during 2D-PAGE would arguably destroy protein function, electroelution would separate the two forms of PCNA from one another allowing for a comparative analysis to be performed by mass spectrometry. Electroelution of the two forms of PCNA was carried out by resolving large amounts of MCF7 P4 fraction (~600 μg) by 2D-PAGE and excising the areas of the gels known to contain PCNA (Figure 4). The gel slices containing PCNA polypeptide were then electroeluted into 14 fractions, with fraction 1 being the most acidic and fraction 14 being the most basic. The electroeluted fractions were then slot blotted for PCNA. Figure 5A showed the presence of the PCNA in fractions 2 through 6 at the acidic end of the gel, which should represent the cancer specific form. The slot blot also showed the presence of PCNA in fractions 13 and 14 at the most basic side of the gel, probably representing the non-malignant form. Interestingly, PCNA also eluted into fraction 8, and seemed to represent a distinct pool of PCNA that has an intermediate pI and was neither with the csPCNA nor the nmPCNA. To confirm that the csPCNA and the nmPCNA had both been eluted, fractions 3 and 14 were scrutinized by 2D-PAGE (Figure 5 B and C). Western blotting showed the presence of csPCNA in the fraction 3, as expected. However, nmPCNA that was eluted into the most basic fraction, 14, no longer resolved at the basic side of the gel like nmPCNA, but instead resolved at the acidic side identical to csPCNA (nmPCNA→csPCNA). These results suggest that whatever modification was responsible for increasing the pI of nmPCNA was lost either during the second dimension (i.e. SDS-PAGE) or during the electroelution. However, because the electroelution was done at 4°C and under very mild conditions (50 mM HEPES, pH 7.5), this change in the pI of the nmPCNA most probably occurred during the second dimension (i.e. SDS-PAGE).

G. MCF7 Cells Contain a Third Form of PCNA.

To obtain more material for mass spectral analysis, larger scale 2D-PAGE gels were performed using an isoelectric focusing cell (IEF cell, Bio-Rad). The advantages of the IEF cell are that the gels or strips are pre-cast and therefore do not have the inconsistencies normally seen with tube gels (mini Protean II Tube Cell, Bio-Rad) that are cast by the user. The strips also have a preformed pH gradient that allows for greater reproducibility and higher resolution in addition to separating proteins over larger pH ranges. The higher resolution of polypeptides using the IEF cell is accomplished by increasing the focusing voltages from 750V to 8000V. I then used this technique to resolve the different forms of PCNA found in MCF7 cells.

MCF7 NE/S3 protein fraction (200 μg) was resolved using the IEF cell followed by electrophoresis in 8-16% polyacrylamide gel. The proteins resolved by 2D-PAGE were then electrophoretically transferred to nitrocellulose and probed with anti-PCNA antibodies. Western blotting confirmed that MCF7 cells contain csPCNA and nmPCNA (Figure 6). However, the increased amounts of protein loaded onto these gels and their greater resolution allowed for the detection of a third form of PCNA. Intriguingly, this third form of PCNA resolved at a pI in between that of csPCNA and nmPCNA, and it was in very low abundance. The novel form of PCNA was interesting because it was the first time three forms of PCNA were seen by 2D-PAGE. Actually, this third form of PCNA had been seen on numerous other 2D gels, but because the intermediate form of PCNA was always seen by itself or only with nmPCNA and never seen with both nmPCNA and csPCNA, this intermediate form of PCNA was thought to be an artifact of the 2D gels, and that somehow this artifact altered the migration of csPCNA to what appeared to be a more basic pI. Identification of this intermediate form now argues that what was visualized by those gels was actually a third intermediate form of PCNA. In fact, close evaluation of data generated in past experiments alluded to a third form of PCNA present in MCF7 cells. For example, scrutiny of the 2D-PAGE electroeluted fractions (Figure 5A) demonstrated the presence of a distinct pool of PCNA in fraction 8. Although originally overlooked, this could have represented the intermediate form of PCNA, and its presence was seen because large amounts of protein were resolved on the gels. Discovery of the intermediate form of PCNA, from now on referred to as iPCNA, added another level of complexity to a multifunction protein. In attempts to unravel some of the mystery surrounding the functions of the different forms of PCNA, the interaction(s) between the different forms of PCNA and p21 were studied.

H. Interaction of p21^{WAF1} with iPCNA.

As mentioned in other chapters of this thesis, the interaction of p21 and PCNA is of great interest to many fields of study. Although this thesis puts emphasis on the role of PCNA in DNA replication, it has multiple other cellular functions as well. In addition to DNA replication, PCNA is found in cyclin and CDK complexes and seems to have a role in cell cycle progression amongst its numerous roles in multiple types of DNA repair (Shivji *et al.*, 1992; Xiong *et al.*, 1992; Matsuoka *et al.*, 1994; Szepesi *et al.*, 1994; Gadbois *et al.*, 1995; Umar *et al.*, 1996; Gary *et al.*, 1997; Levin *et al.*, 1997; Prosperi *et al.*, 1997; Tsurimoto, 1999; Koundrioukoff *et al.*, 2000; Dianova *et al.*, 2001; Karmakar *et al.*, 2001; Kleczkowska *et al.*, 2001; Matsumoto 2001; Paunesku *et al.*, 2001; Stivala *et al.*, 2001). p21 also functions in DNA replication, cell cycle and DNA repair, and interestingly, p21 exerts its effects on DNA replication and repair through its interaction with PCNA (Li *et al.*, 1994; Waga *et al.*, 1994; Luo *et al.*, 1995; Nakanishi *et al.*, 1995; Pan *et al.*, 1995; Chen *et al.*, 1996; McDonald *et al.*, 1996; Oku *et al.*, 1998; Cooper *et al.*, 1999; Stivala *et al.*, 2001; Tom *et al.*, 2001). The interaction between PCNA and p21 is made more complex by the fact that p21 is induced in response to DNA damage. The difference in

p21's ability to inhibit DNA replication but not affect DNA repair was first demonstrated in 1994 by Waga *et al.* They showed that the addition of p21 to cell extracts inhibited DNA replication *in vitro*; however, *in vitro* DNA repair of a UV-irradiated DNA template was not affected. The authors then concluded that p21 was able to inhibit DNA replication in response to DNA damage to allow DNA repair to take place. Although the conclusion was widely accepted, multiple labs soon generated conflicting data. After all how could p21 binding to PCNA inhibit its role in DNA replication but not DNA repair? Our particular answer to this question could be explained by a differential binding of p21 to the different forms of PCNA.

To determine whether p21 was able to differentially interact with one or more of the different forms of PCNA earlier identified, multiple different experimental approaches were undertaken. First, p21 co-immune precipitation studies were performed. MCF7 NE/S3 protein fraction was incubated with anti-p21 antibodies and the antibodies were then precipitated with protein-A-agarose. The precipitated anti-p21/p21/PCNA complex was then resolved by 2D-PAGE. The resolved proteins were then transferred to nitrocellulose membranes and immuno-blotted with anti-PCNA antibodies (Figure 7). The results showed the presence of multiple proteins. Western blotting showed that the form of PCNA that was precipitated with anti-p21 antibodies resolved at a molecular weight of 36 kDa at an intermediate pI near the middle of the gel. Interestingly, the form of PCNA was neither the csPCNA nor the nmPCNA, but instead the third form, iPCNA. The additional spots were the anti-p21 antibody light and heavy chains that were used to precipitate p21/PCNA that are recognized by the secondary Western blotting antibody.

To confirm that p21 specifically interacted with the iPCNA, another technique, a GST pull-down assay, was used to examine the intermolecular association of p21 with PCNA. Recombinant p21 fused to a glutathione-S-transferase affinity tag (GST-p21) was expressed in *E. coli*, and bound to glutathione-Sepharose. GST-p21/glutathione-Sepharose was then incubated with MCF7 NE/S3 protein fraction for 2 hours and precipitated by low speed centrifugation. The precipitated proteins were subsequently resolved by 2D-PAGE and Western blotted for PCNA (Figure 8). The data showed that p21 bound to and precipitated PCNA. Comparison of MCF7 NE/S3 protein fraction resolved by 2D-PAGE (Figure 8A) with the GST-p21 pull-down resolved by 2D-PAGE (Figure 8C) showed that csPCNA and nmPCNA were present in the control gels but were not present in the GST pull-down gels. Instead, the results corroborate the results of the p21 co-immune precipitations and showed that GST-p21 specifically precipitated iPCNA. The GST pull-down assay showed two interesting phenomenon. First, nmPCNA could be seen in the GST-p21 pull-down in addition to the iPCNA, and a faint line connecting them was indicative of conversion of nmPCNA to iPCNA. Secondly, the total absence of csPCNA and nmPCNA in the supernatant suggested that p21 may also be interacting with csPCNA, and that this interaction may also convert csPCNA into iPCNA. However, the amount of p21 used in these assays was enormous, and it was highly unlikely that MCF7 cells would have these levels of p21 without first encountering DNA damage. Therefore, it was feasible that the molar excesses of GST-p21 may have "forced" an interaction with csPCNA and nmPCNA, and this could explain the increased amounts of iPCNA in the GST-p21 precipitation gel (Figure 8C) and the disappearance of csPCNA and nmPCNA in the pull-down supernatant gel (Figure 8D). To further investigate whether p21 specifically interacted with iPCNA, another experimental approach was taken.

Far-Western blotting was used to examine the specific interaction of p21 with iPCNA. Similar to traditional Western blotting, Far-Western blotting utilizes a labeled protein, in this case p21, instead of an antibody for detection of proteins immobilized on membranes. Forty μg

of MCF7 NE/S3 protein fraction was resolved by 2D-PAGE using the 2D cell and transferred to polyvinylidene difluoride (PVDF) support membranes. The MCF7 NE/S3 proteins immobilized on the PVDF membranes were then denatured in hybridization buffer containing 6 M guanidine HCl and slowly re-folded to their native conformation by serial dilution of 6 M guanidine HCl to 0.187 M and finally in hybridization buffer alone. The membranes were then incubated with ^{32}P-labeled GST-p21 at 4°C overnight, and the membranes were then washed three times with hybridization buffer, subsequently dried and exposed to X-ray film at -80^{0}C (Figure 9). Far-Western blotting identified multiple proteins including a protein at 36 kDa. However, this assay did not recognize either csPCNA or nmPCNA. Again, Far-Western blotting showed that p21 interacted specifically with iPCNA, and this suggested that p21, at least in lower concentrations exclusively interacts with iPCNA. Interestingly, unlike the previous experiments, csPCNA and nmPCNA were immobilized on membranes and were unable to change their tertiary structure. In other words, large molecular excesses of p21 in this assay may be unable force an interaction with either csPCNA or nmPCNA, and this could be due to the structural rigidity of the immobilized proteins. The appearance of additional spots was not unexpected, and at least two spots that probably represent a p21 interaction with a higher molecular weight cyclin protein, possibly cyclin A at 65 kDa, and a lower molecular weight CDK, possibly CDK2 at 33 kDa that were present in the MCF7 NE/S3 protein fraction were visualized. A third, highly abundant spot present on the gel was somewhat of a mystery, but it most probably represented a cyclin, CDK, or possibly a cyclin related phosphatase.

The results obtained by co-immune precipitation, GST pull-down assay, and Far-Western blotting identified a form of PCNA, iPCNA, that has a molecular weight of 36 kDa and has an intermediate pI consistent with the data obtained by 2D-PAGE of the MCF7 NE/S3 protein fraction using the IEF cell (Figure 6). Together, these data suggested that iPCNA was <u>not</u> an artifact of the 2D-PAGE methodology, but was a real form of PCNA present in MCF7 cells, and that this form specifically interacted with p21. The experiments also show that p21 was apparently unable to interact with either the csPCNA or the nmPCNA, at least in low p21 concentration. To more definitively demonstrate that p21 preferentially bound to iPCNA; the kinetics of the p21 interaction with PCNA was analyzed by surface plasmon resonance (SPR) biosensor analysis.

I. The csPCNA and iPCNA can be Isolated from MCF7 Cells.

The first step needed in order to analyze the interaction kinetics between the PCNA and p21 was the purification of csPCNA and iPCNA. Purification of csPCNA and iPCNA was accomplished using a series of ion exchange and hydrophobic interaction chromatographic steps (Figure 10). Briefly, MCF7 cells were fractionated to the level of NE/S3 protein fraction (Figure 11A). PCNA was then purified from the NE/S3 fraction by chromatography using a weak anion exchange resin, phosphocellulose (Figure 11B). The PCNA containing flow-through fraction resulting from phosphocellulose chromatography, which is devoid of RF-C, and the DNA polymerases α, δ, and ε (Waga *et al.*, 1994), was further purified by hydrophobic interaction chromatography using phenyl-Sepharose. The csPCNA and iPCNA present in the phenyl-Sepharose eluate were then subjected to strong cation exchange chromatography using SP-Sepharose. The csPCNA having a pI below the pH of the malonate buffer (5.8) was unable to bind the SP-Sepharose matrix and instead flowed through the column, while the positively charged iPCNA bound the SP-Sepharose matrix effectively separating the two forms from one another. The iPCNA was eluted from the SP-Sepharose column using a linear gradient of sodium chloride up to 0.5 M (Figure 11D). CsPCNA was purified from other proteins present in the SP-

Sepharose eluate by Q-Sepharose chromatography (Figure 11E). Figure 11 shows the forms of PCNA that were present at different stages during the purification scheme confirming that the csPCNA and iPCNA have been successfully resolved from one another. The Western blots also suggested that csPCNA and iPCNA present in the Q-Sepharose and SP Sepharose eluates, respectively, were dramatically enriched. (Compare the amount of protein loaded and the intensity of the spots from the NE/S3 panel to the Q-Sepharose and SP-Sepharose panels.) It was also of interest that the iPCNA couldn't be seen in the steps prior to its elution from the SP Sepharose column. The enriched isolated fractions containing csPCNA and iPCNA now allowed for the kinetic analysis of their p21 interactions.

K. p21^{WAF1} has a 10,000-Fold Higher Affinity for iPCNA than csPCNA.

In the previous experiments I have shown that p21 specifically interacted with iPCNA, and I have also demonstrated that csPCNA and iPCNA could be isolated from MCF7 cells. To definitively show that p21 specifically binds iPCNA and not the csPCNA, surface plasmon resonance (SPR) biosensor was used to define the kinetics of the p21 interaction. Using a BIACORE 2000 SPR mass spectrometer, ~160 and 60 response units (RU) of purified csPCNA and iPCNA were immobilized on the surface of a CM-5 sensor chip. Purified recombinant GST-p21 was then passaged over the surface of the sensor chip in varying concentrations (Figure 12). Non-specific interactions of p21 with the sensor chip were controlled for by subtracting the binding responses generated by passage of p21 over a blank (no protein) flow cell and a flow cell containing a non-specific protein. The resulting response curves were evaluated using BIAevaluation software. The BIAevaluation software fit the data obtained using the BIACORE to 1:1 Langmuir binding and 1:1 Langmuir binding with a drifting baseline for the interactions of p21 with csPCNA and iPCNA, and the statistical values (χ^2) for the fit were 1.18 and 1.91, respectively. The K_a and K_d values for p21 binding to csPCNA are 9.6×10^4 M^{-1} and 1.04×10^{-5} M, while the values for p21 binding of the iPCNA were 9.36×10^9 M^{-1} and 1.07×10^{-10} M (Table I). These data demonstrated that iPCNA has a 10,000-fold greater affinity for p21 as compared to csPCNA suggesting that, under normal physiological conditions, p21 exclusively interacts with iPCNA. Most importantly, these data demonstrated that p21 does not interact with the csPCNA and confirmed the data obtained by the interaction studies.

L. Identification of csPCNA by 2D-PAGE and Mass Spectrometry.

To better understand the differences between the three forms of PCNA, we have begun to use mass spectrometry to delineate the structure of the proteins. The techniques we employed are widely used in the study of the proteome or proteomics. The goal of proteomics is to identify all the proteins of individual cells. First, cellular proteins are separated using 2D-PAGE. Spots representing proteins stained with a protein dye are then excised from the gel and then "in-gel" digested using a protease such as trypsin. The proteolytic fragments are subsequently extracted from the gel and analyzed by mass spectrometry.

Using these techniques, csPCNA was identified from a fairly complex mixture of proteins. Briefly, PCNA was purified from the MCF7 NE/S3 protein fraction by phosphocellulose chromatography followed by passage over a phenyl-Sepharose column. The proteins present after the two steps of chromatography were then resolved by 2D-PAGE. The resolved polypeptides were subsequently stained with Bio-Safe Coomassie. For comparison purposes, one of the gels was transferred to nitrocellulose and probed with anti-PCNA antibody. The Western blot localized general areas of the gels that contained PCNA, which directed the excision of spots present in the Coomassie stained gels. Figure 12 shows the image of the Coomassie stained gel, and circled are ten spots thought to be csPCNA. These spots were

removed from the gel and digested with trypsin. In addition, 11 spots thought to correlate to the nmPCNA present in the protein stripe at the right hand side of the gel were also removed for tryptic digestion. The tryptic fragments from the in-gel digestion were analyzed using an LCQ mass spectrometer. The mass spectra obtained were used to search two different databases, Seaquest and Mascot.

The database searches revealed internal sequences of eight peptides that corresponded to the tryptic fragments of csPCNA present in spot A6 (Table II). Analysis of these sequences, however, revealed no post-translational modifications except for oxidized methionines, which are by-products of sample processing. Unfortunately, no PCNA peptide(s) were recovered from the spots removed from the protein streak present on the right of the gel. Although the presence of nmPCNA in the stripe was shown by Western blot, the resolution of these gels was not great enough in the range of pH 7 to 10 to be able to effectively separate it from other proteins that were present in that pI range, which obstructed our ability to identify and excise nmPCNA as an individual spot corresponding to nmPCNA.

Because of the inability to resolve and identify nmPCNA in Figure 12, we began to run higher resolution 2D-PAGE gels using the IEF cell. Large amounts of MCF7 NE/S3 protein fraction (450 μg) was resolved by 2D-PAGE and stained with Bio-Safe Coomassie. One hundred μg of MCF7 NE/S3 was resolved on a parallel gel, transferred to nitrocellulose, and Western blotted for PCNA (Figure 6). The Western blot was again used as a reference to identify the spots corresponding to PCNA. Several spots from the Coomassie gel were removed and subjected to in-gel digestion with trypsin. Unfortunately, analysis of the spots by mass spectrometry yielded no peptides correlating to PCNA.

Although we have only been able to identify PCNA once using mass spectrometry, the results presented here show promise for the eventual elucidation of the structural modifications to the PCNA molecule. In addition to the experiments described here we have begun to explore alternative approaches for the separation and identification of the different forms of PCNA. They include new technologies available such as the robotic spot cutters that are better able to identify and excise spots from 2D gels, and two-dimensional liquid chromatography that is able to separate complex mixtures of proteolytic fragments prior to mass spectrometry. Clearly the elucidation of the chemical modification(s) on PCNA has proven to be an intricate and difficult problem to solve. However, with access to newer and more powerful technologies along with utilization of alternative approaches, the answers to these and other complicated biochemical questions will soon be found.

Key Research Accomplishments

- Determined the pI values for the cancer-specific PCNA (csPCNA) and non-malignant PCNA (nmPCNA)
- Determined the molecular mass differences between the csPCNA and nmPCNA
- Cloned PCNA from MCF7 cells
- Expressed and purified recombinant PCNA (rPCNA) from *E. coli*
- Determined the pI of rPCNA to be identical to nmPCNA
- Electroeluted PCNA from 2-dimensional (2-D) gels
- Demonstrated that nmPCNA lost modification during the 2D-PAGE/electroelution process
- Identified a third or intermediate form of PCNA (iPCNA) present in MCF7 cells
- Co-immune precipitated iPCNA with p21 antibodies suggesting a specific interaction of iPCNA and p21
- Confirmed the interaction of p21 with iPCNA by GST pull-down assay and Far-Western blotting
- Purified csPCNA and iPCNA
- Analyzed the interaction kinetics of p21 with csPCNA and iPCNA
- Identified csPCNA resolved by 2D gels by LC/MS/MS

REPORTABLE OUTCOMES

DEGREES OBTAINED

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INVENTIONS

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II. CONCLUSIONS AND DISCUSSION.

A. Discovery of a Third Form of PCNA: iPCNA

To confirm the presence of two different forms of PCNA in MCF7 cells, a new higher resolution approach to 2D-PAGE was taken. Briefly, this new 2D-PAGE technique utilized IEF strips that are focused at voltages over 1000 times greater than the previous technique enabling the separation of proteins with a significantly higher resolution. The IEF strips also provide a larger and more uniform pH gradient spanning a pH from 3-10 leading to a more effective and accurate separation of proteins by their pI. Analysis of large amounts (200 μg) of MCF7 extracts using this technique followed by Western blotting for PCNA showed an interesting result. MCF7 cells contain not two forms of PCNA but three. The Western blot showed the presence of third form of PCNA at an intermediate pI that was not as acidic as csPCNA and not as basic as nmPCNA. This form of PCNA was therefore named the intermediate form or iPCNA. Interestingly, this was not the first time iPCNA was visualized by Western blotting. In numerous other experiments the intermediate form was noted. For instance, a separate pool of PCNA was identified when PCNA was electroeluted from 2D gels (Figure 5). Sometimes cellular extracts and experiments showed the presence of the intermediate form, but because these analyses never contained what looked like csPCNA, the phenomenon was passed off as an artifact. Somehow the gels did not resolve csPCNA to its usual position, but resolved it at a more basic pI. This artifact also seemed to always occur during the analysis of PCNA in p21 interaction experiments; the intermediate form is not quite malignant, but not normal either. It wasn't realized that the intermediate form of PCNA was real until it was identified with csPCNA and nmPCNA on a single gel, hence iPCNA.

There are some interesting conclusions that can be drawn from this information. First, in MCF7 cells, iPCNA was present in very low abundance, and the low abundance of iPCNA may be the real reason that it was never visualized with the other two forms of PCNA on previous gels. The iPCNA therefore could be the result of A.) a different modification on csPCNA, B.) a loss of one or more modifications from nmPCNA, or C.) all of the above. Examination of these gels gives some insight into the answer. It seems that iPCNA was closely related with csPCNA, and this was seen by the way the proteins "tail" into each other on the gels. In other words, the tear-shaped structure of csPCNA clearly leads to iPCNA as if iPCNA were being converted to csPCNA somehow.

B. Purification of csPCNA and iPCNA.

To more fully understand the differences in the post-translational state of csPCNA and iPCNA, the two forms have been isolated from MCF7 cells. The purification scheme follows closely the protocol established by Waga *et al.* (1994) with some exceptions. Following their methods, total cellular PCNA was enriched using two different types of liquid chromatography phosphocellulose and phenyl Sepharose. This protocol was first developed in our laboratory to separate csPCNA from nmPCNA. However, it was soon realized this method was effective for the isolation and separation of csPCNA and iPCNA. After PCNA was enriched by passage over the two columns, iPCNA and csPCNA were effectively separated from each other by cation exchange chromatography in a buffer with a pH of 5.8. CsPCNA, having a pI of 4.7, has a negative character at a pH of 5.8 and flows through the cation exchange column. The presence of csPCNA in the flow-through of the SP-Sepharose column confirms that this form of PCNA has a pI lower than 5.8. The intermediate form of PCNA could also be isolated by SP-Sepharose

chromatography, but unlike csPCNA, iPCNA bound to the column. Therefore, at a pH of 5.8, iPCNA exhibits positive character, suggesting that iPCNA has a pI value greater than 5.8. Additionally, because iPCNA elutes from the cation column in low concentrations of salt (~300 mM), iPCNA's pI is most likely not considerably greater than 5.8, but above 5.8 nonetheless. This is also confirmed by 2D-PAGE of iPCNA isolated by cation exchange. Isolated iPCNA exhibits an interesting albeit frustrating phenomenon. Once purified from other cellular proteins and other forms of PCNA, iPCNA readily precipitates. Apparently, iPCNA forms aggregates in solution and falls out of solution. In fact, after initial identification of PCNA in the cation exchange eluate, 2D-PAGE of the same fractions yielded no identifiable spots by immunoblotting. It then was concluded by the addition of protease inhibitors that the disappearance of iPCNA from the eluate was not the result of proteolysis. Therefore this form of PCNA is apparently unstable in solution by itself, and this instability is most probably due to a combination of factors. First, PCNA's ability to form multimers could contribute to the formation of aggregates that causes precipitation from aqueous solutions. Also, the pH of the buffer is very near the pI of iPCNA, and this too could lead to solubility problems. Additionally, iPCNA may not be stable in solution by itself it may require another protein to keep it in solution. This solubility problem was eventually overcome by the addition of 20% ethylene glycol to the purified iPCNA, and 2D-PAGE/Western blotting was able to confirm the exclusive presence of iPCNA in the cation exchange eluate. This protocol was therefore effective for the isolation of csPCNA.

Now that three different forms of PCNA have been identified and csPCNA and iPCNA have been isolated, I examined which, if not all, forms of PCNA, were able to interact with the DNA replication inhibitory protein p21.

C. The Interaction of p21 with iPCNA.

Multiple different approaches were taken to analyze the interaction of p21 with PCNA, and all of these experiments provide evidence supporting the interaction with p21 with the intermediate form of PCNA. First, co-immune precipitations clearly identified iPCNA as the form of PCNA that specifically precipitates with anti-p21 antibody, and neither csPCNA nor nmPCNA were detected. This evidence demonstrated that p21 present in MCF7 cells specifically interacted with iPCNA and seemingly does not interact with either the csPCNA or the nmPCNA. Interestingly, the levels of p21 in MCF7 cells in normal culture conditions were very low. This was consistent with the low levels of DNA damage these cells encounter and this observation could directly correlate with the quantity of the iPCNA present in these cells. This suggests that under conditions that lack high levels of p21 or, in other words, in conditions that do not promote DNA damage, p21 is associated with iPCNA, and the iPCNA/p21 complex may be localized with the DNA synthesome. Then, to confirm that the iPCNA specifically interacts with p21 and not csPCNA or nmPCNA, another approach was taken to study the interaction of p21 and PCNA.

The interaction of p21 and iPCNA was scrutinized by affinity precipitation (GST pull-down) of iPCNA with a recombinant p21. This is a more artificial way of analyzing the interaction because it utilizes a p21 protein produced in bacteria. However, it is an interesting experiment in that it somewhat mimics the induction of p21 in response to DNA damage. High levels of p21 bound to Sepharose beads were incubated with MCF7 extracts, and again the GST-p21 pull-down assays demonstrated that p21 specifically

interacts with iPCNA. Therefore, iPCNA precipitated by p21 could be enriched from the extracts, because its presence in the control is undetectable. Amazingly, p21 was able to precipitate nearly all of the PCNA present in the MCF7 extracts. However, all of PCNA precipitated is apparently iPCNA. These results allude to an ability of p21 to convert both csPCNA and nmPCNA to iPCNA. The p21 precipitate also shows the presence of nmPCNA, and, although it is in very low abundance, it is suggestive of an interaction. Nonetheless, the presence of nmPCNA seems to be transient. A trail seems to be present that suggests a conversion of either iPCNA to nmPCNA or, more likely, nmPCNA to iPCNA. On the hand, csPCNA is not detected in the precipitate. These results suggest that p21 is able to interact with nmPCNA and csPCNA, but the interaction causes the conversion of either nmPCNA or csPCNA to iPCNA. Then, to test whether p21 was able to interact with csPCNA and nmPCNA as it interacts with iPCNA, another approach to examine the p21 interaction with PCNA was used. This technique, Far-Western blotting, takes advantage of protein support membranes, and these membranes rigidly hold the conformational states of the immobilized proteins and does not allow for gross conformational changes to occur.

Similar to Western blotting, Far-Western blotting utilizes radiolabeled p21 instead of anti-PCNA antibody to detect PCNA immobilized on the membranes. This technique recognized a low molecular weight protein (~33 kDa.) that corresponded to a CDK, possibly CDK2, a high molecular weight protein (~65 kDa.) corresponding to a cyclin, possibly cyclin A, and a protein at ~58 kDa. that is somewhat of a mystery, but could be CDC25A, a protein phosphatase that is present in cyclin/CDK complexes and is responsible for removal of inhibitory phosphates from CDKs. The most interesting spot however is the spot in the middle of the blot at a molecular weight of 36 kDa and at intermediate pI. This spot corresponds with pI and molecular weight data for iPCNA, again providing evidence for the interaction of p21 with iPCNA. On the other hand, csPCNA and nmPCNA were not visualized, suggesting that they do not interact with p21.

The inability of p21 to interact with csPCNA and nmPCNA by Far-Western blotting could be slightly misleading. Undoubtedly, iPCNA has the highest affinity for p21. However, this does not mean that csPCNA or nmPCNA are unable to interact with p21. It is possible that the conditions of this experiment did not allow for detection of their interaction or their interaction requires a conformational change as suggested by the GST pull-down assay. It can be argued that the inability of p21 to bind to csPCNA and nmPCNA could be the result of the inability of these forms to change their tertiary structure, and this inability to change tertiary structure could mean that epitope on PCNA that is responsible for the p21 interaction may lie buried within the protein. Additionally, the inability of p21 to bind csPCNA or nmPCNA in this assay may be the due to the fact that the labeled p21 is purified. Although p21 in the GST pull-down experiments is purified, it is added to extracts. The purified p21 could then associate with other factors (e.g. modifying enzymes) present in the extracts leading to the modification/de-modification of csPCNA and nmPCNA causing their conversion to iPCNA. It is therefore possible that, as a result of the p21 interaction, csPCNA and nmPCNA change conformation, and this conformational change exposes amino acids that contain charge and/or are available for modification, and these events are responsible for the altered 2D-PAGE migration of iPCNA seen in the previous experiments. It is therefore essential that we explore the PCNA molecule to look for post-translational modifications.

Identification of post-translational modifications present on the different forms of PCNA is a fairly straightforward process, and it encompasses the utilization of the techniques developed in the past 25 years for the study of the proteome. Now that the human genome has been sequenced (Venter *et al.*, 2001; McPherson *et al.*, 2001), the next logical step in life science research is to identify the structures of the encoded proteins and how they function to make a cell viable and give it a certain function. The study of the proteome or proteomics involves structural determinations of proteins present in cells. Proteomics also involves the study of protein/protein interactions, macromolecular complexes, and their functions within cells ultimately leading to better understanding of complex biochemical processes providing a "smarter" approach to the development of diagnostics and therapeutics for the treatment of disease.

D. The Kinetics of p21 Interaction with the csPCNA and iPCNA.

Using a proteomic approach I analyzed the interaction of p21 with csPCNA and iPCNA by surface plasmon resonance (SPR) biosensor analysis. SPR measures changes in mass on the surface of a thin sheet of gold. First, an immobilized ligand (PCNA) was bound to the surface of a dextran-coated gold chip, then protein solutions containing p21 were passaged over the chip and binding events were recorded by measuring the change in the angle of reflection of polarized light on the opposite surface of the gold chip. Therefore, the larger the change in reflective index on the surface of the chip, the greater the mass concentration on the surface of the chip indicating a higher degree of protein binding. I then utilized this technology to analyze the interaction kinetics of the p21 and PCNA.

The SPR experiments were carried out following rules developed by Myzka *et al.* (1999) for accurate biosensor analysis. First, to avoid avidity effects, isolated csPCNA and iPCNA were immobilized onto the surface of SPR chip instead of p21 because PCNA forms trimers in solution. Therefore, if p21 were immobilized a monomer, dimer, or trimer of PCNA could theoretically bind to one p21 molecule skewing the results. Secondly, the effects of "mass action" were minimized. Mass action is the result of multiple binding events with the same molecule. In other words, it is important to minimize the probability that one molecule of p21 could interact with more than one molecule of PCNA as it's passaged over the surface. This was done in several ways first; low quantities of PCNA were immobilized on the chip's surface. Additionally, the amount of exposure time was limited by passage of p21 at high flow rates. Interestingly, the p21/PCNA interaction has previously been analyzed by SPR (Gibbs *et al.*, 1997). However, there were major limitations with the earlier data. For one, the authors used recombinant PCNA, which I have shown to be identical to the modified nmPCNA. Also, the authors did not control for avidity effects or the effects of mass action. The authors immobilized p21 onto the chip surface allowing for up to three binding events to occur for a single p21/PCNA interaction. Also, the authors immobilized a tremendous amount of p21 on the surface of the chip leading to binding data in excess of 2000 RU. Also, the experiments were performed at very low flow rates (5 μl/min) with long exposure times almost guaranteeing multiple binding events. Indeed, these experiments were performed in the early days of SPR mass spectrometry, and these rules may not have been recognized as being important for accurate bio-sensor analysis, but with knowledge of these rules, a more valid experiment was performed.

The analysis of the p21/iPCNA interaction kinetics follows the rules for improved bio-sensor analysis more closely than previously published studies, and therefore may give a more accurate assessment of the interaction kinetics. To begin with, avidity effects were avoided in this experiment by immobilizing PCNA and not p21 onto the chip's surface. Next, even though the analysis with iPCNA has a longer exposure time (>30s), the mass action effects are fairly well controlled by the low amount of immobilized iPCNA (<70 RU) and flow rates of 100 μl/min. The effects of mass action were also controlled for in the studies with csPCNA. Although there were higher RU values than for iPCNA, high flow rates and short exposure time (30 seconds) limited most if not all of the mass action effects in the experiment. Unfortunately, the interaction analysis of p21 and iPCNA is hindered by the presence of a drifting baseline. However, BIAevaluation software, the software that evaluates the experimental binding data generated and fits it to a theoretical binding model is able to correct for the drifting baseline, and both p21 binding to csPCNA and iPCNA were fit to models with χ^2 values of less than 2. The affinity for the interaction of p21 with csPCNA is 10.4 μM. This extremely high K_d corroborates previous results that were unable to detect a p21 csPCNA interaction. Analysis of the p21 iPCNA interaction, on the other hand, showed a very strong affinity, K_d = 0.1 nM. These data again suggest that csPCNA does not interact with p21 under physiological conditions, while iPCNA interact with p21 with an extremely high affinity.

These findings were interesting because they suggested that p21 was able to differentially interact with the different forms of PCNA. Specifically, the interaction kinetics done using SPR demonstrated that p21 bound to iPCNA very strongly, while having a very weak ability to interact with csPCNA. This closely agreed with the previous experiments (co-immune precipitation, GST pull-down, Far-Western) that demonstrated a specific interaction of p21 with iPCNA. The results start to make sense when enzymatic activities are considered.

The ability of p21 to inhibit DNA replication and not DNA repair is controversial. First, Li *et al.* (1994) provided evidence that p21 was able to inhibit DNA replication *in vitro*, and that 60 to 240-fold excesses of p21 were unable to inhibit *in vitro* DNA repair in these same extracts. These results were collaborated by Shivji *et al.* also in 1994. They too showed that concentrations of p21 up to 0.8 μM effectively inhibited DNA replication activity in *Xenopus* egg extracts. However, these same concentrations had no effect on the ability of the extracts to repair a UV-damaged plasmid template. These results were challenged in 1995 by Pan *et al.* who showed that p21 concentrations as low as 0.34 μM inhibited 50% of DNA repair while concentrations reaching 1 μM inhibited 100% of DNA replication. They showed that a mere 3-fold excess of p21 to PCNA was enough to inhibit nucleotide excision repair. This result was surprising because further studies found that a 5 to 10-fold excess of p21 was needed to inhibit DNA replication *in vivo* (Li *et al.,* 1996) and therefore suggested that p21 inhibited DNA repair more effectively than DNA replication. These experiments, however, examined p21's effect on DNA replication and repair *in vitro*, and results looking at the ability of p21 to interfere with DNA repair *in vivo* showed that a 50:1 p21 to PCNA ratio was needed to inhibit DNA repair, while a 100:1 ratio inhibited 80% (Cooper *et al.*, 1999).

Whether the ratios of p21 to PCNA were accurate or not, the results definitely suggested that there is a difference in the ability of p21 to inhibit DNA replication and

repair, and this agreed very closely to the results obtained examining the interaction of p21 and the different forms of PCNA. It could therefore be argued that because p21 has a high affinity for iPCNA, it is the form of PCNA that is responsible for DNA replication inhibition. Alternatively, csPCNA may be the form of PCNA that is required for DNA repair, and because it has a lower affinity for p21 than iPCNA would not be affected by increased concentrations of p21. Interestingly, the results shown by GST pull-down of PCNA in MCF7 cells (Figure 8) showed the absence of csPCNA in the pull-down supernatant. This could very well agree with the results shown by large excesses of p21 *in vivo* (Cooper et al., 1999). Therefore, the inhibition of DNA repair in these cells could be due to the large excesses of p21 "forcing" an interaction with csPCNA. This forced interaction could either change the conformation of csPCNA and/or add modification(s) to PCNA through association with p21, which may thereby convert csPCNA to iPCNA. The intermediate form of PCNA or iPCNA could therefore be inhibitory with respect to DNA replication and DNA repair. This however suggests that iPCNA is not the form of PCNA that functions in DNA replication, but this may be the function of nmPCNA. Interestingly, the non-malignant breast cells only contain nmPCNA. This may suggest that although they are undergoing DNA replication, they are not being exposed to the levels of DNA damage that the malignant MCF7 cells are, and this is why iPCNA and csPCNA are visualized there. Also, the low levels of p21 present in the MCF7 cells could correlate with the amount of iPCNA present in these cells and could therefore be the result of sensing low levels of DNA damage. The presence of the three forms of PCNA in MCF7 cells could therefore be due to a signaling pathway that connects DNA replication and DNA repair through an interaction of p21 and PCNA (Figure 13).

E. Structural Analysis of the Different Forms of PCNA.

As with all other signal transduction pathways, molecular signals are propagated by structural changes of the signaling proteins. It is therefore imperative that we understand the structural modifications present on PCNA. In general, a proteomic approach was taken to elucidate the structural changes in csPCNA, iPCNA, and nmPCNA, and it encompassed the resolution of proteins by 2D-PAGE, excision of the polypeptides from the gels, proteolytic digestion of the polypeptides and mass sequencing of the resulting peptides. This was accomplished with limited results. Although csPCNA was identified using this technique, the analysis did not detect any structural changes in the polypeptide. One possibly explanation of these results is that csPCNA is not modified, as expected. Another reason could be that PCNA is losing its modification through the 2D-PAGE as seen in the electroelution experiments. Therefore, this presents a possible limitation to current proteomic research, and some laboratories have begun exploring two-dimensional chromatographic techniques for the in solution separation of proteins prior to mass spectrometry. Additionally, numerous attempts were made to remove iPCNA and nmPCNA from 2D gels for mass spectrometric analysis. This has proven much more difficult than originally thought. Recently, MCF7 protein fraction was resolved by 2D-PAGE and stained with Coomassie blue. Using a Western blotted 2D gel as reference for localization of the different forms of PCNA in combination analysis using PD-Quest 2D-PAGE software, over 100 polypeptides were removed using a robotic spot cutter in the areas were PCNA was visualized by Western blotting. Analysis by MALDI-ToF mass spectrometry identified the majority of the proteins, however, none were identified as PCNA. Clearly, this approach has turned out to be more difficult than

originally thought, and in order to analyze the differences in structure of csPCNA, iPCNA, and nmPCNA, alternative approaches for purification of PCNA have begun to be developed.

F. PCNA and p21 Signaling in MCF7 Breast Cancer Cells.

The evidence presented here is suggestive of a signaling pathway that may connect DNA replication and DNA repair (Figure 13). Interestingly, nonmalignant cells contain a single form of PCNA, nmPCNA. This form of PCNA may therefore be responsible for DNA replication, and presence of this form could be the result of cycling cells. CsPCNA is found in malignant cells, and according to Sekowski *et al.* (1998) malignant cells have lower replication fidelities and therefore are exposed to more DNA damage. Therefore, csPCNA may function during DNA repair. If these assumptions are true, it would be expected that p21, having the ability to inhibit DNA replication while not inhibiting DNA repair would be able to bind to nmPCNA and not csPCNA. Intriguingly, I have shown that neither is the case. In fact p21 binds to an intermediate form of PCNA (iPCNA) that is also present in malignant cells. Additionally, I have shown that high levels of p21 seem to be able to convert csPCNA and nmPCNA to iPCNA. Therefore, the presence of iPCNA in malignant MCF7 cells could be due to an interaction of p21 with PCNA. Additionally, because previous studies done using recombinant PCNA have demonstrated a high affinity for p21 (Gibbs *et al.*, 1997), the initial interaction of p21 with PCNA in cells that have undergone DNA damage could be with nmPCNA. The interaction of p21 with nmPCNA could lead to the conversion of nmPCNA to iPCNA as seen with the "tailing" of nmPCNA to iPCNA in the GST-p21 pull-down experiments. This could implicate iPCNA in the initial steps of DNA repair. Alternatively, csPCNA may function in the DNA re-synthesis step of DNA repair, and the low affinity of p21 for csPCNA would therefore allow DNA re-synthesis to occur unabated in the presence of p21. Therefore, with amounts increasing genetic insult, increasing levels of p53 may induce large molar excesses of p21. The large molar excesses of p21 could then be able to "force" an interaction with csPCNA, and this forced interaction could lead to the inhibition of DNA repair re-synthesis. Indeed, the DNA repair assays used in the previous studies (Shivji *et al.,* 1994; Li *et al.,* 1994; Pan *et al.,* 1995; Cooper *et al.*, 1999) all measure DNA re-synthesis The forced interaction of csPCNA with p21 may result in the conversion of csPCNA to iPCNA, and this conversion would then increase the levels of iPCNA in the cell. This would be of advantage to the cell because it would direct all the available PCNA to the initial steps of DNA repair. The different forms of PCNA demonstrated in this thesis could therefore be due to a intricate molecular signaling cascade that is occurring in malignant cells in response to their ever increasing levels of genetic mutation.

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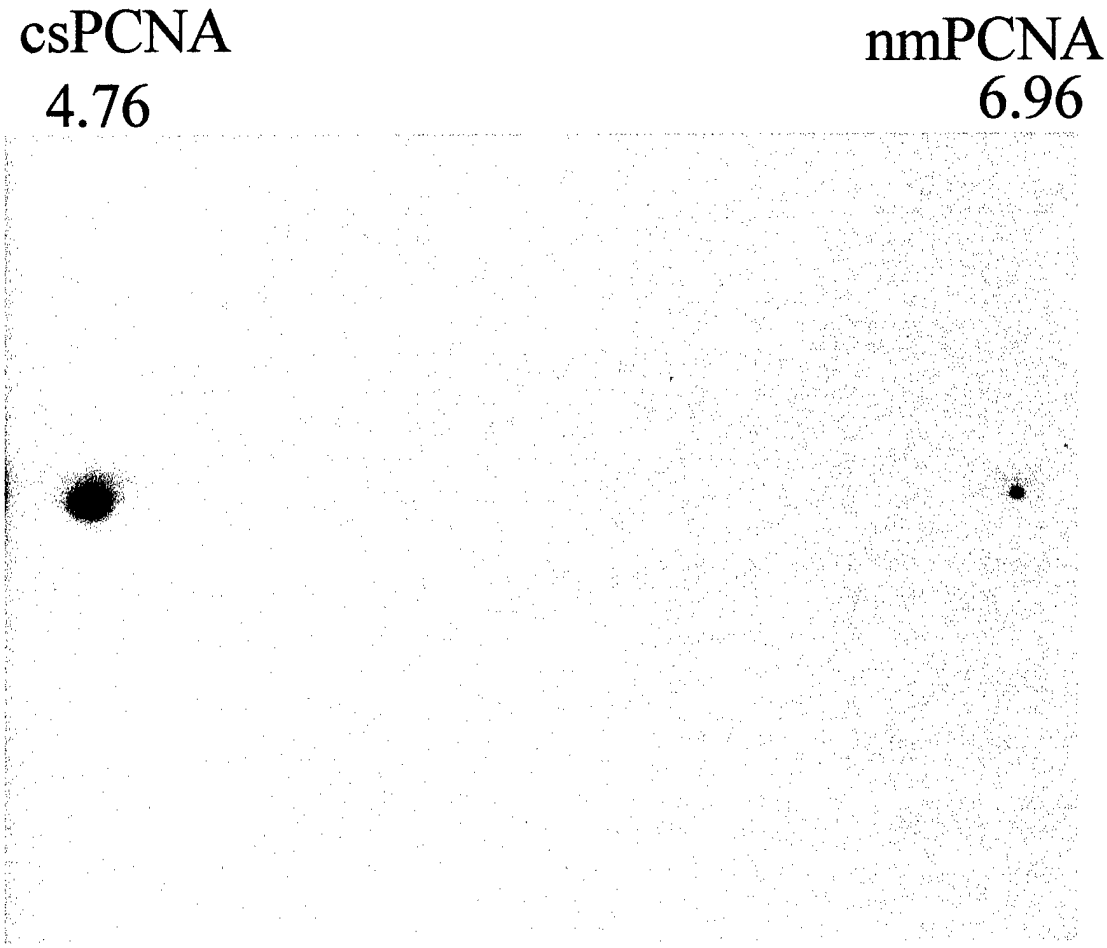


Figure 1. Determination of the pI values for csPCNA and nmPCNA. MCF7 cells were fractionated to the NE/S3 protein fraction according to Malkas *et al.* (1990) and subjected to 2D-PAGE. The resolved polypeptides were then electroblotted to nitrocellulose membranes and subsequently Western blotted with anti-PCNA antibodies. Two distinct species of PCNA were observed. The acidic form of PCNA has a pI of 4.74 (csPCNA), while the basic form has a pI of 6.96 (nmPCNA). **Materials and Methods.** MCF7 cells were fractionated to a clarified nuclear extract and post-microsomal (NE/S3) fraction and PCNA was purified using phosphocellulose and phenyl-Sepharose chromatography as described above. The PCNA containing fraction was then loaded onto a 2D-PAGE and resolved using the mini-PROTEAN II tube cell. The gels were silver stained and imaged using a GS710 scanning densitometer (Bio-Rad). The scanned image was then analyzed using the Melanie II software (Bio-Rad). Using 2D protein standards (Bio-Rad) as pI and molecular weight landmarks, Melanie II was able to assign pI and molecular weight values for the csPCNA and nmPCNA.

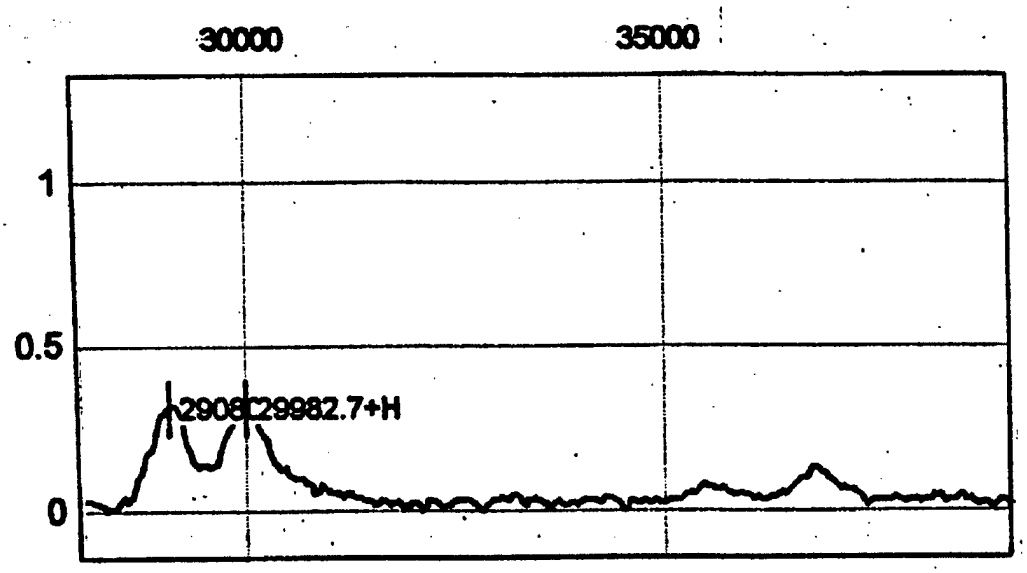


Figure 2. Surface Enhanced Laser Desorbtion/Ionization (SELDI) Mass Spectrometry of PCNA from MCF7 cells. Anti-PCNA antibodies were immobilized onto the surface of a SELDI sample plate and MCF7 P4 protein fraction (Malkas *et al.*, 1990) was added to the surface of the plate. Non-specific proteins were washed away and the proteins binding the anti-PCNA antibody were analyzed by time of flight mass spectrometry using a SELDI mass spectrometer (Ciphergen). The above spectrum shows the presence of two peaks. A peak with a mass of 29,080 and a peak with a mass of 29,982. These data suggest that the modification on PCNA has an approximate molecular weight of 900 Da.

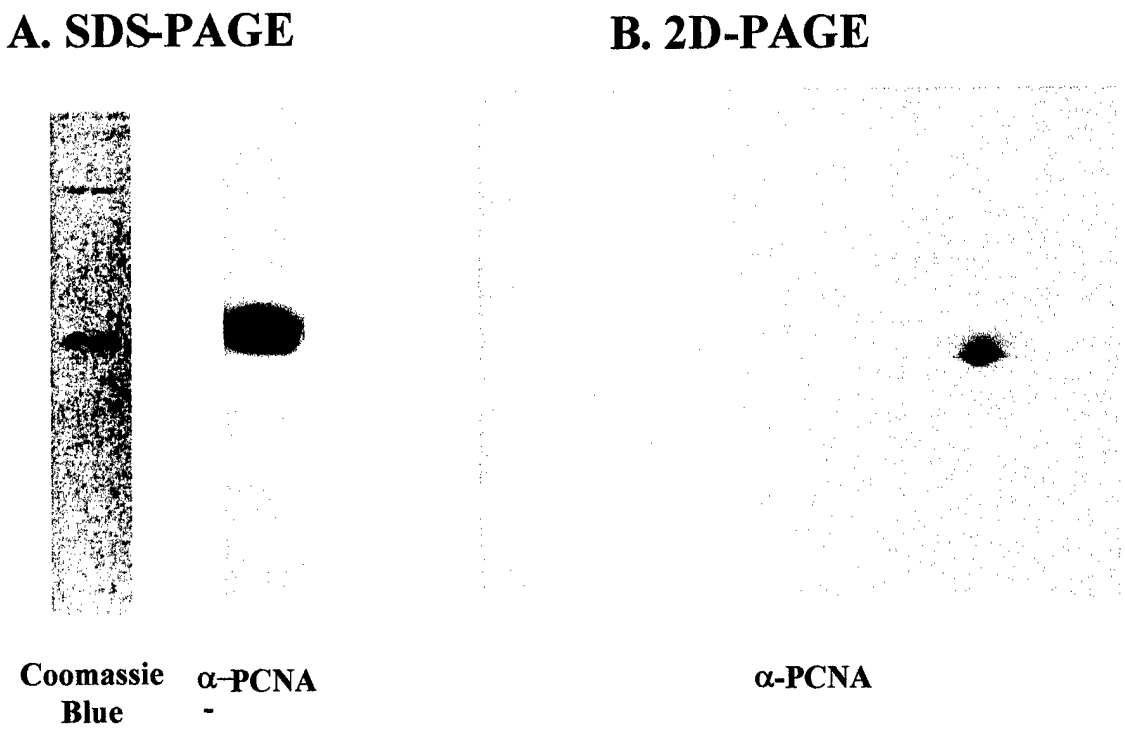


Figure 3. Purification and Analysis of Recombinant PCNA. PCNA was purified from *E. coli* as described in the text and analyzed by 2D-PAGE and Western blotting. 5 ug of purified PCNA was resolved by SDS-PAGE, Coomassie stained and Western blotted using anti-PCNA antibodies (panel A). The Commassie stained gel shows the presence of two bands (arrows) that represent the lower molecular weight PCNA monomer and higher molecular weight band that could represent a minor contaminant or a PCNA multimer. The Western blot recognizes a PCNA monomer at the same molecular weight as the Coomassie band (arrow), however does not show the presence of a higher molecular weight band. The CBP affinity tag was then removed using thrombin and PCNA was resolved by 2D-PAGE and transferred to nitrocellulose. suggested that either rPCNA is not post-translationally modified as initially suspected, or it is losing the modification at some step prior to the sequencing step. Immunoblotting of the nitrocellulose shows the exclusive presence of the nmPCNA (panel B). **Materials and Methods.** PCNA cDNA was cloned from MCF7 cells as described by Bechtel *et al.*, 1998. Briefly, the cDNA sequence encoding the entire PCNA translation unit was prepared using Superscript Preamplification System First Strand DNA Synthesis (Life Technologies, Inc.) followed by cloning of the amplified cDNA into the vector pCR2.1 (InVitrogen) according to the manufacturers instructions. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Second strand DNA synthesis was carried out by priming the first strand cDNA with the oligonucleotide 5'-GCGTTGTTGCCACTCCGC-3' on the 5' end of the cDNA and 5'-GCAGTTCTCAAAGAGCTTAG-3' on the 3' end of the cDNA and amplifying the primed first strand using reverse transcriptase PCR.Subcloning of the cDNA into a protein expression plasmid was done using the pDUAL expression and cloning kit (Stratagene) according to the manufacturer's protocol. Briefly, *Eam1104I* restriction sites were engineered into the 5' ends of the PCR primers 5'-ATGTTCGAGGCGCGCCTGGTCCAG-3' and 5'-

AGATCCTTCATCCTCGATCTTGGGAGC-3', which were used to amplify the PCNA coding sequence. Eam1104I was then used to generate staggered ends, and the PCR product was inserted into the pDUAL expression vector. Expression of PCNA in *E. coli BL21(DE3)* cells (Stratagene) was done according to the procedures of Podust *et al.,* 1995 yielding a full-length PCNA fused with a 6000 Da calmodulin binding protein affinity tag. Purification of PCNA was accomplished using Calmodulin Affinity Resin (Stratagene) which specifically bound a Calmodulin Binding Protein (CBP) tag fused to the C-terminus of PCNA according to the manufacturers instructions.

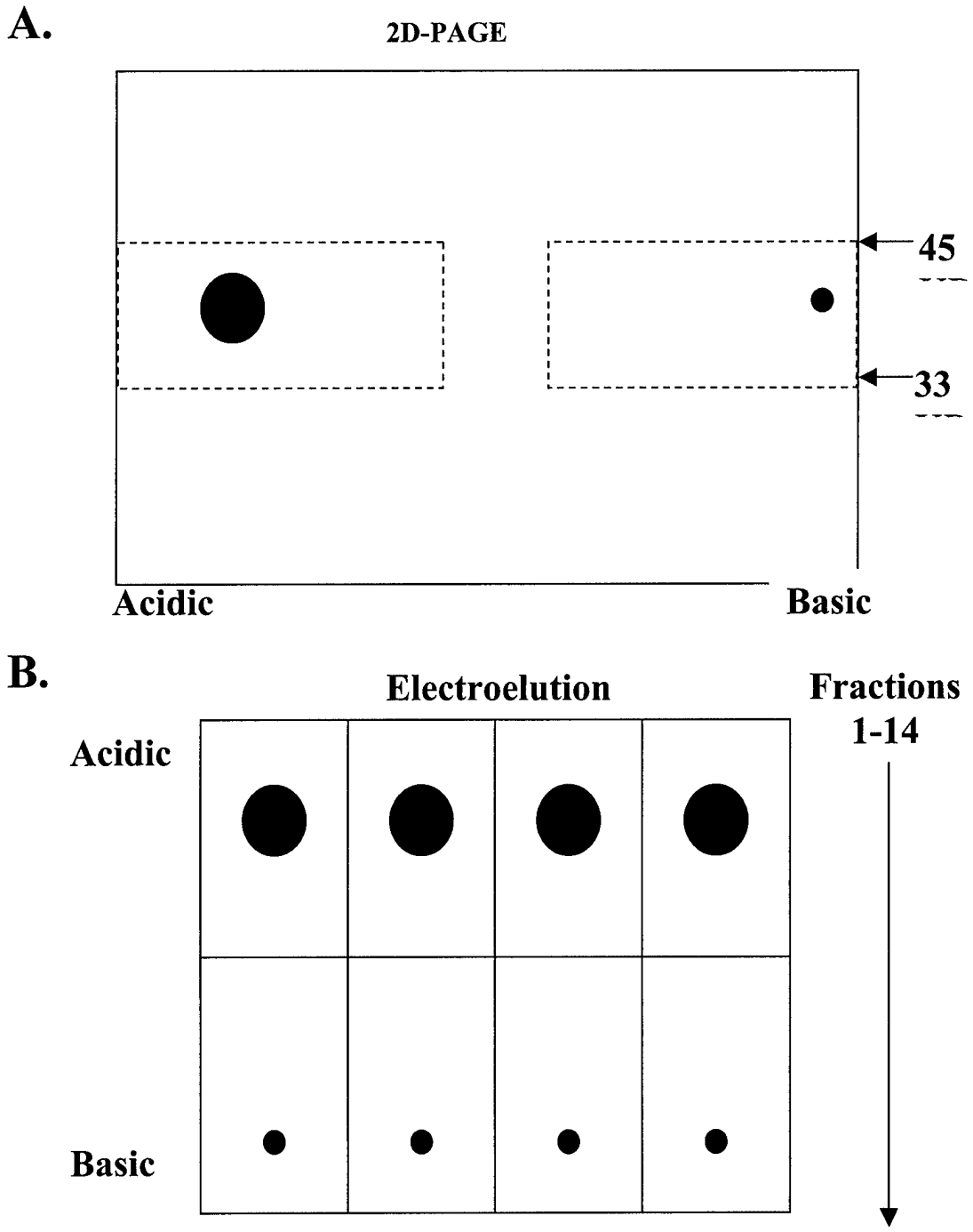


Figure 4. Electroelution Scheme for PCNA. PCNA was excised from 2D-PAGE gels as shown by the dotted lines (A). The excised segments of the gels were rotated 90° clockwise and placed in the electroeluter as shown in B. PCNA was then eluted into 14 fractions from the acidic end of the gel to the basic side of the gel.

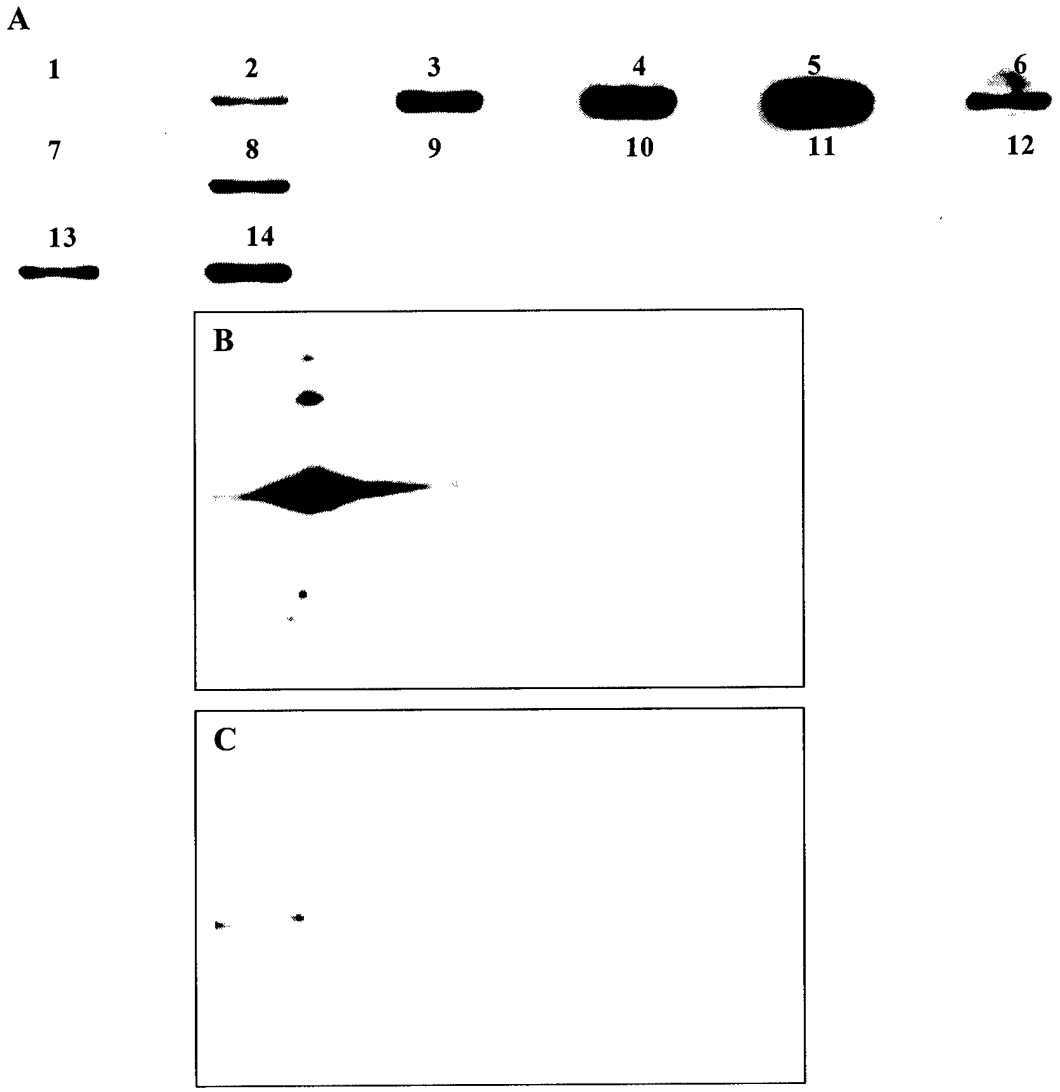


Figure 5. Electroelution of PCNA from 2D-PAGE. MCF7 P4 protein fraction (600 μg) was resolved by 2D-PAGE. The areas of the gels known to harbor PCNA were removed and electroeluted into 50 mM HEPES, pH 7.5, with the most acidic side of the gel eluting into the lower fractions, and the more basic proteins eluting into the higher fractions. The PCNA slot blot of the 14 electroeluted fractions is shown in A. Electroeluted fractions 5 (B) and 14 (C) were then resolved by 2D-PAGE and Western blotted for PCNA. Panel C shows the conversion of the nmPCNA to csPCNA resulting from the 2D-PAGE/electroelution procedures. **Materials and Methods.** The csPCNA and nmPCNA resolved by 2D-PAGE were removed from four parallel 2D-PAGE gels and electroeluted into 25 mM HEPES buffer, pH 7.2 using a Mini Whole-Gel Eluter according to the manufacturer's instructions (Bio-Rad). The presence of PCNA in the electroeluted fractions was determined by slot blotting (Life Technologies, Inc.), and the fractions were subsequently tested for the presence of either csPCNA or nmPCNA by 2D-PAGE.

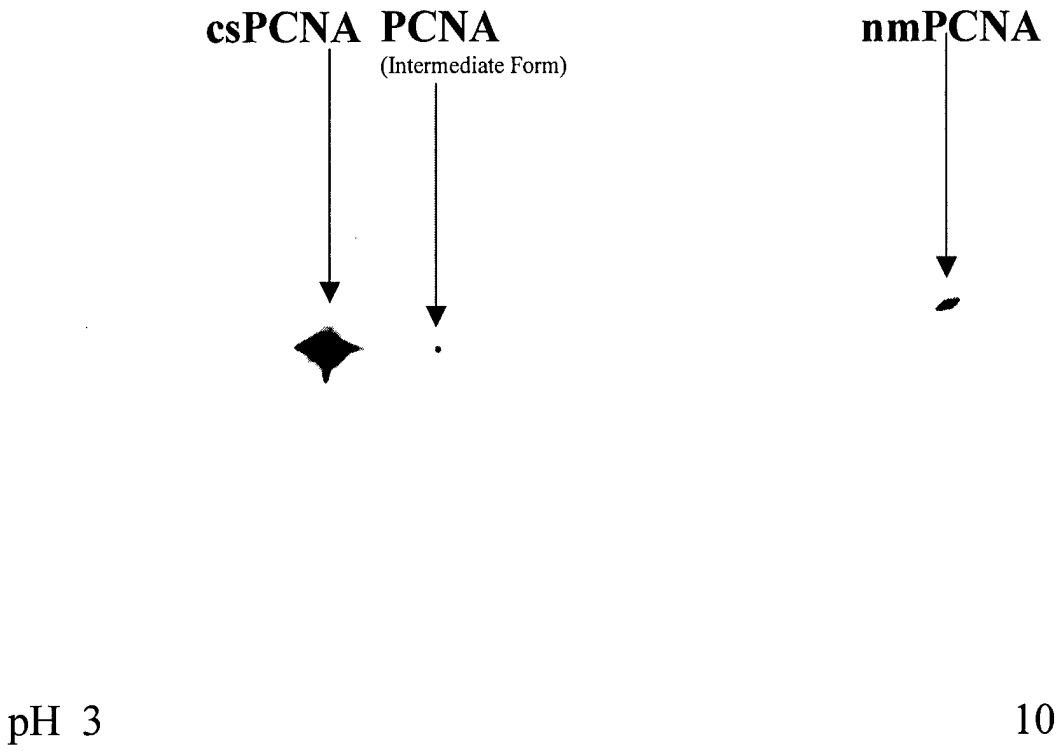


Figure 6. Identification of a Third Form of PCNA in Breast Cancer Cells. MCF7 NE/S3 protein fraction (200 μg) was resolved on pH 3-10 IEF strips (Bio-Rad), then resolved in the second dimension using an 8-16% SDS-PAGE, the resolved polypeptides were then electroblotted to nitrocellulose. The nitrocellulose membrane was then Western blotted using anti-PCNA antibody. The Western blot shows the presence of three forms of PCNA in MCF7 cells, the acidic cancer specific form (csPCNA), the intermediate form of PCNA, and the non-malignant form of PCNA (nmPCNA). **Materials and Methods.** MCF7 NE/S3 fractions were de-salted to dH2O using P6 desalting columns (Bio-Rad) and dried in a speed-vac (ATR Biotechnology). The dry sample was then re-suspended into sample rehydration buffer (Bio-Rad) and 2D-PAGE was performed using a PROTEAN IEF cell (Bio-Rad) following the manufacturer's instructions.

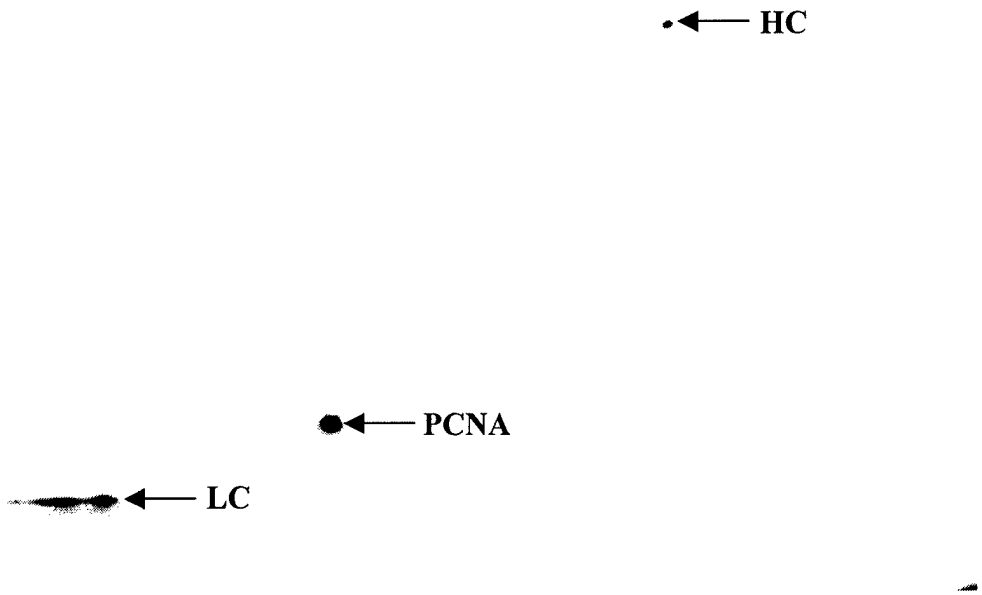


Figure 7. Co-immune Precipitations using anti-p21^{WAF1} Antibodies. MCF7 NE/S3 protein fraction (200 μg) was incubated with purified recombinant GST-p21 bound to glutathione Sepharose. The interacting proteins were precipitated and resolved by 2D-PAGE followed by Western blotting with anti-PCNA antibodies. The gel shows the resolution of low molecular weight antibody light chain (LC) and high molecular weight antibody heavy chain (HC) along with PCNA at 36 KDa (iPCNA). **Materials and Methods.** MCF7 cells were fractionated to a NE/S3 fraction and incubated with monoclonal anti-p21 antibody (DF10, Oncogene Research) at 4°C for 2 hours. The antibodies were then bound to BSA coated Protein-A-Agarose beads (Oncogene Research) and washed three times with buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.1% Triton X-100, and 500 mM NaCl). The protein precipitate was then resolved by 2D-PAGE and the polypeptides transferred to nitrocellulose. Western blotting was done using anti-PCNA antibody

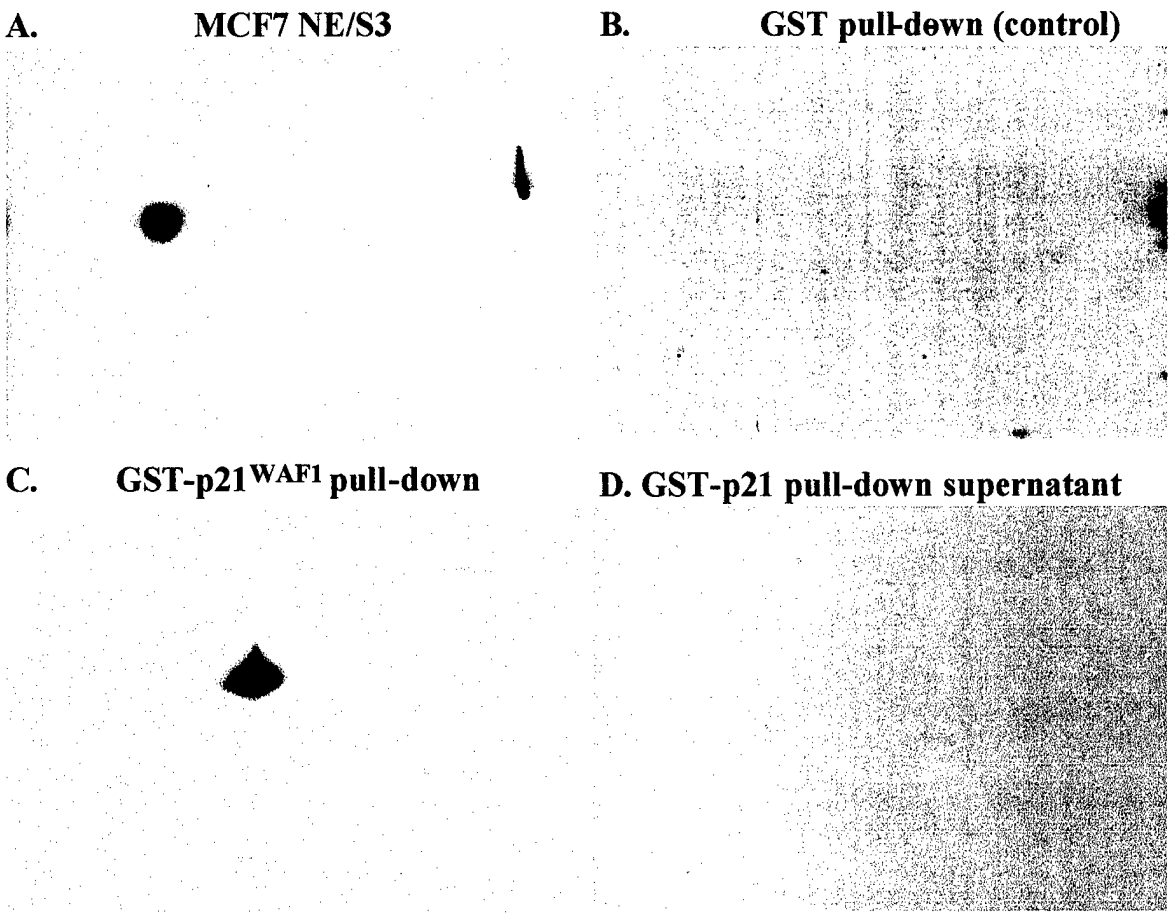


Figure 8. GST-p21^{WAF1} Pull-down Experiments. Recombinant GST-p21 was produced in *E. coli* and batch purified using glutathione-Sepharose. 200 μl of GST-p21 bound glutathione-Sepharose was then incubated with 200 μg of MCF 7 NE/S3 protein fractions. After incubation, the proteins co-precipitating with GST-p21 were resolved by 2D-PAGE and immunoblotted for PCNA. (A) Shows the csPCNA and nmPCNA present in these cells. (B) The GST control shows that GST alone does not precipitate PCNA. (C) GST-p21 precipitates the iPCNA. (Note the small presence of the nmPCNA). (D) The GST-p21 supernatants do not contain PCNA suggesting the possible conversion of the csPCNA and nmPCNA to the iPCNA. **Materials and Methods.** GST-p21 was purified from inclusion bodies present in the BL21(DE3) cells and bound to Glutathione-Sepharose. MCF7 NE/S3 protein fraction was then incubated with the GST-p21 conjugated Glutathione-Sepharose beads at 4°C for 2 hours. The beads were precipitated and then washed 3 times with ice-cold PBS. The precipitated PCNA was then analyzed by 2D-PAGE/Western blotting.

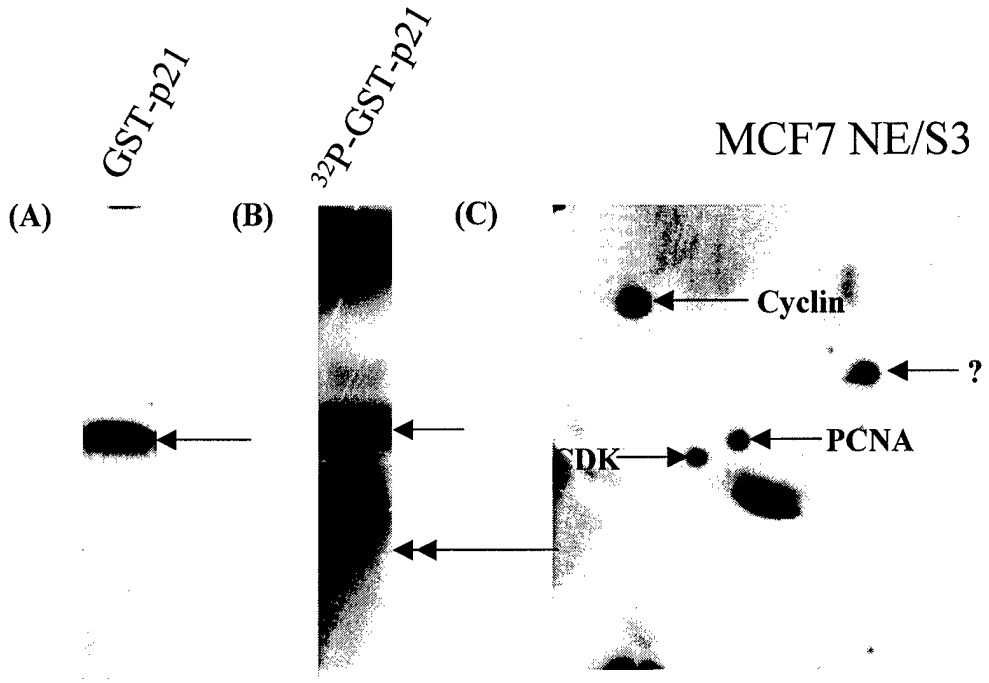


Figure 9. Recombinant p21^{WAF1} purification, labeling, and Far-Western blotting. p21^{WAF1} was produced in *E. coli* DE3 cells and purified as described in the text. 5μg of p21 was loaded onto a 12% SDS-PAGE gel and Coomassie stained showing the presence of a single protein band corresponding to GST-p21 (A). P21 was then labeled with γ^{32}P-ATP in the presence of bovine protein kinase C and run on 12% SDS-PAGE. The gel was then dried and exposed to Kodak XAR-5 film (B). The Far-Western blot of 40 μg of MCF7 NE/S3 protein fraction shows the presence the iPCNA (C, arrow). **Materials and Methods.** Recombinant GST-p21 bound to Glutathione-Sepharose 4B was labeled with α^{32}P-ATP using bovine protein kinase C (Sigma) following the manufacturers instruction (Amersham Pharmacia Biotech.). Unincorporated α^{32}P-ATP was removed from the bound GST-p21 by washing with ice-cold PBS three times, followed by elution into 10mM reduced glutathione (Sigma). Labeled p21 was visualized by SDS-PAGE followed by autoradiography (Figure 6). Far Western blotting was performed according to the protocol developed by Nie *et al.*, 1994. The MCF7 NE/S3 protein fraction was first resolved by 2D-PAGE and transferred to PVDF membrane (Bio-Rad). The immobilized proteins were then denatured in hybridization buffer containing 6 M guanidine HCl for 1 hour. The proteins were then slowly re-natured by serial dilution of guanidine HCl first to 3 M and step-wise down to 0.187 M and then into hybridization buffer alone. The membranes were incubated with ^{32}P-labeled GST–p21 (see above) overnight at 4°C. The membranes were washed three times with buffer, dried, and exposed to X-ray film at –80°C.

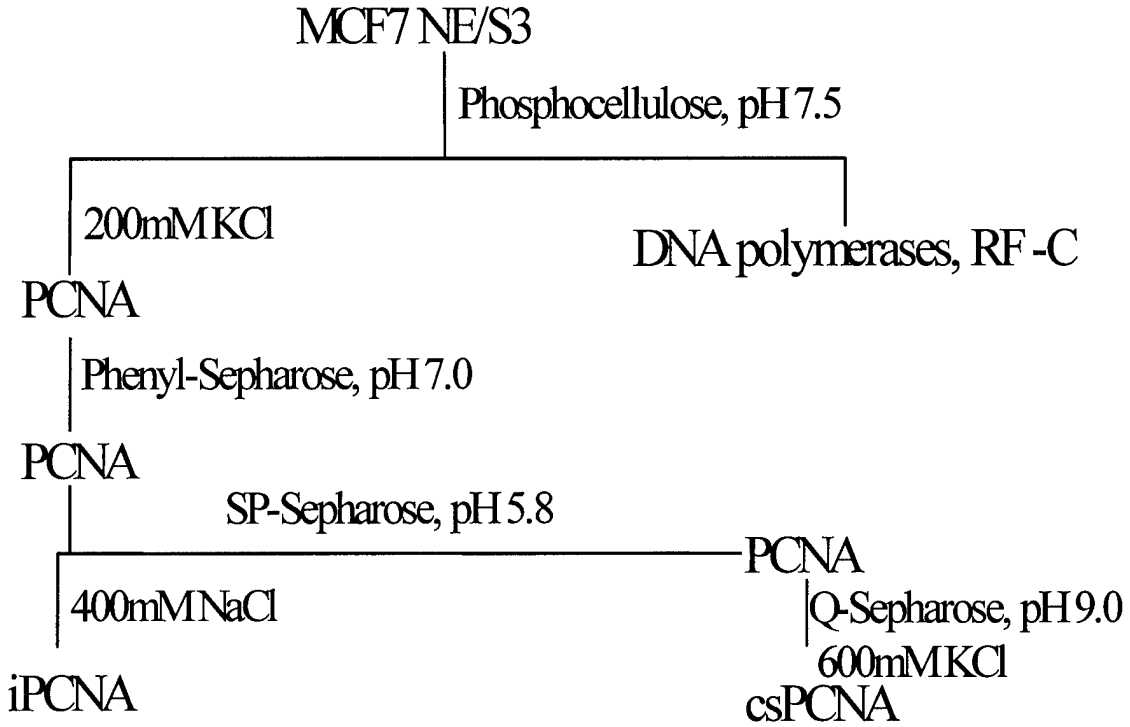


Figure 10. The Purification Scheme for the Isolation of csPCNA and iPCNA. The MCF7 NE/S3 protein fraction was resolved using a phosphocellulose column equilibrated in 200 mM KCl. PCNA contained in the column flow-through was then equilibrated to 1 M NH_4SO_4, and purified further by hydrophobic interaction chromatography on phenyl-Sepharose. The two forms of PCNA, csPCNA and iPCNA were then separated from one another by SP-Sepharose chromatography at a pH of 5.8. The iPCNA was eluted from the SP-Sepharose matrix with 400mM NaCl, while csPCNA was subjected to further purification on a Q-Sepharose column. Purified csPCNA is eluted from the Q-Sepharose column in 600 mM KCl.

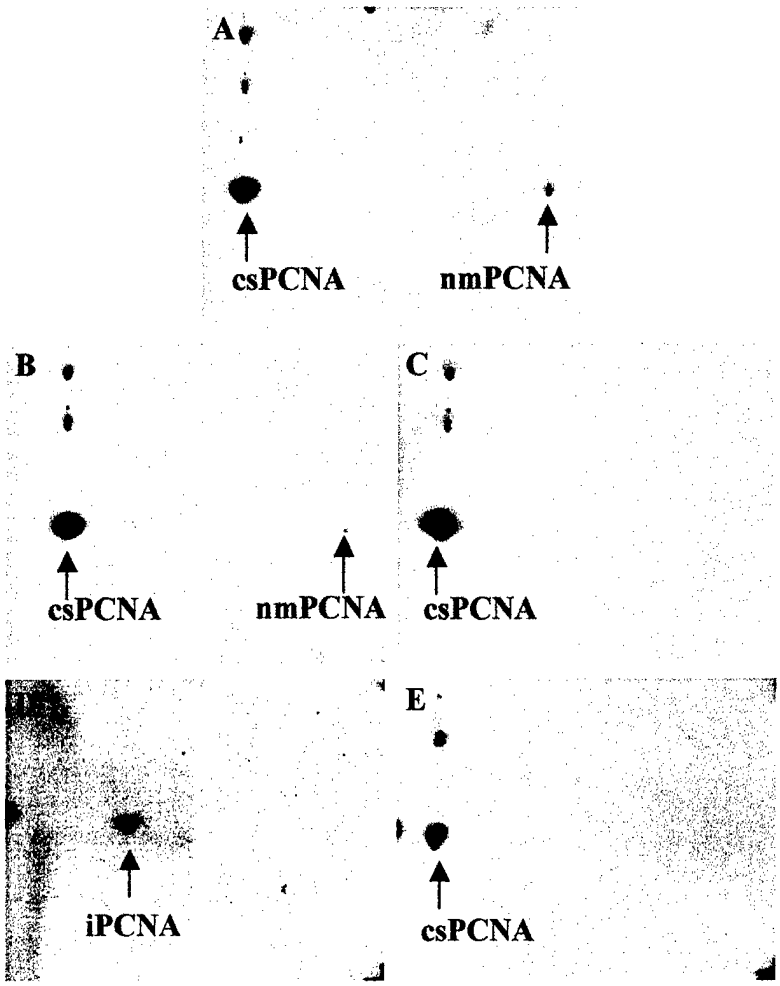
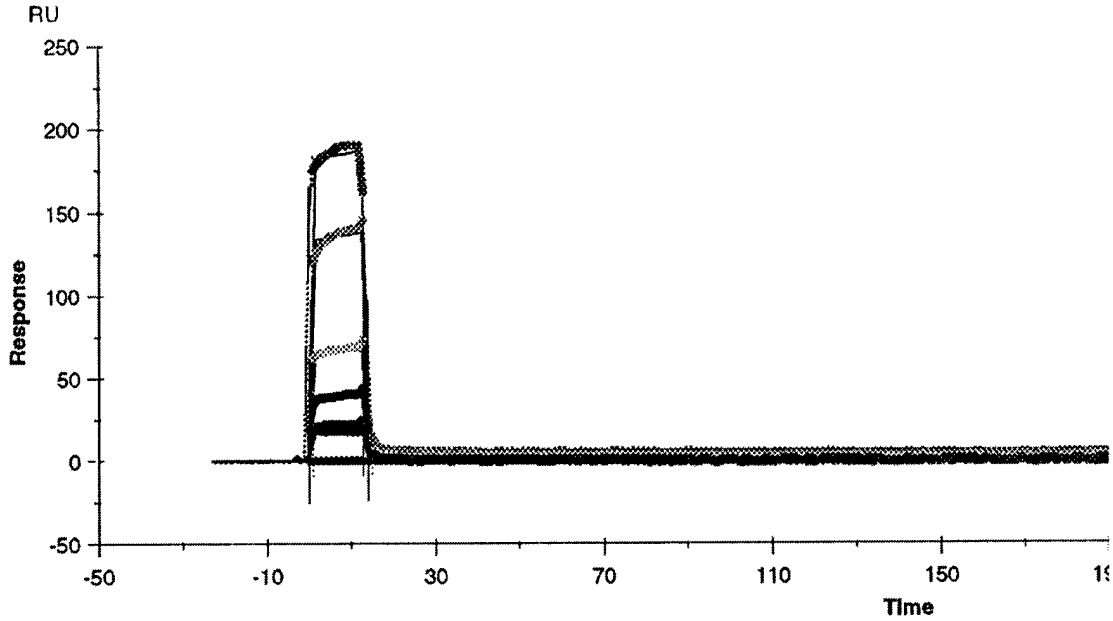


Figure 10. The Purification of the csPCNA and iPCNA. The above PCNA Western blots show the presence of the different forms of PCNA at different stages during the purification scheme of csPCNA and iPCNA. (A) 60 μg of MCF7 homogenate, (B) 60 μg of NE/S3, (C) 40 μg of phosphocellulose flow through, (D) 5 μg of SP-Sepharose eluate, and (E) 5 μg of Q-Sepharose eluate show the separation of the csPCNA and the iPCNA at the level of the SP-Sepharose. The Western intensities of the csPCNA and iPCNA at 5 μg compared to 40 μg of phosphocellulose flow through suggest that they are greatly enriched.

1 1:1 (Langmuir) binding



3 1 1 binding with drifting baseline

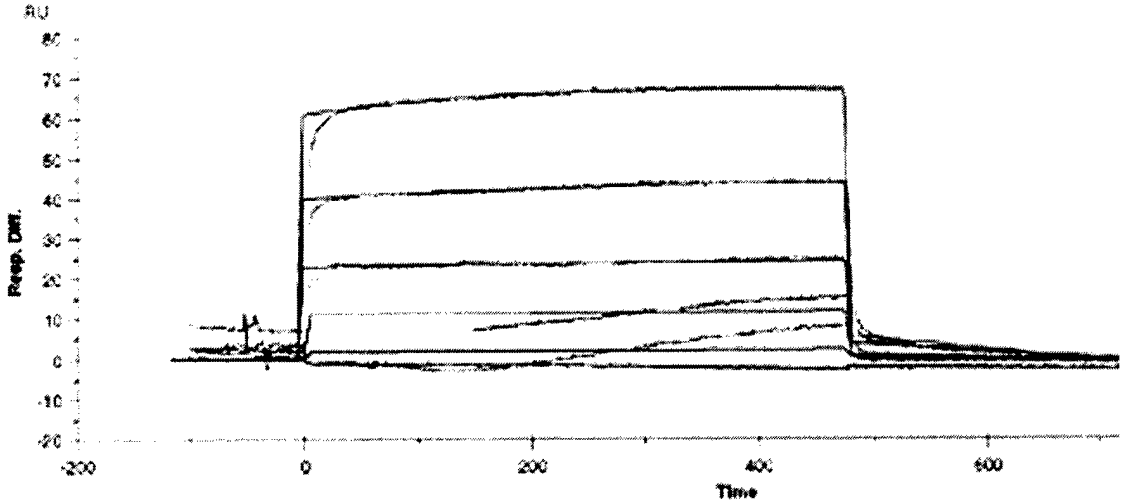


Figure 11. BIACORE Interaction Analysis of p21^{WAF1}and the csPCNA and iPCNA. (A) Sensogram showing the interaction of p21 with csPCNA. The binding responses for the passage of concentrations of p21 at 1 mM, 0.8 mM, 0.4 mM, 0.2 mM, 0.1 mM, 0.05 mM, and 0 mM are shown. (B) Sensogram showing the interaction of p21 with the iPCNA. Concentrations of p21 at of 678 nM, 339 nM, 139 nM, 85 nM, 28 nM, and 0 nM are shown. **Materials and Methods.** The

kinetic analysis of the interactions of p21 with the csPCNA and the intermediate form of PCNA (iPCNA) was done using a BIACORE 2000 surface plasmon resonance (SPR) biosensor (BIACORE, Inc) closely adhering to the rules established by Myszka *et al.* (1999). 2000 response units (RU) of either the isolated csPCNA or iPCNA were bound to separate flow cells of a CM-5 sensor chip (BIACORE, Inc.) according to the manufacturer's protocol. Next, varying concentrations of p21 were passed over the chip surface. Concentrations of p21 at 1000 nM, 800 nM, 400 nM, 200 nM, 100 nM, and 50 nM were passed over the flow cell containing csPCNA, while concentrations of 678 nM, 339 nM, 139 nM, 85 nM, and 28 nM were passed over the flow cell containing immobilized iPCNA at a flow rate of 100 μl/min for 30 seconds. The data generated from the interaction of p21 with csPCNA and iPCNA were then subjected to analysis using BIAevaluation software version 2.1 (BIACORE, Inc.).

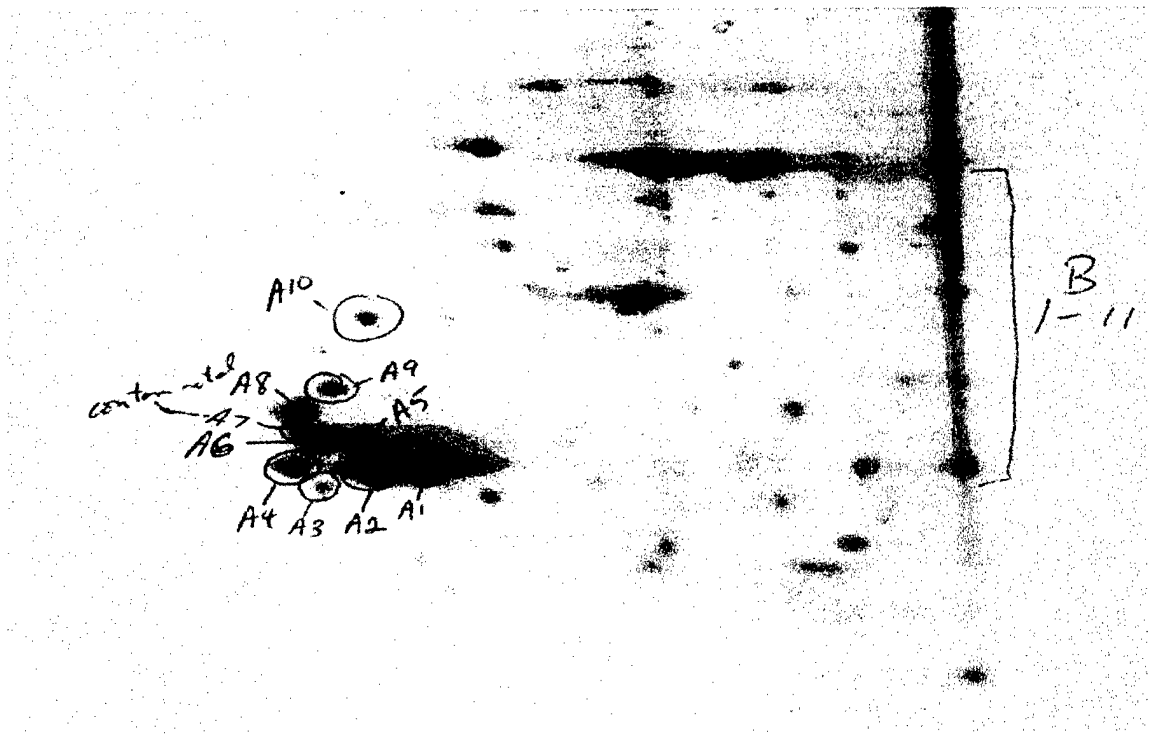


Figure 12. Coommassie stained two-dimensional polyacrylamide gel. The above gel shows proteins (40 μg) resolved by 2D-PAGE that were present in the PCNA-enriched fraction of MCF7 cells. Proteins thought to correspond to PCNA are circled and numbered. Proteins labeled A1-10 are those corresponding to csPCNA while proteins labeled B1-11 correspond to nmPCNA as judged by Western blotting. These proteins were then removed from the gel and subjected to proteolytic digestion with trypsin followed by analysis by mass spectrometry. **Materials and Methods.** The MCF7 NE/S3 protein fraction was resolved by 2D-PAGE and the resolved polypeptides were localized using colloidal Coomassie stain (Bio-Safe Coomassie, Bio- Rad). Polypeptides were removed from the gel using a sterile scalpel and washed three times with de-ionized water (dH_2O) and dried in a speed-vac (ATR Biotechnology, Inc.) followed by re-hydration in 10mM NH_4HCO_3, pH 8.3. The polypeptides present in the gel were then incubated with modified sequencing grade bovine trypsin (Promega). Liquid chromatography/Mass spectrometry (LC/MS/MS) was performed using an ion-trap (LCQ, Thermo-Finnigan, Inc.) mass spectrometer. PCNA was subsequently identified from the mass spectral data using SEQUEST.

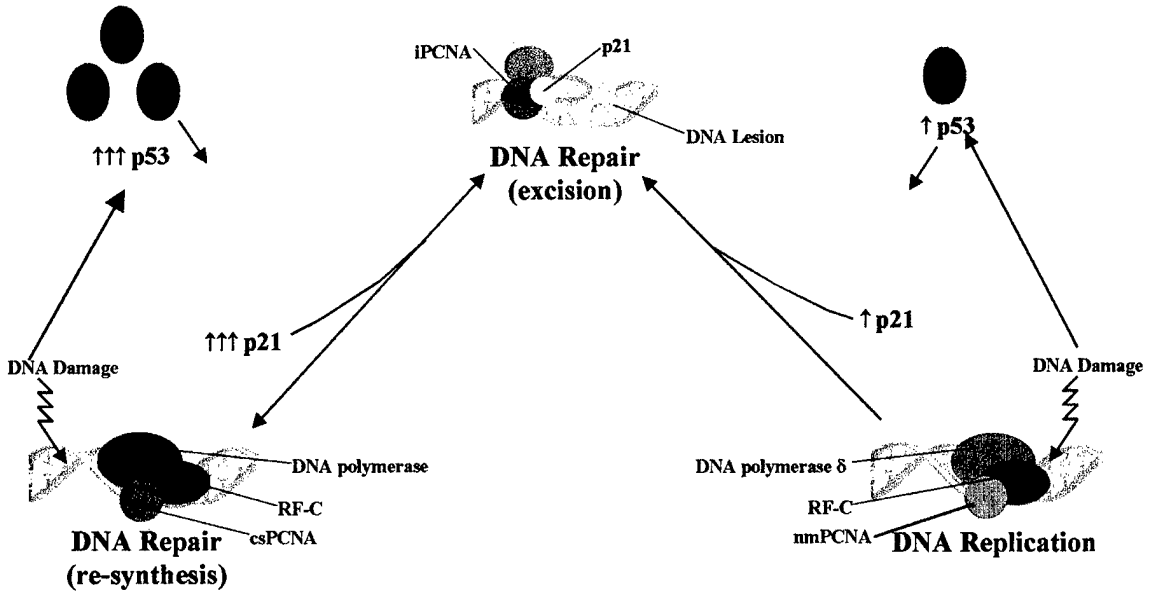


Figure 13. A Model for PCNA Signaling in DNA Replication and DNA Repair. The nmPCNA participates in DNA replication by association with DNA polymerase δ and other factors (i.e. DNA ligase, FEN1, MCMT) as shown in the right of the diagram. A DNA damaging event would lead to the up-regulation of p53, which would increase levels of p21. Through an association with p21, nmPCNA could be converted to iPCNA. iPCNA may now be able to function in the initial steps of DNA repair such as excision of the DNA lesion. After the DNA lesion has been excised, csPCNA may then direct DNA re-synthesis of the excised strand. Because p21 has a low affinity for csPCNA, DNA re-synthesis mediated by csPCNA would not be inhibited by p21. Therefore, by this mechanism, p21 would effectively inhibit DNA replication while allowing DNA repair to occur. However, if large amounts of DNA damage are encountered or more damage is encountered while the cell is repairing damage, p21 levels are further increased to concentrations that "force" an interaction with csPCNA. This interaction abrogates DNA repair re-synthesis and converts csPCNA to iPCNA. Conversion of csPCNA to iPCNA directs the cell away from the later steps of DNA repair and towards the initial steps of DNA repair in effort to overcome an overwhelming amount of DNA damage.

Table I. Binding Constants for the $p21^{WAF1}$ Interaction with PCNA.

PCNA Form[a]	K_a (M^{-1}) [b]	K_d (M) [b]	χ^{2bc}
Cancer Specific	9.6×10^4	1.04×10^{-5}	1.18
Intermediate	9.36×10^9	1.07×10^{-10}	1.91

[a] The cancer specific and intermediate forms of PCNA immobilized on the surface of the CM5 sensor chip were purified as described in the text.

[b] Determined by the analysis of experimental data generated on a BIACORE 2000 SPR mass spectrometer using BIAevaluation software version 2.1.

[c] Statistical values measuring accuracy of fit of the determined binding model to the experimental data.

Table II. Identification and analysis of PCNA by Mass Spectrometry.

Proliferating Cell Nuclear Antigen (PCNA) (Human) [a]	M_r 28,750[b]	Score: 379[c]				
Peptide	Mass (Measured)	Mass (Theoretical)	Δ Mass	Missed[d]	Score[c]	Modification
YLNFFTK	931.17	931.48	-0.31	0	33	
YYLAPKIEDEEGS	1512	1512.7	-0.71	1	46	
DGVKFSASGELGNGNIK	1691.6	1691.9	-0.27	1	35	
AEDNADTLALVFEAPNQEK	2073.6	2074	-0.37	0	92	
ATPLSSTVTLSMSADVPLVVEYK	2422.8	2423.3	-0.42	0	76	+1 Oxidized Met.
AEDNADTLALVFEAPN QEKVSDYEMK	2942.4	2942.4	0.04	1	61	+1 Oxidized Met.
LSQTSNVDKEEEAVTIEMNEPVQLTFALR	3290.6	3290.6	-0.04	1	17	
ATPLSSTVTLSMSADVPLVVEYKIADMGHLK	3303.9	3304.7	-0.78	1	26	+2 Oxidized Met.

[a] Proliferating Cell Nuclear Antigen was identified by searching Mascot and Seaquest databases with data generated from the proteolytic digestion of protein A6 (figure 15) and its subsequent analysis by mass spectrometry.
[b] The theoretical molecular mass for full-length PCNA
[c] Statistical scores were assigned according to Mascot. Scores above 12 are considered statistically significant and suggest extensive homology or identity.
[d] Missed cleavages are the result of incomplete proteolytic digestion.

Curriculum Vitae

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EDUCATION

Institution and location	Degree	Year	Field
Kent State University Kent, OH	B.A.	1995	Biology
University of Maryland, Baltimore Graduate School, Baltimore, MD	Ph.D.	2002	Pharmacology

AWARDS AND RECOGNITIONS

Department of Defense Breast Cancer Fellowship DAMD99-1-9273. Total costs $66,000. The Regulatory Interactions of p21 and PCNA in Human Breast Cancer.
(1999-2002)

Invited participant in the Molecular Biology and Pathology of Neoplasia Workshop, organized by the American Association for Cancer Research Keystone, Co.
(1998)

Graduate Research Assistantship, Department of Pharmacology and Experimental Therapeutics. (1997-2001),

Full athletic scholarship including room and board to Kent State University.
(1990-1995)

Region 7 team member at Junior Olympic Nationals for gymnastics, second place team.(1990)

Top 25 individual at Junior Olympic Nationals and invited member of Top 25 Training Camp, Olympic Training Center, Colorado Springs, Co.
(1990)

CURRENT RESEARCH FUNDING SUPPORT

Department of Defense Medical Research and Development Command Breast Cancer Research Program (1999-2002) The Regulatory Interactions of p21 and PCNA in Human Breast Cancer. Total costs $66,000.

PUBLICATIONS

Hoelz, D.J., Hickey, R.J., Malkas, L.H. (2001) Prokaryotic DNA Replication. *Encyclopedia of Life Sciences,* Nature Publishing.

Hickey, R.J., **Hoelz, D.J.,** Malkas, L.H. (2001) Eukaryotic DNA Replication. *Encyclopedia of Life Sciences,* Nature Publishing.

Malkas, L.H., Bechtel, P.E., Sekowski, J.W., Schnaper, L., Lankford, C.R-V., **Hoelz, D.J.,** Tomic, D., Hickey, R.J. (2001) A Cancer Specific Form of Proliferating Cell Nuclear Antigen (csPCNA) is Present in Malignant Human Breast Cells and Tissues. *Journal of Ligand Science* (in press).

SCIENTIFIC MEETINGS PRESENTATIONS

Hoelz, D.J., Sekowski, J.W., Hickey, R.J., Malkas, L.H. (1997) Identification of Mismatch Repair Proteins in the Human Cell DNA Synthesome. Presented at the University of Maryland Graduate Student Research Day.

Sekowski, J.W., **Hoelz, D.J.,** Hickey, R.J., Malkas, L.H. (1997) Altered Fidelity in Cancer Cells. Presented at the University of Maryland Graduate Student Research Day.

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Tomic, D., Liu, Y., Hickey, R.J., **Hoelz, D.J.,** Bechtel, P.E., Schnaper, L., Malkas, L.H. (2002) Cancer Specific Proliferating Cell Nuclear Antigen as a Novel Diagnostic Marker for the Detection of Breast Cancer. 43: 1041.

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INVENTIONS

Hoelz, D.J., Malkas, L.H., Hickey, R.J. (2001) The Isolation of Modified and Non-modified Forms of Proliferating Cell Nuclear Antigen (PCNA) for Diagnostic/ Drug Development. (UMAB disclosure, patent pending)

RESEARCH SKILLS

Molecular Biology

The skills I have developed include molecular biological techniques such as agarose gel electrophoresis, the polymerase chain reaction (PCR), enzymatic assays (ligation, de-phosphorylation, restriction digestion, that have enabled me to construct prokaryotic and eukaryotic protein expression constructs that has allowed for production and isolation of mammalian gene products in large quantities. Utilizing these skills I was also able to develop and carry out strategies to construct plasmid DNA templates used for enzymatic assays such as the SV40 DNA replication assay. These skills will also allow me to further clone and develop other mammalian gene expression constructs and create different DNA templates useful in alternative enzymatic assays.

Enzymatic Assays

The enzymatic assays of which I am able to perform are activity assays for the DNA polymerases α, δ, and ε, the polymerases responsible for the synthesis of new DNA during mammalian DNA replication. Briefly, the assays involve the incorporation of radiolabeled deoxynucleotides into DNA primed templates followed by isolation of the DNA template and quantitation of the incorporated radioactivity by liquid scintillation. In addition, I am also able to perform the SV40 DNA replication assay. The assay encompasses all three phases of DNA replication and requires the SV40 virus large T-antigen and a DNA template containing the SV40 origin of replication in addition to mammalian DNA replication proteins. I am also skilled at performing topoisomerase assays, and using these enzymatic assays I have effectively been able to study the proteins responsible for DNA replication in mammalian cells and tissues. Additionally, I

have performed *in vitro* transcription/translation assays to produce ^{35}S-methionine labeled recombinant proteins.

Protein Chemistry

I am also proficient at analyzing protein structure using a variety of techniques. I have extensive knowledge and experience with multiple types of chromatography (ion-exchange, hydrophobic interaction, chromatofocusing, affinity, and size exclusion) in addition to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). These techniques have allowed me to compare the 2D-PAGE patterns of proteins isolated from malignant cells to those from non-malignant cells, and differences in the isoelectric patterns are seen in malignant cells as compared to non-malignant cells. I am also skilled at identification of proteins separated by chromatography, SDS-PAGE, and 2D-PAGE by proteolytic digestion of individual proteins followed by analysis by matrix assisted laser desorbtion/ionization time-of-flight mass spectrometry (MALDI-ToF) in addition to analysis of internal peptide sequence by the HPLC separation of peptides followed by electrospray tandem mass spectrometry (LC/MS/MS). I am also skilled in the immune and co-immune precipitation of proteins and protein complexes. In addition to co-immune precipitations, I am also skilled at GST pull-down assays and Far-Western blotting for the analysis of protein/protein interactions. I am also adept at detecting proteins by Western blotting.