2108–2114 *Nucleic Acids Research, 1999, Vol. 27, No. 10*

Isolation and identification of the third subunit of mammalian DNA polymerase δ by PCNA-affinity chromatography of mouse FM3A cell extracts

Patrick Hughes*, Isabelle Tratner, Manuelle Ducoux, Karine Piard and Giuseppe Baldacci

Centre National de la Recherche Scientifique (CNRS), UPR9044, Institut de Recherches sur le Cancer, 7 rue Guy Moquet BP 8, 94801 Villejuif, France

Received February 3, 1999; Revised March 16, 1999; Accepted March 26, 1999

ABSTRACT

Using proliferating cell nuclear antigen affinity chromatography and glycerol gradient centrifugation of partially purified fractions from mouse FM3A cells we have been able to isolate novel complexes of DNA polymerase δ and DNA ligase 1 containing clearly defined subunit compositions. In addition to the well known catalytic subunit of 125 kDa and accessory subunit of 48 kDa, the DNA polymerase δ complex contained three supplementary components, one of which reacted with antibodies directed against the p40 and p37 subunits of RF-C. Of the two remaining components, one termed p66 turned out to be coded by a gene whose putative C-terminal domain displayed significant homology with that of the Cdc27 subunit of *Schizosaccharomyces pombe* polymerase δ. On the basis of these and other observations, we propose p66 to be the missing third subunit of mammalian DNA polymerase δ. The DNA ligase 1 complex was made up of three novel components in addition to the 125 kDa catalytic subunit, two of which, p48 and p66, were common to DNA polymerase δ. We discuss the implications of our findings within the current framework of our understanding of DNA replication.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is a highly conserved and essential protein functioning in a number of transactions on DNA in eukaryotic cells. These include DNA replication and two forms of DNA repair: excision and mismatch repair. In DNA replication, PCNA directly interacts with a large number of proteins including the multi-subunit replication protein RF-C, DNA polymerase δ, Flap endonuclease 1 (FEN-1) and DNA ligase 1. In excision repair, PCNA interacts with XPG and stimulates the re-synthesis step of nucleotide excision repair mediated by DNA polymerase ε. The observation that PCNA interacts with the mismatch-repair proteins MLH1 and MSH2 and that a yeast PCNA mutant displays faulty mismatch repair indicates PCNA involvement in mismatch-repair. PCNA would also appear to be involved in the re-methylation of cytosine residues in CG sequences after DNA replication as the cytosine methylase MCMT has been shown to interact with PCNA (reviewed in 1,2).

Besides these multiple interactions with enzymes of the replicative and DNA repair apparatuses, PCNA also interacts with a number of proteins important for cell cycle control. Chief among these is the cyclin-dependent kinase inhibitor p21, which inhibits DNA replication by interacting with PCNA (3). Others are Gadd45 (4) and its homolog MyD118 (5), cyclin D (6) and a wide variety of other cyclin/cyclin-dependent kinase complexes (7).

These multiple interactions suggest that PCNA is a central element mediating signals between DNA replication and cell cycle control mechanisms. We were interested in identifying novel components of PCNA complexes and, in particular, the indirect interactions predicted to exist between PCNA and other currently unknown elements of the DNA replication and cell cycle control machinery. To this end, we chromatographed partially purified FM3A mouse cell extracts known to contain DNA polymerase δ on PCNA-affinity columns and subsequently established the relationship between the bound proteins by separating them as complexes on glycerol gradients. Basically, two novel complexes of DNA ligase 1 and DNA polymerase δ with novel subunit compositions were isolated. One of the proteins of the DNA polymerase δ complex, migrating with an apparent molecular weight of 66 kDa on SDS–polyacrylamide gels, was found to display significant homology in its C-terminal domain to that of *Schizosaccharomyces pombe* Cdc27 (8). We propose this protein to be the missing third subunit of mammalian DNA polymerase δ.

MATERIALS AND METHODS

Reagents and enzymes

Poly(dA)$_{300}$ and oligo(dT)$_{12–18}$ (used at a ratio of 10:1) were purchased from Pharmacia. [^{3}HTTP] was obtained from Amersham. Anti-DNA polymerase δ p125 (C20) and p50 antibodies were kind gifts from Robert Hindges and Ulrich Hübscher (9). Anti-DNA ligase 1 was a kind gift of Jean-Michel Rossignol (10). The anti-p40 and anti-p37 RF-C antibodies were kind gifts from Jerard Hurwitz. The anti-cdc2 PSTAIRE antibody was obtained from Santa Cruz Biotechnology Inc. Mouse FM3A cell cultures were obtained from Computer Cell Culture, Belgium. Total RNA from HeLa cells was a kind gift of

*To whom correspondence should be addressed. Tel: +33 1 49583330; Fax: +33 1 46789980; Email: hughes@infobiogen.fr

Dr Michèle Ernoult-Lange (this Institute). The pBluescript SK$^+$ vector containing the cDNA KIAA0039 was provided by Kazusa DNA Research Institute (Japan). Immuno-pure anti-KIAA0039 antibody was obtained from rabbits inoculated with a synthetic peptide containing the sequence KQMLYDYVERKRKENS-GAQ present within the N-terminal region of the putative coding sequence of KIAA0039 (unpublished methods).

Expression and purification of recombinant *S.pombe* PCNA

The vector pEPT containing the entire *S.pombe* PCNA coding sequence (11) was digested with *Bam*HI and *Hin*dIII. Two partially complementary oligonucleotides, 5′-TATGCTTGAAGCTAGATT-TCAGCAGGCTGCTTTGTTGAAAA and 5′- AGCTTTTTCA-ACAAAGCAGCCTGCTGAAATCTAGCTTCAAGCA, were annealed together to produce 5′ and 3′ located *Nde*I and *Hin*dIII cohesive ends, respectively. The *Hin*dIII–*Bam*HI fragment containing the PCNA coding sequence, the oligonucleotide fragment and the expression vector, pET 19b, digested with *Nde*I and *Bam*HI, were ligated together to place the PCNA coding sequence downstream of the T7 *lac* promoter and in frame with the His-tag encoding region. For purification of *S.pombe* PCNA, the resulting construct was transformed into *Escherichia coli* BLR/DE3 by electroporation and one colony was used to inoculate 2 ml of LB medium containing 40 mM glucose and 100 µg/ml ampicillin. The culture was grown overnight and 1 ml was used to inoculate 100 ml of the same medium. After 3 h of growth, the culture was centrifuged as described below and the cell pellet was resuspended in the same volume of the same medium but lacking glucose and containing 1 mM ITPG. After growth overnight at 37°C, the culture was centrifuged at 3500 r.p.m. for 10 min at 4°C in a Sorvall GSA rotor. The pellet was resuspended in buffer A containing 20 mM K_2HPO_4 –HCl (pH 7.8) and 300 mM NaCl, sonicated seven times for 30 s and centrifuged for 20 min at 15 000 r.p.m. in a Sorvall SS34 rotor at 4°C. The supernatant was then mixed with 1 ml of washed Ni-NTA resin, incubated for 1 h at 4°C with rotation, and the resin was loaded into a column. The column was washed with 10 ml of buffer A containing 10% glycerol and proteins bound to the resin were eluted with a gradient of 0.01–0.5 M imidazole–HCl (pH 7.2) in 10% glycerol. Fractions containing >100 µg/ml of PCNA were frozen in liquid nitrogen and stored at –70°C until use.

Preparation of mouse FM3A cell extracts

Cell extracts from 5×10^{10} FM3A cells were prepared as described previously (12) except that the 0.4 M NaCl eluate from phosphocellulose was used for PCNA-affinity chromatography after dialysis against interaction buffer containing 30 mM HEPES (pH 7.6), 1 mM EDTA, 2 mM DTT, 20% glycerol, 50 mM NaCl and 0.01% NP-40.

Preparation of PCNA-affinity columns, affinity chromatography and glycerol gradient centrifugation

About 0.5–1 mg of recombinant *S.pombe* PCNA was dialyzed against a buffer containing 30 mM HEPES (pH 7.6), 0.2 M NaCl and 20% glycerol overnight on ice and then incubated at 4°C with 1 ml of Affi-gel 10 equilibrated in the same buffer. Coupling of PCNA to the matrix was allowed to continue for 1 h at 4°C, after which the matrix was introduced into a Bio-Rad column (0.5 cm ϕ) and equilibrated with a 20–100-fold volume of interaction buffer. The column was charged with the dialyzed 0.4 M NaCl eluate from phosphocellulose containing ~100 mg protein at a flow rate of 5 ml/h, washed with the same buffer containing 0.1 M NaCl and eluted with 10 vol each of 0.25 M NaCl and 1.5 M NaCl, and 1 ml fractions were collected. Elution with the latter buffer was allowed to proceed over 48 h in order to remove most of the bound protein. All the fractions containing ~0.3 mg of total protein were concentrated through a centricon-30 microconcentrator and centrifuged through 10–30% glycerol gradients (5 ml) in interaction buffer containing 0.2 M NaCl and protease inhibitors (12) for 13 h at 53 000 r.p.m. in a SW55 Beckman rotor at 3°C. Fractions of ~200 µl were collected from the bottom of the tube, frozen and stored in liquid N_2.

SDS–PAGE and immunoblot analysis

These techniques were done according to standard procedures (13). The silver-Stain Plus kit (Bio-Rad Laboratories) was used to stain proteins separated on 12% SDS–polyacrylamide gels. Immunocomplexes were revealed using the western blot chemi-luminescence reagent of NEN.

N-terminal amino acid sequencing

For N-terminal amino acid sequencing, all of the glycerol gradient fractions comprising either the DNA polymerase δ or DNA ligase complex were precipitated with acetone and separated on one lane of an SDS–polyacrylamide gel. After brief staining of the gel with Coomassie brilliant blue G-250, bands of interest were excised from the gel and subjected to ‘in-gel’ CnBr digestion as described previously (14). After digestion, the resulting peptides were migrated on a Tricine–SDS–polyacrylamide gel (15), transferred to a PVDF membrane (Applied Biosystems), stained with Coomassie brilliant blue G-250 and then well separated bands were cut out from the membrane and subjected to Edman degradation at the University of Newcastle Molecular Biology Unit, Newcastle Upon Tyne, UK.

RNA 5′ extension analysis

Total RNA (HeLa) isolated according to the methods of Chomczynski and Sacchi (16) and Sambrook (13) were extended using a 30mer oligonucleotide complementary to nucleotides 60–90 of the cDNA clone KIAA0039 by standard procedures (2).

Expression of the protein encoded by the KIAA0039 cDNA clone

pBluescript SK+ containing the 3430 bp cDNA fragment with a 34 bp poly(A) stretch was digested with *Nco*I and *Bgl*II, which liberated a fragment of 1490 bp containing the KIAA0039 coding sequence starting just upstream from the first ATG codon. After blunt-ending with the Klenow fragment, the fragment was inserted into the blunt-ended *Xho*I site of pET 19b. This placed the putative coding sequence downstream of the T7 *lac* promoter and in frame with the His-tag coding region.

Escherichia coli BL21 was transformed with the recombinant plasmid containing the insert in the correct orientation by electroporation and the protein was expressed using standard procedures.

DNA replication and DNA ligation assays

DNA replication assays were performed as described previously (17). DNA ligation assays were performed in a volume of 20 µl containing 50 mM HEPES–KOH (pH 7.7), 5 mM DTT, 7 mM $MgCl_2$, 50 µg/ml BSA, 1 mM ATP and 50 µg of DNase I-nicked pUC19 DNA at 37°C for 1 h. At the end of the reaction, 5 µl of 5× loading dye containing 50% glycerol, 1% SDS, 0.25% bromophenol blue and 0.25% xylene cyanol was added and 10 µl were loaded onto a 1% agarose gel in TAE buffer containing 0.5 µg/ml ethidium bromide. The appearance of a band migrating to the position of supercoiled pUC19 DNA was taken as evidence of DNA ligase activity.

RESULTS

PCNA-affinity chromatography

We used recombinant *S.pombe* PCNA to prepare the affinity columns as this protein could be obtained in large quantities in our laboratory and was shown previously to be as efficient as human PCNA for the stimulation of calf-thymus polymerase δ activity (18). Moreover, PCNA proteins from a number of diverse sources display significant structural conservation (8). As a source of mouse DNA replication enzymes, we chose to use a 0.4 M phosphocellulose fraction of mouse FM3A cells as this fraction was used previously as starting material for the purification of DNA polymerase δ (19). In addition, we hoped that the use of a partially purified fraction would reduce the number of proteins binding to the PCNA column and, thereby, render the separation and identification of the bound proteins easier. In Figure 1a we show a silver-stained polyacrylamide gel of the proteins eluted at two salt concentrations, 0.25 and 1.5 M NaCl, from a PCNA-affinity column charged with ~100 mg of protein from the dialyzed 0.4 M NaCl phosphocellulose fraction. As can be seen, all the fractions, with the exception of the first fraction, contained proteins of identical mass, indicating that most of these proteins had similar affinity for PCNA. When we measured the DNA polymerase activity of the fractions, all of them displayed strong incorporation of [^{3}H TTP] but only in the presence of *S.pombe* PCNA (Fig. 1b) or calf-thymus PCNA (data not shown). Thus, we considered that the column had effectively retained DNA polymerase δ, even though the silver-stained gel displayed many more molecular species than the two subunits currently known to constitute mammalian DNA polymerase δ.

Glycerol gradient centrifugation

In order to determine the relationship between the proteins retained on the PCNA-affinity column, all the fractions except for fractions 1 and 2 were mixed together, concentrated and centrifuged on 10–30% glycerol gradients. SDS–PAGE of the different fractions from the gradient showed that the proteins had been separated into three groups (Fig. 2a): a slow sedimenting group of proteins made up of essentially three molecular species of 51, 44 and 35 kDa (fraction 23), a complex of intermediate mass made up of 125, 66, 53, 51 and 37 kDa species (fraction 19) and a fast sedimenting complex made up of 132, 125, 100, 66, 53, 42 and 38 kDa species (fraction 11). The peak level of PCNA dependent DNA replication activity was found to coincide with fraction 12 (Fig. 2b) and DNA ligase activity was detected in fractions 16–24 (Fig. 2c). Some of the proteins were identified by

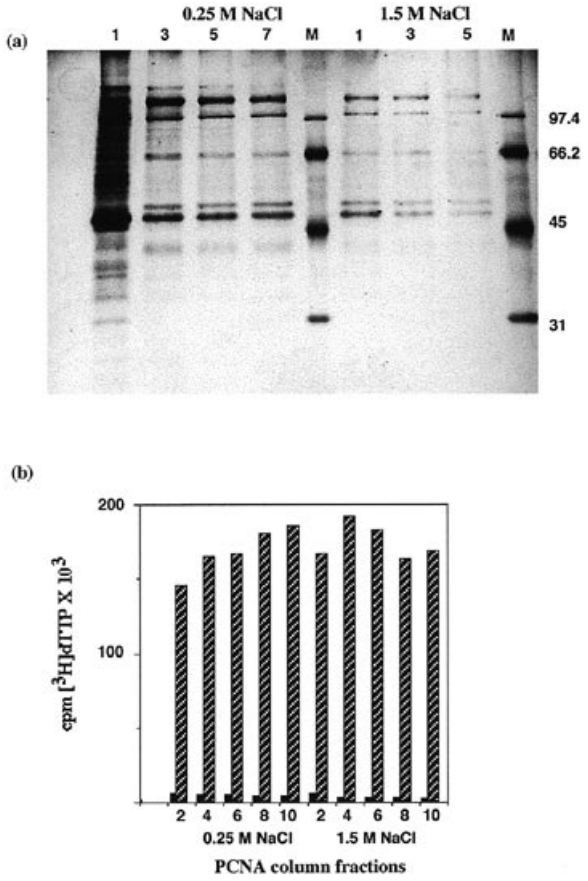

Figure 1. SDS–PAGE and DNA polymerase activity of the fractions eluted from the PCNA column. (**a**) Silver-stained SDS–PAGE gel of the different fractions eluted at two salt concentrations, 0.25 and 1.5 M NaCl. The molecular weights of the protein standards are indicated on the right (M). (**b**) DNA polymerase activity. Hatched bars, presence of PCNA; black bars, absence of PCNA.

immunoblot analyses using specific antibodies (Fig. 3b). The 35 kDa band found mostly in fraction 23 (Fig. 3a) reacted with an antibody directed against the PSTAIRE sequence found in cdc2 (cdk1), cdk2 and cdk3. The 125 and 53 kDa species in fractions 19 and 21 turned out to be DNA ligase 1 and the small subunit of DNA polymerase δ, respectively. In the fast sedimenting complex (fraction 11), the 125 kDa protein was identified as the large subunit of DNA polymerase δ, the 53 kDa band as the small subunit of DNA polymerase δ and the 42 kDa subunit as being either the p40 or p37 subunit of RF-C. Fractions were negative for proteins that cross-react with antibodies against NDH II (DNA helicase), DNA polymerase ε, Topoisomerase I, RPA protein, cdk4, cdk5 and PCNA (data not shown).

N-terminal sequencing

With the exception of the two smallest bands in the DNA polymerase δ and DNA ligase fractions, all of the unknown proteins yielded amino acid sequence data which allowed unequivocal identification of either the corresponding gene or cDNA (Table 1). Thus, the 132 and 100 kDa proteins migrating above and below the 125 kDa subunit of polymerase δ were found to be the products of the genes MSH6 and MSH2, which are

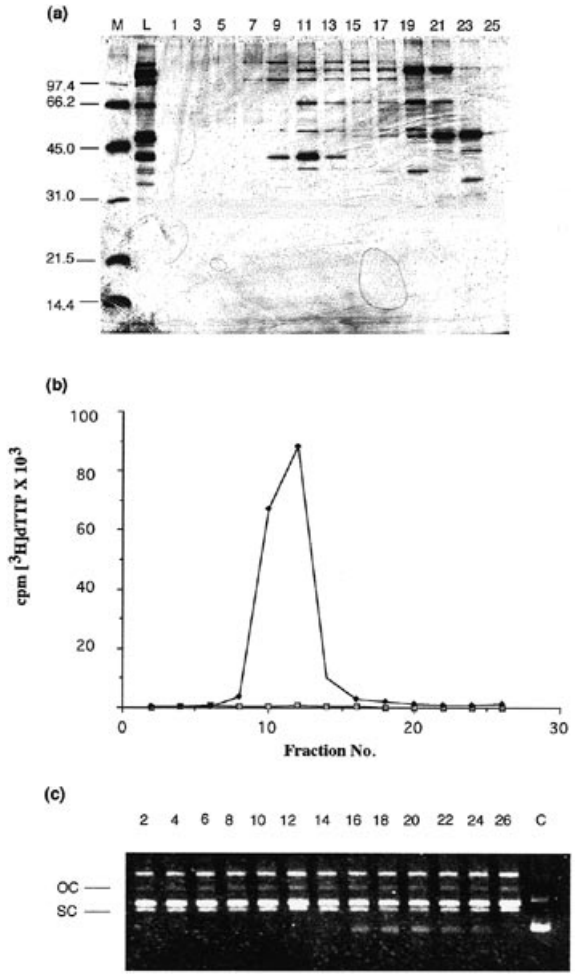

Figure 2. Glycerol gradient centrifugation of fractions from the PCNA column: SDS–PAGE and DNA polymerase activity of the fractions. (**a**) Silver-stained SDS–PAGE gel of 100 µl of every two fractions from the gradient. Lane L, the concentrated PCNA affinity fraction used to charge the gradient. The molecular weights of the protein standards are indicated on the left (M). Fractions are numbered from the bottom of the gradient. (**b**) DNA polymerase activity for 0.5 µl of every two fractions in the presence (◆) and absence (□) of PCNA. (**c**) DNA ligase activity for 1 µl of every two fractions. OC, open circular; SC, supercoiled; lane C, control supercoiled DNA.

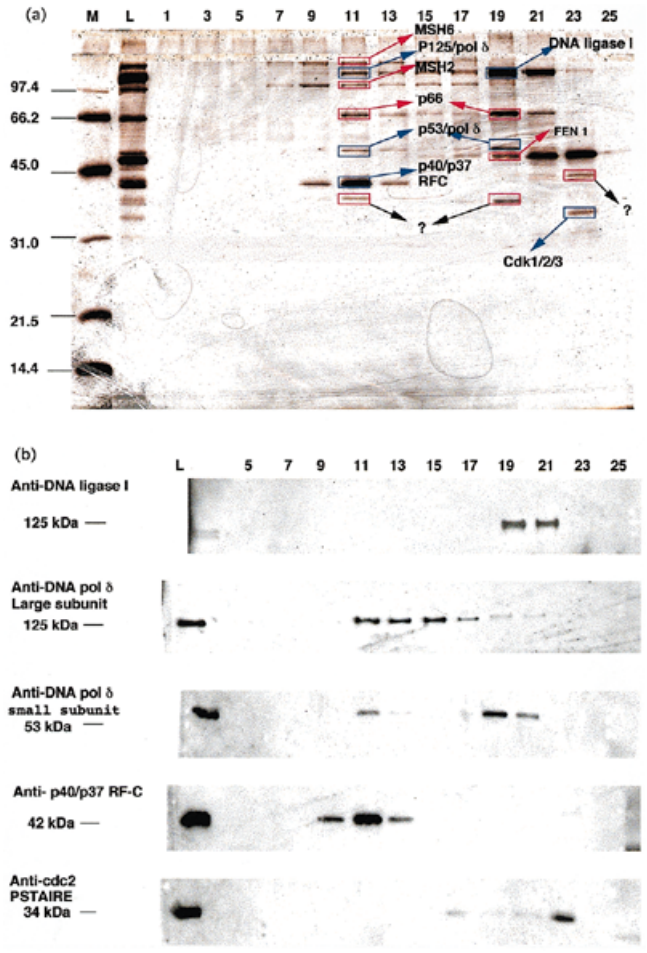

Figure 3. Identity of the proteins as determined by western blot and N-terminal amino acid analysis. (**a**) Same gel as shown in Figure 2 except that the identities of the different bands are indicated. Bands enclosed in red boxes and blue boxes indicate proteins that were identified by internal N-terminal sequencing and western blot analysis, respectively. A question mark indicates the proteins that remain to be identified. (**b**) Results of western blot analysis using a variety of specific antibodies whose identities are indicated on the left.

known to form a heterodimer involved in mismatch repair (20). The protein migrating with an apparent molecular mass of 51 kDa was revealed to be FEM±1. The 66 kDa protein was found to be coded by the cDNA KIAA0039 originally identified by the human large cDNA isolation program of Kazusa DNA Research Institute, Japan (21). The identities of the bands identified by specific antibodies and N-terminal amino acid sequencing are presented graphically in Figure 3a.

KIAA0039

The nucleotide sequence of the KIAA0039 cDNA (GeneBank accession number D26018) contained an open reading frame starting at nucleotide 1 and finishing at nucleotide 1474. However, there was no stop codon at the beginning of the 5′ sequence to allow unequivocal identification of the initiating ATG codon. Also, the entire open reading frame would code for a protein of only 56 kDa, which is much smaller than the 66 kDa mass of the protein estimated from the SDS–polyacrylamide gel. Despite repeated efforts, we failed to isolate cDNA clones larger than the one already present in the sequence databases (data not shown). In order to determine the full length of the 5′ end of the RNA, we carried out 5′ terminal extension of total RNA isolated from human HeLa cells using as primer an oligonucleotide complementary to nucleotides 60–90 of the KIAA0039 cDNA. A major product with a size of 111 nucleotides was detected, indicating that the missing 5′ sequence could be no longer than 21 nucleotides (Fig. 4a). An ATG codon positioned at nucleotide 76 completely obeyed Kozak's rule (22) for translation initiation. When the sequence starting at position 74 and ending at 1565 was inserted into pET 19b and expressed along with the His-tag under the control of the *lac* promoter, a protein with a mass almost identical to that of mouse p66 protein was expressed and detected by antibodies directed against an N-terminal sequence of KIAA0039 after ITPG induction (Fig. 4b). The slightly larger size of the expressed protein in *E.coli* is, in all probability, due to

the added mass of the 10-amino acid His-tag. Antibody detection of a p66 band before the addition of ITPG (Fig. 4b, lane 2) and the appearance of bands smaller than p66 after ITPG addition (Fig. 4b, lanes 3–6) probably result from leakiness of the *lac* promoter and proteolysis, respectively. Thus, it seems that the cDNA clone KIAA0039 contains all the coding sequence for p66 starting at nucleotide position 76 and codes for a protein with a calculated mass of 51.4 kDa.

Table 1. N-terminal amino acid sequences and corresponding gene or cDNA of internal peptides generated by CnBr degradation

Peptide band	Amino acid sequence	Gene/ cDNA	Position
135C	C?–K–V–S–G–L–L–E–E–V	MSH6	866–875
100A	K–Y?–Q–V–K–K–G–V–C?–D	MSH2	814–823
100B	D–R–N–R–I–E–E–R–L–N	MSH2	352–361
66A	N–K–L?–K–V–N–L–D–S–E	KIAA0039	268–277
66B	L(P)–K–D(T)–E(S)–P(G)–P–L(P)–V(F) N(K)–K(R) mixture of two peptides	KIAA0039	66–75 and 133–142
50B	R–H–L–T–A–S–E–A–K–K	FEN-1	192–201
50C	X–G–E–K–Q–F–S–E–E–R	FEN-1	311–320

The BLAST program at http://www.infobiogen.fr/services/menuserv.html was used to search for homology in Genebank and Swissprot databases. A question mark indicates a doubt about the identity of the amino acid residue from the spectrograph and an X indicates that the amino acid could not be determined.

Homology to *S.pombe* Cdc27 and p66 protein motifs

An initial search of the sequence databases failed to reveal any striking homology to other proteins except to acrosin but this involved only the proline-rich motif (data not shown). However, close examination of the C-terminal domain revealed the presence of a well conserved PCNA binding motif (Fig. 5) (2). When the C-terminal sequences of *S.pombe* Cdc27, *Saccharomyces cerevisiae* Pol32 and that of p66 beyond the proline-rich region were aligned, significant homology with 32.4% amino acid identity was observed between Cdc27 and p66 (Fig. 5). Overall homology between both proteins was not >23%. The significance of the C-terminal homology was estimated using the PRDF program (http://www.infobiogen.fr/services/analyseq) which compares the degree of homology between a test sequence and a shuffled sequence (23). The optimal scores (p = 105 for Cdc27/p66, p = 59 for pol32/p66 and p = 51 for pol32/Cdc27) were calculated to occur in 100 sequences on a random basis 0.005 times for Cdc27/p66, 7 times for pol32/p66 and 8 times for Cdc27/pol32. Thus, p66 is much closer to Cdc27 than to the third subunit of *S.cerevisiae* polymerase δ, pol32, and displays a much higher degree of homology towards Cdc27 than does pol32 (Fig. 5).

Besides the conserved PCNA binding domain, the only other significant domains detected by PROSITE were a proline-rich domain (amino acids 374–395) and a bi-partite nuclear localization signal (amino acids 310–324) located on the C-terminal side of the protein.

DISCUSSION

Currently, mammalian DNA polymerase δ is considered to be a functionally active heterodimer composed of a catalytic subunit

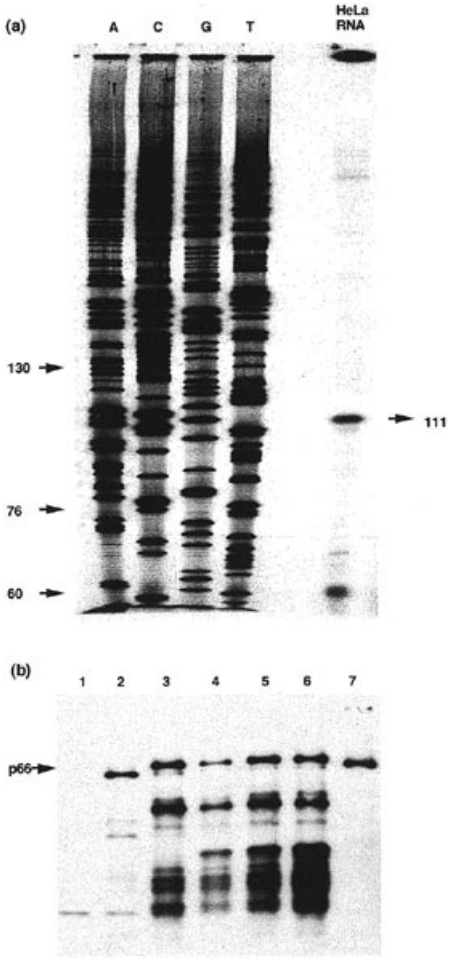

Figure 4. RNA 5′ extension analysis and expression of the KIAA0039 cDNA in *E.coli*. (**a**) Autoradiograph showing 5′ extension analysis of total HeLa RNA. On the left: sizes in nucleotides of bands on the DNA sequence ladder which was derived from the transcription initiation site of the human MZF-1 gene (37). The HeLa RNA lane is indicated. (**b**) Western blot analysis of the expression of the KIAA0039 cDNA sequence in *E.coli*. Lane 1, the strain without the expression vector; lane 2, with the expression vector but without ITPG induction; lane 3, induction with ITPG for 10 min; lane 4, 20 min induction; lane 5, 30 min induction; lane 6, 1 h induction; lane 7, an aliquot of the eluate from the PCNA-affinity column containing mouse p66 protein. Lanes 3–6 contained 10 times less protein than lanes 1 and 2.

of 125 kDa and an accessory subunit of 48 kDa (24). This is in contrast to the *S.cerevisiae* enzyme which is now known to be made up of three subunits of 125 kDa (*POL*3), 58 kDa (*POL*31) and 55 kDa (*POL*32) (25) and the *S.pombe* enzyme which is made up of at least four subunits of 125 kDa (*pol3*$^{+}$), 54 kDa (*cdc*27), 51 kDa (*cdc*1) and 22 kDa (*cdm*1^{+}) (26). Recently, the *POL*32 gene was shown to be the functional equivalent of *cdc*27 on the basis of limited sequence homology and induced structural and enzymatic modifications of the two-subunit (Pol3 and Pol31) polymerase complex (25,27). However, Pol32 is neither essential for yeast DNA polymerase activity *in vitro* nor cell viability but reduces the amount of PCNA needed for processivity and alleviates pausing by the replication complex. In addition, Pol32 induces dimerization of the heterodimeric Pol3–Pol31 core, suggesting that polymerase δ functions as a dimer for both leading

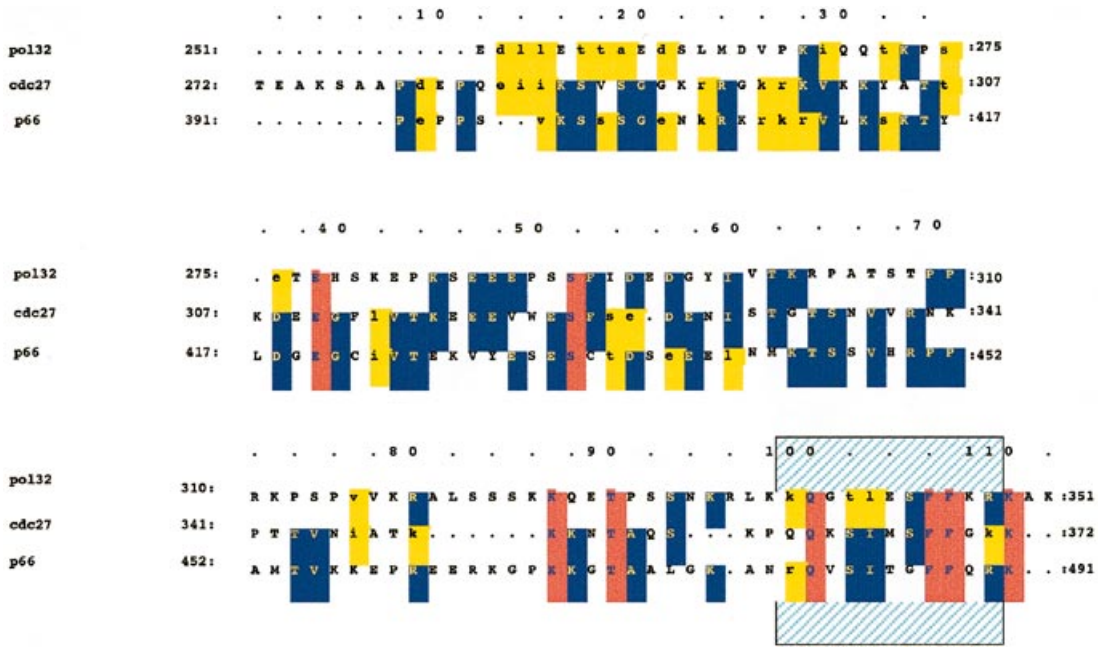

Figure 5. Amino acid sequence alignments A clustal alignment of the C-terminal portions of Pol32, Cdc27 and p66. Yellow shading indicates amino acids of the same group shared by two or more sequences. Blue shading signifies identical amino acids shared by at least two sequences. Orange shading signifies identical amino acids shared by all three sequences. Blue-striped box, the extreme N-terminal sequences of Pol32, Cdc27 and p66 comprising the PCNA consensus motif as defined by Warbrick (2).

and lagging strand synthesis at the replication fork (27). Although similar studies have not been carried out with Cdc27, the fact that cdc27 deletion mutants are unviable (28) would seem to suggest that this subunit is more critical for *S.pombe* DNA replication.

The structure of mammalian DNA polymerase δ remains enigmatic. Unlike its yeast counterparts, a third subunit has never been observed. Here we show that a DNA polymerase δ with five subunits can be isolated from mouse FM3A cells by a novel strategy involving PCNA-affinity chromatography. We propose that one of these, p66, is the counterpart of Cdc27 and the product of the KIAA0039 cDNA on the basis of the following observations. (i) The p66 protein was retained by PCNA-affinity columns, and sedimented with active DNA polymerase δ during glycerol gradient centrifugation (Fig. 2). (ii) Three internal amino acid sequences of p66 are identical to amino acid sequences found within the translated KIAA0039 sequence (Table 1). (iii) Antibodies against a synthetic peptide containing a sequence found within the N-terminal coding sequence of the human cDNA KIAA0039 reacted with the mouse p66 protein (Fig. 4b). (iv) The product of the KIAA0039 cDNA expressed from the first ATG codon migrated with nearly the same mobility as the mouse p66 protein (Fig. 4b, lane 7). (v) The C-terminal sequence of KIAA0039 displayed significant homology to that of Cdc27 and this homology was greater than the homology between Cdc27 and Pol32 (Fig. 5). (vi) The C-terminal sequence of KIAA0039 contains a canonical PCNA binding sequence like Cdc 27 and Pol 32 (Fig. 5). (vii) The product of the KIAA0039 cDNA displays anomalous migration on SDS–polyacrylamide gels (Fig. 3a) like Cdc27 and Pol32 (29). Pol32, with a calculated molecular mass of 40.3 kDa, migrates as a 55 kDa protein on SDS–polyacrylamide gels (25), Cdc27 with a calculated molecular mass of 42.2 kDa migrates as a 54 kDa protein (30) and p66 with a calculated molecular mass of 51.4 kDa migrates as a 66 kDa protein (Fig. 3a). In addition, all three proteins are rich in charged amino acids. Two reasons can be put forward to explain previous failure to detect p66 in purified DNA polymerase δ preparations from calf-thymus. (i) The p66 protein is present but remains undetectable because this subunit is refractory to certain silver-staining reagents. Although we experienced no difficulties using the silver-stain plus reagent of Bio-Rad Laboratories, Pol32 could only be detected reproducibly using the silver-staining reagent of Morrissey (25). (ii) More likely, protein p66, like Pol32, is not absolutely necessary for polymerase activity, and may, therefore, have been lost during lengthy purification procedures which rely solely on the measurement of incorporation of radioactive nucleosides using poly(dA)-oligo(dT) or activated calf thymus DNA as template. Moreover, recombinant human DNA polymerase δ expressed and purified as a two-subunit complex from baculovirus-infected cells was entirely active for DNA replication stimulated by PCNA (31). A more detailed biochemical characterization of the contribution of p66 to DNA polymerase δ activity will be required to resolve this issue.

The polymerase δ fraction also contained two other proteins, one of which reacted with both antibodies against the p40 and p37 subunits of RF-C. However, as these two antibodies were polyclonal and as the small subunits of RF-C all share significant amino acid similarity, the band could be composed of one or more of any of the small subunits of RF-C, all of which migrate to similar positions on SDS–PAGE gels (32). Indeed, the intensity of the 42 kDa band is in accord with the latter possibility. Although RF-C containing a truncated p140 subunit was recently shown to dissociate from DNA after loading of PCNA at the primer terminus or after polymerase δ assembly (33), complexes containing polymerase α, polymerase δ and RF-C have been

reported previously (34) and ulterior interactions between polymerase δ and the small subunits of RF-C necessary for the dissociation of this polymerase during discontinuous Okazaki fragment synthesis or at the end of DNA replication cannot be ruled out. The smallest component of the DNA polymerase δ complex, with an apparent molecular weight of 38 kDa, remains to be identified, as does the small component of the DNA ligase complex. The presence of MSH2 and MSH6 among the proteins binding to the PCNA column is not surprising in view of MSH2 affinity for PCNA and heterodimerization with MSH6 (20). Although it cannot be completely excluded that these proteins are part of the DNA polymerase δ complex, their presence in neighboring fractions nearly devoid of DNA polymerase δ suggests that they sedimented independently on the gradient (Fig. 3a).

Remarkably, two of the proteins sedimenting with DNA ligase 1 turned out to be the p66 (on the basis of the CnBr peptide map and reactivity towards p66 antibodies; data not shown) and p48 subunits of polymerase δ, suggesting that these subunits also contribute to DNA ligase 1 activity (Fig. 3a). However, DNA ligase 1 has already been shown to contain a binding site for PCNA, and as with purified DNA ligase 1 (29), we were unable to demonstrate enhanced DNA ligase activity using this fraction in the presence of PCNA (data not shown). Perhaps novel reaction conditions will have to be established before the contribution that these accessory proteins make to DNA ligase 1 activity can be assessed. The presence of FEN-1 among the proteins binding to the PCNA column confirms FEN-1 interaction with PCNA (35). Recently, PCNA-affinity chromatography and western blot analysis were used to identify a large number of replication and cell cycle proteins having affinity for PCNA. Some of the proteins identified such as the p37 and p40 subunits of RF-C and DNA polymerase δ are the same as those described here. However, others such as RPA, NDH II and DNA topoisomerase 1 were not identified in our study and their study failed to identify DNA ligase 1 (36). This may reflect the fact that we used a 0.4 M phosphocellulose fraction which contains only a small fraction (~10%) of total cellular proteins. Thus, PCNA-binding proteins that fail to bind to phosphocellulose would not have been identified in our study.

We hope to identify other novel PCNA-interacting proteins in the remaining phosphocellulose fractions and to eventually examine the dynamic interactions taking place between PCNA and other proteins during the mammalian cell cycle using this technique.

ACKNOWLEDGEMENTS

We wish to thank Drs Michèle Ernoult-Lange and Michel Kress for their continuing help and support during the analysis of the gene structure of the KIAA0039 clone. This work was supported in part by grant 6704 from the Association pour la Recherche sur le Cancer (ARC) and by contract FMRX-CT97-0125 of the TMR Programme of the European Community.

REFERENCES

1 Cox,L.S. (1997) *Trends Cell Biol.*, **7**, 493–498.
2 Warbrick,E. (1998) *Bioessays*, **20**, 195–199.
3 Flores-Rozas,H., Kelman,Z., Dean,F.B., Pan,Z.-Q., Harper,J.W., Elledge,S.J., O'Donnell,M. and Hurwitz,J. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 8655–8659.
4 Smith,M.L., Chen,I.T., Zhan,Q., Bae,I., Chen,C.Y., Gilmer,T.M., Kastan,M.B., O'Connor,P.M. and Fornace,A.J.,Jr (1994) *Science*, **266**, 1376–1380.
5 Vairapandi,M., Balliet,A.G., Fornace,A.J.,Jr, Hoffman,B. and Liebermann,D.A. (1996) *Oncogene*, **12**, 2579–2594.
6 Matsuoka,S., Yamaguchi,M. and Matsukage,A. (1994) *J. Biol. Chem.*, **269**, 11030–11036.
7 Zhang,H., Xiong,Y. and Beach,D. (1993) *Mol. Biol. Cell*, **4**, 897–906.
8 Kelman,Z. (1997) *Oncogene*, **14**, 629–640.
9 Hindges,R. and Hübscher,U. (1997) *Genomics*, **44**, 45–51.
10 Elder,R.H. and Rossignol,J.-M. (1990) *Biochemistry*, **29**, 6009–6017.
11 Piard,K., Baldacci,G. and Tratner,I. (1998) *Nucleic Acids Res.*, **26**, 2598–2605.
12 Hughes,P. and Baldacci,G. (1997) *Nucleic Acids Res.*, **25**, 3881–3888.
13 Sambrook,J., Fritsch,E. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14 Córdoba,O.L., Linskens,S.B., Dacci,E. and Santomé,J.A. (1997) *J. Biochem. Biophys. Methods*, **35**, 1–10.
15 Schägger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 368–379.
16 Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
17 Tratner,I., Piard,K., Grenon,M., Perderiset,M. and Baldacci,G. (1997) *Biochem. Biophys. Res. Commun*, **231**, 321–328.
18 Arroyo,M.P., Downey,K.M., So,A.G. and Wang,T.S.F. (1996) *J. Biol. Chem.*, **271**, 15971–15980.
19 Melendy,T. and Stillman,B. (1991) *J. Biol. Chem.*, **266**, 1942–1949.
20 Iaccarino,I., Palombo,F., Drummond,J., Totty,N.F., Hsuan,J.J., Modrich,P. and Jiricny,J. (1996) *Curr. Biol.*, **6**, 484–486.
21 Nomura,N., Miyajima,N., Sazuka,T., Tanaka,A., Kawarabayashi,Y., Sato,S., Nagase,T., Seki,N., Ishikawa,K.-I. and Tabata,S. (1994) *DNA Res.*, **1**, 47–56.
22 Kozak,M. (1986) *Cell*, **44**, 283–292.
23 Pearson,W.R. and Lipman,D.J. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 2444–2448.
24 Lee,M.Y.W.T., Tan,C.-K., Downey,K.M. and So,A.G. (1984) *Biochemistry*, **23**, 1906–1913.
25 Gerik,K.J., Li,X., Pautz,A. and Burgers,P.M.J. (1998) *J. Biol. Chem.*, **273**, 19747–19755.
26 Zuo,S., Gibbs,E., Kelman,Z., Wang,T.S.-F., O'Donnell,M., MacNeill,S.A. and Hurwitz,J. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 11244–11249.
27 Burgers,P.M.J. and Gerik,K.J. (1998) *J. Biol. Chem.*, **273**, 19756–19762.
28 MacNeill,S.A., Moreno,S., Reynolds,N., Nurse,P. and Fantes,P.A. (1996) *EMBO J.*, **15**, 4613–4628.
29 Levin,D.S., Bai,W., Yao,N., O'Donnell,M. and Tomkinson,A.E. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 12863–12868.
30 Hughes,D.A., MacNeill,S.A. and Fantes,P.A. (1992) *Mol. Gen. Genet.*, **231**, 401–410.
31 Zhou,J.-Q., He,H., Tan,C.-K., Downey,K.M. and So,A.G. (1997) *Nucleic Acids Res.*, **25**, 1094–1099.
32 Lee,S.-H., Kwong,A.D., Pan,Z.-Q. and Hurwitz,J. (1991) *J. Biol. Chem.*, **266**, 594–602.
33 Podust,V.N., Tiwari,N., Stephan,S. and Fanning,E. (1998) *J. Biol. Chem.*, **273**, 31992–31999.
34 Maga,G. and Hübscher,U. (1996) *Biochemistry*, **35**, 5764–5777.
35 Li,X., Li,J., Harrington,J., Lieber,M.R. and Burgers,P.M. (1995) *J. Biol. Chem.*, **270**, 22109–22112.
36 Loor,G., Zhang,S.J., Zhang,P., Toomey,N.L. and Lee,M.Y. (1997) *Nucleic Acids Res.*, **25**, 5041–5046.
37 Hui,P., Guo,X. and Bradford,G. (1995) *Biochemistry*, **34**, 16493–16502.