

Generation and Characterization of a Rat Monoclonal Antibody Specific for PCNA

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) is a homotrimeric ring-shaped protein that encircles the DNA and acts as a stationary loading platform for multiple, transiently interacting partners participating in various DNA transactions. This essential cellular component, originally characterized as a nuclear antigen of dividing cells, is evolutionary highly conserved from yeast to human. Within the eukaryotic cell, PCNA serves as a processivity factor for DNA polymerase δ and plays a key role in DNA replication, repair, cell cycle regulation, and post-replicative transactions like DNA methylation and chromatin remodelling. All these cellular processes are regulated by a complex network comprising cell cycle dependent changes in expression levels, dynamics, interactions, and localization of PCNA. Here we report the generation and characterization of the first rat monoclonal antibody (MAb) against human PCNA, designated as PCNA 16D10. We demonstrated that PCNA 16D10 is a highly affine and specific MAb suited for ELISA, immunoblotting, immunoprecipitation, and immunofluorescence. The characteristic punctate staining of S phase cells allows the identification of proliferating cells and the monitoring of cell cycle progression.

INTRODUCTION

PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) was originally characterized as a processivity factor for the eukaryotic DNA polymerase δ and belongs to the family of evolutionary highly conserved DNA sliding β clamps.^(1–3) Crystallographic studies revealed that the PCNA clamp constitutes a highly symmetric assembly of three identical head-to-tail arranged monomers, each comprising two similar domains.^(4–6) The inner layer of the homotrimeric ring is composed of positively charged α -helices mediating the association with the DNA, whereas the surface is organized in β -sheets. This three-dimensional structure connects the PCNA ring topologically to the DNA, besides maintaining the ability to slide along the double helix.

The PCNA clamp serves as a stationary loading platform for numerous interacting partners that rapidly and transiently exchange in a mutually exclusive manner.^(7–10) These PCNA

interacting proteins can be classified in three major categories—namely, DNA replication/repair, cell cycle control, and chromatin regulation/transcription.⁽¹¹⁾

The DNA replication machinery is one example for the dynamic complexity in the spatio-temporal interplay of PCNA with multiple interacting proteins. During the replication process, a large number of DNA replication factors like Pol δ , Lig1, FEN1, CAF-1, and Cyclin A interact with PCNA at overlapping binding sites but in highly ordered chronology.^(12–17) In addition, PCNA acts as loading platform recruiting repair factors to sites of DNA damage.^(18,19) Beside presenting a sliding platform for multiple partners and thus facilitating the interaction with DNA, PCNA is capable of stimulating the catalytic efficiency of several enzymes like Flap endonuclease-1 (FEN1) through structural rearrangements caused by direct interaction.^(20,21)

In addition, PCNA serves as a proliferation marker and is therefore an interesting antigen for diagnostic purposes, espe-

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cially in the prognosis of tumor development.⁽²²⁾ Changes in the expression level of PCNA are directly related to the malignancy of various tumors.^(23,24)

So far several poly- and monoclonal antibodies were raised against PCNA.⁽²⁵⁾ To extend the range of applications, especially to enable double staining with monoclonal antibodies, we generated the first rat monoclonal antibody against human PCNA. We could demonstrate the suitability of this highly affine and specific antibody for ELISA, immunoblotting, immunoprecipitation, and immunofluorescence in mouse and human cells.

MATERIALS AND METHODS

Cell lines

Mouse C2C12 myoblasts, human EBNA cells, human HeLa cells, human SHEP neoblastoma cells, rat L6E9 cells, and hamster BHK cells were cultured in DMEM containing 50 μ g/mL gentamicin supplemented with 20% and 10% FCS, respectively.

Expression and purification of human PCNA (antigen preparation)

The human full-length PCNA with an N-terminal His₆-tag was cloned into the pRSET bacterial expression vector and expressed in BL21(DE3) cells (Novagen, Darmstadt, Germany). His-tagged human PCNA was purified with the Talon Superflow Metal Affinity Resin system (Clontec, Saint Germain, France) under native conditions. After freezing for 1 h at -20°C the cell pellet of a 500 mL bacterial culture was resuspended in 20 mL Talon extraction buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole [pH 8.0]) and homogenized for 10 min. The lysate was cleared by centrifugation (12,000 g, 30 min, 4°C), and the supernatant was mixed with 3 mL of Talon Superflow Metal Affinity resin, pre-equilibrated in Talon extraction buffer. After incubation at 4°C for 2 h, the beads were washed with 50 mL Talon extraction buffer, and the PCNA protein was eluted by adding 150 mM imidazole to Talon extraction buffer. Elution fractions were dialyzed against 1x PBS, and the purity of the eluted fractions were analyzed by SDS-PAGE followed by Coomassie staining. The protein concentration was determined by photometrical measurement at 280 nm.

Immunizations, generation of hybridomas, and ELISA screening

Approximately 50 μ g of PCNA-His-tagged protein were injected both intraperitoneally (i.p.) and subcutaneously (s.c.) into Lou/C rats using CPG2006 (TIB MOLBIOL) as adjuvant. After 8 weeks, a boost was given i.p. and s.c. 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed using polyethylene glycol 1500 (PEG 1500, Roche Mannheim, Germany). After fusion, the cells were plated in 96-well plates using RPMI1640 with 20% fetal calf serum, penicillin/streptomycin, and pyruvate, non-essential amino acids (PAA) supplemented by aminopterin (Sigma, St. Louis, MO). Hybridoma supernatants were tested in a solid-phase immunoassay. PCNA-His-tagged proteins were coated overnight at a concentration of 3 μ g/mL

in 0.1 M sodium carbonate buffer (pH 9.6). After blocking with non-fat milk (Frema, Neuform Zarrentin, Germany), hybridoma supernatants were added. Bound rat MAbs were detected with a cocktail of biotinylated mouse MAbs against the rat IgG heavy chains, thus avoiding IgM MAbs (α -IgG1, α -IgG2a, α -IgG2b [ATCC, Manassas, VA] and α -IgG2c [Ascension, Munich, Germany]). The biotinylated MAbs were visualized with peroxidase-labeled Avidin (Alexis, San Diego, CA) and o-phenylenediamine as chromogen in the peroxidase reaction. PCNA 16D10 (rat IgG2b) was stably subcloned and is characterized in this study.

Peptide array synthesis and binding analyses (epitope mapping)

Cellulose membrane-bound peptides were automatically prepared according to standard Spot synthesis protocols⁽²⁶⁾ using a Spot synthesizer (Intavis, Cologne, Germany). Sequence files for the peptide array were generated with LISA 1.71 software. Peptides were synthesized on an N-modified cellulose-aminohydroxypropyl ether membrane (N-CAPE).⁽²⁷⁾

The cellulose-bound peptides were pre-washed once with EtOH for 10 min, with Tris-buffered saline (TBS, [pH 8.0]) three times for 10 min each, and then blocked overnight with blocking buffer (blocking reagent [Sigma-Aldrich] in TBS [pH 8.0], containing 5% sucrose). The membranes were incubated with the anti-PCNA MAb (1:100 in blocking buffer) for 2.5 h at room temperature. The interactions of the MAb with the peptides were detected by anti-rat-HRP (Jackson ImmunoResearch Europe, Suffolk, United Kingdom) (1:10,000 in blocking buffer) for 1.5 h at room temperature. To remove excess of antibody the membrane was washed with TBS (pH 8.0) (3×10 min) after each antibody incubation step. An Uptilight HRP blot chemiluminescent substrate (Uptima-Interchim, Montlucon, France) was applied for detection using a LumiImager (Roche, Mannheim, Germany).

Western blot analysis

For immunoblot analysis EBNA, HeLa, C2C12, L6E9 and BHK cells were harvested in ice cold 1x PBS, washed twice, and subsequently lysed in 60 μ L lysis buffer (1x PBS, DNaseI [1.2 mg/mL], 2 mM PMSF, 5 mM MgCl) for 15 min. Finally the cell extracts were homogenized in SDS-containing loading buffer. A serial dilution ($2.5\text{--}50 \times 10^3$ cells/lane) was separated on a 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). After blocking the membrane for 30 min with 3% milk in PBS containing 0.075% Tween-20, 0.1 μ g of PCNA 16D10 was added and incubation continued for 1 h at room temperature. After washing with PBS containing 0.1% Tween-20, the blots were incubated with a secondary anti-rat-HRP (Jackson ImmunoResearch Europe). Immunoreactive bands were visualized with ECL Western Blot Detection Kit (GE Healthcare, Freiburg, Germany).

Immunoprecipitation

For immunoprecipitation, $\sim 1 \times 10^7$ cells were harvested in ice cold 1x PBS, washed twice, and subsequently homogenized in 200 μ L lysis buffer (20 mM Tris/HCl [pH 7.5], 150 mM

NaCl, 0.5 mM EDTA, 2 mM PMSF, 0.5% NP40). After a centrifugation step (10 min, 20,000 g, 4°C), the supernatant was adjusted with dilution buffer (20 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF) to 500 μ L. Fifty μ L were mixed with SDS-containing sample buffer, referred to as input (I). For immunoprecipitation, 2 μ g of the PCNA antibody was added and incubated 1 h at room temperature. For pull down of immunocomplexes, 25 μ L of equilibrated protein G agarose beads (GE Healthcare) were added and incubation continued for 1 h. After centrifugation (2 min, 5000 g, 4°C), the supernatant was removed and 50 μ L were collected, referred to as non-bound/or flowthrough (F). The beads were washed twice with 1 mL dilution buffer containing 300 mM NaCl. After the last washing step, the beads were resuspended in 2x SDS-containing sample buffer and boiled for 10 min at 95°C.

For immunoblot analysis, 1% of the input and the flowthrough, as well as 10% of the soluble supernatants, were separated on 12% SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and subjected to immunoblot analysis as described.

Immunofluorescence

For immunostaining, C2C12 cells were grown on cover slips. Cells were fixed with 3.7% formaldehyde in PBS and subsequently permeabilized with ice-cold methanol for 5 min. As a primary antibody PCNA 16D10 (diluted 1:20 in PBS containing 2% BSA) was used. The primary antibody was detected with a sec-

ondary anti-rat antibody (diluted 1:400 in PBS containing 2% BSA) conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR). Cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Images of the cells were obtained using a TCS SP2 AOBs confocal laser scanning microscope (Leica, Wetzlar, Germany) using a 63x/1.4 NA Plan-Apochromat oil immersion objective. Fluorophores were excited with the 488 nm line of an argon laser.

RESULTS AND DISCUSSION

Generation of rat monoclonal antibodies against human PCNA

The full-length human PCNA with an N-terminal His₆-tag was expressed and purified from *Escherichia coli* for antigen production. The recombinant protein was highly soluble and the yield of purified protein was ~4 mg/L of bacterial culture. By performing gelfiltration analysis of the recombinant protein, we observed the formation of a homotrimeric complex, which is in accordance to our previous observations of an endogenous trimeric PCNA complex in human and mouse cells (data not shown).

The native recombinant PCNA protein was used to immunize Lou/C rats, and a panel of clonal hybridomas was generated by fusing lymphocytes from immunized animals with the myeloma cell line P3X63-Ag8.653.

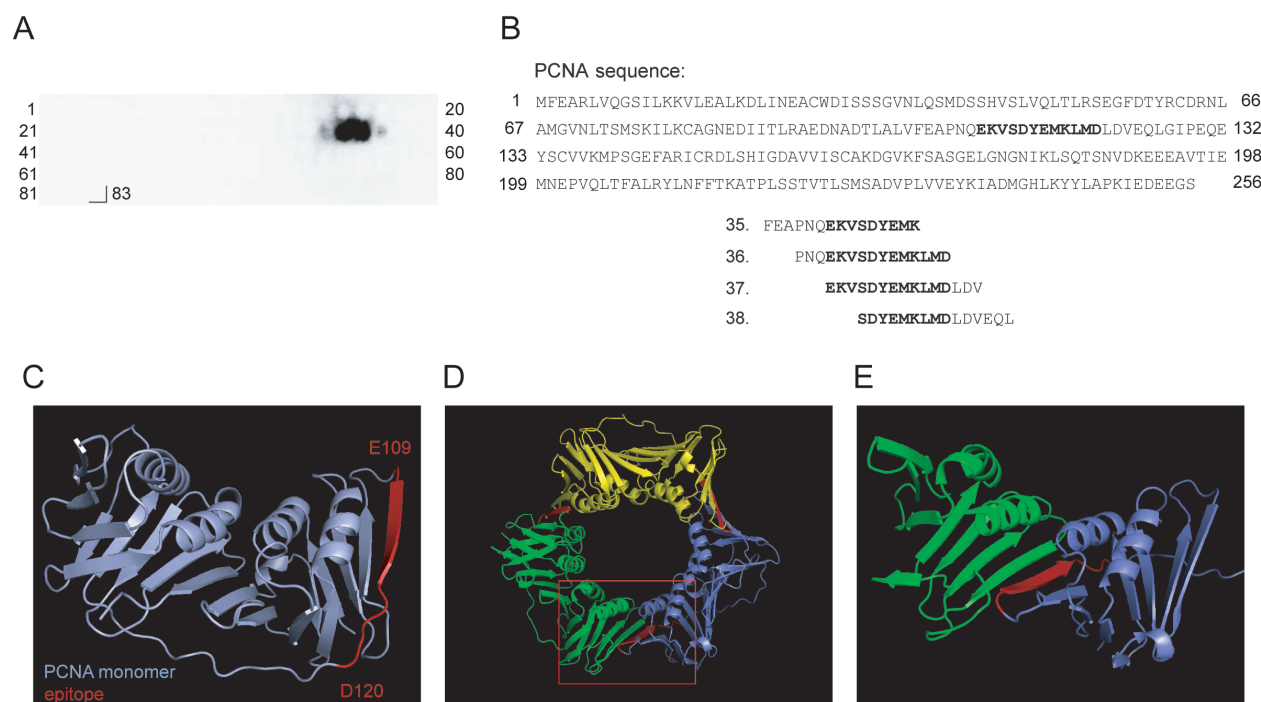


FIG. 1. Epitope mapping of the rat MAb PCNA 16D10. (A) Binding analysis of PCNA 16D10 to a peptide array representing the human PCNA. Binding of PCNA 16D10 to four peptides (35, 36, 37, 38) is detected identifying a continuous epitope. (B) Amino acid sequence of the human PCNA monomer. The recognized core epitope comprises the amino acids E109 to D120 marked in bold letters. (C–E) Three-dimensional structure of the PCNA monomer and the trimeric PCNA ring. The recognized epitope is part of a β -sheet at the outer surface of the PCNA monomer (highlighted in red). The PCNA monomers forming the trimeric ring are shown in blue, green and yellow. The figure was generated using PyMOL.⁽²⁸⁾

The antibodies produced by the hybridomas were initially screened in a solid-phase immunoassay (indirect ELISA). Plates coated with His₆-tagged PCNA protein were incubated with the hybridoma supernatants containing monoclonal antibodies. Specific antigen binding was detected with a mixture of biotinylated mouse MABs against the rat IgG heavy chains. In the next step, 17 positive hybridoma supernatants were tested by an immunoblot assay using human recombinant PCNA, as well as whole cell extracts derived from human HeLa cells and mouse C2C12 myoblast cells. After comparative analysis, one single clone designated as PCNA 16D10 was selected, established, and expanded. The isotype of this specific antibody was found to be rat IgG2b.

Epitope mapping

To determine the binding site of PCNA 16D10 within the PCNA protein, we performed a pepscan analysis. To this end, the amino acid sequence of the human PCNA was subdivided into 83 synthetic 15-mer peptides so that each peptide sequence overlaps with the preceding, as well as the following, peptide by the length of 12 amino acids. The peptides were spotted on

an N-modified cellulose-amino-hydroxypropyl ether (N-CAPE) membrane and incubated with PCNA 16D10 (Fig. 1A). A specific binding of the MAB antibody could be observed at four consecutive spots corresponding to the positions 35, 36, 37, and 38. Analyses of these peptides indicate the binding of PCNA 16D10 to a linear epitope ranging from amino acid E109 to D120 of PCNA (Fig. 1B).

To define the minimal epitope of the antibody, we performed a substitution and length analysis of the initially mapped binding site EKVSDYEMKLM. For substitution analysis, synthetic peptides were generated, where each amino acid position within the epitope (ordinate) was exchanged with every possible other amino acid (abscissae). As positive controls, peptides with the wild-type sequence (wt) were used. The substituted peptides were spotted on a cellulose membrane and incubated with the PCNA 16D10 antibody (Suppl. Fig. 1A). The analysis showed that exchange of the central amino acids SDYEMKL severely affected antibody binding, which indicates that these amino acids constitute the minimal core epitope recognized by the antibody.

In a second approach, we performed an epitope length analysis to determine the minimal antibody recognition site. Sin-

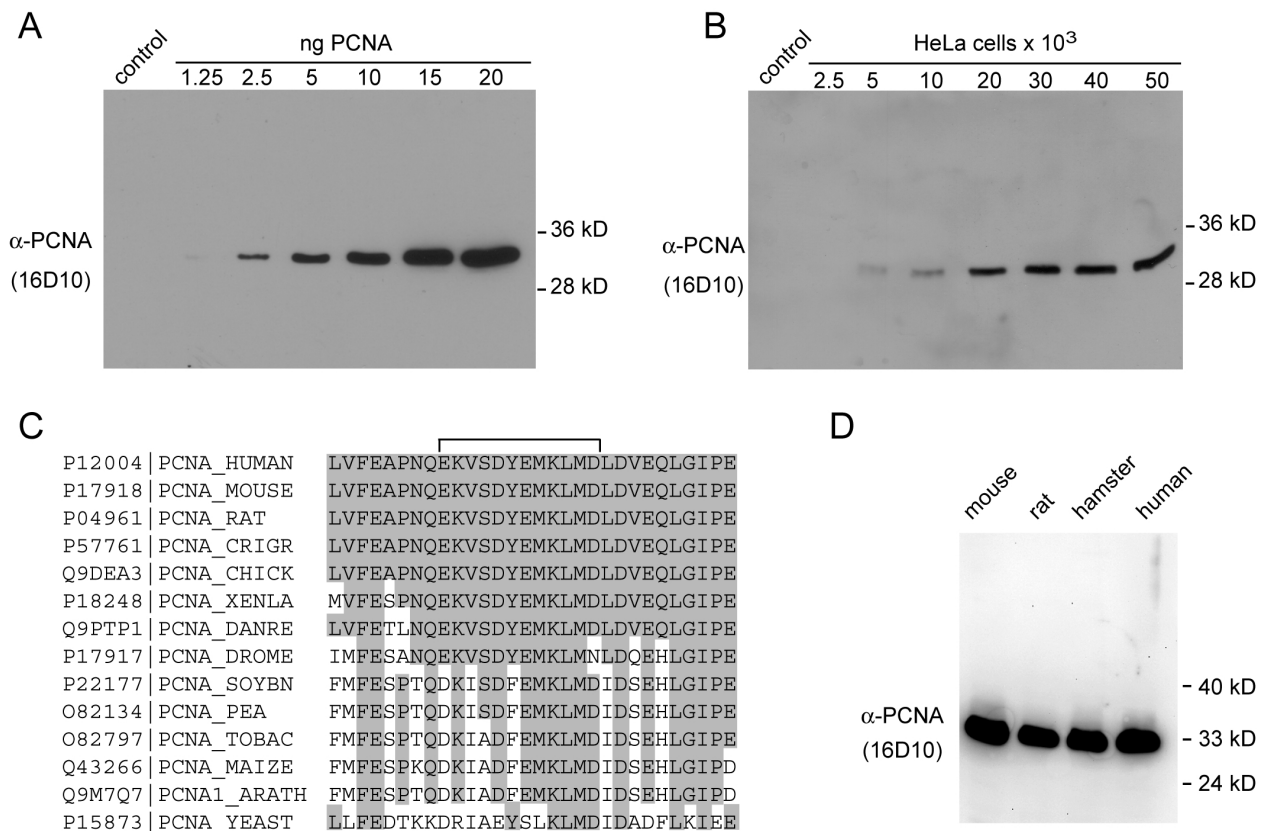


FIG. 2. Immunoblot analyses with the rat MAb PCNA 16D10. Various amounts of purified PCNA and protein extracts from HeLa cells and *E. coli* (control) were loaded to test affinity and specificity. (A) The detection limit of the PCNA 16D10 antibody ranges between 1.25 and 2.5 ng of recombinant purified PCNA. (B) Endogenous PCNA can be detected in extracts from as few as 2500 and 5000 human HeLa cells. The PCNA protein migrates at ~31 kDa. (C) Sequence alignment of the recognized PCNA epitope from different species. The epitope is indicated in parentheses and highly conserved residues are shaded in gray. (D) Multi-species immunoblot. Cell extracts of mouse (C2C12), rat (L6E9), hamster (BHK), and human (EBNA) were loaded in equal amounts. The molecular weight of the PCNA protein is indicated on the right.

gle amino acid residues were removed from the initial epitope peptide EKVSDYEMKLMD, either from the N- or the C-terminus alone or in combination, until a number of four amino acids was reached. Those resulting 45 peptides were spotted on the same membrane as the peptides for substitution analysis, and binding of the MAb was detected as described before (Suppl. Fig. 1B). The length analysis of the epitope shows that further shortening of the core domain SDYEMKL impeded the recognition by the PCNA 16D10 (spot 20–45), which is consistent with the substitutional analysis and confirms that these seven amino acids are sufficient for the specific binding of the PCNA 16D10 antibody.

The visualization of the minimal epitope within the crystal structure of the human PCNA showed that the recognized amino acids are part of a β -sheet structure on the outer surface of the PCNA monomer (Fig. 1C). Interestingly, this linear epitope is located at the head-to-tail interface of the trimeric PCNA ring and therefore important to ensure the correct assembly of the complex trimeric structure of PCNA⁽⁴⁾ (Fig. 1D and E).

Immunoblotting and immunoprecipitation with the PCNA 16D10 antibody

To determine the sensitivity and specificity of the PCNA 16D10 antibody for immunoblotting, a serial dilution of purified PCNA ranging from 1.25–20 ng was blotted onto nitrocellulose membrane and incubated with 0.05 μ g PCNA 16D10. The detection limit of the monoclonal antibody lies between 1.25 and 5 ng, shown by a distinct signal of around 31 kDa. As expected, this signal was not present in the *E. coli* extract, which was used as a negative control (Fig. 2A). Next we tested the ability of the PCNA 16D10 to recognize its endogenous antigen. Soluble protein extracts from different numbers of human HeLa cells were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. As shown in Figure 2B, PCNA 16D10 was able to detect endogenous PCNA in extracts from as few as 5×10^3 HeLa cells. Moreover, the absence of any cross-reacting bands underlines the highly specific antigen binding of this antibody. The sequence alignment showed that the recognized epitope EKVSDYEMKLMD is evolutionarily highly conserved from plants to humans (Fig. 2C). This remarkable sequence similarity in the epitope opens a wide application area for PCNA 16D10. To test the ability of PCNA 16D10 to recognize endogenous PCNA from different species, we performed a multi-species immunoblot. Cell extracts from

mouse C2C12 cells, rat L6E9 cells, hamster BHK cells, and human EBNA cells were subjected to SDS-PAGE, blotted on a nitrocellulose membrane, and incubated with PCNA 16D10 (Fig. 2D). PCNA 16D10 was able to detect specifically endogenous PCNA from different species. Those results demonstrate the high specificity and affinity of the PCNA 16D10 antibody for classical immunoblot applications in different species.

To test whether PCNA 16D10 antibody recognizes the native, folded PCNA, we performed immunoprecipitations of endogenous PCNA from whole cell lysates of human HeLa, human neuroblastoma (SHEP), and mouse myoblast C2C12 cells. For immobilization, the PCNA 16D10 was coupled to protein G agarose beads. After short-time incubation (30 min to 1 h) of the soluble cell extract with the bead-coupled antibody followed by extensive washing of the beads, the bound protein was eluted and subjected to SDS-PAGE electrophoresis and immunoblotting. By Ponceau staining of the nitrocellulose membrane, a protein band of ~ 31 kDa was visible in the bound fraction, which subsequently reacted with the PCNA 16D10 antibody (Fig. 3). A quantitative analysis of the non-bound (F) fraction revealed a nearly complete depletion of PCNA from human cell extracts after immunoprecipitation with PCNA 16D10. This result shows that the PCNA 16D10 recognizes its epitope not only under denaturing conditions, but also when it is part of larger nuclear complexes. The high pulldown efficiency of PCNA represents a significant improvement for the analysis of factors interacting with PCNA.

Immunofluorescence staining and detection of the cell cycle dependent distribution of PCNA with the PCNA 16D10 monoclonal antibody

We tested whether the PCNA 16D10 antibody is also suitable to detect PCNA specific pattern occurring throughout the cell cycle. PCNA, as a key component of the nuclear replication machinery accumulates at sites of DNA replication, the so-called replication foci (RF).⁽¹⁶⁾ During S phase progression, the distribution of replication machineries changes from countless RF localized throughout the entire nucleus corresponding to early-replicating genes, up to a few larger foci of late-replicating heterochromatin. These specific patterns allow a detailed analysis of S phase progression and cell proliferation of eukaryotic cells.

We examined the immunoreactivity of PCNA 16D10 in mouse C2C12 myoblast cells and developed a two-step fixa-

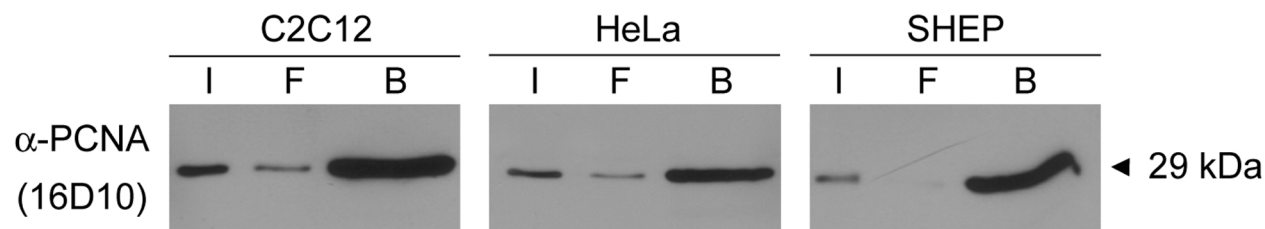


FIG. 3. Immunoprecipitation of endogenous PCNA. Soluble cell extracts of mouse C2C12 myoblasts, human HeLa, and SHEP cells were subjected to immunoprecipitation analysis using the PCNA 16D10 antibody. One percent of input (I) and flowthrough (F) fractions and 10% of the bound (B) fractions were subjected to SDS-PAGE followed by immunoblot analysis. Precipitated PCNA was detected by immunostaining with the PCNA 16D10. The molecular weight of the PCNA protein is indicated on the right.

tion protocol. In the first step, we fixed the cells with formaldehyde to maintain the three-dimensional structure of the nucleus. In the second step, we performed a fixation and permeabilization with methanol (see section on Material and Methods).

The results of the immunofluorescence analyses with PCNA 16D10 are summarized in Figure 4. Antibody specific signals in C2C12 cells are shown in the left panel and the distribution of AT-rich heterochromatic regions is high-

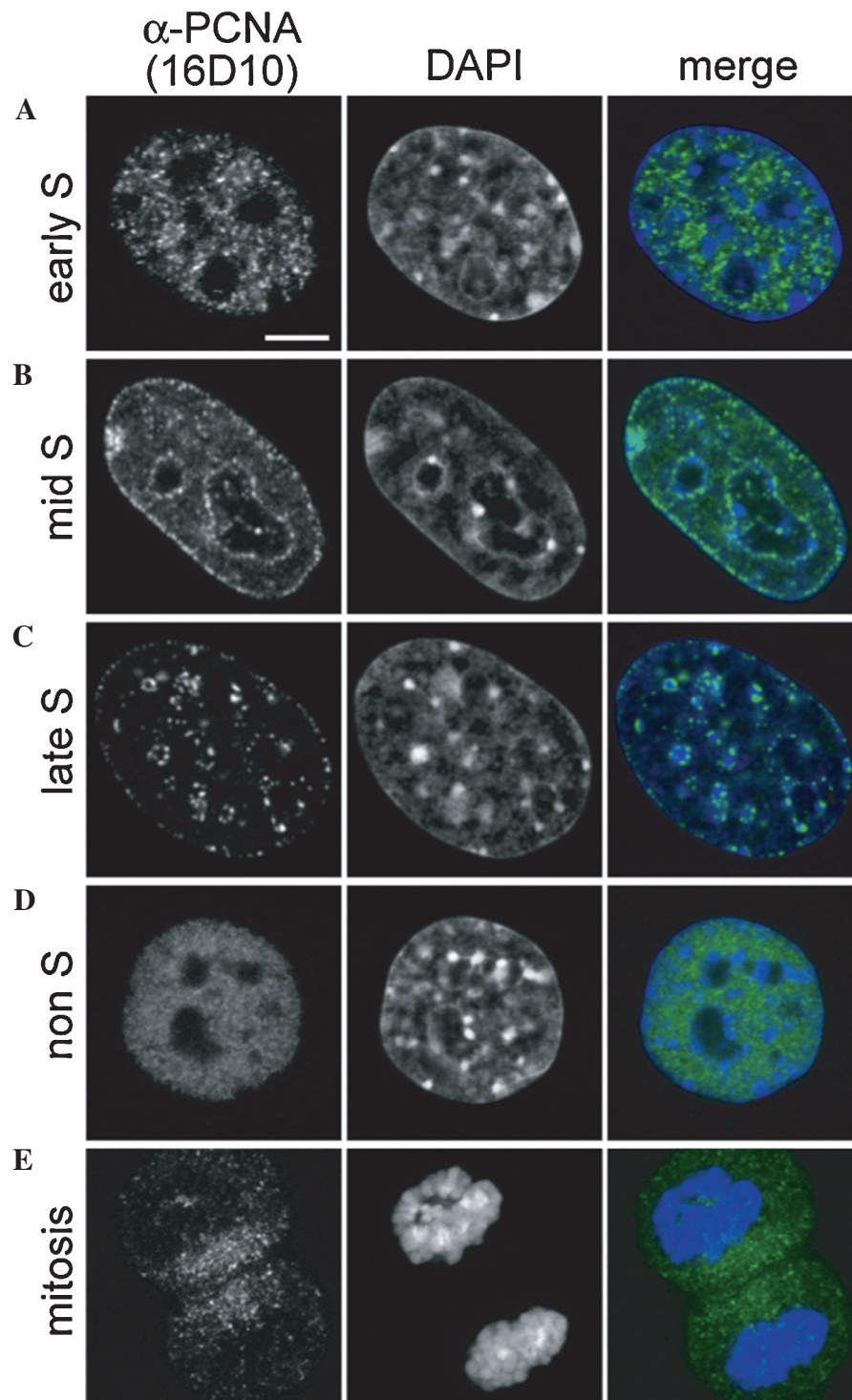


FIG. 4. Cell cycle dependent distribution of PCNA detected with PCNA 16D10 antibody. Shown are confocal mid-sections of mouse C2C12 myoblasts fixed by a two-step fixation with formaldehyde and methanol (A–E). The immunostaining of endogenous PCNA occurs in the cell nucleus and is associated with replication foci (RF) during different stages of S phase (left panel). The DNA was counterstained with DAPI (mid panel). The right panel shows an overlay of both signals in the cell nuclei. Scale bar, 5 μm.

lighted by DAPI staining (mid panel). An overlay of immunostaining and DAPI staining could be seen in the right panel. The image on the first panel shows the detection of initial replication foci in early S phase by immunofluorescence staining of PCNA (Fig. 4A). In mid S phase the facultative heterochromatin mainly gets replicated. At this stage we could detect a PCNA pattern at the nuclear periphery and around the nucleoli (Fig. 4B). A specific localization of PCNA at mouse C2C12 chromocenters could be detected in late S phase when replication of the constitutive heterochromatin takes place (Fig. 4C). In non S phase we could observe a dispersed distribution of PCNA throughout the entire nucleus (Fig 4D), while the immunofluorescence staining shows an exclusion of PCNA from chromatin during mitosis (Fig. 4E).

The detection of the characteristic PCNA localization during different cell cycle stages demonstrates that the PCNA 16D10 antibody is suited to analyse S phase progression and cell cycle dynamic changes of the replication machinery.

In summary, the sliding clamp PCNA plays a central role in DNA replication, repair, cell cycle regulation, and post-replicative modifications by orchestrating the involved proteins and mediating the interaction with DNA. This prominent role in the inheritance of genetic and epigenetic information makes PCNA a highly interesting antigen for antibody production.

We have now generated the first rat monoclonal antibody with a high affinity and specificity for human PCNA. The PCNA 16D10 antibody recognizes PCNA from multiple species and is highly suitable for ELISA, immunoblotting, immunoprecipitation, and immunofluorescence stainings, making it a valuable tool for cell cycle analysis, proliferation assays, and tumor diagnosis.

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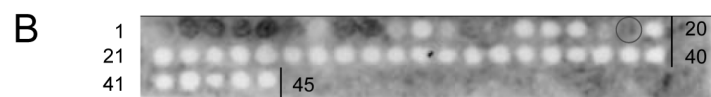
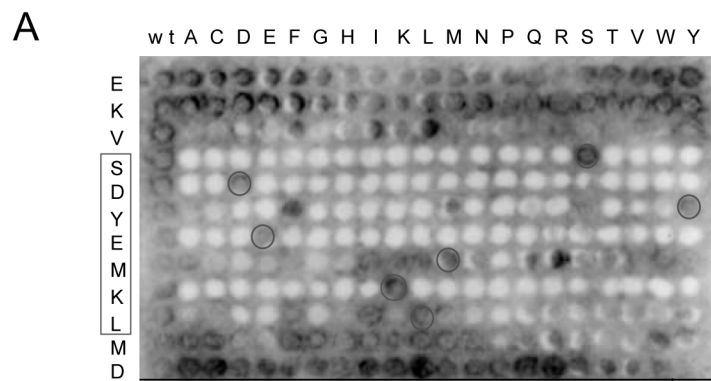
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28	EMKLM
29	EKVSD
30	KVSDY
31	VSDYE
32	SDYEM
33	DYEMK
34	YEMKL
35	EMKLM
36	MKLM
37	EKVS
38	KVSD
39	VSDY
40	SDYE
41	DYEM
42	YEMK
43	EMKL
44	MKLM
45	KLMD

Supplemental Figure 1