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PRINCIPAL INVESTIGATOR: Marietta Y. Lee, Ph.D.

CONTRACTING ORGANIZATION: New York Medical College
Valhalla, New York 10595

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Marietta Y. Lee, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York Medical College
Valhalla, New York 10595

E-MAIL:
marietta_lee@nymc.edu

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13. ABSTRACT ***(Maximum 200 Words)***

We have utilized polymerase δ immunoaffinity chromatography and immunoprecipitation studies to link DNA replication to the cell cycle. Understanding the cell cycle leads to greater understanding of cancer. Tracking down new cell cycle genes and studying their function in the cell cycle will help to increase our understanding of this basic biological process at both the molecular and genetic levels.

Another hypothesis that underlies the proposed studies during this period is that defects in DNA polymerase δ and its accessory proteins contribute to the molecular etiology of sporadic and hereditary breast tumors. Normal and breast cancer cell lines and tissues were screened for genetic alterations in the POLD1 gene. The search for changes were focused on target sequences which could give rise to changes in the fidelity of DNA polymerase δ. The target sequences were the 3'to 5' exonuclease regions of polymerase δ. Mutations that are found will be introduced into the recombinant proteins which will be expressed, isolated and characterized to establish their molecular phenotypes.

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(4) Introduction

The major hypothesis that underlies the proposed studies is that defects in DNA polymerase δ and its accessory proteins could contribute to the molecular etiology of sporadic and hereditary breast tumors. We have been using a polymerase δ immunoaffinity column and immunoprecipitation studies to link DNA replication to the cell cycle. Understanding the cell cycle leads to greater understanding of cancer. Tracking down new cell cycle genes and studying their function in the cell cycle will help to increase our understanding of this basic biological process at both the molecular and genetic levels.

We have recently shown that DNA polymerase δ consists of at least four subunits(Mo et. al., 2000, Liu et al., 2000, Liu et al., submitted) .. We have isolated genomic DNA clones covering the gene for human DNA polymerase δ catalytic p125 (POLD1) and p50 (POLD2) subunits (Chang et al., 1995, Perez et al., 2000).. Normal and breast cancer cell lines and tissues were screened for genetic alterations in the POLD1 gene. The search for changes were focused on target sequences which could give rise to changes in the fidelity of DNA polymerase δ. The target sequences were the 3'to 5' exonuclease regions of polymerase δ. Mutations that are found will be introduced into the recombinant proteins which will be expressed, isolated and characterized to establish their molecular phenotypes.

The p53 tumor suppressor is involved in cell cycle arrest, differentiation and apoptosis. It transactivates many genes that regulate cell cycle and cell growth. The DNA polymerase δ catalytic subunit gene (POLD1) was studied as a transcriptional target of p53 in response to DNA damage. Northern blot analyses showed that POLD1 steady-state mRNA was repressed by about 80% when ectopic wild-type p53 expression was induced to a physiologically relevant level in "tet-off" cultured cells in which p53 was expressed. Moreover, transfection assays demonstrated that p53 was able to repress Sp1-stimulated POLD1 promoter activity, and that this repression was largely due to the loss of the sequence specific interaction between Sp1 protein and an Sp1-binding site which overlaps the P4 p53-binding site. Finally, gel shift and co-immunoprecipitation assays suggested that p53 competes with Sp1 protein for binding to the P4 sequence of the POLD1 promoter. These studies provide a molecular mechanism for the repression of POLD1 expression by p53. These novel findings may be important because many cancers display loss of p53 function, with the implication that the repression of POLD1 expression that is lost may be important to the cancer phenotype.

5. **Body**

During the past year we have accomplished the following:

Technical Objective 1. Determining the activity, protein and mRNA levels of pol δ, PCNA, RPA, RFC in normal and breast cancer cell lines and tissue before and after challenge with DNA damaging agents.

Task 1. Assay of DNA polymerase and exonuclease activities , protein and mRNA levels.

This work has been initiated and has been described in our Sept 1999 progress report. We measured the message level of the p125 catalytic subunit of pol δ after treatment with 30 μM N'-methyl N'nitro-N-nitrosoguanidine (MNNG) and 100 μg/ml methyl methanesulfonate (MMS). Different time points were taken at 0, 0.5 , 1, 2, 3, 4 and 6 hours. We observed that the message levels in MCF 7 cells declined after treatment with MMS. More interestingly, the protein level of p53 increased after damage treatment. Thus there may be a link between the increase of p53 and the decrease in polymerase δ message.

The levels of polymerase δ protein were also determined by Western blotting in MCF7 cells treated with MMS and MNNG. A time cousre from 0 , 0.5, 1, 2, 3, 4 and 6 hours revealed that the pol δ protein decreased from as the p53 protein level increased after treatment with 100 μg/ml MMS. The same was true after treatment with MNNG.

During this grant period we have cultured MCF10A cells to about 80 % confluence. The cells were then serum starved for two hours The cells were treated with MMS or MNNG at different concentrations for four hours. The cells were harvested, lysed and the protein concentrations were determined. 20 μg of total protein were run on SDS-PAGE and Western blotted with antibodies to p125, PCNA, p53 and actin. The results (Fig. 1a) showed that p53 protein increases in response to MMS treatment. p125 protein levels decreased when cells were treated with MMS, which is consistent with the previous data that p53 inhibits the transcription of the polymerase δ POLD1 gene. Our results to date show that both message and proteins levels of polymerase δ catalytic subunit are downregulated in MCF10A and MCF7 cells while the p53 protein level increases upon treatment with MMS.

However, when MCF10A cells were treated with different MNNG concentrations, there was not much change in p125 or PCNA levels (Fig. 1b). This work actually agrees with Fig. 3 of last year's report on the treatment of MCF10A cells with MNNG and the p125 protein level was measured as a function of time. The difference in response of MCF10A cells to these two DNA damaging agents (MMS and MNNG) will need to be further studied.

Task 2. Comparison of DNA replication functions of purified pol δ and PCNA of normal and breast cancer cells.

Experiments in this laboratory have shown that the expression of DNA polymerase δ p125 mRNA increased three fold at the G1/S border in Molt 4 cells. We had previously shown that polymerase δ is a phosphoprotein that is most actively phosphorylated during the S phase (Zeng et al., 1994). The primary sequence of the p125 catalytic subunit of DNA polymerase δ contains a number of sites that are potential targets for different cdk kinases. These include six sites possessing the (S/T)P motif for the cdks (Ser 207, 788 and Thr 83, 150, 238 and 64).

Using commerically available antibodies to Cdk2 and Cdk4 we have found that p125 co-immunoprecipitates with these cell cycle dependent kinases in both MCF10A and MCF7 cells (Fig.2).

We had developed an immunoaffinity column to purify DNA polymerase δ (Mo et al., 2000; Liu et al., 2000). We had utilized this column for preparative scale isolations of pol δ holoenzyme and also to obtain multiprotein complexes that contain DNA polymerase δ. We have succeeded during this grant period to scale down the methodology to isolate these complexes from tissue culture cells so that we can compare the DNA replication functions of normal and breast cancer cells with the least amount of material possible. Preliminary data showed that we can recover DNA polymerase δ activity from both MCF10A and MCF7 cell lysates (Fig. 3, 4). Western blotting with various antibodies was performed on proteins from fractions across the peak of polymerase activity recovered from MCF7 cell extracts (Fig. 5 a, b). As can be seen, Cyclins A,G1, D1, D3 and E , Cdk 2 cdk 4, p21, p27 and p53 coeluted with DNA polymerase δ activity (Table I) confirming the immunoprecipitation data that DNA polymerase δ is associated with cell cycle proteins.

Additional work also showed that a histone kinase activity was associated with the polymerase δ preparations. A histone kinase activity was associated with the highly

purified polymerase δ and this activity phosphorylated both the p125 and p50 subunits on overnight incubation with [γ-^{32}P]ATP (Fig.6). The labeled p125 band in Fig 6 was eluted from the gels and subjected to phosphoamino acid analysis by 2-D separation on thin-layer cellulose plates. The results showed that the labeled p125 using contains only phophothreonine and phosphoserine (Fig.7).

This work was repeated with MCF10A, MCF7 and MDA MB231 cells. MCF10A is a phenotypically normal, nontransformed line. The cells are a spontaneously immortalized line from a culture of human breast epithelial cells from a reduction mammoplasty. The cells are diploid and possess the characteristics of normal breast epithelium. MCF7 is a malignant human breast epitheial cell line that has been widely used for in vitro mechanistic studies. MDA-MB-231 is from an pleural effusion breast adenocarcinoma.

Table II shows the total units of pol δ activity that could be recovered from these extracts after immunoaffinity chromatography and gel filtration. The fractions containing DNA polymerase activity were Western blotted with antibodies to RPA, RFC, cyclins Cdks Topoisomerase, DNA ligase NDHII and DNA dependent protein kinase and were found to be positive.

Thus our studies showed that: 1) we can isolate an active form of DNA polymerase δ multiprotein complex from breast cancer cells by immunoaffinity chromatography and gel filtration; 2) immunoprecipitation and Western blot analysis demonstrate the presence of cell cycle proteins providing a possible ink between cell cycle regulation and DNA replication.; 3) the presence of p53, p21 and p27 in the active DNA polymerase δ complex indicates that they may have a role in the regulation of DNA replication or DNA repair.

We have repeated the immunoblot analysis of components of pol δ complex isolated from our pol δ immunoaffinity column several times using different cell lines. It was found, for example, that cyclin D1 is only found in the phenotypically normal MCF10 A cell lines and not in the cancerous MCF7 and MDA MB231 cell lines. It has been reported by Xiong et al that the cyclin D1 gene is present in a rearranged or amplified form in 20% of all breast tumors (Xiong et al.,1993a). Thus, our biochemical data agreed with their findings. These results are surprising, but are consistent with a redistribution of PCNA from the replication complex to the cyclin complexes as has been proposed by Xiong et al (1993b). A sequestering of PCNA by the D cyclins could explain their absence in the immunoaffinity purified pol delta.

p21 was found in the complex from the normal cell line (MCF 10A) but was absent in MDA MB 231 complexes and was lost from the MCF 7 complex after gel filtration. Our conclusion is that p21 is dissociated from the complex on gel filtration.

More recently a new gene and protein called p16, which has been shown to be damaged in a large proportion of many types of cancer has been identified. (Alcorta et al., 1996) Therefore, immunoblots for p16 will also performed in the coming year. This p16 antibody has been ordered from Santa Cruz Biotechnology Inc.

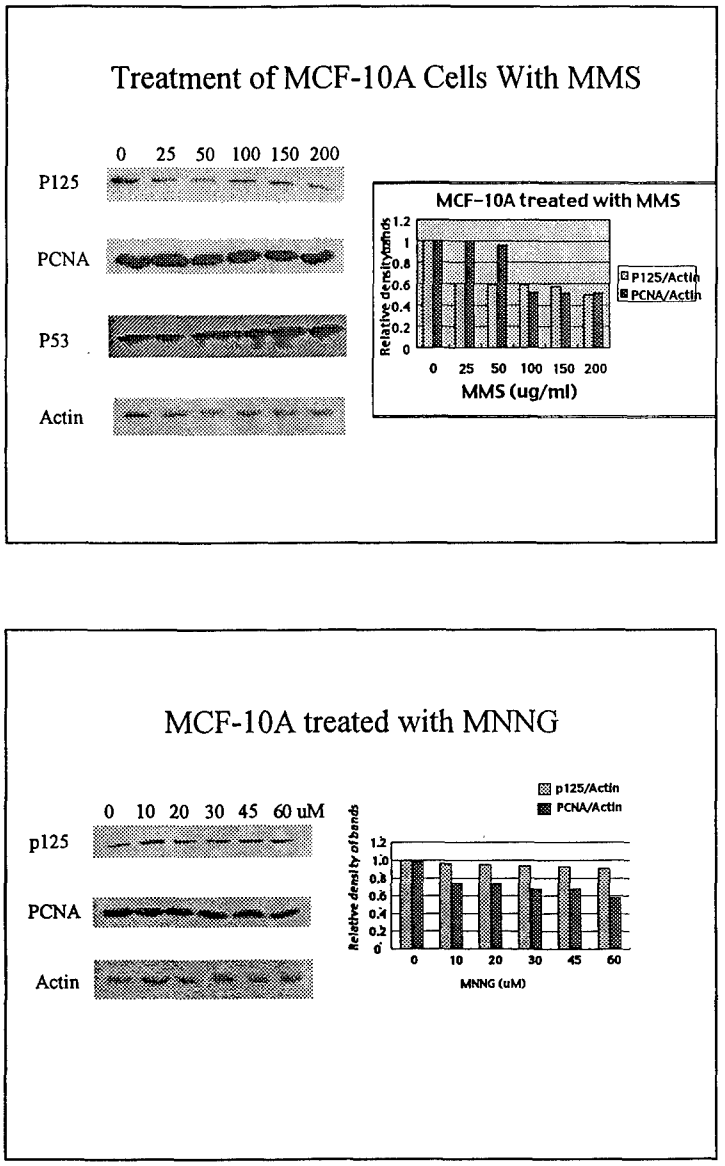


Fig.1. Treatment of MCF10A cells with MMS and MNNG

Upper panel: MCF-10A cells were grown to 90% confluence and treated with varying amounts of MMS for four hours. The cells were harvested, lysed and the protein concentrations were determined. 20 μg of total protein were run on SDS-PAGE and Western blotted with antibodies to p125, PCNA, p53 and actin.

Lower panel: MCF10A cels were treated with varying amounts MNNG. The cells were harvested, lysed and the protein concentrations were determined. 20 μg of total protein were run on SDS-PAGE and Western blotted with antibodies to p125, PCNA, and actin.

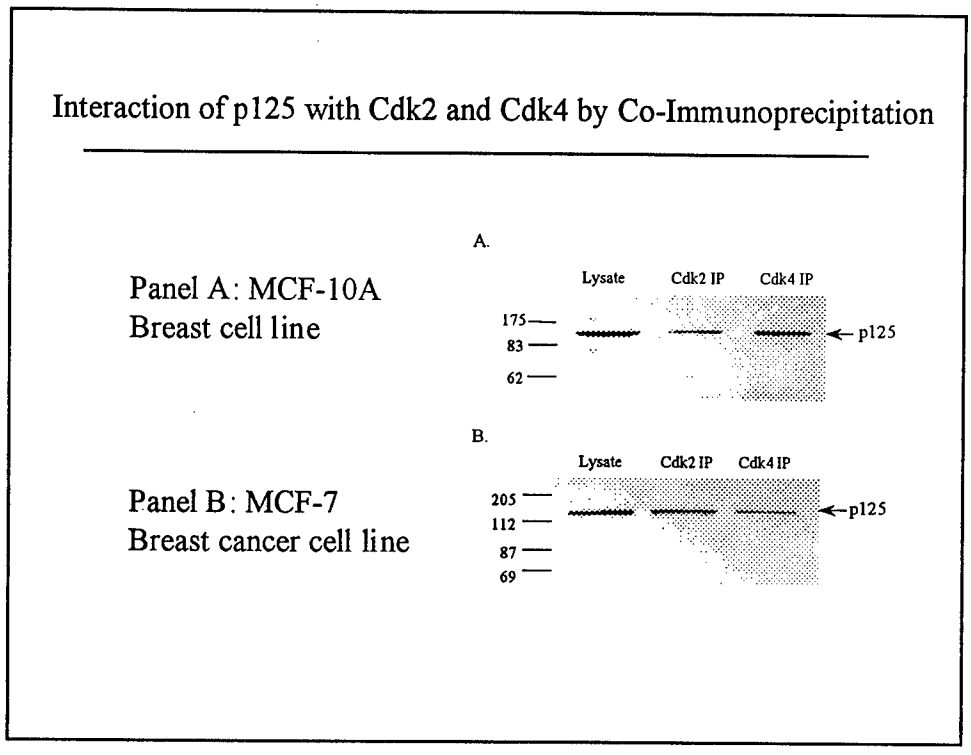


Fig.2 Interaction of p125 with cdk2 and cdk4 by co-immunoprecipitation in MCF-10A breast cell line and MCF-7 breast cancer cell line.

Cell lysates from MCF-10A cells were immunoprecipitated with antibodies against cdk2 and cdk4. The immunoprecipitates were extensively washed with phosphate buffered saline and then subjected to Western blotting with a monoclonal antibody against pol δ, and developed using a chemiluminescence stain. The diagram shows the western blot.

A. From left to right , these are MCF-10A lysate, the immunoprecipitate using cdk2 antibody, and the immunoprecipitate using the cdk4 antibody. Size of the mw standards is shown on the left in kDa.

B. Cell lysates from MCF-7 cells were immunoprecipitated with antibodies against cdk2 and cdk4 as in panel A.

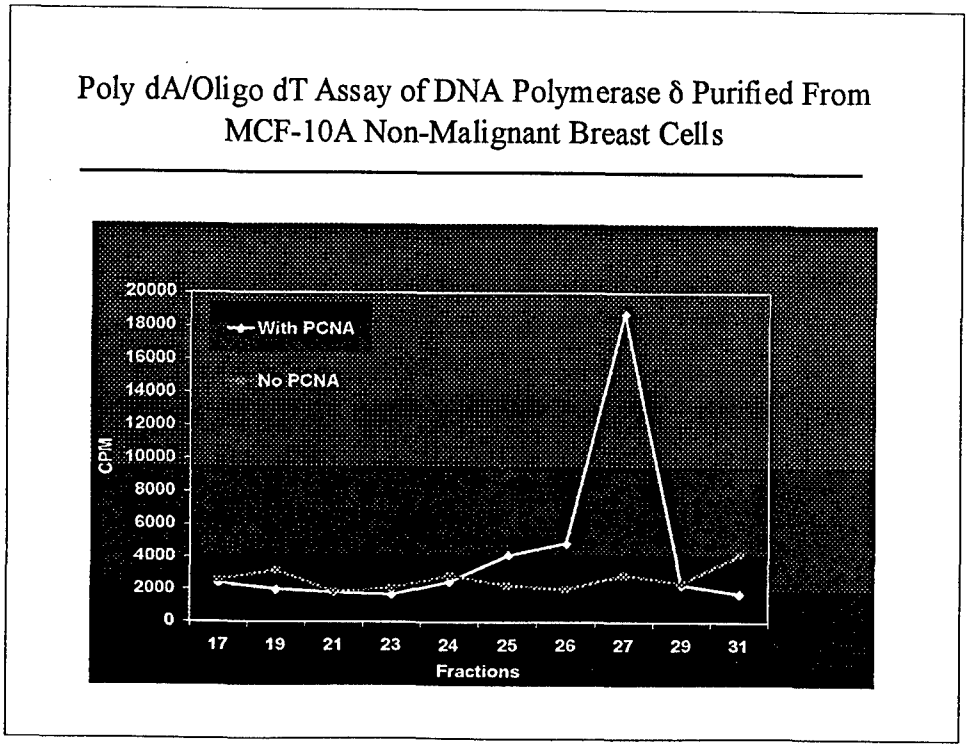


Fig.3. Poly(dA)/Oligo(dT) Assay of DNA Polymerase δ Purified from MCF10A. Cell extracts after pol δ immunoaffinity column were assayed for pol δ activity in CPM using poly dA/oligo dT as a template, in the presence and absence of PCNA per eluted fraction . Sparsely primed poly(dA)/oligo(dT) was used as the template by Lee et al. (Lee et al., 1984). The standard reaction for the poly(dA)/oligo(dT) assay contained 0.25 optical density units/ml poly(dA)/oligo(dT) (20:1), 200 μg/ml bovine serum albumin, 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES, pH 6.0, 100 cpm/pmol [^{3}H]TTP, and 0.2-0.4 unit of pol δ in the presence or absence of 0.2 μg of PCNA in a total volume of 100 μl. Reaction mixtures were incubated for 60 min at 37°C and were terminated by spotting onto DE81 papers that were then washed four times with 0.3 M ammonium formate, pH 7.8, once with 95% ethanol, and counted on a Beckman scintillation counter.

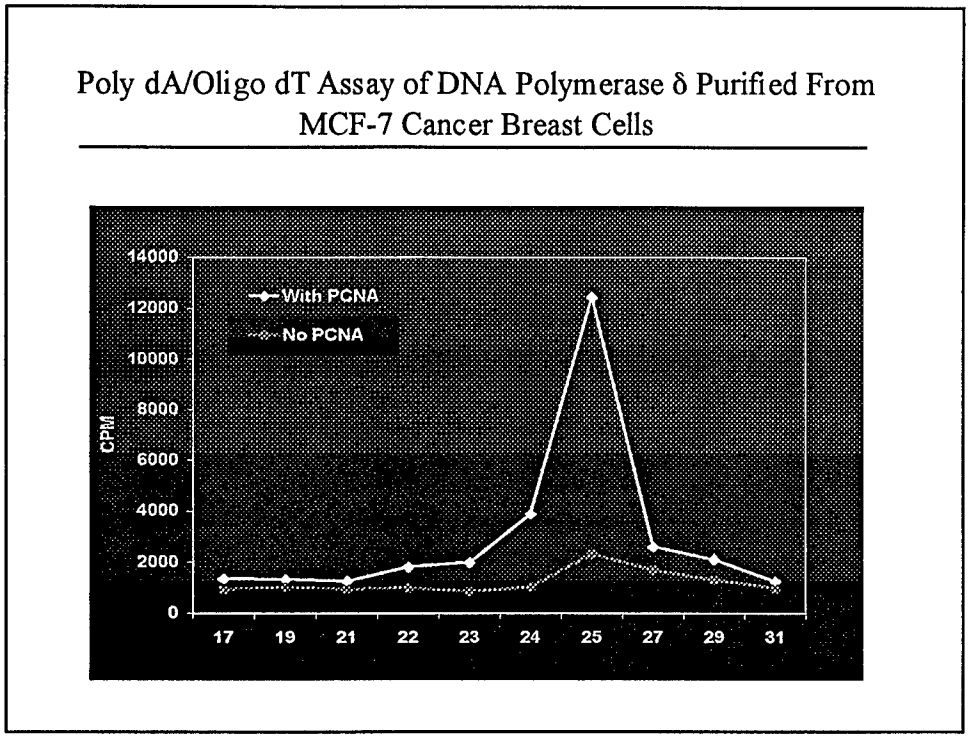
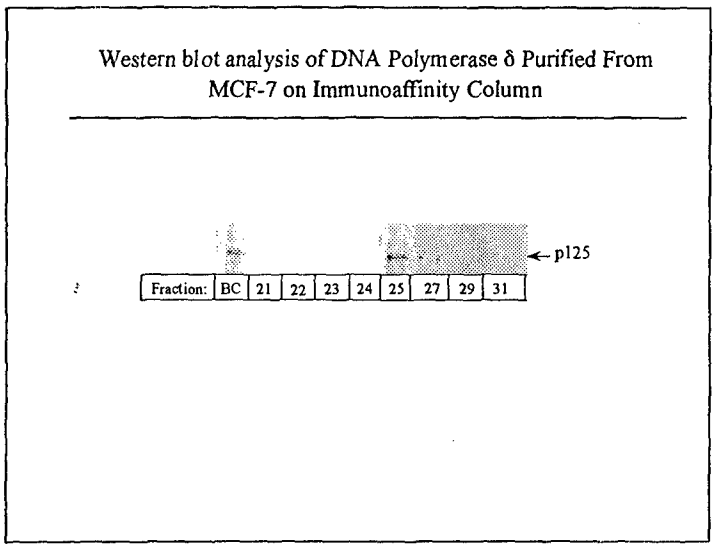


Fig.4. Poly(dA)/Oligo(dT) Assay of DNA Polymerase δ Purified from MCF-7.
Cell extracts after pol δ immunoaffinity column were assayed for pol δ activity in CPM using poly dA/oligo dT as a template, in the presence and absence of PCNA per eluted fraction.



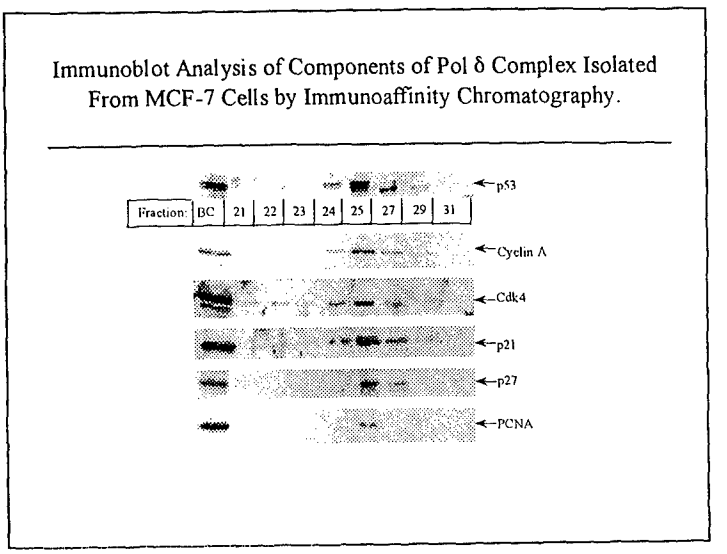


Fig 5 A and B. . Western Blot Analysis.

Extracts of MCF-7 cells prepared as described above were subjected to SDS-PAGE in 5-15% gradient gels that were then transferred to nitrocellulose membranes. Prestained protein standards (Sigma) were used as molecular weight markers and also to provide visual confirmation of efficient transfer. The nitrocellulose blots were blocked with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (V/V) Tween-20 (TBST) for 1 hour at room temperature. The blots were then incubated with monoclonal antibodies for 4 hours at 4°C followed by three 10 min washes in TBST, then incubated with streptavidin-horseradish peroxide anti-mouse conjugate diluted in TBST (1:5,000) and 2% nonfat dry milk for 1 hour at room temperature with constant rocking. The blot was then washed three times with TBST for 30 min each and developed by chemiluminescence (ECL detection system, Amersham-Pharmacia Biotech.). When polyclonal antibodies were used, the secondary antibody used was streptavidin-horseradish peroxide anti-rabbit conjugate diluted in TBST (1:10,000) and 2% nonfat dry milk. Antibodies used were as follows: polyclonal antibodies against cdk 2 and 4, and cyclins A, B1, D1, D3, and E (Santa Cruz Biotech.); monoclonal antibody against the catalytic subnuit of DNA pol δ, p125 and monoclonal antibody against PCNA were produced in the laboratory; monoclonal antibody against RFC 145; polyclonal antibody against RFC 40; monoclonal antibody against DNA pol ε; goat antibody against WRN and Cyclin A1 (Santa Cruz Biotechnology); monoclonal antibody against BRCA1 and monoclonal antibody against mdm2.

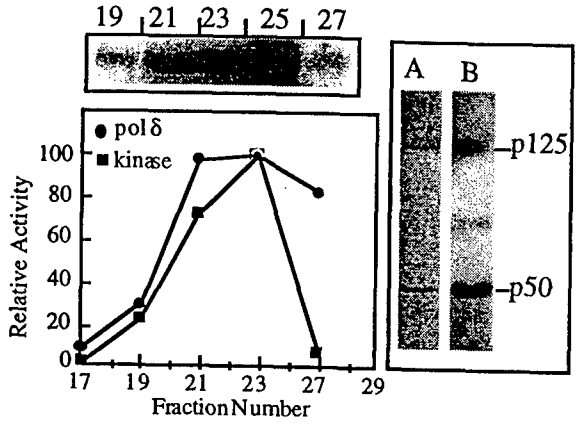


Fig.6. Association of a histone kinase activity with the immunopurified complex.
This figure shows the elution of polymerse delta activity from the immunoaffinity column. The fractions were assayed for polymerase delta activity and for histone knasae activity by incubation with histone H1 in the presence of 32P labelled ATP. The reaction mixtures were autoradiographed (upper panel) and radiolabeled histone H1 bands were quantitatd by densitometry. The relative activities wer plotted against fraction number (lower panel).

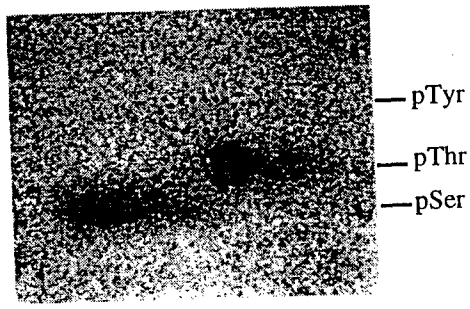


Fig. 7. Phosphoamino acid analysis of p125 by 2D separation on thin layer cellulose plates.

After elution of the 32P labeled band from a SDS-PAGE gel, the protein was digested in 6N HCL at 125 C for 45 min, and then run on a cellulose TLC plate with 5 ug each of phosphotyrosine, phosphothreonine and phosphoserine. First dimensin separation was electrohoresis at pH1.9 at 1.6 kV for 50 min. The second dimension separation was by ascending chromatography in isobutyric acid: 0.5M NH4OH, 5:3 for 6 hours. The standard phosphoaminoacids wer visualized by ninhydrin stainin and the radioactive amino acids were visualized by phosphorimaging on a Molecular Dynamics instrument. The position of pTyr is shown by the dotted circle.

Table I. Immunoblot Analysis of Components of Polymerase δ Complexes Isolated by Immunoaffinity Chromatography.

Cells studied were MCF-7 (breast cancer cells). Pol δ complexes were separated from cell lysates on pol δ immunoaffinity column (Jiang et al. 1995). Peak fractions of pol δ activity were immunoblotted with the indicated antibodies.

Antibodies against:		MCF-7	
	M.W.	BC	Affinity
Pol δ	125	+	+
PCNA	36	+	+
Pol ε	215	+	+
RFC145	145	+	+
RFC40	40	+	+
CDK 2	33	+	+
CDK 4	34	+	+
Cyclin A	58	+	+
Cyclin A1	65	+?	+?
Cyclin B1	35	+	+
Cyclin D1	35	+	+
Cyclin D3	33	+	+
Cyclin E	52	+?	+?
p53	53	+	+
p21	21	+	+
p27	27	+	+

Table II. Poly(dA)/Oligo(dT) Assay of DNA Polymerase δ Purified from MCF-10A ,MCF-7 and MDA MB-231 in the Presence and Absence of PCNA.

Cell extracts of equal numbers of cells from MCF-7 and control cell line MCF-10A after pol δ immunoaffinity column were assayed for pol δ activity using poly dA/oligo dT as a template in the presence and absence of PCNA.

Cells	Fractions	Total Activity (Units)	
		+ PCNA	-PCNA
MCF-10A	Affinity	59.3	7.0
	SW 300	23.5	4.5
MCF-7	Affinity	16.3	7.2
	SW300	10.2	6.5
MDA MB-231	Affinity	19.2	6.2
	SW300	9.8	6.8

Task 3. Study of the response of the pol δ promoter in normal and breast cancer cell lines.

This work is in the process of being written up as a manuscript and will be submitted to J. Biol. Chem. under the title " Transcriptional Regulation of the Human DNA polymerase δ catalytic subunit Gene (POLD1) by p53 tumor suppressor and Sp1" by Baoqing Li and Marietta Y.W. Lee.

Technical objective 2 The multi-protein complexes of pol δ from normal and breast cancer cell lines and tissues will be studied to determine if a) they display functional defects and b) to determine if they exhibit altered behavior in terms of protein-protein interactions.

Task 4. Comparison of DNA replication functions of multi-protein pol δ complexes of normal and breast cancer cells.

Last year we reported an interesting finding. Dr. Krucher showed that the product of the retinoblstoma tumor suppressor gene (pRb) interacted with the catalytic subunit of DNA polymerase δ. This work has been submitted for publication and is in press.

"Interaction of the Retinoblastoma protein (pRB) with the catalytic subunit of DNA polymerase delta (p125) "
Krucher, N., Zygmunt A., Mazloum, N.,Tamrakar, S., Ludlow, J.W. and **Lee**, M.Y.W.T.. *Oncogene*, in press (2000)
Please see appendix.

Task 5. Study of protein-protein interactions within complexes of pol δ .

This is well underway. Five papers result from this work :

1. Characterization of the p125 Subunit of Human DNA Polymeraseδ and Its Deletion Mutants" *J. Biol Chem.* 273, 9561-9569 (1998)
2. Identification of DNA Replication and Cell Cycle Proteins that Interact with PCNA. *Nucleic Acids Research* 25, 5041-5046 (1998)
3. Direct Interaction of Proliferating Cell Nuclear Antigen with the p125 Catalytic Subunit of Mammalian DNA Polymerase δ. *J.Biol. Chem.* 274, 26647-26653(1999)

4. Evidence that DNA polymerase δ isolated by immunoaffinity chromatography exhibits high molecular Weight characteristics and is associated with the KIAA0039 protein and RPA. *Biochemistry*, 39, 7245-7254 (2000)
5. Identification of a Fourth Subunit of Mammalian polymerase δ. *J.Biol. Chem* 275, 18739-18744 (2000)

We have developed a native gel electrophoresis technique (Jaime et al., 2000). This, coupled with PCNA overlay technique will be very powerful to study protein - protein interaction between pol δ and PCNA complexes.

Using a yeast two hybrid analysis we have also identified a new subunit of DNA polymerase delta, p38 (Liu et al., 2000)

Technical Objective 3. The ability of extracts of the breast cancer cell lines to carry out nucleotide excision repair will be compared to that of normal breast cell lines using an in vitro repair assay.

Task 6. Assay of repair activities of nuclear extracts of normal and breast cancer cell lines.

Nayef Mazloum, a graduate student, has been setting up the repair assays. He has also been collected MCF7 and MCF10A cell extracts that were not treated and treated with UV or methyl methanesulfonate (MMS). We expect to perform this work during the remaining funding period.

Task 7. Study of repair synthesis of multi-protein polδ complexes from normal and breast cancer cells.

Nayef Mazloum has recently initiated these studies.

Technical objective 4. Normal and breast cancer cell lines and tissues will be s creened for genetic alterations in the pol δ and PCNA genes

Task 8. RT-PCR and genomic PCR analyses of 3' to 5' exonuclease and N2 domains of pol δ .

Last year we reported that in order to detect the mutation of the POLD1 gene, we have isolated genomic DNA from twenty-three breast cancer tissues and three breast cancer lines.

Dr. Heng Xu has been working on this part of the project. He has written up the results in a manuscript format. With some editing , the manuscript will be ready for submission .

"Mutational Analyses of the Exo Motif of the POLD1 Gene in Human Breast Cancer Cells. " Xu, H., Zhang, P., Mazloum, N., and Lee, M.Y.W. (in prep).

To obtain the true mutants without false, the three repeated PCR products amplified from the POLD1 genomic DNA from each breast tissue or cell line were analyzed by the NIRCA. And, each mutant of POLD1 gene detected from NIRCA was repeated three times to make true detection of mismatches.

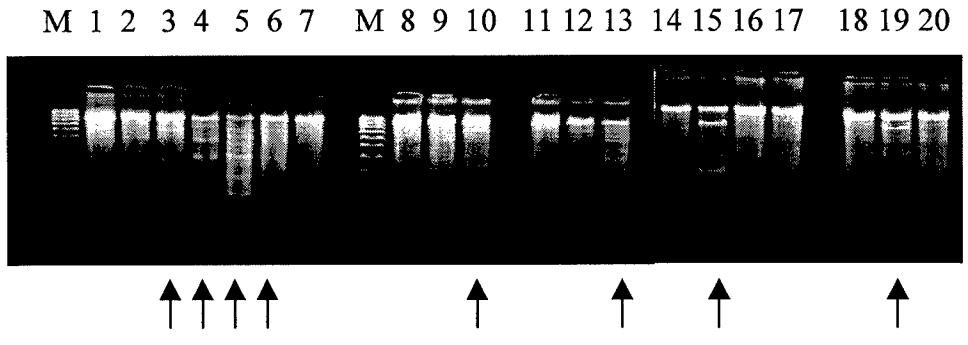


Figure 8. Detection of mutation in POLD1 genomic DNA target by NIRCA.

A nonisotopic RNase cleavage assay was used to analyze POLD1 gene specimens for mutations. Representative results are illustrated. Lanes 2-7, 9-19, patient samples; lanes 1 and 8, normal breast tissue control; lane 20, normal breast cell line control. Samples 3, 4, 5, 6, 10, 13, 15, and 19 are abnormal and indicate mutants in POLD1 gene.

Determination of NIRCA detected mutations by DNA sequencing

NIRCA detected mutations were cloned and subjected to DNA sequencing in order to confirm that the NIRCA was not producing any false positives results in the detection of POLD1 gene mutations. The inserts for these cloning experiments were the PCR products from which positive NIRCA cleavage products were observed.

After ligating mutant PCR products into the TOPO vector, the topoisomerase was disassociated from the DNA to increase the yield of transformants. The TOPO clone was

transformed to the TOP10 One Shot competent *E. coli* cell. Then, each transformation was spread onto the LB plate containing kanamycin. Cells that contain non-recombinant vector are killed upon plating. After culturing overnight, the plasmid DNA from each clone was isolated using the QIAprep miniprep kit (QIAGEN), digested with *EcoR I*, and analyzed by running 1% agarose gel. Then, the purified PCR amplified clone inserts (POLD1 gene mutations confirmed by NIRCA) were subjected to DNA sequence determination that was performed by Davis Sequencing Company (Davis, CA). Finally, all sequences of NIRCA detected mutants are aligned and analyzed with the cDNA sequences of POLD1 gene in Enterz database.

Table III lists the mutants of POLD1 gene from breast cancer samples detected by NIRCA and DNA sequencing. Mutations causing a change in the amino acid sequences of DNA polymerase δ were identified in some tissues and cell lines. Of twenty-one breast cancer tissues and two breast cancer cell lines, we found two silent mutants and five point mutants.

One silent mutation was detected in the breast cancer from patients KID121, KID202 and 3783 (ACC_ACT; Thr_Thr) in codon 495. This silent mutant also was found in human sporadic colorectal cancers [21]. Another silent mutation was detected in the breast cancer from patient 6443 (CAG_CAA; Gln_Gln) in codon 461.

Table III. Summary of mutation identified in the Exo motif of POLD1 Gene from breast cancer.

Patient/Cell line	Nucleotide position	Codon number	Nucleotide change	Amaino acid change	domain
3783	980	327	CCT_ CTT	Pro_**Leu**	Close Exo I
3783	1485	495	ACC_ACT	Thr_Thr	Region A
KID121	1432	478	AGC_GGC	Ser_**Gly**	Region A
KID121	1485	495	ACC_ACT	Thr_Thr	Region A
KID202	1485	495	ACC_ACT	Thr_Thr	Region A
6446	1402	468	AAG_ATG	Lys_**Stop codon**	
6443	1380	461	CAG_CAA	Gln_Gln	Region A
4173	1520	507	CGC_CAC	Arg _**His**	Exo III
Hs578T	1334	445	GAC_GGC	Asp_**Gly**	After Exo II

In addition to the silent mutation (ACC_ACT; Thr_Thr) in codon 495, the breast cancer tissue from patient 3783 had a CCT_CTT transition in codon 327 causing the replacement of Pro by Leu, and the breast cancer tissue from patient KID121 also had a AGC_GGC transition in codon 478 causing the replacement of Ser by Gly. The breast

cancer tissue from patient 6446 has a deletion mutation, that is, a AAG_ATG transition in codon 468 led to a stop codon. The deletion shifted the reading frame and converted codon 468 into a premature termination codon (the normal termination codon has the number 1108). The premature termination predicts that the C-terminal segment including the DNA binding domain was lost in the mutated protein. The breast cancer tissue from patient 4173 had a point mutation in the Exo III motif, a CGC_CAC transition in codon 507 resulted in the replacement of Arg by His. Breast cancer cell line Hs578T exhibited a GAC_GGC transition in codon 445 located between Exo II and region A, causing the replacement of Asp by Gly.

Figure 9 shows the point mutations found in the breast cancer tissues from patient KID121 in codon 478 (Fig. 9a), in the breast cancer cell line Hs578T in codon 445 (Fig. 9b), in the breast cancer tissue from patient 4173 in codon 507 (Fig. 9c), in the breast cancer tissue from patient 3783 in codon 327 (Fig. 9d), and in the breast cancer tissue from patient 6446 in codon 468 (Fig. 9e). The arrows indicate mutated nucleotide positions at which the breast cancer tissues and cell line show G (KID121), G (Hs578T), A (4173), T (3783), and T (6446), respectively.

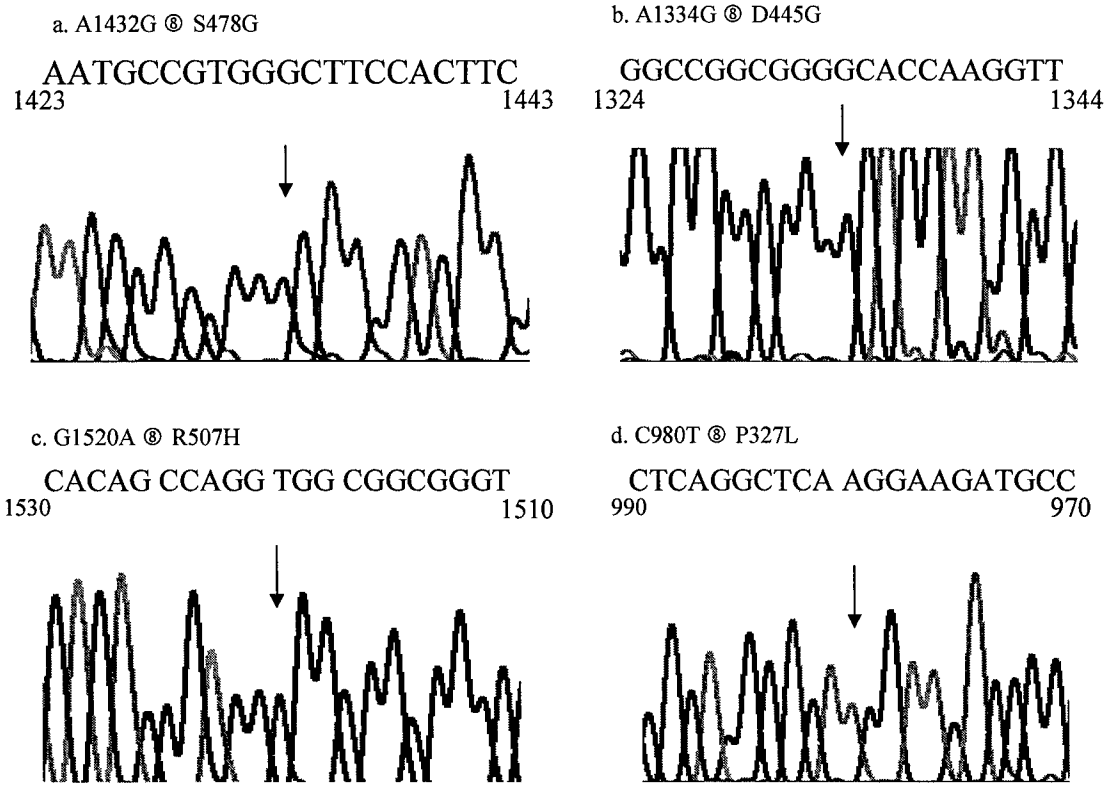


Figure 9. Mutation identified in the POLD1 gene in breast cancer patients and cell lines. Genomic sequences are presented in the 5' to 3' direction. The arrow indicates the nucleotide position at which the sequence in breast cancer is mutated.

Task 9. Analysis of functional properties of mutations found in breast cancer cell lines and tissues.

Dr. Heng Xu is in the process of designing primers and overexpress these mutants that he has found in the breast cancer cell lines and tissues in the baculovirus expression system. The recombinant mutants will be purified and functions analyzed by in vitro DNA replication assays.

Task 10. PCR analyses of the promoter region of pol δ in breast cancer cell lines and tissues.

Using NIRCA and DNA sequencing stated in Task 8, we are also investigating the mutants of the POLD1 promoter. We have amplified a length of 2 kb of the POLD1 genomic DNA fragment covering the full length 1.8 kb from different breast cancer tissues and different breast cancer cell lines If we find some interesting mutated sites in the POLD1 promoter, we will introduce these mutations into the pGL2 delta reporter plasmid. The function of the mutated promoter will be compared to that of the normal promoter.

Task 11. RT-PCR and genomic PCR analyses of human PCNA.

Not yet initiated.

6. Key Research Accomplishments

We have used breast cancer cell lines to probe for changes in proteins associated with polymerase δ complexes isolated by immunoaffinity chromatography.

1) In the case of the transformed breast cell lines, significant differences were observed in that several of the protein ligands were not present in the affinity purified fraction consistent with the findings of other investigators. Of significant note is that after immunoaffinity column and after gel filtration p21 is present in MCF10A cells but absent in MCF7 and MDAMB231 breast cancer cell lines.
2) There is a correlation between accumulated p53 and POLD1 mRNA inhibition in cellular response to DNA damaging agents.
3) The protein levels of pol δ catalytic subunit and p53 are also correlated in response to DNA damaging agents. As the protein level of p53 goes up, the pol δ protein level goes down

4) We are the first to show that the tumor suppressor Rb interacts with pol δ.

5) Using breast cancer cell lines and tissue samples we have found 6 mutations leading to changes in the amino acid sequences of p125 that are in the Exo regions.

7. Reportable Outcomes

Manuscripts.

1. Wu S.M., Zhang, P., Zeng, X.R., Zhang, S.J., Mo, J., Li, B.Q., and **Lee,** M.Y.W.T.
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4) Mo, J., Liu, Li, Leon, A., Mazloum, N., and **Lee**, M.Y.W.T.
   Evidence that DNA polymerase δ isolated by immunoaffinity chromatography exhibits high molecular Weight characteristics and is associated with the KIAA0039 protein and RPA.
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5. Liu, L., Mo, J., Belmonte, E., and **Lee**, M.Y.W.T.
   Identification of a Fourth Subunit of Mammalian polymerase δ.
   J. Biol Chem. 275, 18739-18744 (2000)
6. Krucher, N.A., Zygmunt, A., Mazloum, N., Tamrakar, S., Ludlow, J.W., and **Lee**, M.Y.W.T.
   Interaction of the Retinoblastoma protein (pRB) with the catalytic subunit of DNA polymerase δ (p125).
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   Transcriptional Regulation of Human DNA polymerase δ catalytic subunit gene (POLD1) by p53 and Sp1
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Cloning and characterization of a novel protein p38 which interacts with DNA polymerase δ and p50 small subunit .
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Mutational Analysis of the Exo Motif of POLD1 gene in human Breast Cancer cells (in preparation)

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1) Li, B.Q., Chen, X., and **Lee**, M.Y.W.T. Transcriptional repression of DNA polymerase delta catalytic subunit Gene (POLD1) by p53 tumor suppressor. Abstract #443 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999

2) Liu, L., Rodriquez-Belmonte, E., and **Lee**, M.Y.W.T. Cloning the putative human DNA polymerase δd thilrd subunit, which binds to the small subunit p50 and PCNA Abstract #337 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999

3) Mazloum, N., **Lee**, MY.W.T. , and Zhang, P. The Examinatin of protein-protein interaction between the catalytic subunit of DNA polymerase δ (p125) and the proliferating cell nuclear Antigen (PCNA) Abstract #343 _mechanisms in DNA Replication and Recombination . Keystone Symposia 1999

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7) Zhang, P., Liu, L., Mo, J., Mazloum, N., Xu, H., and **Lee**, M.Y.W.T. Direct Interaction of PCNA with p125, the catalytic subunit of mammalian DNA polymerase delta. Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999

8) Xu, H., and **Lee**, M.Y.W.T. Analyzes of POLD1 gene mutation and study of its transcriptional regulation in Breast Cancer Cells. Breast Cancer Research Program Era of Hope 2000 p391

Oral Presentations

1. Liu, L., Rodriquez-Belmonte, E., and **Lee**, M.Y.W.T. Cloning the putative human DNA polymerase δ thilrd subunit, which binds to the small subunit p50 and PCNA Abstract #337 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999
2. Zhang, P., Liu, L., Mo, J., Mazloum, N., Xu, H., and **Lee**, M.Y.W.T. Direct Interaction of PCNA with p125, the catalytic subunit of mammalian DNA polymerase δ. Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999.

Degrees Obtained:

1. Li Liu Ph.D Dissertation Thesis "Cloning and Characterization of Novel Proteins which associate with Mammalian Dna Polymerase δ" 1999

2. Baoqing Li Ph.D Dissertation Thesis" Transcriptional Regulation of Human DNA polymerase δ catalytic subunit gene (POLD1) by p53 and Sp1" 2000

Employment opportunities received on experiences/training supported by this award

1. Li Liu	DOD postdoctoral fellow, New York Medical College
2. Baoqing Li	postdoctoral fellow Columbia University College of Physicians and Surgeons
	3. Nancy Krucher Assistant Professor, Department of Biological Sciences, Pace University , Pleasantville, New York

8. Conclusions.

DNA polymerase δ , the principal replicative DNA polymerase in mammalian system is responsible for the elongation of leading strand and for the completion of Okazaki fragment on lagging strand synthesis. We have used an immunoaffinity column to the p125 subunit and co-immunoprecipitation experiments to show that polymerase δ is linked to cell cycle and repair enzymes. We have linked polymeraseδ to p21,p27 and to the tumor suppressor genes p53 and Rb. The finding that p53 represses polymerase δ is a novel route for its regulation of DNA replication after p53 is induced by DNA damaging agents. Mutational analyses of the Exonuclease regions of POLD1 gene in

human breast cancer cell lines and tissues led to the finding of 6 mutations confirming the hypothesis that polymerase δ may acquire mutator properties during tumor development.

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10. Appendices.

Mo, J., Liu, Li, Leon, A., Mazloum, N., and Lee, **M.Y.W.T.**

Evidence that DNA polymerase delta isolated by immunoaffinity chromatography exhibits high molecular Weight characteristics and is associated with the KIAA0039 protein and RPA.

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Submitted to J. Biol. Chem. (2000)

Evidence That DNA Polymerase δ Isolated by Immunoaffinity Chromatography Exhibits High-Molecular Weight Characteristics and Is Associated with the KIAA0039 Protein and RPA†

Jinyao Mo, Li Liu, Argentina Leon, Nayef Mazloum, and Marietta Y. W. T. Lee*

Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York 10595

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ABSTRACT: DNA polymerase δ, the key enzyme for eukaryotic chromosomal replication, has been well characterized as consisting of a core enzyme of a 125 kDa catalytic subunit and a smaller 50 kDa subunit. However, less is known about the other proteins that may comprise additional subunits or participate in the macromolecular protein complex that is involved in chromosomal DNA replication. In this study, the properties of calf thymus pol δ preparations isolated by immunoaffinity chromatography were investigated. It is demonstrated for the first time using highly purified preparations that the pol δ heterodimer is associated with other polypeptides in high-molecular weight species that range from 260000 to >500000 in size, as determined by FPLC gel filtration. These preparations are associated with polypeptides of ca. 68−70, 34, 32, and 25 kDa. Similar findings were revealed with glycerol gradient ultracentrifugation. The p68 polypeptide was shown to be a PCNA binding protein by overlay methods with biotinylated PCNA. Protein sequencing of the p68, p34, and p25 polypeptide bands revealed sequences that correspond to the hypothetical protein KIAA0039. KIAA0039 displays a small but significant degree of homology to *Schizosaccharomyces pombe* Cdc27, which, like *Saccharomyces cerevisiae* Pol32p, has been described as the third subunit of yeast pol δ. These studies provide evidence that p68 is a subunit of pol δ. In addition, the p68−70 and p32 polypeptides were found to be derived from the 70 and 32 kDa subunits of RPA, respectively.

DNA replication is a vital cellular process in which the basic synthetic reactions are performed by the DNA polymerase enzymes. These enzymes are the central components of larger assemblies of proteins that are required for cellular DNA replication. DNA polymerase δ, the key enzyme for eukaryotic chromosomal replication, has been well characterized as consisting of a core enzyme of a 125 kDa catalytic subunit and a smaller 50 kDa subunit. In *Escherichia coli*, the DNA polymerase III holoenzyme consists of at least 10 different polypeptides (*1*, *2*). Biochemical and genetic studies have enabled the formulation of models in which these form a multiprotein assembly that functions to coordinate both leading and lagging strand DNA synthesis at the replication fork (*2*). This includes a mechanism whereby this complex contains two DNA polymerase molecules linked by a dimerization protein (tau). This model has been proposed for T4 and *E. coli* as a means of concurrent replication of both template strands at the replication fork (*3*−*5*). Similar models have been proposed for eukaryotic systems, although the details of the proteins or their macromolecular assembly are still vague (*6*).

DNA polymerase δ is the main replicative polymerase involved in the duplication of eukaryotic cell chromosomal DNA (*7*−*11*). Despite the central importance of this enzyme, the delineation of its subunit structure to this date is far from complete. Rigorously purified mammalian pol δ has been extensively characterized as a tightly associated heterodimer consisting of a 125 kDa catalytic subunit and a small 50 kDa subunit (*12*−*14*). The pol δ catalytic subunit is highly conserved in eukaryotic cells, e.g., between human and yeast (*15*). In recent work, *Schizosaccharomyces pombe* pol δ has been isolated in a form that contains two additional subunits (*16*), while in *Saccharomyces cerevisiae*, pol δ has been shown to consist of three subunits (*17*). In addition, the recombinant *S. cerevisiae* pol δ heterotrimer can dimerize when analyzed on Superose 6 gel filtration columns (*18*). This third subunit is encoded by the POL32 and Cdc27 genes in *S. cerevisiae* and *S. pombe*, respectively (*17*, *19*), while the fourth subunit, only identified in *S. pombe*, is encoded by the Cdm1 gene (*16*, *19*).

The task of identifying new subunits of mammalian DNA polymerases is made difficult by the low amounts of enzyme in mammalian tissues and the instability of the enzyme (*12*, *13*). Very little is known of the polypeptides that are associated with mammalian pol δ at the level of the demonstration of physical complexes that are more highly organized than the heterodimer. In an effort to facilitate the identification of proteins and enzymes that interact with pol δ and PCNA, we have developed PCNA affinity (*20*) and

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* To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595. Telephone: (914) 594-4070. Fax: (914) 594-4058. E-mail: Marietta_Lee@NYMC.edu.

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pol δ immunoaffinity chromatography methods (*14*). A pol δ heterodimer consisting of p125 and p50 was readily obtained when immunoaffinity-purified pol δ is subjected to single-stranded DNA cellulose chromatography (*14*). Using a PCNA overlay technique, we have identified another subunit of polymerase δ, p68. A partial protein sequence and a BLAST search identified this polypeptide as KIAA0039 (*21*). In this study, we have investigated the behavior of the immunoaffinity-purified pol δ from calf thymus, and demonstrated that it exhibits the behavior of a higher-order complex with other polypeptides at a high level of purification.

EXPERIMENTAL PROCEDURES

Materials. Single-stranded DNA cellulose and heparin−agarose were obtained from Sigma Chemical Co. Hydrazide Avid gel F was from Unisyn Technologies. Poly(dA)$_{2000}$ was obtained from Midland Certificate Co. Superose 12 columns, a protein biotinylation system, and the ECL chemiluminescence detection reagents were purchased from Amersham-Pharmacia Biotech Inc. Fetal calf thymus glands were obtained from Animal Technologies Inc. The lysis buffer used for homogenization of calf thymus consisted of 50 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25 M sucrose, and 5% glycerol. TGEED buffer consists of 50 mM Tris-HCl (pH 7.8 or 8.5), 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol. TGEE buffer is the same as TGEED buffer except for the omission of dithiothreitol. KGEED buffer is 20 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol.

Purification of DNA Polymerase δ from Calf Thymus. All steps were carried out at 0−4 °C using the procedures described by Jiang at al. (*14*), unless otherwise indicated. Eight hundred grams of frozen calf thymus tissue in 4 L of lysis buffer was homogenized in a Waring blender. The suspension was centrifuged at 5000 rpm at 4 °C for 1 h. The supernatant was filtered through glass wool.

Batchwise DEAE-Cellulose Adsorption. DE-52 cellulose (1.5 L, Whatman) was equilibrated with TGEED buffer (pH 7.8). The supernatant was mixed with the DE-52 cellulose and stirred for 30 min. The mixture was filtered through a Buchner funnel. The DE-52 cellulose was washed with 10 L of TGEED (pH 7.8), and the pol δ was stripped with 3 L of 20% ammonium sulfate in TGEED (pH 7.8).

Phenyl−Agarose Hydrophobic Chromatography. The DE-52 cellulose fraction was loaded onto a phenyl−agarose column (bed volume of 500 mL) equilibrated with KGEED buffer [20% ammonium sulfate (pH 7.0)]. The phenyl−agarose column was washed with 1 L of KGEED and eluted with 1 L of TGEED (pH 8.5).

Immunoaffinity Chromatography. The peak fractions from step 2 were pooled and precipitated by addition of 0.32 g/mL ammonium sulfate. The suspension was stirred for 30 min and kept on ice for an additional 30 min, and then centrifuged at 10000*g* for 45 min. The precipitate was resuspended in TGEE buffer, and the conductivity was adjusted to that of TGEE buffer with 80 mM NaCl. The solution was divided into two equal batches, which were individually subjected to immunoaffinity chromatography. The immunoaffinity column (bed volume of 20 mL) was equilibrated with TGEE buffer (pH 7.8). After the sample had been loaded, the column was washed with 60 mL of TGEE buffer containing 0.4 M NaCl. Pol δ was eluted with 30% ethylene glycol and 0.4 M NaCl in TGEE. The peak fractions from two batches of immunoaffinity chromatography were then combined.

Single-Stranded DNA Cellulose Chromatography. The fractions from the immunoaffinity column were combined, and the conductivity was adjusted to that of TGEED buffer containing 50 mM NaCl and loaded onto a ssDNA cellulose column (bed volume of 20 mL). The column was washed with 50 mL of TGEED buffer (pH 7.8). The enzyme was eluted with a 200 mL gradient from 50 to 700 mM NaCl.

Heparin−Agarose Chromatography. The fractions from the ssDNA cellulose column were combined and the conductivities adjusted to that of TGEED buffer containing 50 mM NaCl, and the fractions were loaded onto a heparin−agarose column (bed volume of 2 mL). The column was washed with TGEED buffer (pH 7.8), and the pol δ was eluted with 0.4 M NaCl in TGEED buffer.

FPLC Gel Filtration Chromatography. A preparation obtained after immunoaffinity chromatography was dialyzed against TGEED buffer (pH 7.8) with two changes over a period of 16 h, and then concentrated to 300 μL by centrifugal concentration (5000*g* at 4 °C) using Centricon 30 filters (30 000 MW cutoff, Millipore). The concentrated pol δ was then chromatographed on a FPLC Superose 12 column (HR 10/30, Pharmacia) equilibrated with TGEED (pH 7.8) containing 150 mM NaCl at a flow rate of 0.25 mL/min. A total of 72 fractions of 0.25 mL each were collected.

Glycerol Gradient Ultracentrifugation. Sedimentation analysis was carried out using a Beckman ultracentrifuge with a SW 41 rotor. The buffer was TGEED (pH 7.8) with 150 mM NaCl. The proteins were laid on the top of glycerol gradients in 12 mL tubes and centrifuged at 30 000 rpm for 16 h. After centrifugation, fractions were withdrawn from the bottom of the tubes. The sedimentation velocities were estimated by the use of standard proteins.

SDS−Polyacrylamide Gel Electrophoresis. Fractions were precipitated with 10% trichloroacetic acid, washed with cold 80% acetone, and dissolved in SDS loading buffer. The samples were loaded on a 10% SDS−polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue or silver (Bio-Rad).

Western Blotting. Western blotting was performed using 78F5 and 38B5 pol δ monoclonal antibodies (*14*). Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and also to provide for visual confirmation of efficient transfer. Nitrocellulose blots were blocked in 5% w/v nonfat dry milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% (v/v) Tween 20 (TBST) for 1 h at room temperature. The blot was then incubated with monoclonal antibodies against pol δ for 12 h at 4 °C. After three 10 min washes in TBST, the blot was incubated with the streptavidin−horseradish peroxidase conjugate diluted in TBST (1:10000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence (ECL detection system, Amersham-Pharmacia Biotech Inc.).

Nondenaturing Polyacrylamide Gel Electrophoresis. The samples were run on a 5 to 15% gradient gel with a 3.5% stacking gel at 4 °C for a sufficient length of time such that all the protein markers and the pol δ band had reached limiting mobilities. An 18 h period of electrophoresis at 200 V was found to be suitable in preliminary trial experiments. SDS and 2-mercaptoethanol were excluded from the gel. In this experiment, a crude calf thymus extract which has been subjected to batchwise purification on DEAE-cellulose was analyzed. The DEAE sample was concentrated via ammonium sulfate precipitation, and the sample was subsequently desalted on a desalting column. The sample (1 mg of protein) was electrophoresed for 18 h at 4 °C along with the protein standards. Proteins were transferred to a nitrocellulose membrane at 12 V for 12−14 h at 4 °C. The membranes were immunoblotted with 78F5 pol δ p125 monoclonal antibody.

Overlay Blotting with Biotinylated PCNA. Recombinant PCNA was expressed in *E. coli* and purified to near homogeneity as previously described (*22*). PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester (Amersham-Pharmacia Biotech Inc.). The reaction mixtures contained 0.5 mg of PCNA and 0.5 mg/mL biotinamidocaproate *N*-hydroxysuccinamide ester in a total volume of 0.5 mL in 20 mM bicarbonate buffer (pH 8.6). After reaction for 60 min at room temperature, the PCNA was purified on a Sephadex G25 column (bed volume of 5 mL) equilibrated with phosphate-buffered saline containing 1% bovine serum albumin. Samples (1−5 μg of protein) to be overlayed were run onto a 10% SDS−PAGE gel and transferred to nitrocellulose membranes. Prestained protein standards (New England Biolabs) were used as molecular weight markers. The nitrocellulose membrane was blocked with 5% nonfat dry milk in TBST for 45 min at room temperature followed by three washes of TBST for 10 min each. The blot was then incubated with biotinylated PCNA (1 μg/μL) diluted in TBST (1:900) at 4 °C overnight. The blot was washed five times with TBST for 15 min. It was subsequently incubated with the streptavidin−horseradish peroxidase conjugate diluted in TBST (1:5000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence.

DNA Polymerase Assay. Sparsely primed poly(dA)$_{2000}$/oligo(dT)$_{16}$ was used as the template. The assays contained poly(dA)$_{2000}$/oligo(dT)$_{16}$ (20:1, 0.25 OD$_{260}$ unit/mL), 200 μg/mL BSA, 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES (pH 6.0), 20 μM [^{3}H]TTP (100 cpm/pmol, 5 μCi/nmol), and 0.2−0.4 unit of pol δ, in the presence or absence of 0.2 μg of PCNA in a total volume of 100 μL. When poly[d(AT)] was used as a template, assays were performed in the absence of PCNA as described by Lee et al. (*13*). The reaction mixtures were incubated for 60 min at 37 °C, and the reactions were terminated by spotting onto DE-81 papers which had been washed four times with 0.3 M ammonium formate (pH 7.8) and once with 95% ethanol and counted as previously described (*13*). One unit of DNA polymerase activity is the amount that catalyzes the incorporation of 1 nmol of dTMP per hour at 37 °C.

Protein Quantitation. Protein was quantified by the Bradford method with BSA as a standard (*23*).

Peptide Sequencing. Sequence analyses were performed by the Harvard University Microchemistry Facility using single microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer.

RESULTS

Immunoaffinity-Purified Pol δ Behaves as a High-Molecular Complex That Is Much Larger Than the Heterodimer. A number of methods have been reported for the rigorous isolation of pol δ from mammalian sources by conventional methods. These generally lead to the isolation of a tightly associated heterodimer of 125 and 50 kDa subunits, although there are reports of the isolation of the free p125 catalytic subunit (*24*, *25*). The amounts recovered are generally very small (tens of micrograms). We reported a more facile procedure that involves batchwise purification of pol δ from calf thymus through DE-52 and phenyl−agarose supports, followed by immunoaffinity chromatography using a monoclonal antibody directed against the p125 catalytic subunit (*14*). This procedure yields about 1 mg of protein, which on SDS−PAGE contains the p125 and p50 polypeptides of pol δ but also a number of other polypeptide bands. However, a persistent association of a ca. 70 kDa band was also noticed. Final purification to the heterodimer was achieved by ssDNA cellulose chromatography (*14*). The specific activity of the preparation was similar to that reported previously (*13*, *14*).

The question which arose was whether any of the polypeptide bands found with the pol δ heterodimer after immunoaffinity chromatography represented replication proteins, i.e., whether the immunoaffinity-purified enzyme contains additional pol δ subunits or other replication proteins that contribute to a higher-order complex. FPLC gel filtration analysis was used to determine if the immunoaffinity-purified enzyme behaved as a higher-molecular weight complex(es) than could be ascribed to a heterodimer. To do this, the immunoaffinity-purified enzyme was concentrated before FPLC analysis. This was done as any higher-order complexes were more likely to be dissociated on dilution. The column fractions were assayed for DNA polymerase δ activity using poly(dA)/oligo(dT) as a template in the absence and presence of PCNA. The fractions were also assayed with poly[d(AT)] alternating copolymer as a template in the absence of PCNA as described previously (*13*). The enzyme activity behaved in a polydisperse manner, but the pol δ activity eluted with a molecular weight higher than that found for the heterodimer (M_r = 175 000), as shown in Figure 1. The major peaks of pol δ activity ranged from a relative molecular weight of 230000 to >500000 as determined from calibration of the column with protein standards. In Figure 1, the identities of the 125 and 50 kDa polypeptides as the subunits of pol δ were confirmed by Western blotting. SDS−PAGE of the peak fractions of the high-molecular weight fractions of immunoaffinity-purified pol δ revealed that the preparations contained the 125 and 50 kDa subunits as major components; i.e., these represent very highly purified preparations. Typical SDS−PAGE profiles for the fractions obtained on FPLC gel filtration of the immunoaffinity-purified enzyme are shown for two separate preparations in Figure 2. In addition to the p125 and p50 polypeptides, we consistently observed the presence of a number of other polypeptides. These included a ca. 70 kDa band, which often appeared as a doublet, and

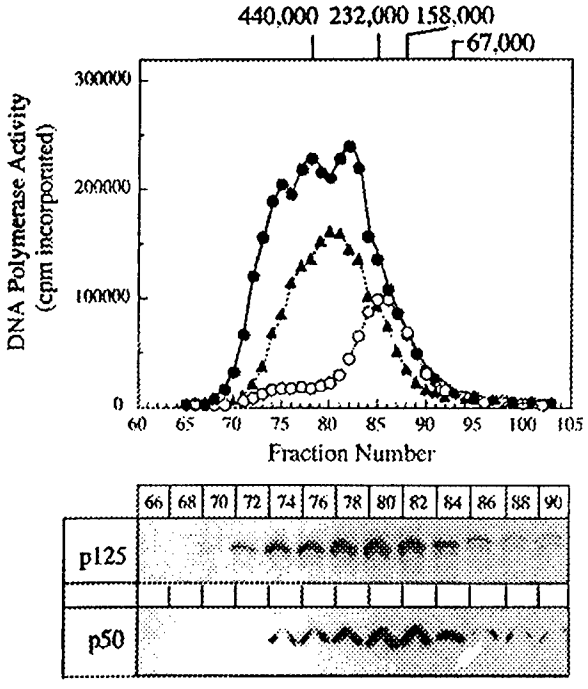

FIGURE 1: FPLC gel filtration analysis of immunoaffinity-purified fetal calf thymus DNA polymerase δ. Calf thymus pol δ was purified by immunoaffinity chromatography and concentrated by centrifugation on Centricon filters as described in Experimental Procedures. The concentrated preparation was run on a Superose 12 HR10/30 column on a Bio-Rad Biologics FPLC system. Fractions of 0.15 mL were collected. The column was calibrated with protein standards (ferritin, catalase, aldolase, and albumin). (Top) Five microliters of each fraction was assayed with poly(dA)/oligo(dT) in the presence (●) and absence (○) of added calf thymus PCNA at 37 °C for 30 min. Activated poly(dAT) was also used as a template (▲) and assayed in the absence of PCNA. (Bottom) Samples of the active fractions were subjected to SDS−PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The membrane was blotted with antibodies against p125 and p50.

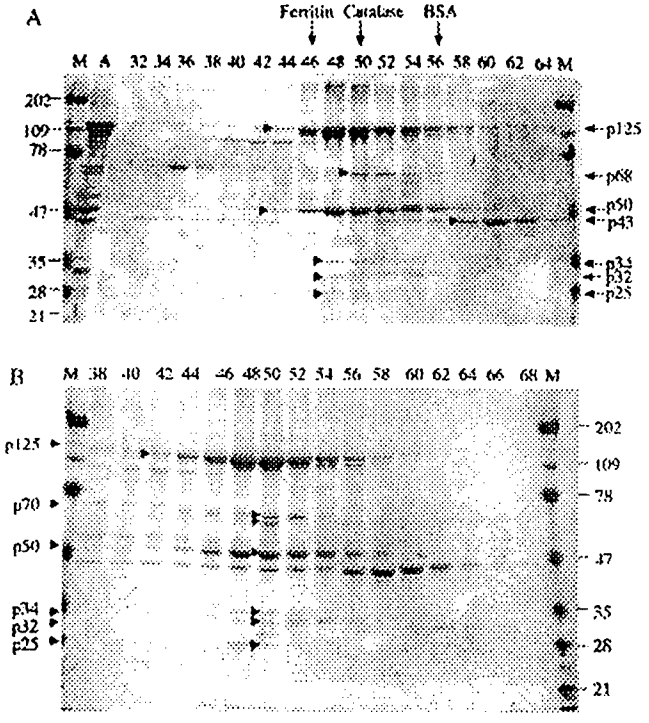

FIGURE 2: SDS−PAGE of immunoaffinity-purified pol δ after FPLC gel filtration. Calf thymus pol δ was purified to the immunoaffinity chromatography step as described for Figure 1, and subjected to FPLC gel filtration using a Superose 12 column. In these experiments, fractions of 0.25 mL were collected. Samples of the active fractions were run on SDS−PAGE and stained with Coomassie Brilliant Blue, and the diagram (A and B) shows the results from two separate representative experiments. Protein standards were as indicated on the left of the diagram and were run in the lanes marked M. A sample of the immunoaffinity-purified enzyme preparation before gel filtration was run in the lane marked A (panel A). The numbers refer to the column fractions which were analyzed. The position of elution of ferritin (MW of 440 000), catalase (232 000), and bovine serum albumin (BSA, 67 000) are shown by the arrows above the gel which indicate the fraction numbers at which these standards eluted.

bands at 43, 34, 32, and 25 kDa. These experiments were repeated for at least 20 preparations, and the appearance of these polypeptides was consistent, although the amounts were variable from preparation to preparation. These bands did not appear in stoichiometric amounts with p125 and p50.

In the following experiments, (a) additional evidence was obtained which shows that the immunoaffinity-purified enzyme behaves as a larger physical entity than can be ascribed to the size of the heterodimer, (b) the identities of the ancillary polypeptides were investigated by protein sequence determination, and (c) the PCNA response of the immunoaffinity-purified enzyme is shown to differ from that of the heterodimer.

Comparison of Physical Properties of the Immunoaffinity-Purified Pol δ with Those of the Heterodimer. The immunoaffinity-purified enzyme was passed through a single-stranded DNA cellulose column and subsequently onto a heparin−agarose column as described in Experimental Procedures, leading to the isolation of a heterodimer of p125 and p50 (not shown) as previously reported (*14*). Calibration of the FPLC gel filtration column on which the immunoaffinity preparations were chromatographed (Figure 2) indicated that the p125 polypeptide (based on SDS−PAGE and protein staining) eluted with a peak between fractions 49 and 50, corresponding to relative molecular weights between 250 000 and 300 000 (not shown). The behavior of the enzyme was not due to aggregation since similar findings were obtained when the amounts of enzyme loaded were reduced 10-fold. Previous studies have shown that the heterodimer behaves as a protein with a relative molecular weight of 173 000 (*13*). This was confirmed with the FPLC gel filtration column used in these studies (not shown). A plot of the Stokes radii of the immunoaffinity-purified enzyme as determined by FPLC chromatography on Superdex 200 gave a value of 57 Å based on the peak fraction containing the p125 band as determined by SDS−PAGE. This is larger than the value of 53 Å that we have previously determined for the heterodimer by conventional purification (*13*).

Similar results were obtained by using glycerol gradient ultracentrifugation (Figure 3); i.e., the immunoaffinity-purified enzyme sedimented with a much higher velocity ($S_{20,w}$ = 9.2) than the heterodimer which under the same experimental conditions migrated as a species of about 7.0 S (not shown). The fractions of pol δ activity obtained after glycerol gradient ultracentifugation were run on SDS−PAGE and stained with silver (Figure 3, inset). It may be noted that p68, p34, p32, and p25 polypeptides also cosedimented with the core enzyme. These experiments demonstrate that the immunoaffinity-purified calf thymus pol δ activity

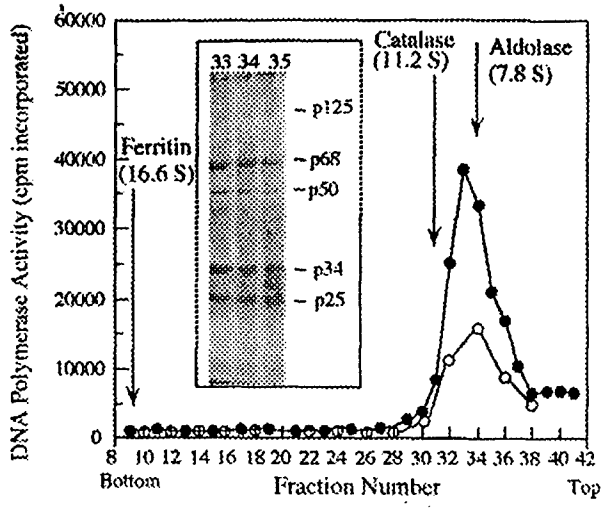

FIGURE 3: Glycerol gradient centrifugation of the immunoaffinity-purified pol δ. Pol δ was purified to the immunoaffinity chromatography step. The peak fractions were concentrated to 150 μL on Centricon filters (Experimental Procedures), laid on the top of a 10 to 45% glycerol gradient (11 mL) containing 150 mM NaCl, 1 mM DTT, and 50 mM Tris-HCl (pH 7.8), and centrifuged for 16 h at 25 000 rpm. After centrifugation, fractions of 0.25 mL each were withdrawn from the bottom of the tubes and assayed for polymerase activity in the presence (●) and absence (○) of PCNA using poly(dA)/oligo(dT) as the template. Migration positions of protein standards (ferritin, catalase, and aldolase) are shown by the arrows. In the inset, the proteins from the peak fractions of activity (fractions 33−35) were concentrated using Centricon 30 filters and loaded onto a 10% SDS−polyacrylamide gel. The gel was visualized by silver staining. The lines show the p125, p68, p50, p34, and p25 polypeptides.

Table 1: Molecular Weights of Mammalian DNA Polymerase Preparations

ref	source	Stokes radius (Å)	sedimentation coefficient	molecular mass
Goulian et al. (*24*)	mouse	54	8.0	178
		43	6.3	112
Lee et al. (*13*)	calf thymus	53	7.9	173
this work	calf thymus	57	9.2	215

behaves as a larger complex than the heterodimer. These findings are the first demonstration that highly purified pol δ in which the core enzyme is the principle component behaves as a macromolecular complex. The calculated molecular weights are shown in Table 1, and are larger than those previously reported for the calf thymus (*13*) and mouse (*24*) pol δ preparations.

Sequence Analysis of Polypeptides in the Pol δ Complex. The identities of the polypeptides that are associated with pol δ preparations were investigated by sequence analysis of the bands excised from the preparations shown in Figure 2, i.e., of the fractions obtained on FPLC gel filtration of immunoaffinity-purified pol δ. A list of the protein sequences obtained from polypeptides excised from SDS−PAGE gels from the two preparations (Figure 2) is shown in Table 2. The p125 and p50 bands were also excised, and the peptide sequences that were obtained exactly matched the known sequences of p125 and p50 (Table 2).

We have previously shown that the doublet of proteins of 68−70 kDa that are associated with high-molecular weight form of affinity-purified pol δ are PCNA binding polypeptides (*21*). Sequencing of bands from this region was performed by MS/MS peptide sequencing, yielding sequences that are identical to the KIAA0039 cDNA sequence (*21*). Additional analysis showed that the peptide sequences obtained from the p68/p70 band (Figure 2 and Table 2) could be arranged into two groups, showing that there were two polypeptides that were migrating in this region. Five sequences (Table 2) were exactly identical with the open reading frame encoded by the human cDNA (Genbank entry D26018) for the hypothetical protein KIAA0039 (*26*). The cDNA encoded by KIAA0039 contains an open reading frame of 466 amino acids. The hypothetical KIAA0039 protein has a predicted molecular mass of 51.4 kDa. The extreme C-terminus contains a consensus PCNA binding site, consistent with the results of the PCNA overlay experiments (*21*). The latter observation makes this protein a strong candidate for the human homologue of the "third" subunits of yeast pol δ, which are encoded by the Cdc27 and POL32 genes in *S. pombe* and *S. cerevisiae*, respectively (*16*, *17*). The second of the groups of sequences obtained from the p68 polypeptide was found to be a match for the 70 kDa subunit of RPA, the eukaryotic single-stranded DNA binding protein.

The p34 and p25 bands also yielded sequences identical to those in the KIAA0039 sequence. Five peptide sequences obtained from the p34 polypeptide, and one peptide sequence obtained for the p25 polypeptide, were found to be derived from KIAA0039. This indicates that these two bands are proteolytic fragments of the p68 full-length KIAA0039 protein. The association of these KIAA0039 fragments with the high-molecular weight fraction of pol δ strongly suggests that they may be associated with nicked species of KIAA0039 which retained an ability to associate with pol δ. This could also explain failure to find a consistent stoichiometric association of KIAA0039 in these particular experiments.

The p43 polypeptide, which is present in the immunoaffinity-purified preparation but is clearly separated from the heterodimer on gel filtration (see Figure 2), was also sequenced and was identified as actin.

PCNA Overlay of Immunoaffinity-Purified Pol δ. We have shown that biotinylated PCNA can be used to identify PCNA binding proteins, and have used this method to demonstrate that PCNA interacts with pol δ p125 (*21*). The p50 subunit does not bind PCNA by this method (*21*). The results of a typical overlay experiment in which pol δ preparations at different stages of purification were examined are shown in Figure 4A. It is seen that there are a number of PCNA binding polypeptides in the crude extract, and that the prominent ones are p125 and a doublet at around 68 kDa. The partial protein sequence of this 68 kDa band was obtained, and a BLAST search identified this polypeptide as KIAA0039 (*21*). This protein is retained in the preparation up to the immunoaffinity step, and is retained with the p125 band during subsequent FPLC gel filtration on Superose 12 (Figure 4A). Thus, highly purified pol δ preparations are associated with a ca. 68 kDa polypeptide which is the mammalian counterpart of *S. pombe* Cdc27. If the immunoaffinity-purified pol δ enzyme is first chromatographed on ssDNA−cellulose, this polypeptide is removed and only the p125 band can be detected by PCNA overlay (Figure 4B).

Native Gel Electrophoresis. The nature of the complex(es) involving the p125 catalytic subunit of pol δ was investigated by Western blot analyses of partially purified pol δ on

Table 2: Sequence Analysis of Polypeptides in Immunoaffinity-Purified Calf Thymus Pol δ^a

excised polypeptide band (kDa)	amino acid sequence	residue numbers	identity	Genbank accession no.
125	TEGGEDYTGATVIEPLK	574−590	p125 subunit of pol δ	M80395
	GLLPQILENLLSAR	653−666		
50	YIHPDDELVLEDELQR	126−141	p50 subunit of pol δ	U2109
	QAASVEAVKMLDEIL	269−283		
	YSSMEDHLEILEWTL	355−369		
70	VVILMELEVLK	93−103	RPA 70 kDa subunit	M63488
	LFSLELVDESGEIR	221−234		
	NEQAFEEVFQNANFR	554−568		
68	WLSYTLGVHVNQAK	25−38	KIAA0039 protein	D26018
	QMLYDYVER	39−47		
	DSGPLFNTDYDILK	110−123		
	FSAIQCAAAVPR	132−143		
	GIMGMFASK	191−199		
34	WLSYTLGVHVNQAK	25−38	KIAA0039 protein	D26018
	QMLYDYVERK	39−48		
	AMLKDSGPLFNTDYDILK	106−123		
	DSGPLFNTDYDILK	110−123		
	GIMGMFASK	191−199		
32	IGNVEISQVTIVGIIR	66−81	RPA 32kDa subunit	gi|4506585|
	IDDMTAAPMDVR	94−105		
	KSLVAFK	139−145		
	PRGLNFQDLK	222−231		
	NQLKHMSVSSIK	232−243		
25	DSGPLFNTDYDILK	110−123	KIAA0039 protein	D26018

^{a} Protein sequences from polypeptides extracted after SDS−PAGE were obtained as described in Experimental Procedures.

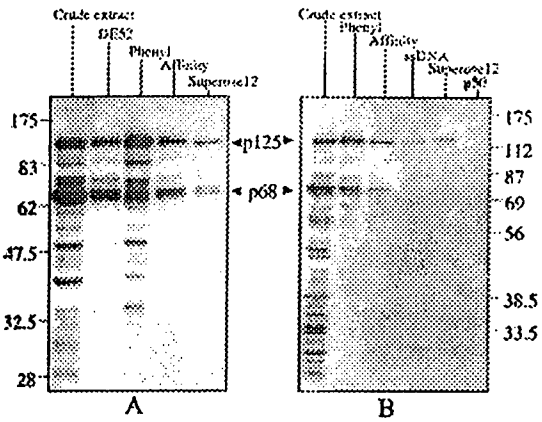

FIGURE 4: PCNA overlay analysis of DNA pol δ at different stages of purification. (A) Samples of a pol δ preparation from the crude extract, DE-52, phenyl−agarose, immunoaffinity, and Superose 12 gel filtration steps were run on SDS−PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The membrane was blotted using biotinylated recombinant PCNA, and visualized using the streptavidin−horseradish peroxidase conjugate and a chemiluminescence method (ECL detection system). (B) Samples of a pol δ preparation at the crude extract, phenyl−Sepharose, immunoaffinity, ssDNA cellulose, and Superose 12 gel filtration steps were subjected to SDS−PAGE and analyzed using biotinylated PCNA as described above. Recombinant p50 (2.5 μg) was also run on this gel, showing that it is not overlayed by PCNA. The positions of the prestained protein standards are marked on the sides. The positions of the p125 and p68 bands are shown by the arrowheads.

nondenaturing gel electrophoresis in gradient gels under conditions where limiting mobility of the proteins was reached. Under these conditions, the migration of proteins or stable protein complexes can be correlated with their relative molecular weights. Our analyses of calf thymus pol δ purified through the initial DE-52 column revealed a single high-molecular weight complex ($M_r \sim 520\,000$) by Western blotting against a pol δ p125 antibody (Figure 5A, lane 1). Similar results were obtained when the material was purified through a subsequent Q-Sepharose column (Figure 5A, lane 2). This monodisperse behavior of the pol δ complex on native gel electrophoresis is striking and argues for the maintenance of a very discrete complex under these conditions. Examination of the purified heterodimeric form of pol δ under the same conditions gave a single band at 175 000 (not shown). Further analysis was performed by excision of the 520 kDa bands obtained from nondenaturing gel electrophoresis. These were subjected to SDS−PAGE, and assessed by PCNA overlay analysis. The results revealed the presence of polypeptide bands at 125 and 68 kDa, and a band at ca. 58 kDa. The latter is not the small subunit of the pol δ (p50) which does not interact with PCNA in the overlay analysis (*21*). This work further confirmed that p68 is strongly associated with high-molecular weight forms of pol δ. The results of these experiments support those shown above for the presence of a PCNA binding protein of ca. 68 kDa which is associated with the pol δ heterodimer in a high-molecular weight complex. In addition, these experiments indicate the possible existence of yet another novel PCNA binding protein of about 58 kDa that is associated with the complex, although we cannot rule out the possibility that it is a proteolytic product of the 125 kDa catalytic subunit.

Sensitivity to PCNA. When the response of the immunoaffinity- and gel filtration-purified enzyme to PCNA was compared to that of the heterodimer, it was observed that the former consistently exhibited a higher response than the latter. To eliminate the possibility that this was due to

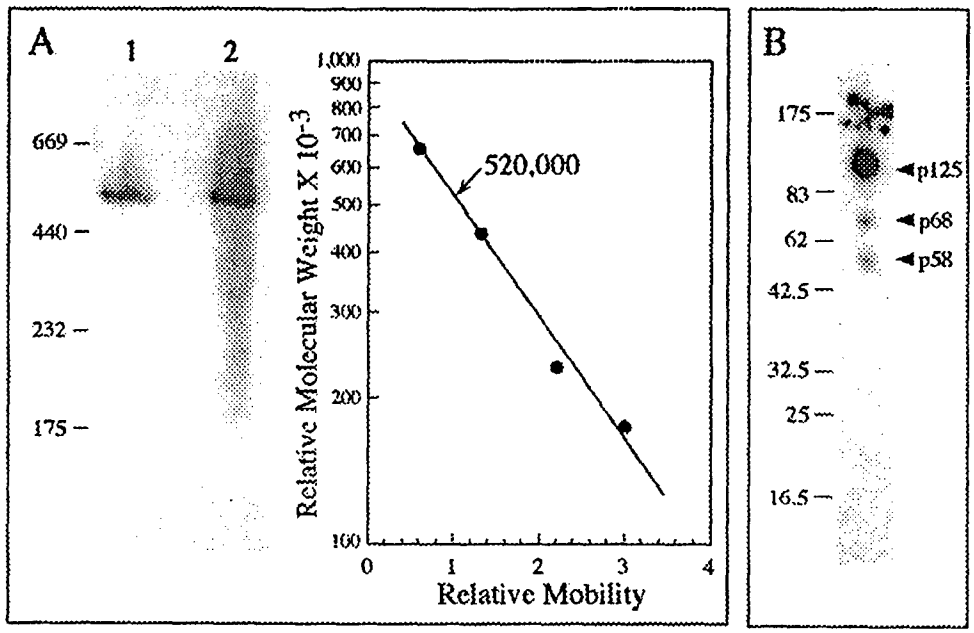

FIGURE 5: Native gel electrophoresis of pol δ. (A) Nondenaturing gel electrophoresis of pol δ. Calf thymus pol δ that was purified through the DE-52 step (lane 1) and then further purified on a Q-Sepharose column (lane 2) was subjected to electrophoresis on a gradient gel (5 to 15% acrylamide) under nondenaturing conditions and electrophoresed until the marker proteins had reached a limiting mobility (Experimental Procedures). The proteins were then transferred to nitrocellulose membranes which were then Western blotted with an antibody against the p125 subunit of pol δ. The diagram on the right shows the estimation of the size of the pol δ complex. Protein standards were used to estimate the size of the complex containing the p125 subunit of pol δ. These were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The arrow shows the migration position and estimated size of the protein complex containing the pol δ p125 subunit. (B) Two-dimensional PCNA overlay analysis. The gel slice from the first dimension corresponding to the 520 kDa band was cut with reference to the Western blot, immersed in SDS–PAGE sample buffer without shaking for 2 h at 37 °C, and then secured on top of the stacking gel of a 5–15% SDS–polyacrylamide gradient gel with 0.5% agarose in SDS sample buffer without glycerol. The proteins were transferred onto a nitrocellulose membrane, and the PCNA overlay was performed as described in Figure 4. The positions of the p125, p68, and p58 bands are marked by arrows.

differences in assay conditions, a systematic comparison was made of the PCNA responses of (a) the recombinant human p125 catalytic subunit, (b) a p125 mutant in which the N-terminus containing the PCNA binding region was deleted (*10*), (c) the recombinant heterodimer produced by coexpression in Sf9 cells, (d) the calf thymus heterodimer isolated as described previously by immunoaffinity and ssDNA cellulose chromatography (*14*), and (e) the immunoaffinity-purified calf thymus pol δ preparation. Different time points and concentrations of PCNA were used to optimize the assay conditions, and the same preparation of human PCNA was used for all the assays. The results (Figure 6) show that the p125 catalytic subunit has a small but detectable response to PCNA, which is eliminated by deletion of the N-terminus. The recombinant human p125/p50 heterodimer and the calf thymus heterodimer isolated to near homogeneity were activated by PCNA, to comparable extents, about 12–16-fold, in contrast to the immunoaffinity-purified enzyme, which was activated by nearly 40-fold. This suggested that components required for the full response to PCNA were removed from the immunoaffinity-purified pol δ during its purification to the heterodimer.

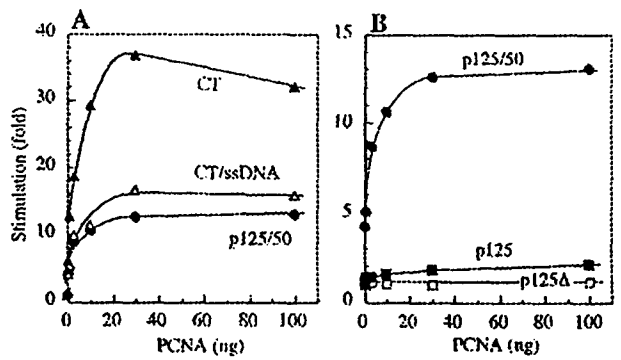

FIGURE 6: PCNA sensitivity of pol δ after immunoaffinity and ssDNA cellulose chromatography. Pol δ activity was determined using poly(dA)/oligo(dT) as the template–primer complex, and results are shown as the fold stimulation of activity in the presence of varying amounts (nanograms) of PCNA per 50 μL assay. Linearities of the assays were determined by time course measurements (not shown). (A) The black triangles show the response of immunoaffinity-purified calf thymus pol δ (CT). The white triangles show the same preparation after subsequent purification to a homogeneous heterodimer on ssDNA cellulose and heparin–agarose chromatography. The black circles show the PCNA response of recombinant human pol δ p125/p50 obtained by coexpression of the two subunits in Sf9 cells. (B) The response of the recombinant human pol δ p125/p50 heterodimer is compared with that of the recombinant p125 subunit (■) and its deletion mutant (p125Δ) in which the 186 N-terminal amino acids were deleted (□). All recombinant human pol δ proteins were expressed in Sf9 cells and purified as previously described (*40*). In these assays, the same preparation of purified calf thymus PCNA was used rather than recombinant PCNA, and all assays were conducted in the same experiment.

DISCUSSION

DNA polymerase δ is now well recognized as the key DNA polymerase in eukaryotic DNA replication, and the p125 and p50 subunits are well conserved between mammals and yeast (*15*, *27*). There are questions as to the complete polypeptide composition of pol δ, as well as that of other

associated replication proteins that together with pol δ form the multiprotein assembly that functions in chromosomal replication. In addition, evidence which shows that pol δ is also involved in DNA repair processes (*28*, *29*) indicates that the pol δ holoenzyme may associate dynamically with different proteins to form more than one type of higher-order assembly. As noted in the introductory section, extensive investigation in prokaryotes of *E. coli* DNA polymerase III has led to the identification of a number of polypeptides which have been studied in various subassemblies. The investigation of the identity and functions of replication proteins in mammalian systems is difficult because of the small amounts of material available, and because these protein complexes are likely to be dissociated upon most conventional methods of isolation, including gel filtration. In this study, we have taken advantage of a method for the immunoaffinity purification of pol δ from calf thymus, which yields relatively large amounts of enzyme, sufficient for both a proteomics approach and the possibility that the methods might be gentle enough to allow isolation of total or partial assemblies of the pol δ heterodimer associated with its natural protein partners.

Investigation of the behavior of the immunoaffinity-purified pol δ revealed that it can be shown to exist in protein complexes that are much larger (>250000) than the heterodimer. Moreover, these complexes represent assemblies that are highly purified and contain the heterodimer as the primary polypeptide component. Our studies indicate that the immunoaffinity purification does in fact lead to isolation of the heterodimer with at least some of its associated proteins. For the preliminary purification steps, we have used batchwise, rather than gradient elution, methods to minimize separation of associated proteins, and used the DEAE-cellulose and phenyl−Sepharose supports originally devised for the purification of pol δ (*13*). We have also found that ssDNA cellulose, as well as heparin−agarose chromatography, which are efficient for the isolation of the heterodimer, may do so by removing other associated proteins.

Comparison of the gel filtration behavior of the heterodimeric and the immunoaffinity-purified pol δ revealed a shift in the native molecular weight of 175 000 of the pol δ core to more than 500 000. As observed by Maki et al. (*4*), gel filtration of diluted pol III* leads to its dissociation from 800 kDa to fractions corresponding to 530, 480, and 380 kDa in size. The studies reported here provide the first evidence for high-molecular weight forms of mammalian pol δ in a highly purified state.

In other studies, we have found a number of replication proteins bound to PCNA Sepharose (*20*). As with other highly complex protein assemblies, e.g., *E. coli* DNA polymerase or RNA polymerase, conventional methods of isolation seldom yield complete stoichiometric complexes. Examination of the polypeptide compositions of the immunoaffinity-purified material revealed that while the p125 and p50 polypeptides were the major constituents, the preparation contained a number of other polypeptides which remain associated with the heterodimer during gel filtration or glycerol gradient ultracentrifugation. Clearly, some of these may represent impurities, while others may represent bona fide components of a mammalian replication complex. In this study, microsequencing was used to identify some of the associated polypeptides. The significant findings are the identification of KIAA0039 and its proteolytically derived peptides, as well as the 70 and 32 kDa subunits of RPA, as components that are associated with the high-molecular weight form of pol δ. During the course of this study, Hughes et al. (*30*) also identified KIAA0039 as a PCNA binding protein by using PCNA affinity chromatography, and have proposed that this is the third subunit of mammalian DNA polymerase δ, on the basis of the similarity of the sequence with that of Cdc27, and the association of the protein with pol δ on glycerol gradient ultracentrifugation. In their studies, they noted that recombinant KIAA0039 protein exhibited an anomalous migration on SDS−PAGE as a protein of 66 kDa, while its calculated molecular mass is 51.4 kDa. This is consistent with our observations on the behavior of the protein. Two reasons were put forth by Hughes et al. (*30*) to explain the previous failure to detect p66 in purified pol δ preparations. One was that this subunit is refractory to certain silver-staining reagents, and the other was that p66 is not absolutely required for polymerase activity and may have been lost during the lengthy purification procedures. The studies presented here provide stronger evidence that the 68 kDa polypeptide is a likely subunit of pol δ. The presence of the proteolytic products of p68 indicates that the protein is susceptible to nicking, and provides a reasonable explanation for the variability of its appearance in highly purified pol δ preparations. In previous work in this laboratory, p68 was found to be a persistent "impurity" that is associated with the immunoaffinity-purified enzyme and is only removed by a combination of ssDNA cellulose and heparin−agarose chromatography (*14*). The native gel electrophoresis experiments described in this work provide additional evidence for the association of p68 with pol δ. Pairwise alignments using the Clustal W 1.8 program show that there is only 15−16% of sequence identity between Pol32p and Cdc27, Pol32p and p68, and Cdc27 and p68. However, evaluation of the significance of the alignment score for p68 and Cdc27 using the PRSS program (http://www.expasy.ch/tools) provided a score of 0.4; i.e., the alignment score would be attained by chance against the randomly shuffled Cdc27 sequence only 0.4 time in 100 attempts. This indicates that the degree of similarity between these two proteins is significant. Interestingly, all three sequences possess a PCNA binding motif at their C-termini. The finding that p68 is a PCNA binding protein may be responsible for the observations that the immunoaffinity-purified enzyme shows a greater PCNA response than the p125/p50 heterodimer.

If p68 were to associate with the pol δ heterodimer in a stoichiometric fashion, the expected molecular mass of the complex would be 226 kDa; given that the KIAA0039 protein behaves on SDS−PAGE with an anomalous molecular mass of 68 kDa, an upper limit for a relative molecular weight for the complex of 243 000 can be projected. This is at the lower limit of the size range (from 250000 to >500000) that was found for the behavior of the immunoaffinity-purified enzyme on gel filtration, and much smaller than the size of pol δ found on nondenaturing gel electrophoresis (ca. 520 000). The explanations for this could be that there are additional subunits or associated proteins in the complex, or that the trimeric species is capable of dimerization. The current experimental information cannot distinguish between these possibilities. However, it is relevant

that in *S. cerevisiae*, the cognate third subunit encoded by POL32 has been shown to be able to form a hexameric protein with the two classical subunits of pol δ, so that it has been suggested to be a dimerization factor (*17*, *18*). This is not inconsistent with the behavior of pol δ that was observed here, since a hexameric complex would have a molecular weight of about 490 000, but more rigorous studies using reconstituted subunits will be needed to establish this. Gerik et al. (*17*) reported no other subunits in the most purified preparation of pol δ in the yeast *S. cerevisiae* besides p125, p58, and p55. In *S. pombe*, one additional polypeptide was identified as a potential subunit of pol δ that is encoded by the Cdm1 gene (*16*).

The issue of whether there is a subunit of eukaryotic pol δ that confers the property of dimerization is of some significance, since there is evidence that pol δ functions in both the leading and lagging strand synthesis (*6*, *29*). This would suggest that the mechanism for concerted DNA synthesis that depends on a dimerization factor that couples two DNA polymerase enzymes as in the *E. coli* system is conserved in eukaryotic systems (*6*). In *E. coli*, the tau protein serves to maintain a dimeric DNA polymerase, while the *S. cerevisiae* heterotrimer has been shown to dimerize, with the implication that the third subunit may serve a parallel function (*2*, *29*). At the present time, we cannot eliminate the possibility that the larger forms of pol δ activity that we observe may be due to the presence of a dimerization factor.

The second significant finding that was made was the presence of two of the three subunits of RPA in the immunoaffinity-purified pol δ preparations. The finding that RPA, or HSSB (human single-stranded DNA binding protein), is also present in the high-molecular weight form of pol δ after FPLC gel filtration suggests that there may be an interaction of RPA with the replication complex. RPA is an abundant multimeric protein consisting of three subunits (p70, p34, and p14) which is essential for DNA replication and is also involved in DNA repair and recombination (*31*). It binds tightly to single-stranded DNA and affects the activity of other replication proteins, e.g., T antigen, DNA polymerases α (*32*) and δ (*33*), p53 (*34*), and transcriptional initiators, e.g., GAL4 and VP16 (*35*). These findings are consistent with previous studies in which the presence of RPA could be detected by Western blotting of pol δ preparations isolated by PCNA affinity chromatography (*20*). While the presence of RPA in highly purified pol δ complexes may be fortuitous, there are a number of studies which indicate the likelihood that RPA itself may interact with the DNA polymerases in the replication complex. Mutation of the zinc finger domain of RPA has been shown to eliminate DNA replication activity (*36*). Genetic evidence in yeast indicates that the p70 subunit interacts with both pol α and pol δ (*37*). In the complete SV40 DNA replication system, neither prokaryotic, yeast, nor viral SSBs (single-stranded DNA binding proteins) can replace human RPA, thus suggesting that it may participate in specific protein−protein interactions with other replication proteins (*38*). More recently, Yuzhakov et al. (*39*) demonstrated a direct interaction between the pol δ heterodimer and RPA. The p70 subunit of RPA bound to the pol δ heterodimer, but not the p34−p14 subcomplex of RPA. RFC, the clamp loader for PCNA, also binds to pol δ. The binding of pol δ and RPA was found to compete for binding to RFC. In studies of the interactions of these proteins, Yukazhov et al. (*39*) have proposed that RPA forms an important touchpoint for the assembly of the pol δ replication complex. Our findings that RPA is present in a purified high-molecular weight complex of pol δ provide further evidence that the interaction of RPA and pol δ is a significant one.

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Identification of a Fourth Subunit of Mammalian DNA Polymerase δ*

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Li Liu‡, Jin-yao Mo‡, Esther M. Rodriguez-Belmonte, and Marietta Y. W. T. Lee§

From the Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York 10595

A 12-kDa and two 25-kDa polypeptides were isolated with highly purified calf thymus DNA polymerase δ by conventional chromatography. A 16-mer peptide sequence was obtained from the 12-kDa polypeptide which matched a new open reading frame from a human EST (AA402118) encoding a hypothetical protein of unknown function. The protein was designated as p12. Human EST AA402118 was identified as the putative human homologue of *Schizosaccharomyces pombe* Cdm1 by a tBlastn search of the EST data base using *S. pombe* Cdm1. The open reading frame of human EST AA402118 encoded a polypeptide of 107 amino acids with a predicted molecular mass of 12.4 kDa, consistent with the experimental findings. p12 is 25% identical to *S pombe* Cdm1. Both of the 25-kDa polypeptide sequences matched the hypothetical KIAA0039 protein sequence, recently identified as the third subunit of pol δ. Western blotting of immunoaffinity purified calf thymus pol δ revealed the presence of p125, p50, p68 (the KIAA0039 product), and p12. With the identification of p12 mammalian pol δ can now be shown to consist of four subunits. These studies pave the way for more detailed analysis of the possible functions of the mammalian subunits of pol δ.

DNA polymerase δ (pol δ) is the key polymerase that is involved in the replication of chromosomal DNA in eukaryotic cells. Studies of the *in vitro* replication of SV40 DNA have established that pol δ plays a central role in mammalian DNA replication (1). Proliferating cell nuclear antigen (PCNA),[1] the molecular sliding clamp of pol δ, is a processivity factor for pol δ and ε (2). PCNA was first identified as an activating factor for pol δ (3, 4) and is essential for replicative DNA synthesis. Several other factors have been identified as being required for SV40 DNA replication. Replication factor C (also known as activator-1) binds to the primer-template terminus, following which it recruits PCNA and then pol δ (5, 6) onto the DNA template. Replication Protein A, the single stranded DNA-binding protein, is involved in both initiation and elongation and also stimulates pol δ activity when replication factor C and PCNA are present (7, 8). The current view of DNA replication at the replication fork is that the pol δ complex is responsible for synthesis of the leading strand and that pol δ also participates in synthesis of the lagging strand (1). DNA polymerase α/primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, and the actual elongation of the primers is performed by DNA polymerase δ in a process that requires "polymerase switching" (9). Additional proteins, including topoisomerase and helicase activities, are also involved in the movement of the replication fork (1).

Mammalian pol δ has been rigorously isolated by conventional methods as a heterodimer consisting of two subunits, p125 and p50 (3). The subunit structure of pol δ has been the focus of recent investigations in yeast, and these have led to the identification of additional subunits. In *Schizosaccharomyces pombe*, pol δ is believed to consist of at least four subunits: a large catalytic subunit (Pol3) and three smaller subunits (Cdc1, Cdc27, and Cdm1) (10, 11). Pol δ purified from *Saccharomyces cerevisiae* is composed of three subunits: Pol3p, Pol31p/Hys2, and Pol32p (12–14). The pol δ core purified from calf thymus consists of two subunits: p125 and p50 (3). However, we have found that recombinant p125 catalytic subunit alone can only be stimulated by PCNA by 2-fold at most, while the overexpressed p125/p50 heterodimer is stimulated much less than pol δ purified by immunoaffinity chromatography (15, 16). These findings suggest that additional factor(s) which may be removed during protein purification are required for a full PCNA response in our assay. This is consistent with the hypothesis that mammalian pol δ may also contain additional subunits.

Using the proteomics approach, by peptide sequencing of polypeptides associated with the core pol δ in highly purified preparations isolated by p125 immunoaffinity chromatography, we have previously identified a 68-kDa polypeptide that is encoded by KIAA0039 and which is associated with the pol δ core. The p68 polypeptide is the third subunit of mammalian pol δ (17). Using a combination of proteomic approaches and GenBank searches, we have identified a novel subunit of pol δ that is the mammalian homologue of Cdm1, which in *S. pombe* is the fourth subunit of pol δ. Mammalian pol δ may thus consist of at least four subunits.

EXPERIMENTAL PROCEDURES

Materials—cDNA AA402118 was obtained from ATCC (Rockville, MD). Calf thymus tissue was obtained from Animal Technologies (Tyler, TX). Q-Sepharose, SP-Sepharose, heparin-Sepharose, Mono Q columns, and Mono S columns were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Purification of Calf Thymus Pol δ—The immunoaffinity purification was performed as described previously by Jiang *et al.* (18).

Conventional Purification of Calf Thymus Pol δ—The following buffers were used: lysis buffer consisted of 50 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25 M sucrose, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin, 10 mM benzamidine. TGEED buffer consisted of 50 mM Tris-HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and

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‡ Contributed equally to the results of this work.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology Valhalla, NY 10595. Tel.: 914-594-4070; Fax: 914-594-4058; E-mail: marietta_lee@nymc.edu.

[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; pol, polymerase; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

TABLE I
Purification of calf thymus DNA polymerase δ

Fraction	Total volume	Protein	Total units	Specific activity
	ml	*mg*		*units/mg protein*
DE 52	3,615	18,075	397,000	22
Q-Sepharose	470	2,585	295,000	113
SP-Sepharose	176	722	468,000	648
Mono Q	34	76.5	56,000	736
Heparin-Sepharose	17	12.2	39,000	3,211
Mono S	3.3	2.1	9,400	4,500
Source Q15	3.0	0.15	900	6,000
Superdex 200	0.8	0.022	200	8,900

5% glycerol. KGEED buffer consisted of 20 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol.

All steps were carried out at 0–4 °C. Pol δ activity was assayed using poly(dA)/oligo(dT) as a template (19). Eight hundred grams of frozen calf thymus tissue in 4 liters of lysis buffer were homogenized in a Waring blender. The suspension was centrifuged at 5,000 rpm at 4 °C for 1 h and filtered through glass wool. The supernatant was mixed with 1.5 liters of DE52-cellulose equilibrated with TGEED buffer and stirred for 30 min. The mixture then was filtered through a Buchner funnel. The DE52-cellulose was washed with TGEED and the pol δ activity was stripped off with 20% ammonium sulfate in TGEED buffer.

The 3.5 liters of DE52-cellulose fraction were precipitated by the addition of 320 g/liter of ammonium sulfate. The suspension was stirred for 30 min, kept on ice for an additional 30 min, and then centrifuged at 10,000 × *g* for 45 min. The precipitate was resuspended in TGEED and dialyzed against TGEED buffer containing 50 mM NaCl with two changes and applied on to a 70-ml Q-Sepharose column. The bound proteins were eluted with a linear gradient of 50–750 mM NaCl in TGEED. The peak fractions containing pol δ activity were pooled and dialyzed against KGEED buffer containing 25 mM KCl. The Q-Sepharose fraction was loaded on to a 50-ml SP-Sepharose column. Pol δ was eluted with a linear gradient of 25–650 mM KCl in KGEED. The fractions containing enzyme activity were pooled and applied to a 10-ml Mono Q column, which was equilibrated with TGEED buffer containing 25 mM NaCl. The column was washed with 40 ml of TGEED buffer containing 25 mM NaCl. The activity was eluted with a gradient of 25–650 mM NaCl in 100 ml of TGEED at a flow rate 0.4 ml/min. The Mono Q fractions were pooled and dialyzed against TGEED buffer containing 25 mM NaCl and applied to a 5-ml heparin-Sepharose column. The column was washed with 2 column volume of TGEED containing 25 mM NaCl and eluted with a 50-ml gradient of 25–750 mM NaCl in TGEED at a flow rate 0.5 ml/min.

The heparin-Sepharose fraction (17 ml) was dialyzed against two changes of KGEED buffer containing 50 mM KCl and loaded onto a 1-ml Mono S column equilibrated with KGEED buffer. The column was washed with 5 ml of KGEED buffer and then eluted with a 20-ml linear gradient of KGEED buffer from 50 to 700 mM KCl. The active fractions were combined and dialyzed against TGEED buffer until the conductivity reached that of TGEED containing 50 mM NaCl. The fraction was applied to a Source Q15 column. The enzyme was eluted with a linear gradient of 50–650 mM NaCl in TGEED. The fractions with enzyme activity were pooled (3.0 ml) and concentrated to 270 μl using Centricon 30 (30,000 MW cutoff, Amicon). The concentrated enzyme (270 μl) was chromatographed on a FPLC Superdex 200 column equilibrated with TGEED buffer containing 150 mM NaCl. Fractions above 50% of the maximum peak of activity were pooled.

Protein Sequence Analysis—Polypeptide bands excised from a Coomassie Blue-stained gel were used for protein sequence analysis by the Harvard Microchemistry Facility using a microcapillary reverse-phase high performance liquid chromatography nano-electrospray tandem mass spectrometry (μLC-MS-MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer.

Antibodies—Peptide rabbit polyclonal antibodies against p12/hCdm1 and p68 were generated from a commercial source (SynPep, Dublin, CA) and purified by a peptide affinity column made from the same peptide antigen. For p12, the peptide contains amino acid residues 77 to 94 of p12 (H_2N-GLEPPPEVWQVLKYHPGD-COOH). For p68 (encoded by KIAA0039) the 19-amino acid peptide from near the extreme N terminus of p68 was used (H_2N-TDQNKIVTYKW-LSYTLGVH-COOH).

Western Blot Analysis—Proteins were transferred to 0.45 μM nitrocellulose membranes (Bio-Rad) after SDS-PAGE in transfer buffer (25 mM Tris-HCl, 192 mM glycine containing 10% v/v methanol) in a Genie blotter (Idea Scientific, Minneapolis, MN) for 75 min for 0.8-mm thick gels using a constant voltage of 12 volts. The membrane was incubated in TBST buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% Tween 20) containing 5% fat-free dry milk for 1 h at room temperature and washed briefly with TBST. The membrane was incubated with primary antibody for 1 h at room temperature or overnight at 4 °C. The membrane was washed 3× with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Pierce, Rockford, IL) for 1 h. The membrane was washed 3× with TBST. SuperSignal West Pico Chemiluminescent Substrate was used for signal production (Pierce) and the signal was captured on a Blue Bio film (Denville Scientific, Metuchen, NJ) after exposure for 15 s to 30 min and developed.

RESULTS

Demonstration That p68 and p12 Are Subunits of Mammalian Pol δ—We have previously devised a conventional procedure for the rigorous isolation of the pol δ core enzyme containing p125 and p50 (3). In order to isolate a multisubunit form of mammalian pol δ, a new purification scheme was devised, which allowed the isolation of pol δ core that retained associated polypeptides. This involved successive chromatographies on DE52, Q-Sepharose, SP-Sepharose, Mono Q, heparin-Sepharose, Mono S, Source Q15, Superdex 200 supports, including four FPLC chromatography steps (Mono Q, Mono S, Source Q15, and Superdex 200). Table I shows the purification of pol δ by this means. The specific activity of the preparation (about 9,000 units/mg) was comparable with that of pol δ purified by immunoaffinity chromatography (18). Review of a number of preparations isolated by the latter method gave an average specific activity about 10,000 units/mg, with a PCNA stimulation of 20–40-fold. The PCNA stimulation of 30-fold was found for the preparation obtained by the new procedure. This is similar to that of the immunoaffinity purified enzyme. The average specific activity of the purified recombinant pol δ heterodimer in our hands is about 2000 units/mg, with maximum PCNA stimulations of 6–10-fold. These results indicate that rigorously purified pol δ p125/p50 heterodimer has lost a significant fraction of its ability to respond to PCNA.

The final purification step used was FPLC gel filtration on Superdex 200. Calibration of the column showed that the peak of pol δ activity was eluted at a position indicating a much higher molecular weight (280,000) than can be accounted for by the two-subunit core. The Coomassie Blue staining of this pol δ complex is shown in Fig. 1. There were six major bands in the peak fractions of pol δ from fractions 48 to 50; these were of 125 kDa, 50 kDa, a doublet at about 25 kDa and a doublet at about 12 kDa. These polypeptide bands were excised from the Coomassie Blue-stained gel and sequenced at the Harvard Microchemistry Facility using LC/MS/MS methods. The sequencing results are displayed in Table II. Both the 25-kDa polypeptides were identified as the proteolytic products of KIAA0039, which was recently found to be associated with pol δ by a PCNA overlay assay (17). The KIAA0039 product was also eluted with pol δ from a PCNA affinity column (20). Our data support the view that p68 is the mammalian third subunit of pol δ but

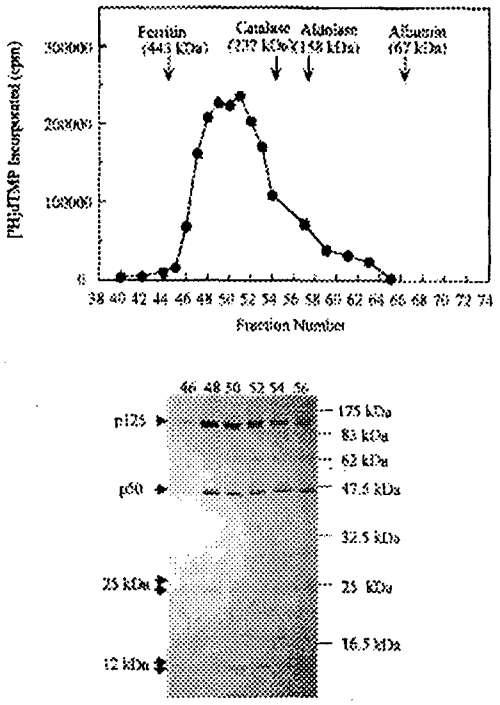

FIG. 1. **Chromatography of the purified calf thymus pol δ complex on Superdex 200.** The figure shows the elution profile of the calf thymus pol δ at the final purification step ("Experimental Procedures"). *Upper panel*, the activity of pol δ in the fractions of Superdex 200 gel filtration chromatography was assayed using poly(dA)/oligo(dT) as the template in the presence of PCNA. *Lower panel*, the peak fractions from the Superdex 200 column were separated on a 10% SDS-polyacrylamide gel and stained for protein with Coomassie Blue. 25-kDa upper and lower and 12-kDa upper and lower, as well as the p125 and p50 pol δ core subunits are marked by *arrows*.

TABLE II
Peptide sequences data from calf thymus pol δ complex

Identity	Peptide mass on SDS-PAGE	Peptide sequences obtained
Pol δ p125	125 kDa	IFEPILGEGR
		DPGSAPSLGDRVPYVIISAAK
Pol δ p50	50 kDa	FLGTSGQNVSDIFR
		AMPLQPSILR
		TQAASVEAVK
Pol δ p68[a] (KIAA0039)	25-kDa upper	VAVVREDKLEAVK
		QMLYDYVER
Pol δ p68[a] (KIAA0039)	25-kDa lower	WLSYTLGVHVNQAK
		FSAIQCAAAVPR
		DSGPLFNTDYDILK
		GIMGMFASK
		QMLYDYVER
Keratin 16	12-kDa upper	ADLEMQIENLR
Pol δ p12	12-kDa lower	QFDLAWQYGPCTGITR

[a] The protein has a size of 68 kDa present in p125-immunoaffinity purified calf thymus pol δ. In this purification scheme, it was proteolysed into two 25-kDa species.

indicate that it is highly susceptible to proteolysis.

The sequence of the upper 12-kDa band showed that this was derived from keratin. The second 12-kDa polypeptide was found to be a novel protein. The partial sequence obtained from this protein was QFDLAWQYGPCTGITR (Table II). This sequence was searched against the known protein data bases, which did not provide a match. A tBlastn search of the EST data base showed a match with one human EST sequence, AA402118, which, however, did not have a well defined open reading frame. Concurrently, the *S. pombe* Cdm1 protein sequence, which represents the fourth subunit of pol δ, was used for a tBlastn search at NCBI. This also retrieved the human EST sequence, AA402118. The EST cDNA clone was obtained from ATCC and was resequenced and corrected. The corrected sequence contained an open reading frame that encoded a protein of 107 amino acid residues, with a predicted molecular mass of 12.4 kDa. This protein was designated as p12. The corrected DNA sequence has been deposited in GenBank with the accession number AJ179890 (Fig. 2). The peptide sequence obtained from p12 shows a perfect match with residues 51–65 of the open reading frame of AJ179890.

```
1                                                       ctttctgcgcccca

15  ctggccggatcctcctcttccccgcagttgccccttctctgcctgcctgggtggccgcc

75  atgggccggaagcggctcaacactgattcctaccggttgtgaaggaggaggaggcccc
    M  G  R  K  R  L  I  T  D  S  Y  F  V  V  K  R  R  E  G  P

135 gctgggcaacagcaaggggagctggcaccgagctagggaggagcccagcccgcgac
    A  G  H  S  K  G  E  L  A  P  E  L  G  E  E  P  Q  P  R  D

195 gaggaggaagcggaggctggagctgctgaggcagtttgacctggcctggcagtacggccc
    E  E  E  A  E  L  E  L  L  R  Q  F  D  L  A  W  Q  Y  G  P

255 tgcaccgggatcacacgggctgcagcgctggtgtcgggccaagcagatgggcttggagct
    C  T  G  I  T  R  L  G  R  W  C  R  A  K  Q  M  G  L  E  P

315 ccccccagaggtgtggcaggtgctgaagacccaccccggagaccccgcttccagtgcagt
    P  P  E  V  W  Q  V  L  K  T  H  P  G  D  P  R  F  Q  C  S

375 ctctgggcatctctatccctatgaggcaccacgtaagacctctgccctagctctcttg
    L  W  H  L  Y  P  L  *

435 cccaccacccaagaacctcaggacagaagcgagagcccattgctcctgctcagctccagcc

495 cggctgcggaggaacct
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FIG. 2. **Nucleotide sequence and predicted amino acid sequence of the smallest subunit of mammalian DNA polymerase δ.** This figure shows the nucleotide sequence of the human EST AA402118 which was corrected by resequencing (AJ179890). The insert of the human EST A402118 in the cloning vector pT7T3D is 512 base pairs, and encodes a protein of 107 amino acids. The amino acid sequence of the peptide derived from the 12-kDa polypeptide in the calf thymus pol δ preparation (Fig. 1) is shown in ***bold***.

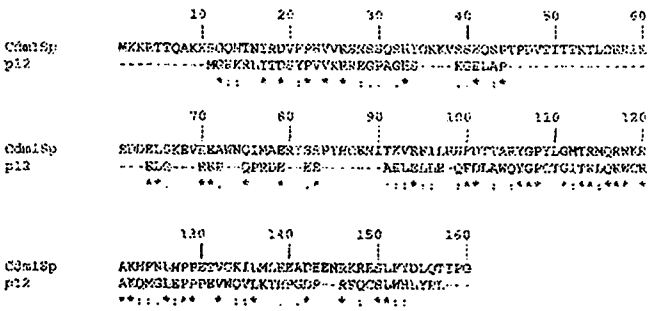

FIG. 3. **Alignments of human p12 with *S. pombe* Cdm1.** Human p12 (AJ179890) and *S. pombe* Cdm1 (emb AJ006032) were aligned with the Clustal 1.74 program. The identity between these two proteins is 25% and the similarity is 39%.

These results indicate that p12 is a likely human homologue of the *S. pombe* Cdm1 protein, which has been reported to be the fourth subunit of *S. pombe* pol δ (10, 21). The *S. pombe* Cdm1 protein has a calculated molecular mass of 18.5 kDa and an apparent size of 22 kDa on SDS-PAGE, and is significantly larger than human p12. Sequence alignments were performed to assess the possible relationships between these two proteins. Protein sequence alignment indicates that the identity between p12 (107 residues) and *S. pombe* Cdm1 (160 residues) is 25% and the similarity is 39% (Fig. 3). It can be seen that the main region of identity of p12 is with the C-terminal half of *S. pombe* Cdm1. Alignment of amino acid residues 96–142 of Cdm1 with

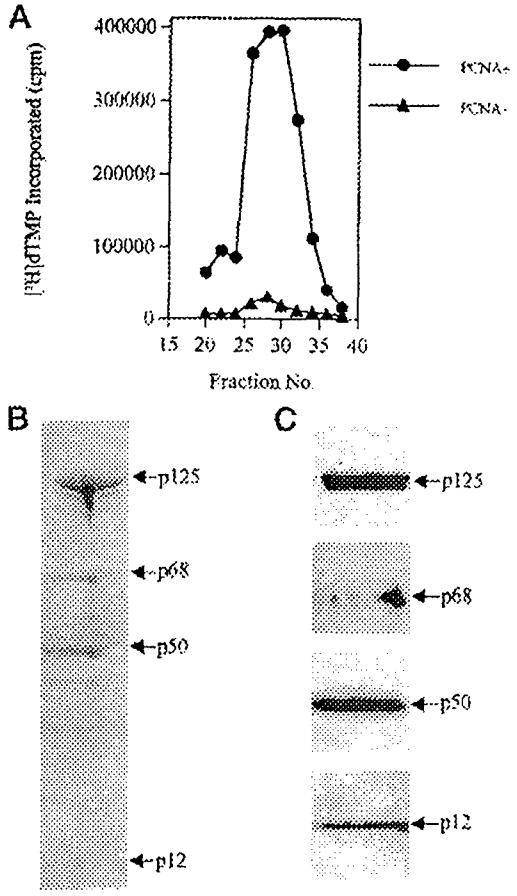

FIG. 4. **Western blot analysis of pol δ subunits purified by p125-immunoaffinity chromotography from calf thymus.** Calf thymus pol δ was purified through DE52, phenyl-agarose, and p125 immunoaffinity column chromatographies in the presence of protease inhibitors (18). *Panel A*, activity assay of the fractions eluted from the immunoaffinity column using poly(dA)/oligo(dT) as the template in the absence (*closed triangles*) and presence (*closed circles*) of PCNA. *Panel B*, Coomassie Blue staining of peak fraction number 28. *Panel C*, Western blotting of peak fraction number 28 using monoclonal antibody 78F5 against p125, 13D5 against p50, and rabbit peptide polyclonal antibodies against p68 and p12.

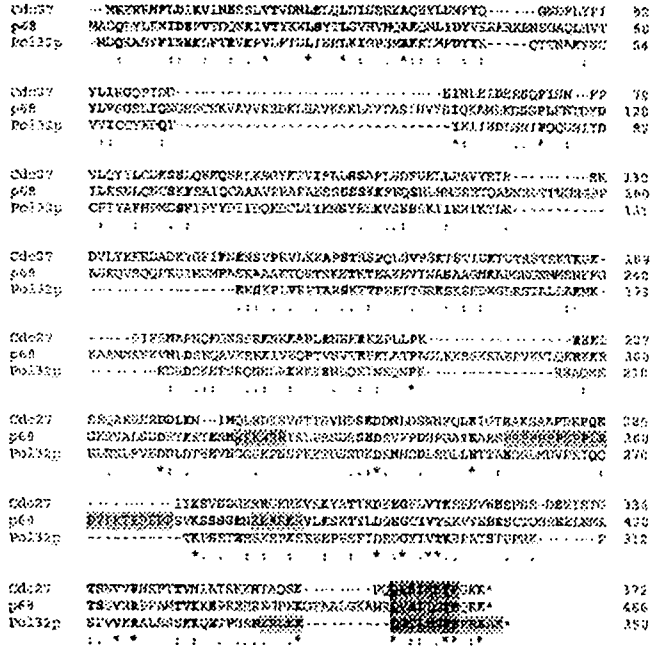

FIG. 5. **Multiple sequence alignment of p68, Pol32p, and Cdc27.** p68 (the protein product of KIAA0039, BAA05039) with *S. pombe* Cdc27 (P30261) and *S. cerevisiae* Pol32p (CAA89571) were analyzed using the Clustal 1.8 program. The PCNA binding motif is highlighted in *dark gray*. The putative nuclear localization signals of p68 and Pol32p are highlighted in *light gray*. The unique proline-rich motif in p68 is also highlighted.

TABLE III
Summary of pol δ subunits

DNA polymerase δ	Mammalian	*S. pombe*[a]	*S. cerevisiae*[b]
The catalytic subunit	p125	125 kDa, Pol3	125 kDa, Pol3p
The second subunit	p50	55 kDa, Cdc1	58 kDa Pol31p
The third subunit	p68[c]	54 kDa, Cdc27	55 kDa Pol32p
The fourth subunit	p12[d]	22 kDa, Cdm1	Not found

[a] Isolated from *S. pombe* (10).
[b] Reconstituted as a three-subunit enzyme (13).
[c] Identified as bovine p68 (encoded by KIAA0039) in p125 immunoaffinity purified pol δ (17). Isolated by PCNA affinity chromatography of mouse FM3A cell extracts (20).
[d] Identified in this study.

residues 48 to 94 of p12 shows that there is a 44% identity. This degree of similarity is sufficient for p12 to be regarded as the mammalian homologue of *S. pombe* Cdm1. Taken together with the sequence identification of the p12 and its co-purification with the calf thymus pol δ core through eight chromatography procedures, these findings provide strong evidence for the identification of p12 as a novel subunit of mammalian pol δ.

Western Blot Analysis of Immunoaffinity Purified Pol δ—We had previously shown that pol δ isolated by immunoaffinity chromatography contains the pol δ core in association with a number of other polypeptides (18), and also displayed a much higher molecular weight than could be accounted for by the core on gel filtration analysis (22). The failure to observe p12 in these studies could be due its small size and the fact that it migrated close to the dye front under the conditions used. A preparation of pol δ was purified from calf thymus using immunoaffinity chromatography (18) and the preparation was assessed for the presence both of the p68 and p12 subunits. The presence of these two polypeptides on SDS-PAGE gels of the preparation are shown in Fig. 4. Polypeptides corresponding to 68 and 12 kDa were prominent components of the preparation, and their identity as the p68 and p12 polypeptides was confirmed by Western blotting (Fig. 4). Thus, the presence of all four subunits of pol δ (p125, p50, p68, and p12) were demonstrated in this preparation (Fig. 4). The KIAA0039 product in the Western blot was 68 kDa.

p68 Is the Third Subunit of Mammalian Pol δ, the Homologue of S. pombe Cdc27 and S. cerevisiae Pol32p—The p68 sequence has a conserved $p21^{Waf1}$-like PCNA binding motif at the extreme C terminus, as does *S. pombe* Cdc27 and *S. cerevisiae* Pol32p, the yeast third subunits of pol δ (13). The p68 sequence encoded by KIAA0039 was aligned with the sequences of Cdc27 and Pol32p (Fig. 5). Analysis of the alignments showed that p68 shares little sequence identity with Cdc27 and Pol32p. The only sequence conservation was the C-terminal PCNA binding motif in these three sequences. p68 and Pol32p both have nuclear localization motifs. p68 also has an unique proline-rich motif. Pairwise alignments using the Clustal W 1.8 program show that between Pol32p and Cdc27, Pol32p and p68, or Cdc27 and p68 there is only 15 to 16% sequence identity (not shown). However, evaluation of the significance of the alignment score for p68 with Cdc27 using the PRSS program provided a score of 0.4, *i.e.* the alignment score (% identity) would be attained by chance against the randomly shuffled Cdc27 sequence only 0.4 times in 100 attempts. This indicates that the similarity between these two proteins is significant.

TABLE IV
The third subunits and putative third subunits of pol δ

Organism[a]	Accession No.	Protein	Length	PCNA binding motif
1	BAA05039	p68	466 amino acids	^{396}QVSITGFFQRK^{466}
2	CAA98958.1		445 amino acids	^{435}NSMITSFFKT^{445}
3	AAD30571.1		509 amino acids	^{499}QGNIMSFFKKV^{509}
4	AAD38629		431 amino acids	^{420}QAGIMNFFSKK^{431}
5	P30261	Cdc27	372 amino acids	^{362}QKSIMSFFGKK^{372}
6	CAA89571	Pol32p	350 amino acids	^{338}QGTLESFFKRKA^{350}

[a] 1. *Homo sapiens*; 2. *C. elegans*; 3. *A. thaliana*; 4. *D. melanogaster*; 5. *S. pombe*; 6. *S. cerevisiae*.

DISCUSSION

The thrust of the earlier studies of pol δ was the rigorous isolation of the enzyme which culminated in the isolation of a two-subunit enzyme, containing a catalytic subunit of 125 kDa and a second subunit of 50 kDa (3, 23). The major difficulties in this process were the small amounts of protein available and the likelihood of proteolysis using extensive purification schemes due to the fragile nature of the mammalian system compared with other systems, *i.e.* prokaryotic or lower eukaryotes such as yeast. Recently, expression systems for the p125 (15, 24, 25), the p50 subunit (16), and the recombinant heterodimer have been developed (26). The p50 subunit has no known enzymatic functions, but has been shown to be required for the response of the p125 subunit to PCNA (16, 26). In this study we have shown for the first time the isolation of a four-subunit mammalian pol δ enzyme. This newly isolated pol δ contains the third subunit p68 and a previously unknown subunit, p12. The latter two are the mammalian homologues of *S. pombe* Cdc27 and Cdm1, respectively.

The association of p68 and p12 with pol δ was demonstrated by their isolation with p125 and p50 from calf thymus through extensive purification involving multiple conventional column chromatographies as well as by immunoaffinity chromatography. The conventional procedure included several FPLC steps including gel permeation chromatography. The strong association of the p68 and p12 polypeptides with the pol δ core provide very strong evidence for the proposal that these represent subunits of pol δ. p68 has also been isolated from mouse cell extracts using a PCNA affinity column in association with the pol δ core consisting of the p125 and p50 subunits (20). There are extensive technical problems associated with the identification of subunits of mammalian pol δ. As encountered in our studies, these include the susceptibility of the p68 polypeptide to proteolysis and the difficulties of isolation of pol δ from animal tissues to study stoichiometries of pol δ subunits in native enzyme preparations. Nevertheless, in these studies it is demonstrated that it is possible to rigorously isolate pol δ from calf thymus in a form which retains the p68 and p12 polypeptides. A key difference in the new method from the older procedure (18) was the avoidance of single-stranded DNA cellulose chromatography.

The identification of the fourth subunit of pol δ in mammalian systems now provides a parallel for the situation found in yeast. A comparison of the subunit structures of pol δ from the mammalian and the two yeast models is shown in Table III. The catalytic subunit of mammalian pol δ is strongly conserved in evolution, and shares a high degree of homology with the corresponding catalytic subunits in *S. pombe* and *S. cerevisiae*, the identity being greater than 48% (27). The p50 subunit is less conserved than the catalytic subunit, the identity between p50 and *S. pombe* being 33% (11). Furthermore, the finding that PCNA from human or yeast origin can activate the heterologous pol δ preparations strongly suggests that the pol δ complex is functionally conserved to a high degree (28). The functions of these subunits are still incompletely understood. The third subunit of *S. pombe* pol δ was only recently identified (10) and is encoded by the *cdc27*$^+$ gene, which is needed for the transition of G_2/M in the cell cycle (11). The third subunit of *S. cerevisiae* pol δ is Pol32p, was isolated and identified in 1998. It was proposed as a candidate for dimerization factor of pol δ (13) based on the finding that the recombinant three-subunit enzyme could be shown to behave as a dimer on gel filtration (13). In addition, Pol32p was found to interact with the pol α catalytic subunit by the yeast two-hybrid method (29). These results suggest that Pol32p can (*a*) dimerize pol δ at the replication fork, and (*b*) provide a means for the proposed "polymerase" switch at the lagging strand through the interaction with pol α as suggested by Waga *et al.* (1).

p68, the mammalian homologue of *S. pombe* Cdc27, KIAA0039 was isolated from a PCNA affinity column (20) and from an immunoaffinity column of pol δ p125 (17). The third subunits of pol δ share a very low degree of similarity. In fact, Blast searches with Cdc27 failed to identify either p68 or Pol32p. tBlastn searches using Pol32p only identified a *Drosophila melanogaster* third subunit of pol δ. Similarly, using p68 the putative *Caenorhabditis elegans* and *Arabidopsis thaliana* third subunit of pol δ were identified (Table IV). As already noted ("Results"), the third subunits of human, *S. pombe*, and *S. cerevisiae* are poorly conserved, although the relationships based on the alignments can be shown to be significant. The third subunits of pol δ from different species all contain a putative p21^{waf1}-like PCNA binding motif (30, 31) at the extreme C terminus. An important aspect of the third subunit is that it interacts with PCNA, and also with the yeast p50 homologues (11, 13, 32). The ability of p68 to bind to PCNA (17, 20) may account for the loss of sensitivity to PCNA shown by pol δ p125/p50 heterodimer. In addition, all share in common a high content of charged amino acids which ranges from 29 to 35%. The calculated isoelectric points for these proteins are all basic, with the exception of the *S. pombe* Cdc27, which has an acidic isoelectric point. This common property suggests that p68 is likely to have an extended structure in solution, which is also consistent with its apparent liability to proteolysis. A third property of the third subunit may be an ability to interact with the p50 second subunit, which has been demonstrated in *S. pombe* and *S. cerevisiae* (11, 13) and also in mammalian pol δ.[2] One speculative function of p68 may be to act as a linker protein between p50 and PCNA, which would provide additional stabilization of the pol δ-PCNA interaction. This possibility is consistent with the higher sensitivity to PCNA of the pol δ preparations which contain p68 compared with that of the heterodimer.

Thus far, the fourth subunit has only been identified in mammalian sources in this present work, and previously as Cdm1 in *S. pombe*. Interestingly, data base searches have failed to identify a homologue in *S. cerevisiae*, despite the fact that its entire genome has been cloned. This may be due to a lack of evolutionary conservation. The functions of this newly

[2] L. Liu and M. Y. W. T. Lee, unpublished observations.

described subunit also remain to be determined.

In summary, this work provides evidence for the identification of a novel subunit, p12, as a component of mammalian pol δ, as well as evidence for the isolation of pol δ in a form that contains the core heterodimer in association with both p12 and the third subunit, p68.

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Interaction of the Retinoblastoma protein (pRb) with the catalytic subunit of DNA polymerase δ (p125)

Nancy A. Krucher[1], Adam Zygmunt[1], Nayef Mazloum[2], Sama Tamrakar[3], John W. Ludlow[3], and Marietta YWT Lee[2]

[1]Department of Biological Sciences, Pace University, Pleasantville, NY and [2]Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY and the [3]University of Rochester Department of Biochemistry and Biophysics, Rochester, NY.

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Key words: DNA polymerase δ, retinoblastoma, cyclin dependent kinase, cell cycle

corresponding author:
Nancy A. Krucher
Department of Biological Sciences
Pace University- Pleasantville
861 Bedford Rd.
Pleasantville, NY 10570
telephone: (914) 773-3770
fax: (914) 773-3634
email: nkrucher@pace.edu

Abstract

The Retinoblastoma gene product (pRb) interacts with many cellular proteins to functions in the control of cell division, differentiation, and apoptosis. Several pRb binding proteins complex with pRb through an amino acid sequence called the LXCXE motif. The catalytic subunit of DNA polymerase δ (p125) contains a LXCXE motif. To further study the biochemical function of this polymerase, we sought to determine if p125 interacts with pRb. Experiments using GST-pRb fusion proteins showed that p125 from breast epithelial (MCF10A) cell extracts associates with pRb. In addition, GST-p125 fusion proteins bound pRb from the same cell extracts. The pRb that associated with GST-p125 was largely unphosphorylated. Coimmunoprecipitation experiments using cell cycle synchronized cells revealed that p125 and pRb form a complex predominantly during G_1 phase, the phase during which pRb is mostly unphosphorylated. In vitro phosphorylation of GST-pRb by the cdks reduced the ability of p125 to associate with GST-pRb. Addition of the LXCXE containing protein SV40 large T antigen to GST-pRb blocks the ability of p125 to associate with pRb, suggesting that it may be through a LXCXE sequence by which p125 interacts with pRb.

Introduction

The growth suppressive activity of the product of the retinoblastoma susceptibility gene, pRb, is manifested through its interactions with many cellular proteins. The finding several years ago that pRb forms specific complexes with several viral transforming proteins, including E1A from adenovirus, SV40 large T antigen and papilloma virus E7 (DeCaprario, 1988; Whyte et al., 1988; Dyson et al., 1989) led to the search for cellular proteins that associate with pRb under normal physiological conditions. These studies led to the discovery that members of the E2F transcription factor family are inhibited by binding to pRb (Chittenden et al., 1991; Bagchi et al., 1991; Chellappan et al., 1991). This ability of pRb to inhibit E2F mediated transcriptional activity is dependent upon the phosphorylation state of pRb, which fluctuates during the cell division cycle. pRb is largely unphosphorylated (hypophosphorylated) during G_1 phase of the cell division cycle, until members of the cyclin dependent kinase (cdk) family phosphorylate pRb in mid to late G_1 (Sherr, 1996). In S phase, pRb exists in its hyperphosphorylated state which is unable to bind E2F. Thus, it is during G_1 that unphosphorylated pRb binds to and sequesters E2F transcriptional activity, until phosphorylation of pRb releases E2F thereby stimulating gene transcription required for S phase progression. Since E2F belongs to a family of transcription factors (E2F 1-5) that possess transcriptional activity mediated by binding to members of the pRb family of "pocket" proteins (ie: p107 and p130), it is not surprising that their binding is also regulated by cell cycle phase specific phosphorylation (Grana et al., 1998 for review).

Recently, several additional pRb binding proteins have been identified. However, the physiological relevance of such interactions have been elucidated for only a few (Taya, 1997). The cellular processes that pRb has been found to be involved are diverse. In addition to its role

in controlling cell division cycle progression, pRb directly affects the processes of transcription, differentiation and DNA replication. To inhibit transcription, pRb binds to histone deacetylase (HDAC) which promotes assembly of DNA into nucleosomes, resulting in repression of transcription (Luo et al., 1998, Brehm et al., 1998). pRb has also been found to bind to myogenic and lymphoid specific transcription factors which control progression into the differentiated state in these cell types (Wang et al., 1993, Gu et al., 1993). In addition, pRb has been reported to interact with molecules involved in DNA replication, such as DNA polymerase α, pur α and MCM7. DNA polymerase α is involved in both leading and lagging strand synthesis (Wang, 1991) and is inhibited by the binding of phosphorylated pRb (Takemura et al., 1997). Pur α is a sequence specific single stranded DNA binding protein involved in gene transcription and DNA replication. The interaction between pur α and pRb has been shown to modulate the binding of pur α to its single stranded recognition element (Johnson et al., 1995). The yeast two-hybrid technique was used to identify MCM7 as a putative pRb binding protein. MCM7, a member of the minichromosome maintenance family of proteins, is a component of licensing factor required for DNA replication. It was further shown by coimmunoprecipitation that MCM7 and pRb form an in vivo complex in cells and that the amino terminal portion of pRb somewhat inhibited replication in a Xenopus DNA replication system (Sterner et al., 1998). However, the interaction of MCM7 and pRb has not been shown to be a direct one.

The site of viral transforming protein interaction with pRb has been identified as a short stretch of amino acids called the LXCXE motif (Moran et al., 1986; Lillie et al., 1987; DeCaprario et al., 1988; Smith and Ziff, 1988; Moran, 1988; Cherington et al., 1988; Whyte et al., 1989). The

cellular proteins that interact with pRb and contain this sequence or a related sequence include the D-type cyclins, Protein phosphatase 1, UBF, and BRG1 (Dowdy et al., 1993, Taya, 1997, Durfee et al., 1993, Dunaief et al., 1994). The amino acid sequence of the catalytic subunit of the eukaryotic DNA polymerase δ (pol δ) also contains the LXCXE pRb-binding sequence Pol δ is the central enzyme in DNA replication and plays a role in DNA repair (Waga and Stillman, 1994, Zeng et al., 1994a). This enzyme is distinguished from DNA polymerase α and β by its intrinsic 3' to 5' exonuclease activity. Pol δ isolated from calf thymus or human cells consists of at least 2 core subunits of 125kDa (catalytic subunit) and 50 kDa (Lee et al., 1984, Lee et al., 1991). Since the molecular weight of the DNA polymerase holoenzyme predicted from gel filtration chromatography analysis exceeds 400 kDa, it is anticipated that DNA polymerase δ is a large, multi-subunit protein complex.

In this report we describe the interaction of the catalytic subunit of DNA polymerase δ with the tumor suppressor protein pRb. The association of these two proteins is dependent upon pRb phosphorylation state, and is regulated dependent upon cell cycle phase. Our data also supports the idea that p125 binds to pRb through its LXCXE amino acid sequence.

Results

The amino acid sequence of DNA polymerase δ catalytic subunit (p125) contains the LXCXE motif (residues 711-715) found in various pRb-binding proteins (DeCaprario et. al., 1988; Whyte et al., 1988; Wang et al., 1993; Ewen et al., 1993; Dunaief et al., 1994; Woitach et al.,1998). We undertook this study to determine whether p125 could indeed bind to pRb and, if so, whether this motif is involved in the interaction. Towards this goal, in vitro pull-down assays were performed using bacterially expressed full length GST-pRb (residues 1-928) using lysates from the breast epithelial cell line, MCF10A, and the breast cancer cell line, MCF7. After purification on glutathione sepharose, GST-pRb interacting proteins were eluted with excess glutathione and analyzed by Western blotting using monoclonal (78F5) antibodies that recognize p125 (Jiang et al., 1995). As shown in figure 1, p125 found in MCF10A and MCF7 cell lysates was able to bind to GST-pRb (lanes 3 and 6), but not to GST alone (lanes 2 and 5). In attempt to determine the region of pRb required for binding to p125, we also performed pull-down assays using fusion proteins encoding partial pRb amino acid sequences. The fusion proteins employed consisted of the amino terminus (aa 1-379), small pocket (aa 379-772) and large pocket (aa 379-928) domains of pRb. These partial sequence fusion proteins were unable to bind to and precipitate p125 (data not shown), suggesting that p125 requires more than one region of pRb for interaction.

Since the in vitro interaction of p125 with pRb was found in both MCF7 and MCF10A cells, subsequent studies utilized phenotypically normal MCF10A cells to further characterize the association of pRb with p125. We performed reciprocal GST fusion pull-down assays using GST-p125. GST-p125 interacting proteins were subjected to Western Blotting and probed

sequentially with 2 different antibodies directed toward pRb. The first, shown in figure 2 (top panel, lane 3), recognizes unphosphosphorylated pRb (Pharmingen G99-549) and the second, shown in the lower panel of figure 2, recognizes the phosphorylated forms of pRb (Pharmingen G33-245). Unphosphorylated pRb is found to be associated with GST-p125, whereas the phosphorylated form of pRb is not. In fact, there is significantly more phosphorylated Rb (lane 1 lower panel), relative to the amount of unphosphorylated pRb (lane 1 upper panel), in the cell lysate, which is characteristic of actively dividing cells. This result suggests that the unphosphorylated form of pRb preferentially binds to p125.

To determine whether the interaction of p125 and pRb occurs *in vivo*, coimmunoprecipitation experiments were performed. Figure 3A shows the coprecipitation of p125 with pRb antibody (lane 3) which comigrates with p125 immunoprecipitated by the 78F5 antibody (lane 2). The association of p125 and pRb was further verified by subsequent immunoprecipitation using polyclonal antibodies to p125. As shown in figure 3B, immunoprecipitation using antibodies raised to either the C-terminus or the N-terminus of p125 (lanes 2 and 3 respectively), coimmunoprecipitates pRb with p125. The fact that p125 coprecipitates with pRb and pRb with p125 utilizing normal mammalian cells suggests that these two proteins form a complex in vivo.

Because it appeared from the pull-down assays that it was the unphosphorylated form of pRb that could bind to p125, and the fact that the phosphorylation state of pRb varies during the cell cycle, we next sought to determine whether pRb-p125 complex formation was regulated in a cell cycle fashion. To do this, we isolated pRb-p125 coimmunocomplexes from cells enriched in the G_1 and S phases of the cell cycle. MCF10A cells were synchronized in either G_1 or S phase

of the cell cycle and assayed for pRb phosphorylation state by Western blotting. pRb isolated from G_1 synchronized cells is largely unphosphorylated, whereas pRb found in S phase synchronized cells is largely hyperphosphorylated. Figure 4A shows pRb isolated from G_1 arrested cells (lane 1) and S phase arrested cells (lane 2). The phosphorylated form of pRb is detected in the S phase arrested cells and not in the G_1 arrested cells. Using the G_1 and S phase synchronized cell lysates, coimmunoprecipitation using the monoclonal p125 and pRb antibodies was performed. Figure 4B shows the detection of p125 in p125 immunoprecipitates during G_1 and S phases (lanes 3 and 4) and the detection of p125 coprecipitating with pRb only in G_1 (lane 5) and not in S phase (lane 6). This result, taken together with the result shown in figure 2, stregthens the argument that p125 forms a complex with pRb when pRb is unphosphorylated during G_1.

We next sought to determine whether phosphorylation of pRb would lead to the inhibition of binding of p125. To address this, 0.5ug of GST-pRb fusion protein was phosphorylated in vitro by cdk2 or cdk4 protein kinases immunoprecipitated from CV-1P monkey kidney cells. The cdk2- and cdk4-phosphorylated GST-pRb was subsequently utilized in the pull-down assay using MCF10A cell extracts. GST-pRb associated p125 was detected by Western blotting. As shown in figure 5, GST alone does not associate with p125 (lane 1). Unphosphorylated GST-pRb (lane 2) binds p125. However, GST-pRb phosphorylated by cdk2 (lane 3) and phosphorylated by cdk4 (lane 4) brings down only approximately 50% of the amount of p125 that unphosphorylated GST-pRb copurifies. This result suggests that phosphorylation of GST-pRb inhibits the binding of p125.

Finally, we addressed the site of pRb interaction with p125. The LXCXE amino acid motif of p125 may comprise part of the interaction domain between p125 and pRb. Therefore, we attempted to block the interaction between pRb and p125 using competition assays. First, we tested the ability of SV40 large T antigen, which has been shown to interact with pRb through its LXCXE domain, to block p125 interaction with pRb. In addition, we utilized a synthetic LXCXE peptide to block the interaction of p125 with GST-Rb consisting of the following sequence: QPGTTDLYCYEQLNDSS. This peptide has been utilized previously to demonstrate the importance of the LXCXE region in protein binding to pRb (Jones et al., 1990, Wang et al., 1993). As shown in figure 6, large T antigen completely blocks p125 interaction with GST-pRb (lane 3 vs. lane 2) whereas the LXCXE peptide partially blocks this interaction (lanes 6 vs. lane 5). It may be that the LXCXE motif of p125 makes up only part of the interaction domain between p125 and pRb.

Attempts to elucidate the functional significance of pRb-p125 binding have been conducted using our well characterized polymerase assays (Lee et al., 1984). This assay utilizes p125 catalytic subunit purified from calf thymus, which during the purification process loses some of its interacting subunits. We were able to detect some inhibition of p125 catalytic subunit polymerase activity upon addition of GST-Rb compared to the addition of GST alone (Figure 7). However, these results do not conclusively reveal the physiological role of pRb binding to p125, since the multimeric DNA pol δ enzyme has not yet been fully characterized.

Discussion

The activity of DNA polymerase δ (pol δ) is required during the S phase of the cell cycle when cellular DNA is replicated in preparation for mitosis. The activity of pol δ thus must be regulated in a cell cycle dependent fashion. Expression of DNA polymerase δ is elevated 3-fold at the G_1/S border compared to other phases of the cell cycle (Zeng et al., 1994b). In addition, the catalytic subunit of pol δ (p125) is phosphorylated in a cell cycle dependent manner, with its highest phosphorylation state detected during S phase. The amino acid sequence of p125 contains consensus phosphorylation sites for the cdks as well as DNA-dependent protein kinase (Yang et al., 1992). Although work to date has not revealed that phosphorylation of p125 affects polymerase activity, it is interesting to note that specific combinations of cyclins and cdks preferentially phosphorylate p125 when coexpressed in sf9 cells (Wu et al., 1998). For example, cdk2-cyclin E phosphorylates p125 whereas cdk2-cyclin A does not. In addition, cdk4 phosphorylates p125 only when coexpressed with cyclin D_3, but not when coexpressed with cyclin D_1 or D_2. In molt4 cells, immunoprecipitation experiments have shown that p125 is capable of forming an in vivo complex with cdk2. Thus, even though changes in p125 phosphorylation state do not affect the polymerase activity of the catalytic subunit in an in vitro assay, this does not preclude the possibility that phosphorylation of p125 regulates the function of the DNA polymerase δ holoenzyme, perhaps through modulation of protein complex formation.

In this report, we present evidence suggesting that the catalytic subunit of DNA polymerase δ (p125) binds to and is potentially regulated by the retinoblastoma protein. In vitro

pull down experiments determined the specificity of the association between p125 and GST-Rb, as well as the association of unphosphorylated Rb with GST-p125 (Figures 1 and 2). Since GST fusion proteins expressed in bacteria are not subjected to post-translational phosphorylation, GST-Rb was subsequently phosphorylated in vitro using active cdk2 and cdk4 enzyme to determine the effect of pRb phosphorylation on p125 binding capability (Figure 5). As cells pass through G_1 phase into S phase, pRb becomes phosphorylated on several serine and threonine residues first by cdk4-cyclin D enzymes followed by the cdk2-cyclin E enzyme. This modification leads to the release of pRb-binding proteins such as E2F, or in virally transformed cells, the large T antigen or E7 proteins. Therefore, we speculated that phosphorylation of GST-Rb might inhibit p125 binding. In fact, phosphorylation by cdk2 or cdk4 each reduced p125 binding to GST-Rb by approximately 50%. This result suggests that phosphorylation of pRb by cdks which occurs as cells pass from G_1 phase into S phase may inhibit binding of p125 to pRb.

Coimmunoprecipitation experiments have often been utilized to demonstrate in vivo association of pRb binding proteins with pRb. Our results show that p125 coimmunoprecipitates with pRb using pRb antibodies, and that pRb coimmunoprecipitates with p125 using two different polyclonal antibodies raised to p125 (Figure 3A and 3B). Furthermore, the association of p125 with pRb preferentially occurs during G_1, when pRb is predominantly unphosphorylated (Figure 4). These data are consistent with the view that pRb may bind to and inhibit p125 during G_1, in a manner yet to be defined, until phosphorylation of pRb allows the release of p125, which allows p125 to be active in S phase.

Our approach to identify the regions of pRb required to associate with p125 using partial sequence pRb GST fusion proteins suggest that more than one region of pRb is required for p125 binding. However, the blocking experiments shown in figure 6 indicate that p125 associates with pRb through a similar sequence as does the SV40 large T antigen, most likely through the LXCXE amino acid motif found in many pRb-binding proteins.

While suggestive, in vitro polymerase assays using the p125 catalytic subunit purified from calf thymus need to be further investigated before the effect of pRb on p125 catalytic activity can be concluded. This is due in part the fact that the multimeric DNA pol δ enzyme has not yet been fully characterized, and the in vitro assay cannot faithfully replicate conditions found within cells during analysis of complex multi-component enzymes. Indeed, the p125 catalytic subunit derived from calf thymus loses some of its interacting subunits during purification. Nevertheless, the evidence presented in this report supports the idea that pRb and p125 interact during G_1 of the cell cycle, that phosphorylation of pRb by the cdks inhibits their association, and that these proteins may interact through the pRb binding motif, LXCXE, found in p125 of DNA polymerase δ.

Materials and Methods

Cell Growth conditions

MCF7 and CV-1P cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum or 10% newborn bovine serum at 37°C in a humidified, 5% CO_2-containing atmosphere. MCF10A cells were grown in DMEM-F12 media supplemented with 5% horse serum, 20ng/ml recombinant human EGF (Gibco), 100ng/ml cholera toxin (Gibco), 500ng/ml hydrocortisone (Sigma), and 0.01mg/ml human insulin (Lilly). Cells were maintained below 80% confluent throughout the analysis. For cell cycle synchronization in G_1, MCF10A cells were grown for 3 days in methionine free DMEM supplemented with 2% dialyzed calf serum (Gibco). To enrich cell populations for cells in S phase, MCF10A cells were grown in methionine free DMEM for 2 days followed by a 16 hour incubation with 0.5mM hydroxyurea (Sigma). After hydroxyurea incubation, cells were trypsinized off the dishes and replated in fresh, drug free medium. Three to four hours later cells were removed and subjected to analysis.

GST-fusion protein expression and pull down assays

Full-length pRb fused to glutathione-S-transferase (GST-pRb$^{(1\text{-}928)}$) was expressed and purified from E. coli strain BL21pLys using glutathione sepharose beads as described (Zarkowska and Mittnacht, 1997). GST-pRb$^{(1\text{-}928)}$ was then eluted from the beads using excess glutathione, and estimates of the amount of eluted GST-pRb were made by comparing silver stained band intensities with those of known quantities of bovine serum albumin. GST was expressed and purified from E. coli similarly. The coding sequence of p125 was inserted into the GEX-5X-3 vector which was used to transform BL21pLys and GST-p125 expression was

performed as described for GST-pRb. For pull down assays, cells were washed twice with ice cold TBS (25mM Tris-HCl pH 8.0, 150mM NaCl) and lysed for 20 minutes with 50mM Tris-HCl pH 7.5, 150mM NaCl, 0.5% Nonidet P-40) containing 10ug/ml of the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonylfluoride (PMSF). The lysates were cleared by centrifugation at 14,000 x g for 10 minutes. Protein concentration was determined (Bradford, 1976). 200ug of cellular lysate was incubated with 0.5ug of fusion protein and rocked at 4°C for 1 hour. Glutathione-sepharose 4B beads (Pharmacia) were washed with buffer, and added to the pull down reactions for 1 hour. Beads were isolated by centrifugation and washed with buffer 4 times. Specifically bound material was eluted by incubation with 50mM glutathione pH 7.5, boiled in 2X SDS-PAGE sample buffer (4% SDS, 20% (v/v) glycerol, 0.2M dithiothreitol, 0.24M Tris-HCl, pH 6.8) and subjected to electrophoresis and Western blotting. Blocking experiments were performed with bacterially expressed large T antigen (0.5ug) or 100ng LXCXE peptide by preincubation with GST-Rb for 1 hour, rocking, at 4°C prior to the pull down assay as described above.

SDS-PAGE, Western blotting

Electrophoresis was performed in 6%, 8% or 10% SDS-polyacrylamide gels using 100ug total cell lysate or total immunoprecipitate for each sample lane. After electrophoresis, the proteins were transferred to nitrocellulose paper in buffer containing 25mM Tris-HCl, 192mM glycine, 20%v/v methanol, and 0.01% SDS, pH 8.5. Residual protein binding sites on the membrane were blocked by incubation for 30 min in TBST (25mM Tris-HCl, pH 8.0, 150mM NaCl, 0.5% Tween-20) containing 4% non-fat dry milk. Next, the nitrocellulose was incubated in TBST containing 2% nonfat dry milk containing 1ug/ml of one of the following antibodies:

monoclonal 78F5 to p125 (Jiang et al., 1995); antibody to pRb (PMG3-245; Pharmingen, San Diego, CA); antibody to underphosphorylated Rb (G99-549; Pharmingen) overnight at 4°C. Following 3 washes of 10 minutes each with TBST, membranes were incubated with horse-radish peroxidase conjugated anti-IgG (Southern Biotechnology) and developed using chemiluminescence detection (Pierce, Rockford, IL) according to the manufacturer's instructions.

Coimmunoprecipitation

Cells were collected by trypsinization and lysed in ice cold kinase buffer (20mM MOPs, pH 7.2, 25mM B-glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol) containing protease inhibitors (10ug/ml of aprotinin, leupeptin, and PMSF) followed by sonication. Cell lysates containing 0.5-1.0 mg were normalized to the same volume (approximate protein concentration 1mg/ml) and following a 1 hour preclearing with Staphylococcus protein A- or protein G- Sepharose beads (Sigma), were immunoprecipitated for 1.5 to 2 hours with primary antibody at 4°C. Next, 100ul of a 1:1 slurry of protein A or protein G beads was added and the precipitations were incubated an additional hour. After three washes with kinase buffer, immunoprecipitates were analyzed by SDS-PAGE.

DNA polymerase assays

DNA polymerase δ was purified from calf thymus as described (Lee et al., 1984). The poly(dA)oligo(dT) assay performed to measure DNA polymerase δ activity contains 0.25 optical density units/ml poly(dA)oligo(dT) (20:1), 200ug/ml BSA, 5% glycerol, 10mM $MgCl_2$, 25mM HEPES, pH 6.0, 100cpm/pmol [^{3}H]TTP, in the presence of 0.2ug PCNA. One or ten micrograms of bacterially expressed GST or GST-Rb was added to each polymerase reaction. After incubation at 37°C for 15 minutes the reaction is stopped by the addition of cold 5% TCA

and the precipitate is collected on Whatman filters (DE81) and analyzed by liquid scintillation counting.

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Figure legends

Figure 1: GST-Rb pull down assay. GST or GST-Rb (0.5ug) was added to MCF10A or MCF7 cell lysates and the bound protein was analyzed by Western blot using antibodies to the catalytic subunit of DNA polymerase δ (p125). p125 is detected in GST-Rb pull downs (lanes 3 and 6) but are not detected in GST pull downs (lanes 2 and 4).

Figure 2: GST-p125 pull down assay. GST (lane 2) or GST-p125 (lane 3) was added (0.5ug) to MCF10A cell lysates and the bound protein was analyzed by Western blot using antibodies to unphosphorylated pRb (top panel). The same blot was subsequently reprobed with an antibody raised to phosphorylated pRb (lower panel). Lane 1 shows the relative amounts of unphosphorylated and phosphorylated pRb in the MCF10A lysates.

Figure 3A: Coprecipitation of p125 with pRb antibodies. Immunoprecipitation with antibodies raised to p125 (lane 2) or pRb (lane 3) was performed as described in the methods section and analyzed by Western blot using p125 antibodies. Lane 3 shows p125 coprecipitation with pRb antibodies, which comigrates with p125 immunoprecipitated by p125 antibody (lane 2).

Figure 3B: Coprecipitation of pRb with p125 antibodies. Immunoprecipitation of MCF10A lysates was performed using antibodies raised to 2 regions of the p125 protein (lanes 2 and 3). The immunoprecipitates were analyzed by Western blot using antibodies to p125 or pRb. Lanes 2 and 3 show coimmunoprecipitation of pRb with p125.

Figure 4: Association of p125 with pRb in G_1 phase of the cell cycle. MCF10A cells were synchronized in the G_1 or S phase of the cell cycle as described in the methods section. To detect the phosphorylation state of the pRb protein, 100ug of cell lysate from synchronized populations of cells were subjected to 6% SDS-gel electrophoresis and Western blotting. The top panel shows that the G_1 arrested cell lysates (lane 1) contain predominantly unphosphorylated pRb, whereas the S phase arrested cell lysates (lane 2) contain predominantly phosphorylated pRb. These cellular lysates were subjected to immunoprecipitation with p125 (lanes 3 and 4) or pRb (lanes 5 and 6) antibodies, followed by Western blot analysis using antibodies raised to p125. Coprecipitation of p125 with pRb antibodies is detected within the G_1 phase arrested cellular lysates (lane 5), but is not detected in the S phase arrested cellular lysates (lane 6).

Figure 5: Phosphorylation of GST-Rb reduces p125 association. GST-Rb pull down assays were performed using GST (lane 1), GST-Rb (lane 2), or GST-Rb <u>in vitro</u> phosphorylated by cdk2 (lane 3) or cdk4 (lane 4). Bound protein was analyzed by Western blot using antibodies to p125. GST-pRb pulls down p125 (lane 2) whereas GST alone does not (lane 1). Phosphorylation of GST-Rb by cdk2 or cdk4 reduces the amount of p125 that associates with GST-Rb by approximately 50% (lanes 3 and 4).

Figure 6: Blocking of p125 binding to GST-Rb by large T antigen and the LXCXE peptide. GST-Rb pull down assays were performed using GST (lanes 1 and 4) or GST-Rb (lanes 2 and 5)

or GST-Rb preincubated with SV40 large T antigen (lane 3) or the LXCXE peptide (lane 6), followed by Western blotting using antibodies to p125. SV40 large T antigen blocks the binding of p125 to GST-Rb (lane 3 vs. lane 2) whereas the LXCXE peptide partially blocks the binding of p125 to pRb (lane 6 vs. lane 5).

Figure 7: Effect of GST-Rb on p125 catalytic activity. Polymerase assays were performed as described in the methods section. Inhibition of thymidine incorporation was observed when the assay contained 1.0ug GST-Rb relative to 1.0ug GST (3 vs. 2) and when the assay contained 10.0ug GST-Rb relative to 10.0ug GST (5 vs. 4).

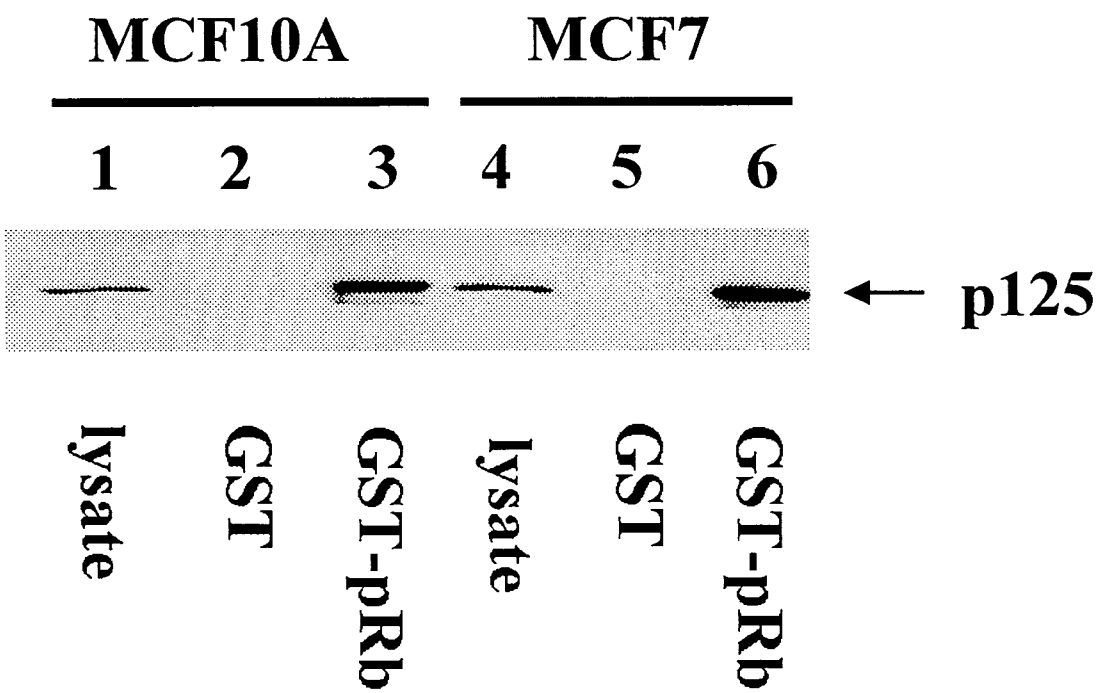
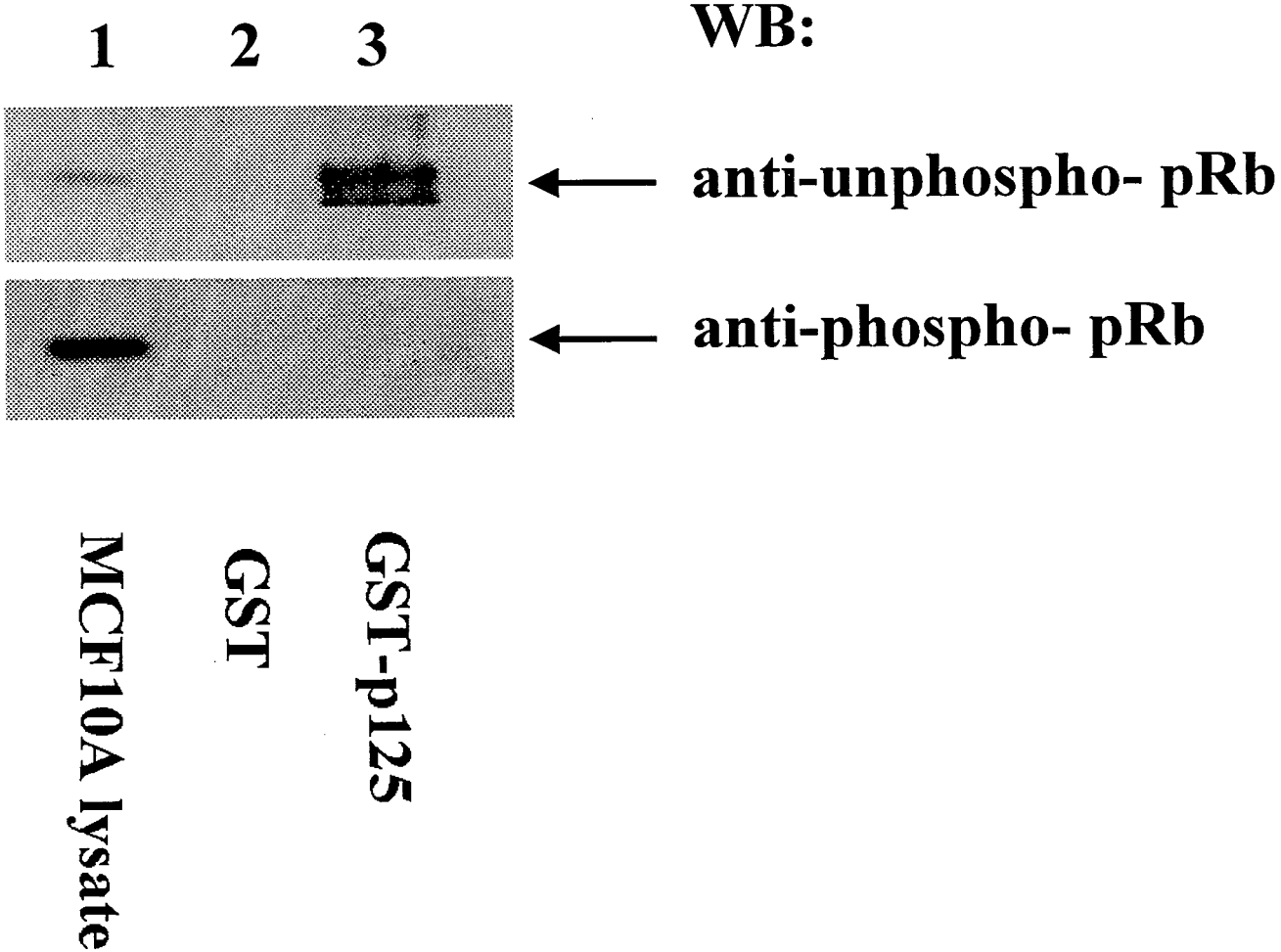

MCF10A
MCF7
1
2
3
4
5
6
p125
lysate
GST
GST-pRb
lysate
GST
GST-pRb

Figure 1


1
2
3
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anti-unphospho- pRb
anti-phospho- pRb
MCF10A lysate
GST
GST-p125

figure 2

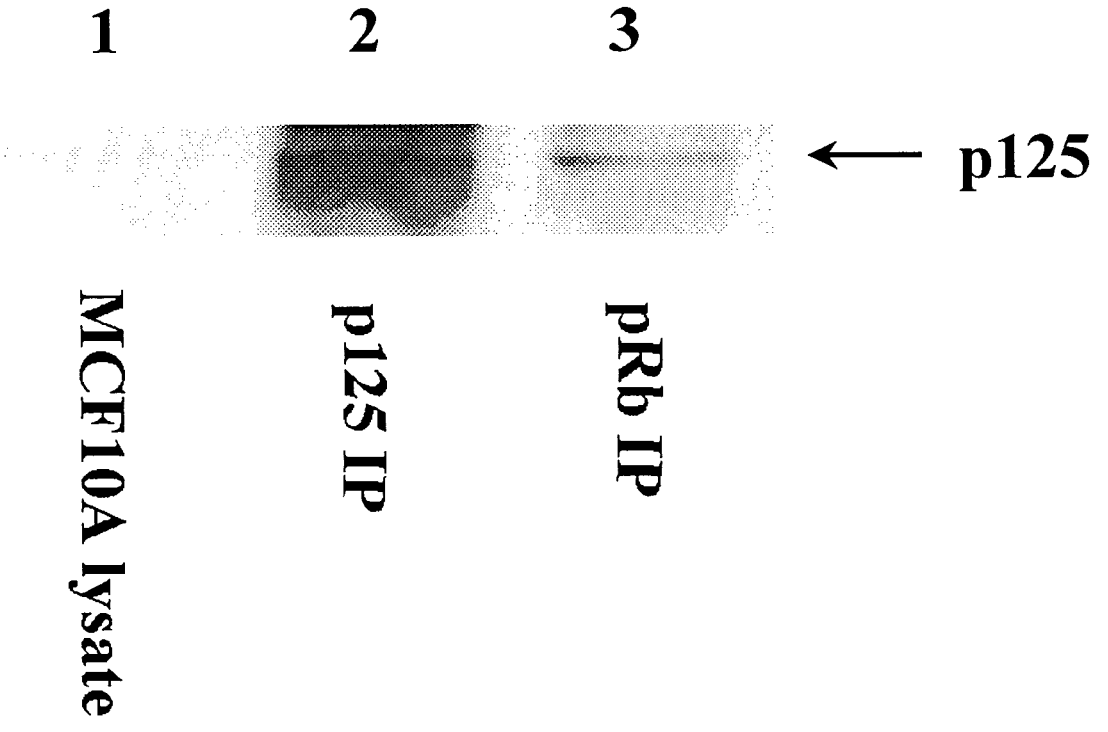

Figure 3A

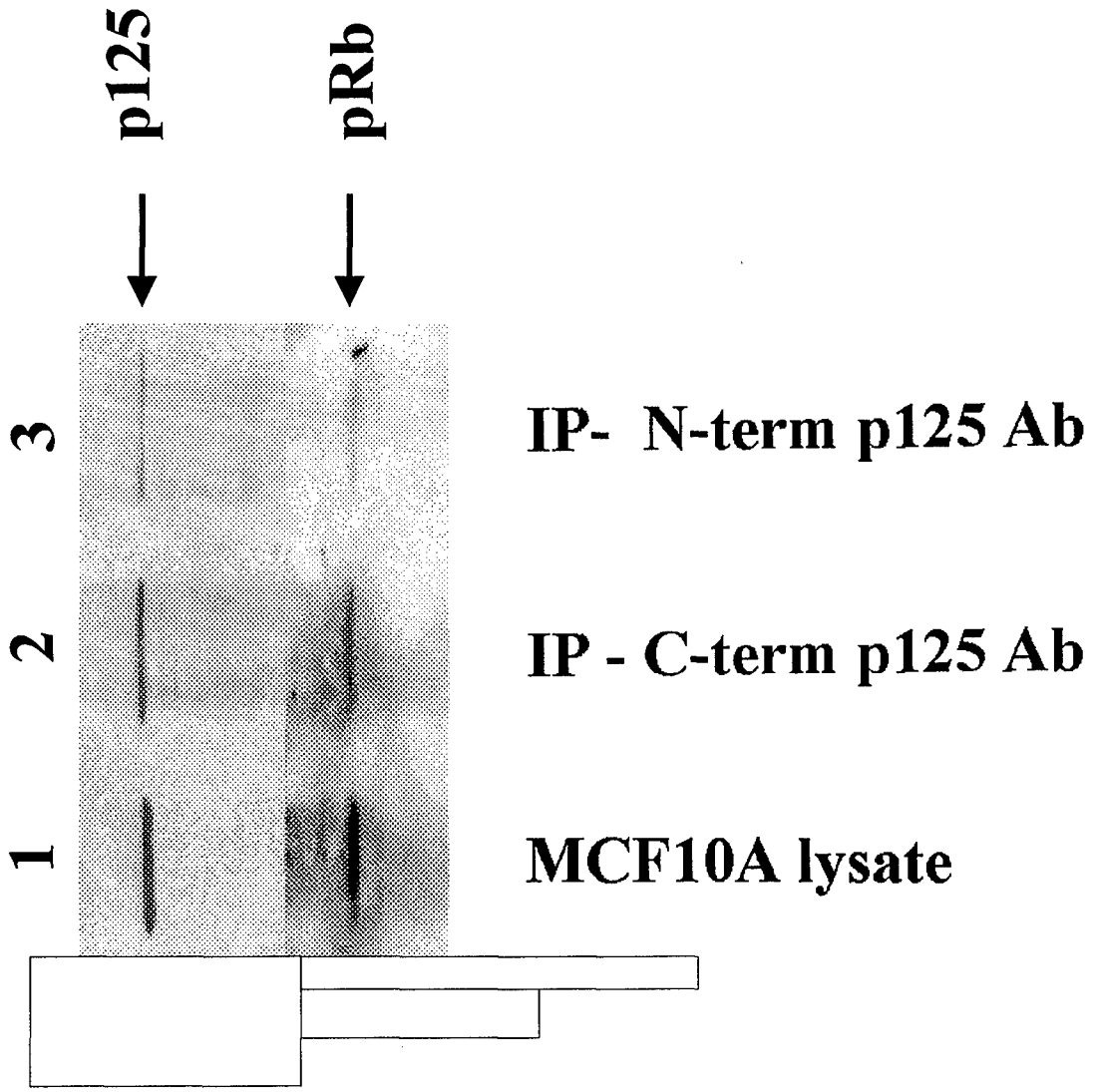

Figure 3B

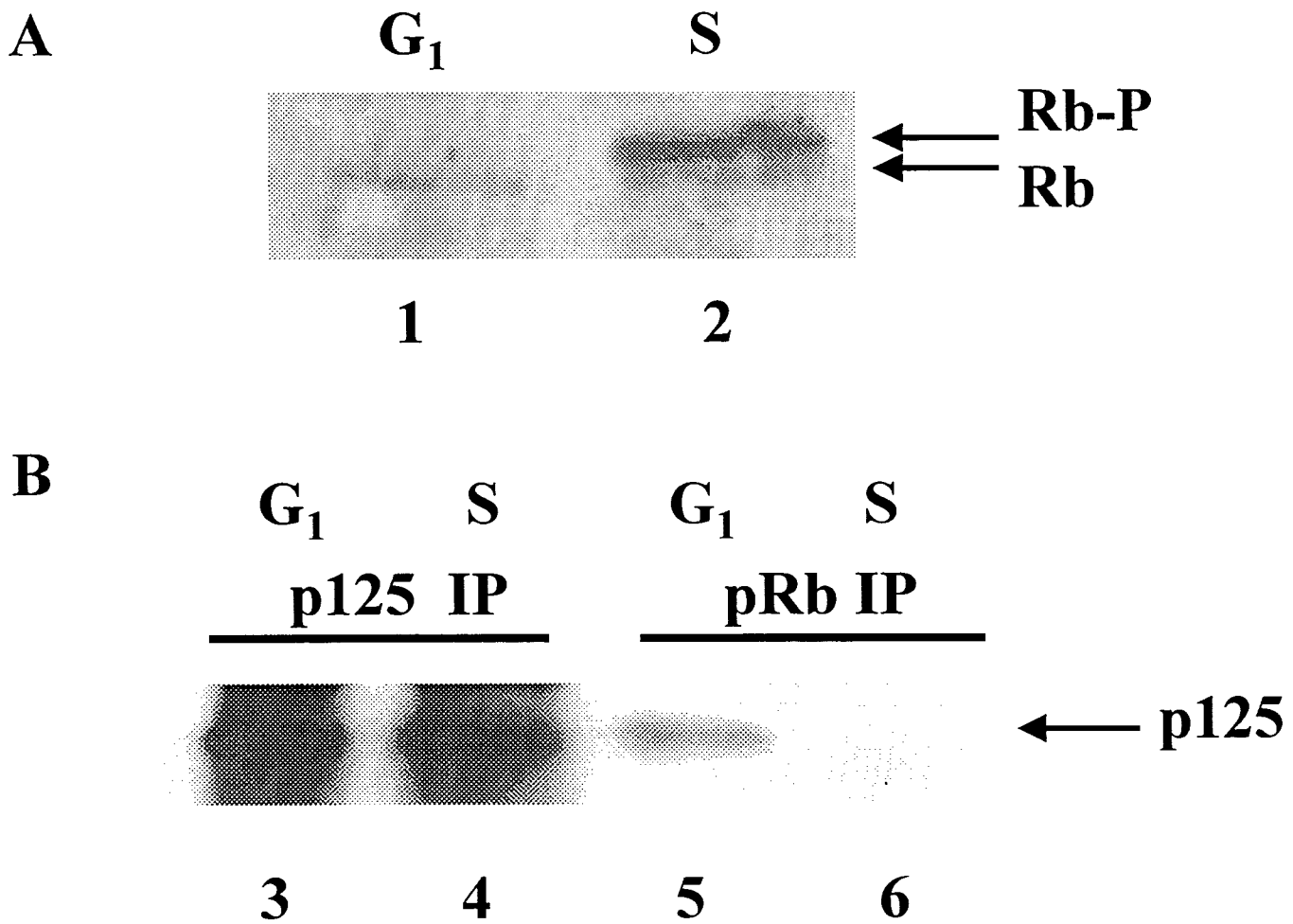

Figure 4

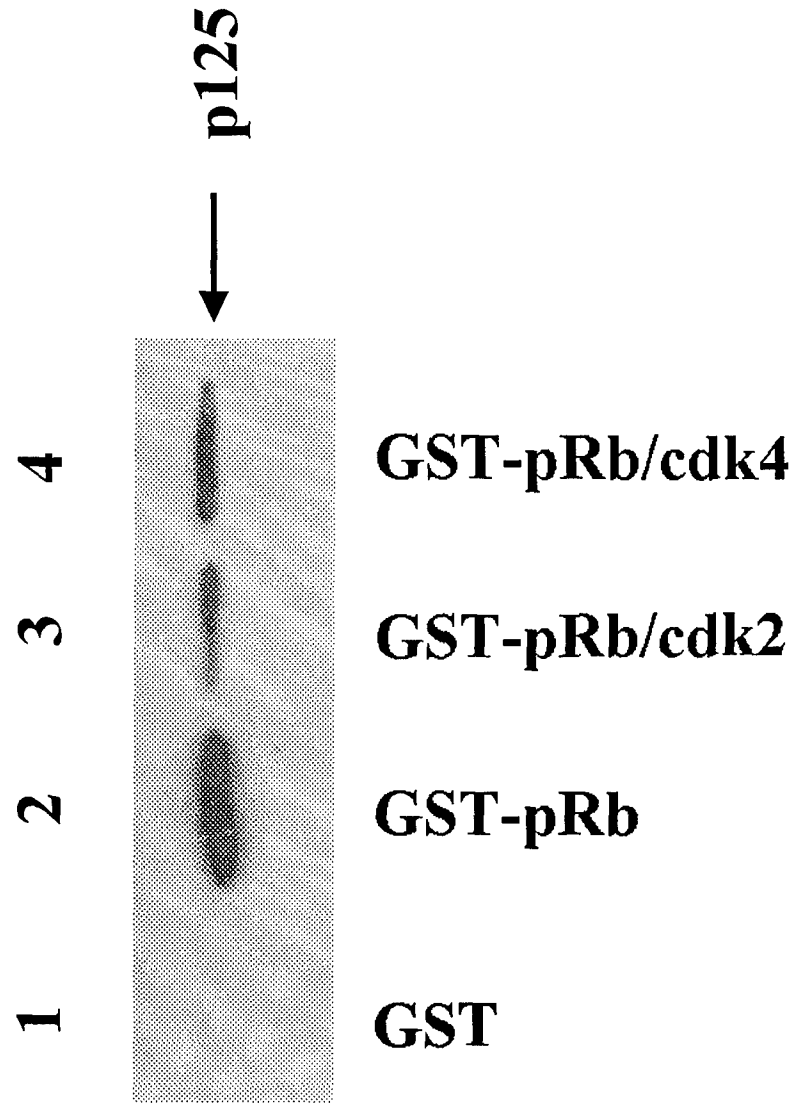

Figure 5

p125
6
GST-Rb+ 100ng LXCXE peptide
5
GST-Rb
4
GST
3
GST-Rb + large T antigen
2
GST-Rb
1
GST

figure 6

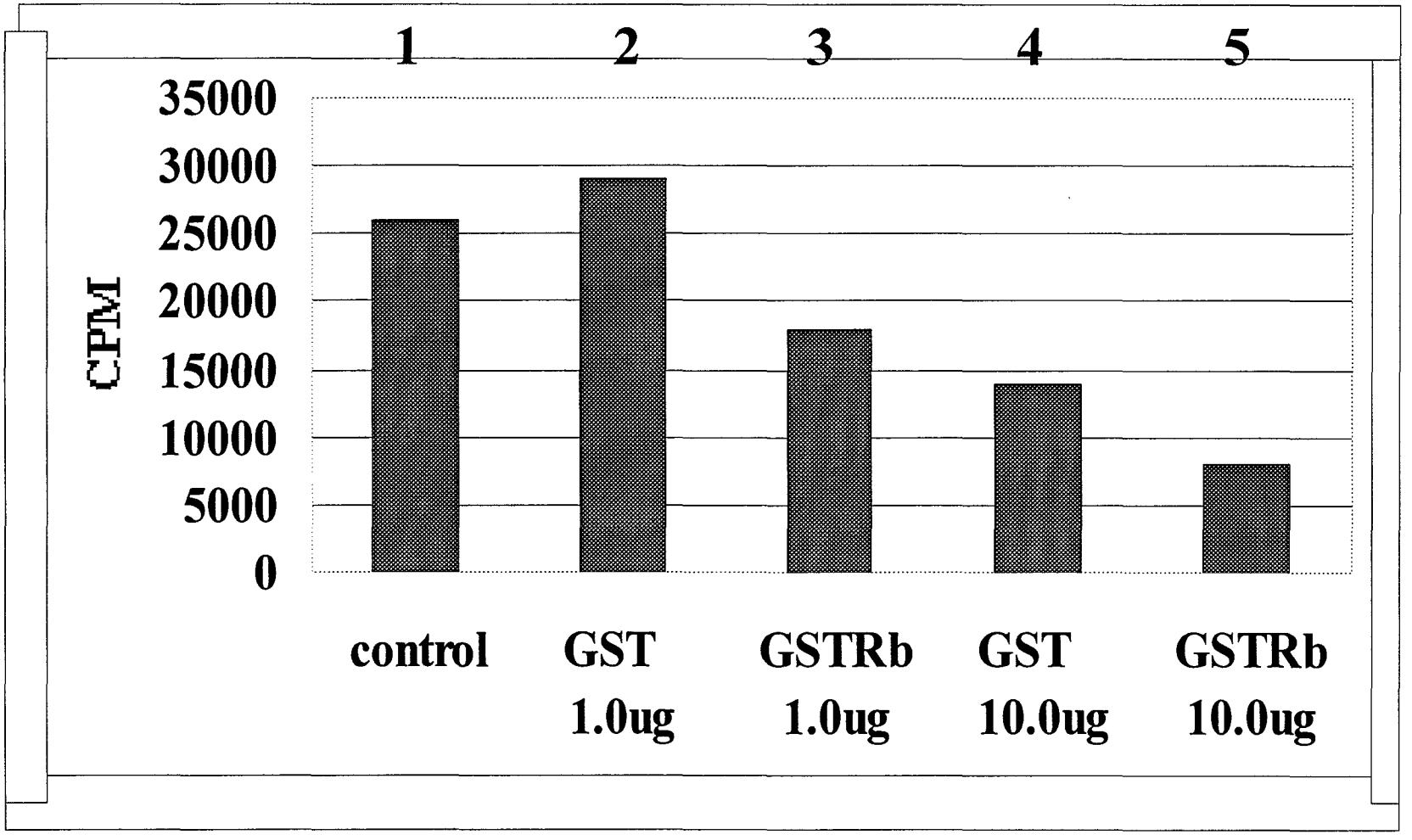
1
2
3
4
5
35000
30000
25000
20000
15000
10000
5000
0
CPM
control
GST 1.0ug
GSTRb 1.0ug
GST 10.0ug
GSTRb 10.0ug

Figure 7

Cloning and characterization of a novel protein, p38, which interacts with the p50 subunit of DNA polymerase delta and PCNA*.

Li Liu, Esther M. Rodriguez-Belmonte, Nayef Mazloum, Heng Xu, and Marietta Y.W.T. Lee¶

Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY, 10595

Running title: p38,a novel Pol δ associated protein

Correspondence to:
Marietta Y.W.T. Lee, Ph.D
Department of Biochemistry and Molecular Biology
Valhalla, NY, 10595
Tel: 914-594-4070
Fax: 914-594-4058
E-mail: marietta_lee@nymc.edu

The yeast two-hybrid screening method was used to identify novel proteins that associate with the human DNA polymerase δ (pol δ)-PCNA complex. Three baits were used in this study. These were the large (p125) and small (p50) subunits of the core pol δ heterodimer, and PCNA. p50 was the only positive isolated with p125 as the bait. $p21^{wafl}$ and DNA (cytosine-5) methyltransferase were the positives from the PCNA screen. Two novel protein partners named p38 and p46 were identified from the p50 screen. It was found that p38 also interacts with PCNA. The ability of p38 to interact with both the p50 subunit of pol δ and with PCNA was confirmed by pull-down assays using glutathione S-transferase (GST)-p38 fusion proteins. The PCNA-p38 interaction was also demonstrated by PCNA overlay experiments. The association of p38 with pol δ was shown to occur in tissue and cell extracts by p125 immunoaffinity chromatography, GST-p38 pull-down and coimmunoprecipitation experiments. p38 was also associated with highly purified pol δ isolated by immunoaffinity chromatography and subsequent gel filtration. These results show that p38 is associated with pol δ. The C-terminus of p38 is homologous to *E. coli* APAG protein and *C. elegans* putative ubiquinone biosynthesis methyltransferase. p46 was identified as a homologue of *S. pombe* MLO3..

DNA replication is essential not only for duplication of the genome but also for maintenance of genomic integrity during DNA repair (1, 2). Chromosomal DNA replication in eukaryotic cells requires three distinct DNA polymerases - α, δ, and ε (1-6). Pol[1] δ is required for replication of the leading strand and for completion of the lagging strand synthesis at the replication fork (7). The core mammalian pol δ enzyme consists of a tightly associated heterodimer of 125 and 50 kDa subunits. Its action as a processive enzyme requires its interaction with PCNA (proliferating cell nuclear antigen) which functions as a molecular sliding clamp (2). Pol δ has recently been shown to consist of at least four subunits, the core enzyme containing the p125 and p50 subunits, and two additional subunits in both yeast and mammalian systems (8-14). In the yeast *S. pombe*, Cdc27 and Cdm1 have been identified as the third and the fourth pol δ subunits, respectively, (8,9). In *S. cerevisiae* Pol32p has been identified as the homologue of the *S. pombe* third subunit (10). A human homologue of Cdc27, the KIAA0039 gene product (11-13) and p12, a human homologue of Cdm1 (14) have recently been identified, and can be considered to be the third and fourth subunits of human pol δ. Our laboratory has been interested in the identification of additional protein components that are involved in the formation of the pol δ replication complex. In this study we report the results of a systematic approach to identifying proteins that interact with pol δ by the yeast two-hybrid screening method (15,16). Two novel proteins of previously unknown function (p38, p46) were identified by the yeast two hybrid screen through an interaction with the p50 subunit of pol δ.

EXPERIENTAL PROCEDURES

Yeast Two-hybrid Screening- The Matchmaker system 2 and human placental cDNA library (HL4025AH) were purchased from Clontech (Palo Alto, CA). The procedures for culture media and plates for yeast growth and selection were as described in the manufactor's protocols. Full-length cDNAs corresponding to the human pol δ catalytic subunit p125, small subunit p50 and PCNA were ligated in frame with GAL4 DNA-binding domain (amino acids 1-147) at the multiple cloning sites of the pAS2-1 vector. Sequential transformations of *S. cerevisiae* Y190 were performed. Y190 cells containing pAS2-1-p125 from three hundred ml of culture were transfected with 50 μg of a mixture of the human placenta cDNA library in the pACT2 vector and 1 mg of salmon sperm carrier DNA. After overnight recovery in 300 ml of Trp^-Leu^- medium, the transfected yeast cells were plated on 10 x 15 cm $Trp^-Leu^-His^-$/3-AT (containing 25 mM 3-amino-1, 2, 4-triazole) plates to select for histidine prototrophy. Histidine-positive colonies were lysed for 20 seconds in liquid nitrogen and assayed for β-galactosidase activity on nitrocellulose filters (Millipore, Bedford, MA). Positive colonies were further plated on Leu^-/cycloheximide plates to rescue the library plasmids. The library plasmid DNA was transformed into *E. coli* DH5α and retrieved from DH5α, and then grouped by *Bgl*II and *Hind*III digestion. Positive clones were confirmed by transfection of the plasmid DNA into Y190 cells containing pAS2-1–p125 for pairwise protein-protein interactions. After confirmation by pairwise yeast two hybrid assays, the positive clones were sequenced. Primer 1 (5'-AGA TTA CGC TAG CTT GGG TGG TC-3') was used for automated sequencing. Blast searches were performed against the NRdb and ESTdb in NCBI. The same procedures were used for pol δ p50 and PCNA screenings.

Pairwise yeast two-hybrid interactions- Pairwise yeast two-hybrid interactions were performed as described previously (11).

Northern blot analysis- Total RNA from human HeLa or MCF7 cells were extracted by the use of the RNA STAT-60 reagent TEL-TEST (Friendwood, TX) . After electrophoresis, RNA was transferred onto a Gene Screen Plus nylon membrane (DuPont) by the upward capillary method using 20 x SSC buffer (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). The p38 probe was prepared by addition of high prime solution (Boehringer Mannheim, Indianapolis, IN) and [α^{32}P]-dCTP (ICN) to the Bgl II-EcoR I 323 bp fragment of human p38. After labeling with [α^{32}P]-dCTP, the probe was passed through a G-50 Sephadex column to remove free [α^{32}P]-dCTP. The p46 probe was prepared from a 382 bp Xho I-EcoR I fragment of p46 (corresponding to amino acids 288-415). The membrane was placed in 5 ml of Northern hybidization solution (50% formamide, 6 x SSC, 5 x Denhardt's solution, 100 μg/ml salmon sperm DNA, 0.1 % SDS) for 2 hr at 42°C. The labeled p38 or p46 probe was added and hybridized overnight at 56°C. The membrane was washed twice for 15 min each at 56°C for p38, 60°C for p46, with 2 x SSC plus 0.01% SDS, then once each with 1 x SSC, 0.5 x SSC and 0.1 x SSC containing 0.01% SDS at room temperature. The membrane was exposed to Kodak X-OMAT LS film for 6 hrs (p46) or 60 hrs (p38) and developed.

p38 antibody- The C-terminal 20 amino acids (H_2N-PPFSLESNKDEKTPPSGLHW-COOH) of human p38 were used to generate rabbit polyclonal antibodies and purified by a peptide affinity column from (SynPep, Dublin, CA).

Purification of pol δ from HeLa cell extracts by immunoaffinity chromatography. Pol δ was purified through p125 immunoaffinity chromatography using a modified

procedure (17). Frozen cells from twenty one liters of mid log phase HeLa cells (Cell Culture Center, Cellex Biosciences Inc) were lysed by sonication in 140 ml of TGEE buffer (50 mM Tris-HCl, pH 7.8, 10% glycerol, 1 mM EDTA, 0.5 mM EGTA) containing 10 mM benzamidine, 10 mM sodium bisulfite, 1 mM PMSF, 7 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysate was centrifuged for 10 min at 27,000 x g. The supernatant was loaded onto a 20 ml pol δ p125 immunoaffinity column. The column was washed with 120 ml of TGEE buffer containing 100 mM NaCl, and eluted with TGEE buffer containing 400 mM NaCl and 30% ethylene glycol. Fractions of 1.5 ml in volume were collected. The fractions were assayed for pol δ activity with polydA/oligodT as the template and loaded onto a 5-15% gradient SDS-polyacrylamide gel for Western blot analysis (14).

Immunoprecipitation- Fresh HeLa cell pellet was extracted by high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 20 mM EDTA, 0.2 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 10 mM benzamidine) for 20 min on ice, sonicated and diluted with 2 volumes of wash buffer without NaCl (50 mM This-HCl, pH 7.8, 20 mM EDTA, 150 mM NaCl, 0.5% NP-40). The diluted nuclear extract was precleared with protein A plus protein G beads and 5 μg rabbit preimmune serum at 4°C for 30 min. After centrifugation, the protein A and protein G beads were discarded. To the precleared HeLa extract, 10 μg p38 rabbit polypeptide antibody or normal rabbit serum, protein A and protein G beads were added and incubated for 2 hrs at 4°C. After incubation, protein A plus G beads were washed 5 x with wash buffer, and the bound proteins were immunoblotted with p38 rabbit polyclonal antibody, PCNA mouse monoclonal antibody 74B1 or pol δ p125 monoclonal antibody 78F5.

PCNA overlay- PCNA overlay was performed as described previously (11).

GST pull-down assays- The p38-GST fusion protein expression vector was constructed by double digestion of pACT2-p38 with EcoR I and Xho I and ligated to pGEX-5X-3 vector (Amersham/Pharmacia). The fusion protein was expressed in *E. coli* strain BL21(DE3, pLysS) (Stratagene). The GST lysate or GST-p38 fusion protein lysate (240 µl) in GST binding buffer (50 mM Tris-HCl, pH7.8, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.2 mM PMSF) was mixed with 200 µl of cell lysate containing recombinant pol δ p50 or PCNA. To this was added 100 µl of 50% suspension of glutathione-Sepharose 4B beads. The suspension was incubated with gentle rocking for 2 hrs at 4°C. After collection by centrifugation, the beads were washed five times with GST binding buffer.

DNA sequencing- The entire insert of p38 and p46 in the pACT2 vector was sequenced.

RESULTS

Yeast Two-Hybrid Screening – A yeast two hybrid screen of a human placental cDNA library was performed (Experimental Procedures) using human p125, p50 and PCNA as the baits. Table 1 summarizes the results from the screens. With pol δ p125 as a bait, 18 of the 24 positive clones were identified as the pol δ p50 subunit. This is consistent with previous data on the strong interaction between these two pol δ subunits which still co-elute after 3.4 M urea treatment (17). Two separate screens were performed with PCNA as the bait. $p21^{waf1}$ emerged as the dominant group of positive clones in the PCNA screens. Forty-one of 55 positives isolated were identified as $p21^{waf1}$ in the first screen, and 18 out of 25 in the second screen. All of the $p21^{waf1}$ clones were nearly full-length cDNAs of 2.0 kb or 2.2 kb in length. This is consistent with the strong binding affinity of $p21^{waf1}$ to PCNA (K_D =1.5 to 4.0 x 10^{-9} M) (18-20). Two of the positives isolated with PCNA as the bait were identified as human DNA (cytosine-5) methyltransferase (MCMT). This enzyme has been identified as containing a $p21^{waf1}$ like PCNA binding motif (21,22). The insert length was 900 bp, which includes the PCNA binding motif at amino acid residues 164 to 171 of the MCMT protein (Genbank Accession No. X63692).

The major advance as a result of this work was the identification of two novel proteins that interact with p50. These will be referred to as p38 and p46. Twelve identical p46 and 6 identical p38 clones were isolated in the screening with p50 as the bait. $p21^{waf1}$ was also found to interact with pol δ p50.

Further tests of the interactions of p50 with $p21^{waf1}$, p38, and p46 were carried out using a pairwise yeast two hybrid analysis followed by a liquid assay for β-galactosidase activity (11). The results confirmed the ability of p50 to interact with $p21^{waf1}$, p38, and

p46 (Fig.1). Several additional important results were obtained in this experiment. The interaction of p38 with PCNA was tested, and was shown to be positive. However, a negative result for an interaction of p38 with p125 was obtained (data not shown). The finding that p38 interacted with PCNA is highly significant, since p38 also interacts with p50. This suggested that p38 could interact both with the pol δ heterodimer and with PCNA. Tests for pairwise interactions between pol δ p125-p46 and PCNA-p46 were negative. Yeast cells cotransfected with p125-p46 or PCNA-p46 did not grow, as would be expected if there was any pairwise interaction. p50-p50 interaction and p50-PCNA interactions were weak, and the strength of the interaction was similar to negative controls when only p50-pAS2-1(the p50-GAL4 DNA binding domain fusion construct), or p50-ACT2 (the p50-GAL4 activation domain fusion construct) were present in the yeast cells which grew in Trp^- or Leu^- media respectively.

The identity of p38- The entire insert of p38 in the pACT2 vector was sequenced and found to consist of 1967 base pairs. BLAST searches of the NRdb revealed that the p38 sequence matched three genomic sequences: AC002094, from human chromosome 17, AC002324 from mouse Chromosome 11 and L14429 from *C. elegans* cosmid ZK652.9, which encodes a putative ubiquinone biosynthesis methyltransferase (YOY9_CAEEL). It also matched over 100 EST sequences from the ESTdb. The human genomic sequence AC002094 was found to have one error (Fig. 2A), in that it is missing a cytosine at position 43699, which results in an incorrect open reading frame (ORF). The corrected AC002094 sequence (Fig. 2A) was used for ORF prediction and allowed the construction of an exon-intron map of the p38 gene (Fig. 2B). The predicted ORF matched the cDNA sequence derived from the clone isolated from the yeast two hybrid

screen, and was also consistent with a number of the EST clones found by BLAST searches. The derived human p38 cDNA (Fig. 3) encodes a protein of 368 aa. The p38 insert in the pACT2 vector contained the entire coding sequence except for the first three N-terminal amino acids. The cognate mouse p38 ORF was derived from the mouse genomic clone (AC002324). The predicted ORF also consists of 368 amino-acid residues and has an identity of 95% with human p38 at the amino acid level. The exon-intron map of the p38 genes for both mouse and human are shown in Fig. 2B.

Analysis of the p38 amino acid sequence. BLAST searches using the p38 amino acid sequence revealed some interesting relationships. p38 from either mouse or human (amino acids 61-363) exhibits significant homology to the C-terminal half of YOY9_CAEEL (296 residues, amino acids 265-561). There is 33% identity in the alignment, which is significant (not shown). The other match found in the Blastp search was APAG, an *E. coli* protein of unknown function, which also aligned with the C-termini of p38 and YOY9_CAEEL. In the Prodom release 99.2 database, APAG is listed as Domain PD031761 with two members, APAG ECOLI and APAG SALTY. A multiple sequence alignment was made with the conserved region of p38 (residues 243-353) and the C-terminus of YOY9_CAEEL with APAG *E. coli*. Also included in this alighment are sequences of p38 from bovine, xenopus and drosophila (Fig. 4). A striking feature of this alignment is a conserved region which conforms to the motif G-x-G-V-V-G-x-x-P-x-[LI]. A third APAG protein, APAG ret (*Rhizocium etli* (TrEMBL Q9ZES3) was found by searching the combined Swissprot and TrEMBL database using the motif G-x-G-V-V-G-x-x-P-x-[LI] and is also shown in Fig. 4. The APAG domain encompasses 110 of the 125 amino acids of APAG, and is strikingly conserved (17% identity and

34% similarity) with the p38 proteins across a wide evolutionary range, arguing that it is likely to have some significant function. This relationship rises to 28% identity and 47% similarity when only the human and mouse p38 sequences are compared to APAG ECOLI. The conserved GxGxxG signature is a well studied motif found in NAD and FAD binding proteins (23,24). The GxGxxG motif is strictly conserved in NAD binding proteins for the binding of ADP moiety (25). We suggest this conserved region in p38 be called the APAG domain.

Northern blot analysis of p38 expression. Northern blot analysis revealed a single major transcript of ca. 2.0 kb of p38 in both HeLa and MCF7 total RNA extracts (Fig. 5) using a 323 bp probe (436-759, Fig. 3) from the human p38 sequence. The size of the transcript is in good agreement with the sequence information. At this point it cannot be ascertained if the minor transcript (1kb) represents a second transcript of the same gene, the existence of a related gene or a degradation product of the 2 kb transcript.

Demonstration of protein-protein interactions between p38 with PCNA or p50 by pull down assays. The existence of protein-protein interactions between human p38 and PCNA and p38 and p50 were further investigated by GST pull-down experiments. The coding sequence of p38 was inserted into the pGEX-5X-3 vector and the recombinant GST-p38 was expressed (Experimental Procedures). Both PCNA and p50 were detected in pulldown assays using GST-p38, but not with GST alone (Fig. 6). These experiments indicate that p38 directly interacts with PCNA and also with the pol δ p50 subunit. Purified GST and GST-p38 adsorbed onto glutathione-Sepharose beads were used in a variation of the pull-down assay to determine if pol δ p125 could be bound from partially purified calf thymus pol δ preparations. The GST pull down assay was negative, while

GST-p38 pull down assay was positive (Fig. 6). This ability of GST-p38 to bring down p125 is presumably because of the tight association of 125 with p50.

An interaction between human PCNA and p38 was also demonstrated by two PCNA overlay assays which showed a positive reaction with GST-p38 (Fig. 7. Panel B and C, Lane 2) and a negative reaction for GST alone (Fig. 7, panels B and C, Lane 1).

Demonstration of interactions between p38 and PCNA or p50 by co-immunoprecipitation. The association of p38 with p50, p125 and PCNA in HeLa cell extracts was demonstrated by coimmunoprecipitation experiments using C-terminal peptide polyclonal antibodies against human p38. The p38 antibody was able to immunoprecipitate p38 from HeLa lysates. The immunoprecipitates were then Western blotted with antibodies against either PCNA or p125 (Fig. 8). The results for the Western blot with PCNA showed strong signals. This is consistent with evidence for a direct interaction of p38 and PCNA obtained by the overlay and GST pull-down experiments (Fig. 6, Fig. 7). Weaker signals were obtained in the coimmunoprecipitate with p125. Nevertheless, the results indicate that p38 is associated with components of the pol δ complex. The most likely interpretation for the coimmunoprecipitation of p125 with p38 is that p38 is bound to the pol δ heterodimer via p50.

p38 is associated with pol δ purified by immunoaffinity chromatography and subsequent gel filtration. Pol δ was purified from HeLa cells by a modification of the immunoaffinity chromatography method used for the isolation of pol δ from calf thymus tissues (17). The HeLa cell lysate was directly chromatographed on a p125 immunoaffinity column without passage through DE52 and phenyl agarose steps, and a lower salt buffer (0.1 M NaCl) was used to wash the immunoaffinity column. The

column was eluted with TGEED containing 0.4 M NaCl and 30% ethylene glycol. A polyclonal antibody to a p38 peptide (Experimental Procedures) was used for the detection of p38 by Western blotting. p38 was found to co-elute with all of the four subunits of pol δ (p125, p50, p68 and p12) in the peak fraction of pol δ activity as judged by pol δ activity assay and Western blot analyses (Fig. 9). The peak fraction was concentrated by centrifugation through a Centricon membrane and then subjected to Superpose 6 gel filtration chromatography. p38 again co-eluted with the pol δ activity (Fig. 10).

Commassie blue staining showed that the major polypeptide components of the preparation after this two step procedure were the p125 and p50 polypeptides, and that a protein band correponding to the p38 immunoreactive band was also present. This indicated that the presence of p38 was not due to adventitious traces of the polypeptide, but that the levels of p38 were comparable to those of the pol δ core polypeptides. The continued association of p38 with the pol δ heterodimer is striking in the face of the loss of two other known subunits, p68 and p12. Moreover, the molecular weight of the pol δ enzyme after concentration and gel filtration was about 200,000, consistent with a complex composed of one of each p125, p50 and p38. Surprisingly, p68 and p12, the third and fourth subunits of pol δ, were not detected either by Coomassie blue stain or Western blot analysis (Fig. 10) after gel filtration. The reason for the loss of the p68 and p12 subunits was unexpected, and may be due to either dissociation during the concentration step on gel filtration or loss through proteolysis, similar to previous problems that were encountered (14). In separate experiments pol δ was purified from calf thymus by conventional chromatography, and similar findings were obtained in that

the p38 polypeptide could be found to be present with pol δ activity after gel filtration (data not shown).

Analysis of the p46 clone. The p46 isolate from the two hybrid screening was sequenced. A sequence of 1950 bp was obtained. The sequence matched THC132984 and THC212521 in the TIGR Tentative Human Consensus (THC) sequences at Blast NCBI website and had over 100 hits in the ESTdb. A search of the NRdb provided additional clones: a human genomic sequence HS222E13 and a protein sequence named Isoform 1 (GenBank Accession No: CAB77058).

The expression of p46 in human cells was analyzed by Northern blotting of HeLa and MCF7 cell mRNAs. Both showed two bands. The major band was 3.7 kb, corresponding to the cDNA length of p46, which is about 3.5 kb in the pACT2 vector. The minor band was 1.1 kB (Fig. 11). Compared to Isoform 1, the cloned p46 in the pACT2 vector is missing 19 amino acids from the N-terminus, i.e. the first 19 amino acid residues was not required for p46-pol δ p50 interaction. This protein is composed of 421 aa.

A Blastp search with p46 matched the C-terminus of p46 to MLO3 (GenBank CAB10980) from *S. pombe*, a protein which when overexpressed blocks normal chromosome segregation (26). In a PFAM search, both p46 and MLO3 have the "PF00076; RNA recognition motif" (27). Full-length MLO3 consists of 199 amino acids. In a partial alignment, the identity between the p46 and MLO3 is 33% over a span of 110 amino acids (Fig. 12).

DISCUSSION

The motivation for these studies was to identify novel proteins which interact with DNA polymerase δ, using p125, p50 and PCNA as the baits in the yeast two hybrid system. Pol δ, rigorously isolated from mammalian tissues, has been shown to consist of a tightly associated heterodimer of the p125 and p50 subunits. Two less tightly associated subunits, p68 and p12, have recently been identified as homologues of the so-called third and fourth subunits in *S. pombe* (11-14). Interestingly, in *S. cerevisiae*, evidence for the presence of the third subunit has been obtained, but so far a homologue of the *S. pombe* fourth subunit has not been identified. A systematic screen using the p125 and p50 subunits, as well as PCNA, was carried out. This did not reveal any new protein-protein interactions for the p125 subunit, or for PCNA, but did reveal three previously unknown interactions involving the p50 subunit. These interactions involved two novel genes, which were termed p38 and p46, and $p21^{wafl}$. The interaction of $p21^{wafl}$ with PCNA has previously been well characterized, and the finding that p50 may also interact with $p21^{wafl}$ is surprising.

Obviously, protein-protein interactions revealed by the yeast two hybrid system do not necessarily indicate that the native (non-fusion) proteins are capable of interaction, nor do they provide any evidence that such interactions take place in a cellular context. In these studies, we have further characterized the interaction of p38 with the p50 subunit of pol δ. The p38 polypeptide migrates on SDS-PAGE with an apparent size of 38 kDa, although its predicted sequence indicates a calculated molecular mass of 42 kDa. The reason for this difference is unknown.

The ability of p38 to interact with p50 and also with PCNA was demonstrated by co-immunoprecipitation and GST-pulldown assays. The results showed that p125 was also detected, indicating that p38 is able to bind to p50 when the latter is in association with p125. These studies strongly support the view that the interaction of p38 with the pol δ complex is physiological, i.e. that this interaction takes place in a cellular context. More significantly, an association of p38 with pol δ activity during immunoaffinity chromatography and the subsequent gel filtration chromatography was established. With regard to these studies, it is also important to note that p38 could be detected by protein staining of the SDS-PAGE gels of the purified pol δ, in amounts that were significant, i.e. the association is not one of trace amounts. This leads to a consideration of whether p38, like p68 and p12, represents a subunit of the pol δ complex. In this regard, there are significant technical difficulties associated with the attempts to isolate a pol δ "holoenzyme", since only the p125 and p50 subunits demonstrate a tight association that allows their facile isolation by conventional chromatography methods. It was also found that the association of p38 could be demonstrated in pol δ preparations from which both p68 and p12 has been removed (Fig. 10). It should be noted that in our hands, attempts to purify stoichiometric complexes of pol δ are hampered by technical problems, i.e. proteolysis, or dissociation during chromatography (13,14).

A summary of pol δ subunits or associated proteins are shown in Table 2. The p125-p50 heterodimer clearly represents a core that is part of a large multiprotein complex. Studies from this laboratory (13,14) and others (28,29) have shown that pol δ can be partially purified as large high molecular weight forms. It is important to consider this in the context that pol δ can be regarded as taking part in a number of cellular functions

that include a) synthesis at the leading strand, b) synthesis at the lagging strand, and c) DNA repair processes. Each of these processes may require a specific and likely different assembly of proteins surrounding the pol δ core heterodimer. This issue is also important in considerations of attempts to isolate a pol δ replication complex from tissues or cells, since there may existent different multiprotein complexes involving pol δ. The current findings, which provide evidence for three novel protein-protein interactions for p50, add to the number of known protein-protein interactions that involve the pol δ system.

Equally important in understanding the nature of the replication complex is the definition of the functions of these subunits/associated proteins. In the case of pol δ, only the function of the catalytic subunit is known for certain. The third subunits of *S. pombe* (28) and *S. cerevisiae* pol δ (29) have been shown to be involved in the formation of dimeric assemblies of the pol δ core enzyme, may function as the eukaryotic dimerization protein, by analogy to the tau protein of the E. coli DNA polymerase III complex. A dimeric model of pol δ is suggested in the current model for eukaryotic DNA replication (2,7). The function of the fourth subunit function is unknown, and has been shown in *S. pombe* to be nonessential (9). In *S. pombe*, genetic studies have indicated that it may play a role in the processing of Okazaki fragments (9), and in addition, enzymological studies have shown that *S. pombe* cdm1 is tightly associated with pol δ and also may stabilize pol δ (28).

p38, like the yeast third subunit of pol δ (30) binds to both p50 and PCNA. p68, the mammalian third subunit of pol δ, was also shown to bind to PCNA (11-13) and interacts with pol δ p50 subunit by the yeast two hybrid assay (Liu and Lee, unpublished

observations). While the functions of p38 are at this point unknown, as already noted for p68 (14), a protein that binds to both p50 and PCNA could serve as a structural role to strengthen the interaction of the pol δ heterodimer with PCNA by binding to pol δ and PCNA. This possibility is consistent with studies of *S. pombe* Cdc27, where the three subunit enzyme in which Cdc27 is absent was found to bind much less strongly to PCNA that did the four subunit pol δ (28) Given the trivalent nature of PCNA, there is also another possibility that either p38 or p68 could serve as a bridging protein between two pol δ - PCNA complexes. This could be envisioned as a linking of two pol δ - PCNA assemblies via p38 (or p68) in which it is attached to one assembly via PCNA and to the other via p50.

While the functions of the APAG protein or of the members of the family (Fig. 12) that includes p38 are unknown, the presence of the G-x-G-x-x-G motif suggests that the presence of a pyrophosphate binding domain, i.e. that they may be able to bind either pyrophosphate or nucleotide triphosphates. Thus, exploration of the possible binding of these compounds to p38 may provide clues to its function.

The possible binding of pyrophosphate to p38 provides only hints to what its functions might be. In this regard, the recent findings that the small subunit (the homologue of p50) of archaeal DNA polymerase II may possess intrinsic phosphoesterase activity may be relevant, and it has been speculated that the p50 subunit of archeal DNA polymerase II could be used for pyrophosphate hydrolysis (27). This could serve an important function during DNA synthesis, as the product of the reaction is pyrophosphate, so that pyrophosphate hydrolysis would shift the reaction equilibrium towards nucleotide polymerization. However, the conserved amino acid residues for the metal binding

ligands important for phosphoesterase activity are absent in members of the eukaryotic p50 family (27). It is possible that during evolution that p50 has become specialized for protein-protein interaction and lost its own enzymatic activity. A purely speculative possibility is that p38 may provide a pyrophosphatase activity for this function.

With regard to p46, the second p50 binding protein that was identified in this study, much further work needs to be performed to demonstrate that this occurs in a cellular context. Nevertheless, the observation that the C-terminus of p46 has significant homology to *S. pombe* MLO3, which blocks normal chromosomal segregation (26), suggests that it may have some role in DNA replication. The presence of the PF00076 RNA recognition motif (31) in both p46 and MLO3 suggests that they may bind RNA. It is relevant that the N-termini of D Tok Pol (an archaebacteria DNA polymerase), DNA pol δ, and pol ε, like T4 polymerase, have RNA-binding domains (32).

In summary, we have identified two novel proteins, p38 and p46, which interact with the p50 subunit of pol δ. The interaction of p38 with p50 was extensively characterized, and while its functions are currently unknown, these findings indicate that the number of proteins that may be involved in the formation of the pol δ enzyme complex may involve additional proteins besides the four known subunits.

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Figure legends

Fig. 1. Demonstration of the interactions between p50-p38, PCNA-p38 and p50-p46 by pairwise Y2H assays. Y190 cells were transfected with pol δ p125, pol δ p50 or PCNA and grown in Trp dropout synthetic media. These cells were transfected with p38 or p46 cDNA, selected for His auxotrophy and assayed for β-galactosidase activity (Experimental Procedures).

Fig. 2. Analysis of the human and mouse genomic sequences of p38. A. The 5' end of the p38 transcript was aligned with the sequences available in the current ESTdb (C06428, U46408 and AL079399) and NRdb (AC002094). The missing cytosine at position 43699 of AC002094 is highlighted. B. Organization of the human and mouse p38 genes. The diagram shows the organization of the human p38 gene, starting from nt 43620 of the AC002094 genomic clone, and ending at nt 53879. The organization of the mouse p38 gene, starting from the beginning of the open reading frame at nt 33275 and ending at nt 23952 of the mouse AC002324 genomic clone, is shown below that of the human gene.

Fig. 3. The cDNA and open reading frame of human p38. The open reading frame of human p38 was deduced from the genomic DNA clone AC002094 after correction of the sequence (Fig. 2A). The GenBank accession number for the cDNA of human p38 is AF179891.

Fig. 4. Multiple alignments of p38 with APAGs. A. The C-termini of human and mouse p38 (residues 243 to 353) proteins and the C-terminal of YOY9_CAEEL(residues 440-551) were aligned with the sequences of three bacterial APAG proteins. Also included are cognate sequences for bovine, xenopus and drosophila p38 obtained by searches of the EST database. The residues that are identical to APAG ECOL1 are shown in bold and are shaded, and residues that are conserved are shaded, in order to show the relationships with APAG ECOL1. The Clustal 1.74 program was used for the alignments. The identity over the 115 residue span is 17% and the similarity is 34%. GenBank accession numbers for the sequences are as follows: human p38, AF179891; mouse p38, AC002324; Drosophila p38, AC008190.3; bovine p38, AW418288.1 and AW446589.1; Xenopus p38, AW460795; APAG ECOLI and APAG SALTY, Prodom release 99.2 database, Domain PD031761; APAGret, TrEMBL Q9ZES3)

Fig. 5. Northern blot analysis of human p38 mRNA. Total RNA from human HeLa (lane 1) or MCF7 cells (lane2) was extracted and northern blotted using a probe for human p38 (Experimental Procedures). The size makers are 28S ribosomal RNA, 4.7 kb and 18S ribosomal RNA, 1.9 kb.

Fig. 6. Demonstration of the interaction of human p38 with PCNA, pol δ p50 and pol δ 125 by GST pull-down assays. Panel A and B. Lane 1 and 2, cell lysates of *E. coli* cells expressing GST or GST-p38 were mixed with lysates of *E.coli* cells containing overexpressed PCNA (Panel A) or pol δ p50 (Panel B) and pulled down with glutathione Sepharose 4B beads (Experimental Procedures). Western blots of the bound proteins

were performed using monoclonal antibody 74B1 against PCNA (Panel A) or with monoclonal antibody 17D2 against pol δ p50. Lane 3 shows the input recombinant PCNA or recombinant pol δ p50. Panel C. Lane 1. calf thymus pol δ purified to DE52 column chromatography step (14) . Lane 1, input calf thymus pol δ, lanes 2 and 3, calf thymus extracts mixed with *E. coli* lysates containing GST and p38-GST fusion proteins, respectively, and pulled down with glutathione Sepaharose. Western blot analysis was performed with monoclonal antibody 78F5 against pol δ p125.

Fig. 7. Demonstration of the interaction of human p38 with PCNA by overlay assay. Panel A, GST (lane 1) and p38 GST fusion protein (lane 2) were purified by affinity chromatography on glutathione-Sepharose and stained with Coomassie blue after SDS-PAGE electrophoresis. Panel B, overlay using biotin-labeled PCNA. Panel C. overlay using digoxigenin labeled PCNA.

Fig. 8. Demonstration of the interaction of human p38 with PCNA and pol δ 125 by coimmunoprecipitation experiments. HeLa cell extracts were immunoprecipitated with p38 peptide polyclonal antibody and protein A, protein G mixture. The bound proteins were analyzed by immunoblotting with p38 rabbit polyclonal antibody (lanes 1-3) , PCNA monoclonal antibody 74B1 (lanes 4 and 5) and pol δ p125 monoclonal antibody 78F5 (lanes 6,7), respectively. Lane 1, mock immunoprecipitation with rabbit pre-immune serum; lane 2, 4 and 6, HeLa input; lanes 3, 5, and 7, immunoprecipitate with p38 antibody.

Fig. 9. p38 is present in pol δ isolated by immunoaffinity chromatography purified pol δ from HeLa cells. A HeLa cell lysate from twenty one liters of HeLa cell culture was fractionated on a pol δ immunoaffinity column (Experimental Procedures). Panel A. Assay of the fractions eluted from the column for pol δ activity on a polydA•oligodT template in the presence (solid circles) and absence (open circles) of PCNA. The peak fractions (fractions 43,46, and 49) of pol δ activity were analyzed by Western blot for the presence of p125 and p38 (Panel B, left panel) and for the presence of p68, p50 and p12 (Panel B, right panel).

Fig. 10. Association of p38 with pol δ during gel filtration. Panel A. Three ml of the immunoaffinity purified HeLa pol δ (Fig. 9) was concentrated to 0.5 ml using Centricon (30 kDa cutoff from Amicon). 250 μl of this was chromatographed on a 24 ml Superpose 6 column (Pharmacia). Fractions of 250 μl were collected and assayed for pol δ activity on a polydA•oligodT template in the presence (solid circles) and absence (open circles) of PCNA. Panel B. SDS-PAGE analysis of the peak fractions followed by staining for protein with Coomassie Blue. Panel C. Western blotting of the peak fractions for the presence of p125, p50 and p38. Western blots for p68 and p12 were also performed and were not detected.

Fig. 11. Northern blot analysis of human p38. Total RNA from human HeLa (lane 1) or MCF7 cells (lane2) was extracted and northern blotted using a probe for human p46 (Experimental Procedures). The size makers are 28S ribosomal RNA, 4.7 kb and 18S ribosomal RNA, 1.9 kb.

Fig. 12. Alignment of p46 with MLO3. The C-terminal half of human p46 (CAB77058) was aligned to MLO3 (CAB10980) by Clustal W 1.74 program. The RNA Recognition Motif (RRM) of both proteins is underlined.

FOOTNOTES

* This work was supported by National Institute of Health Grant GM31973 and in part by the United State Army Medical Research and Material Command under DAMD-17-96-1-6166. The costs of publication of this article were defrayed in part by marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1]The abbreviations used are: Pol, DNA polymerase; PCNA, proliferating nuclear antigen; ESTdb, expressed sequence tag database; NRdb, non-redundant nucleotide database; NCBI, National Center for Biotechnology Information; PMSF, phenyl methylsulfonyl fluoride

Table 1. Summary of yeast two-hybrid screening

Bait	Transformants	Clones	Identity
Pol δ p125	$6x10^5$	18	Pol δ p50
		8	n.c.
Pol δ p50	$3x10^5$	12	p46, novel
		6	p38, novel
		4	$p21^{WAF1}$
		8	n.c.
PCNA	$1.4x10^6$	41	$p21^{WAF1}$
		2	MCMT
		12	n.c
PCNA	$4.5x10^5$	18	$p21^{WAF1}$
		7	n.c.

n.c., not characterized.

Table 2. Summary of pol δ subunits and associated proteins

	Mammalian	*S. pombe*[a]	*S. cerevisiae*[b]
The catalytic subunit	p125	125 kDa, Pol3/Cdc6	125 kDa, Pol3p
The second subunit	p50	55 kDa, Cdc1	58 kDa Pol31p/Hys2
The third subunit	p68[c]	54 kDa, Cdc27	55 kDa Pol32p
The fourth subunit	p12[d]	22 kDa, Cdm1	unknown
The fifth subunit?	p38[e]	unknown	unknown
Associated proteins	p46[f]	MLO3[f]	Unknown
	PCNA	Pcn1	Pol30

a) Isolated and reconstituted as a four-subunit pol δ enzyme in *S. pombe* (8,28)

b) Reconstituted as a three-subunit pol δ enzyme in *S. cerevisiae* (29)

c) Identified as bovine p68 from pol δ p125 immunoaffinity chromatography (11,13). Isolated by PCNA affinity column from mouse FM3A cell extracts (12).

d) Identified as the fourth subunit of pol δ (14)

e) Identified in this study.

f) Identified in this study.

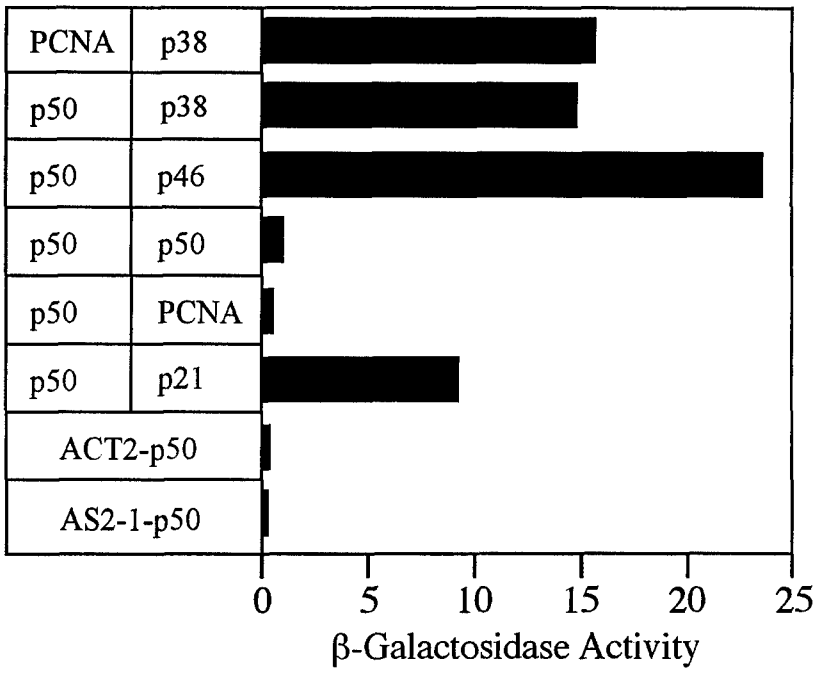

Fig. 1. Liu et al.

A

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AL079399  GCCCGGTGGC CAAGGCCGCT CTGTGCGGCG GCCGGAGCTG GAGCCTTCTC
U46408    GCCCGGTGGC CAAGGCCGCT CTGTGCGGNG GNCGGAGCTG GAGCCTTCTC
Consensus GCCCGGTGGC CAAGGCCGCT CTGTGCGGCG GCCGGAGCTG GAGCCTTCTC
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B

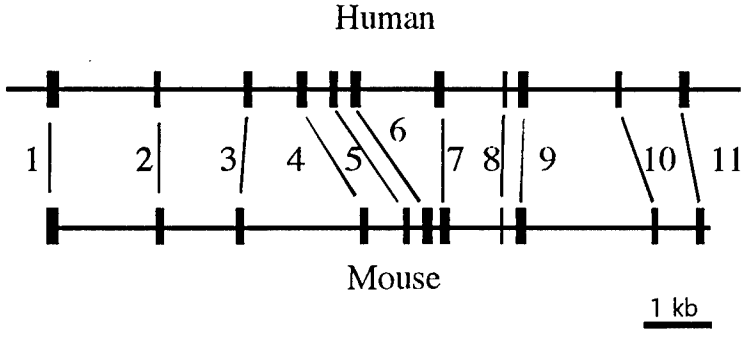

Fig. 2, Liu et al.

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       L  T  G  A  R  W  P  K  P  L  C  A  A  A  G  A  G  A  F  S   40
121   ccagcgtcgaccacgacgacgcggaggcacctctcgtcccgaaaccgaccagagggcaaa
       P  A  S  T  T  T  T  R  R  H  L  S  S  R  N  R  P  E  G  K   60
181   gtgttggagacagttggtgtgtttgaggtgccaaaacagaatggaaaatatgagaccggg
       V  L  E  T  V  G  V  F  E  V  P  K  Q  N  G  K  Y  E  T  G   80
241   cagcttttccttcatagcatttttggctaccgaggtgtcgtcctgtttccctggcaggcc
       Q  L  F  L  H  S  I  F  G  Y  R  G  V  V  L  F  P  W  Q  A  100
301   agactgtatgaccgggatgtggcttctgcagctccagaaaaagcagagaaccctgctggc
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421   cgtgactgcccacatatatctcagagatctcagacagaagctgtgaccttcttggctaac
       R  D  C  P  H  I  S  Q  R  S  Q  T  E  A  V  T  F  L  A  N  160
481   catgatgacagtcgggccctctatgccatcccaggcttggactatgtcagccatgaagac
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541   atcctcccctacacctccactgatcaggttcccatccaacatgaactctttgaaagattt
       I  L  P  Y  T  S  T  D  Q  V  P  I  Q  H  E  L  F  E  R  F  200
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       G  S  H  F  D  V  R  I  P  P  F  S  L  E  S  N  K  D  E  K  360
1081  acaccaccctcaggccttcactggtaggccagctgaggccccaagtgcccaggcttggtc
       T  P  P  S  G  L  H  W  *
1141  accgggaagaacaactctcatcccacaattgctgcagaactcttctctccccatcatggg
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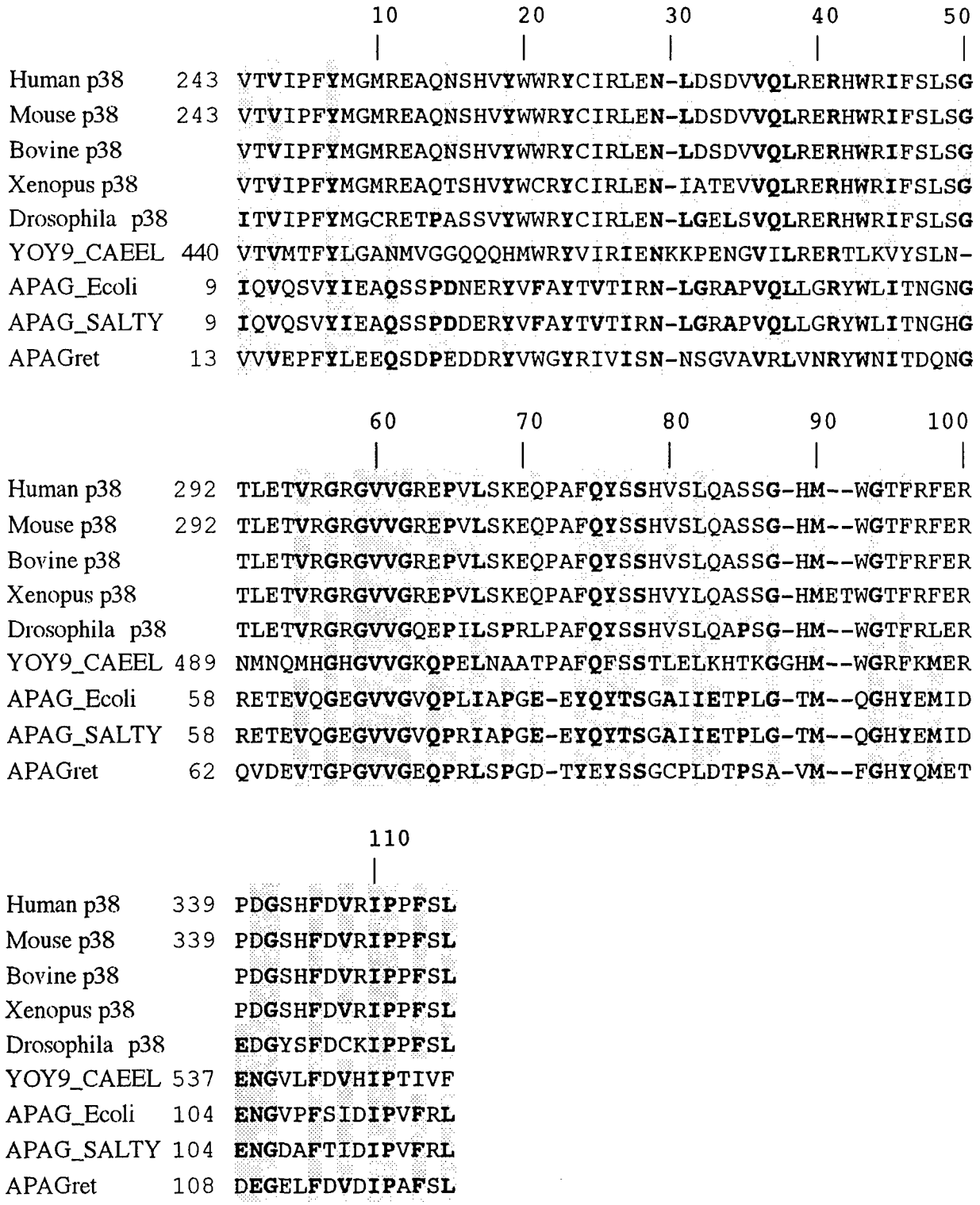
Fig. 3, Liu et al.



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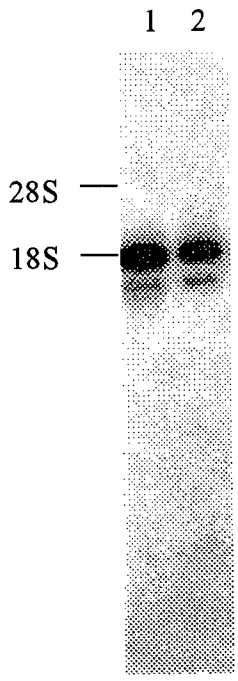

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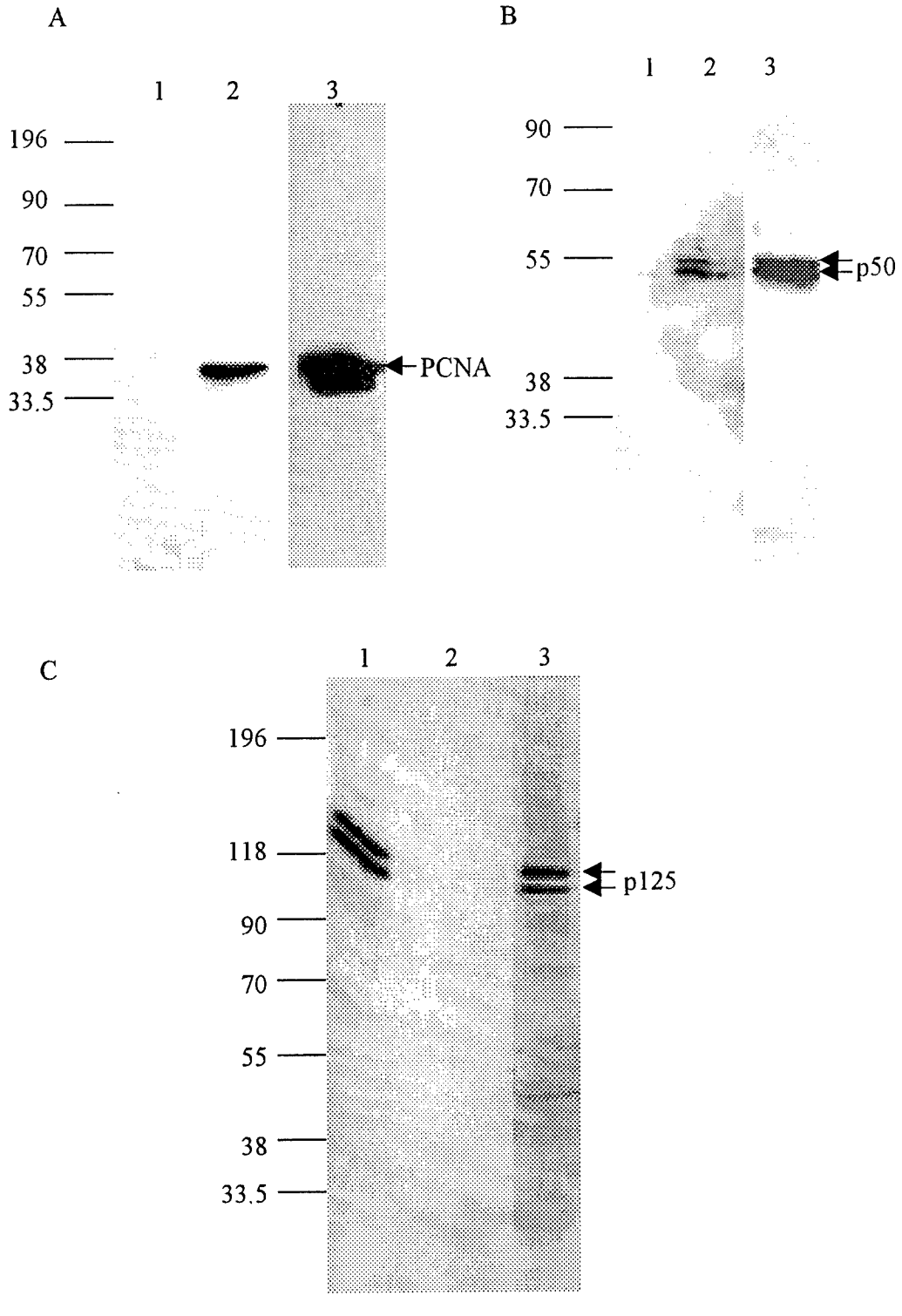

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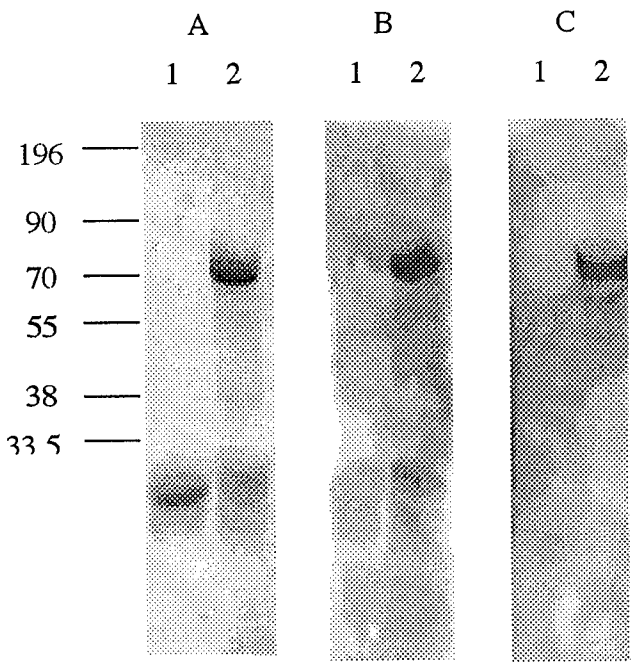

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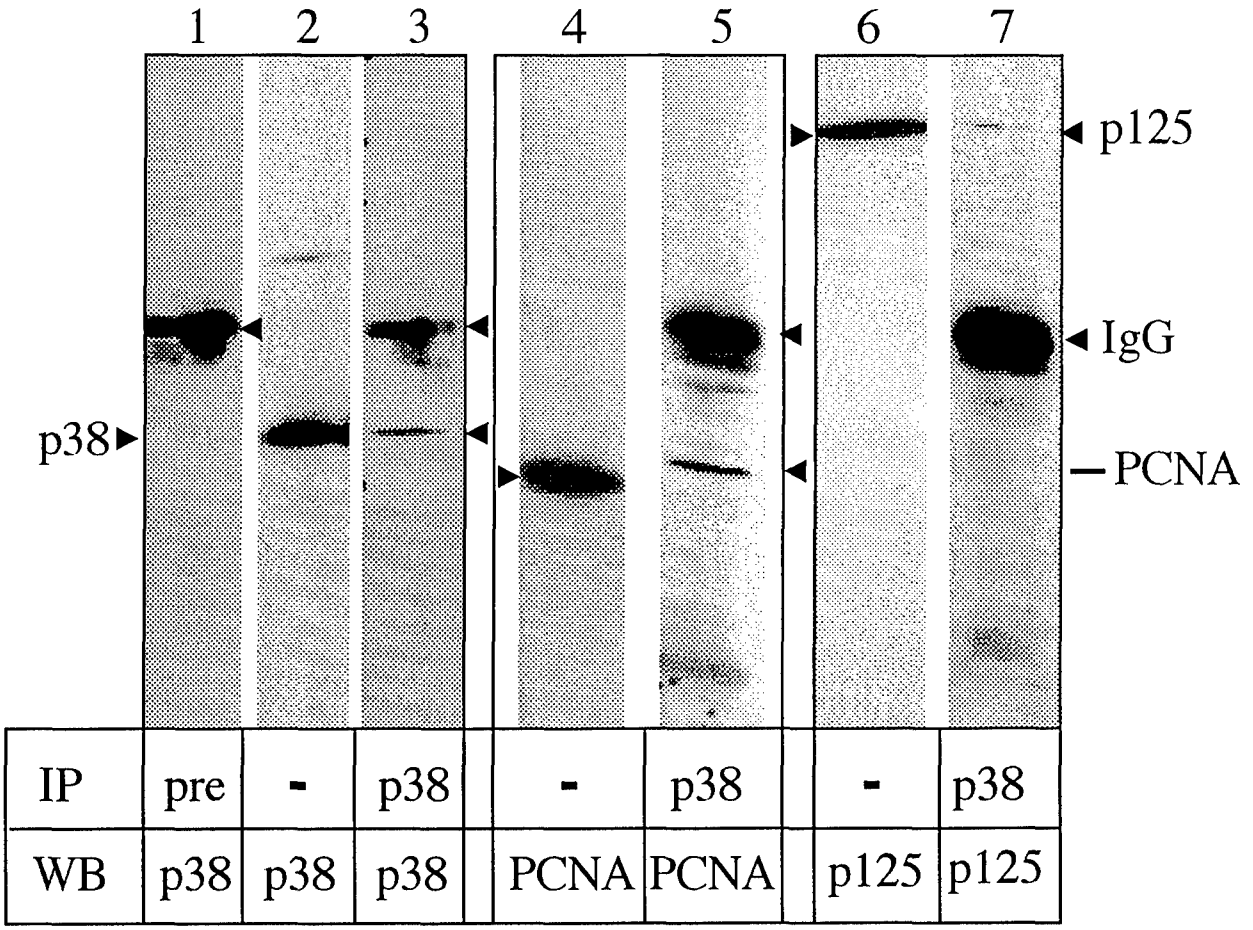
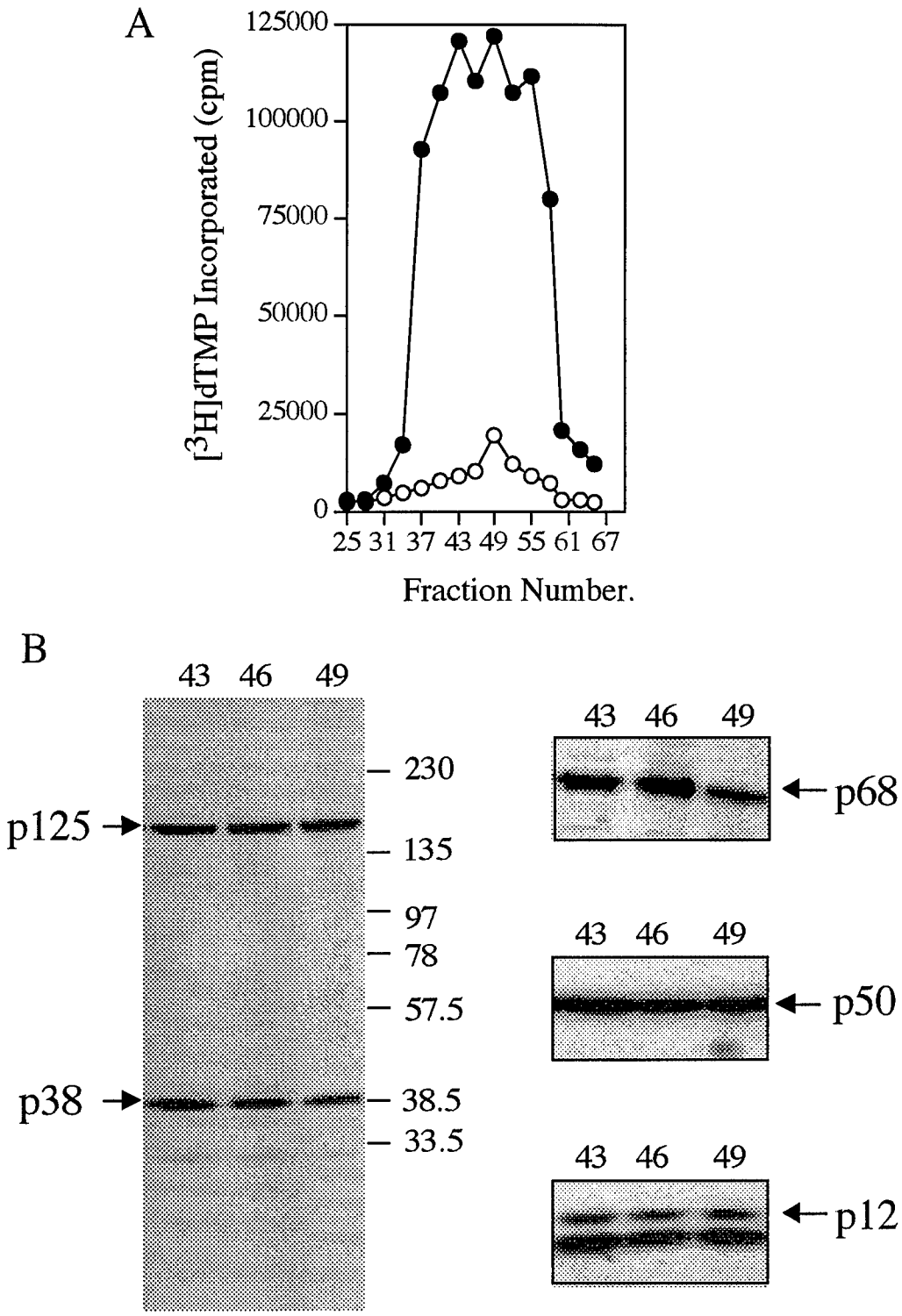


Fig. 8, Liu et al.



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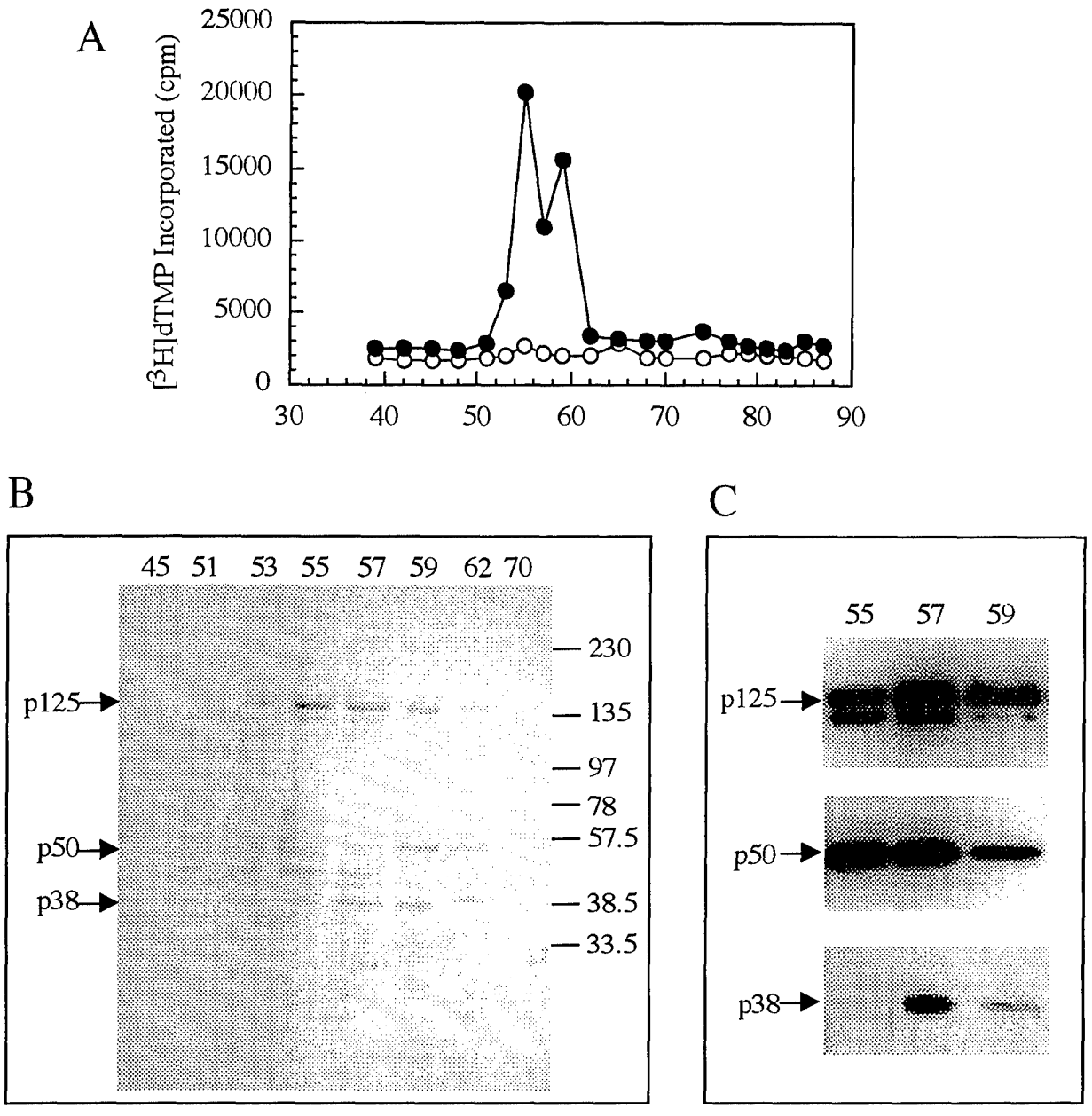

Fig. 10, Liu et al.

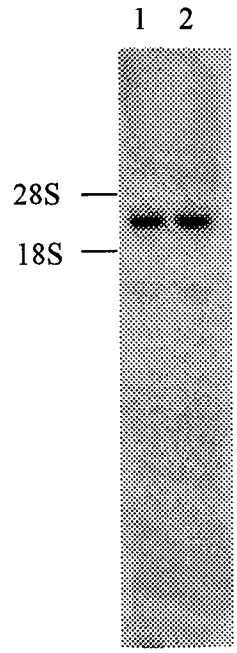

Fig. 11, Liu et al.

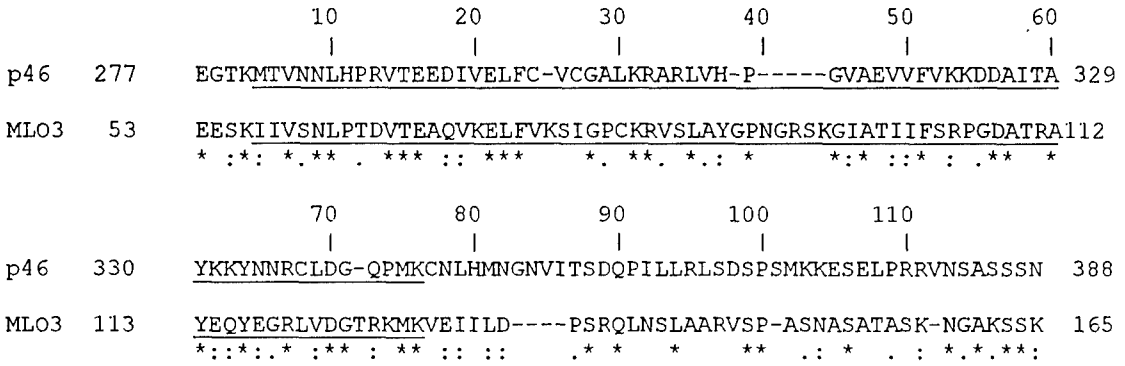

Fig. 12, Liu et al.

MCMR-RMI-S (70-1y) 21 Feb 03

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