Reactivity of Anti-Proliferating Cell Nuclear Antigen (PCNA) Murine Monoclonal Antibodies and Human Autoantibodies to the PCNA Multiprotein Complexes Involved in Cell Proliferation

Yoshinari Takasaki, Toshiaki Kogure, Ken Takeuchi, Kazuhiko Kaneda, Tetsuro Yano, Kaoru Hirokawa, Sachiko Hirose, Toshikazu Shirai and Hiroshi Hashimoto

*J Immunol* 2001; 166:4780-4787; doi: 10.4049/jimmunol.166.7.4780

http://www.jimmunol.org/content/166/7/4780

References

This article cites 53 articles, 19 of which you can access for free at:

http://www.jimmunol.org/content/166/7/4780.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Reactivity of Anti-Proliferating Cell Nuclear Antigen (PCNA) Murine Monoclonal Antibodies and Human Autoantibodies to the PCNA Multiprotein Complexes Involved in Cell Proliferation

Yoshinari Takasaki, 2* Toshiaki Kogure, ‡ Ken Takeuchi, * Kazuhiko Kaneda, † Tetsuro Yano, † Kaoru Hirokawa, * Sachiko Hirose, † Toshikazu Shirai, † and Hiroshi Hashimoto *

Proliferating cell nuclear Ag (PCNA) 3 was originally identified as an autoantigen that reacted with autoantibodies in patients with systemic lupus erythematosus (SLE) (1). When we purified and characterized this Ag from cell extracts, we found it to be a 34-kDa intranuclear polypeptide, existing mainly as a homotrimer (2, 3) whose expression increased in the late G1 to S phases of the cell cycle immediately before DNA synthesis (4). These findings suggested an involvement of PCNA in DNA replication, and, indeed, PCNA was later identified as an auxiliary protein of DNA polymerase (Pol)-δ, playing an essential role in DNA replication and repair (5–9). Further analysis of the structure and function of PCNA showed that it forms a toroidal-shaped trimer, termed a “DNA sliding clamp,” enabling individual polymerases to efficiently synthesize long DNA strands (10). Replication factor C (RFC) serves as a “clamp loader,” binding to PCNA to open the closed trimeric ring (11).

Recent studies have shown that the proteins binding to PCNA are not limited to enzymes involved in the mechanics of DNA replication and repair but also include molecules associated with cell cycle regulation, including p21, cyclin D, and Gadd45 (12–14). In addition, PCNA binds to human DNA-(citosine-5)-methyltrasferase (MCMT), a protein associated with DNA methylation (15), and to CAF1, an essential factor for the coupling of chromatin assembly (16). These data suggest that, through its interaction with other proteins, PCNA plays key roles in the regulation of DNA synthesis, in the reorganization of replicated DNA at replication forks, and in the regulation of the cell cycle.

Autoantibodies against PCNA isolated from the sera of lupus patients have frequently been used to analyze the cellular functions of PCNA (2, 4, 17–19). However, sera from lupus patients often contain other autoantibodies, and it has been difficult to obtain sufficient amounts of monospecific sera for PCNA because of its low incidence in lupus patients (1). To solve this problem, several investigators have generated a group of anti-PCNA mAbs (20–22), among which are TOB7, TO17, and TO30, produced in our laboratory (22). Using these mAbs, we developed a new sandwich-type ELISA and showed it to be useful for measuring PCNA in lymphocyte extracts after mitogenic stimulation (22) and in sera of patients with malignant lymphoma (23). In addition, these mAbs have been used with immunofluorescence microscopy and flow cytometry to detect proliferating and blastoid cells and in the diagnosis and evaluation of the disease activity and prognosis of SLE and cancer patients (24–28).

Received for publication August 7, 2000. Accepted for publication January 30, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a 1998 research grant from Mixed Connective Tissue Disease Research Committee of Japan, Ministry of Health and the Welfare.

2 Address correspondence and reprint requests to Dr. Yoshinari Takasaki, Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, Tokyo, Japan; and Department of Japanese Oriental (Kampo) Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

3 Abbreviations used in this paper: PCNA, proliferating cell nuclear Ag; SLE, systemic lupus erythematosus; RFC, replication factor C; RNP, ribonucleoprotein; KL, proteasome activator 28 γ; MCM, DNA-(citosine-5) methyltrasferase; PCNA complex, multiprotein complex with PCNA; DDI, double immunodiffusion; CIE, counterimmunoelectrophoresis; IB, immunoblotting; IP, immunoprecipitation; Top, topoisomerase I; RPA, replication protein A; NDH II, DNA helicase II; CDK, cyclin-dependent kinase; RTE, rabbit thymus extract; ALP, alkaline phosphatase; T-PBS, Tween 20 PBS; Pol, DNA polymerase.
We recently found that TOB7, TO17, and TO30 immunoprecipitated a variety of proteins in addition to the 34-kDa PCNA polypeptide, despite their high specificity for the PCNA polypeptide (22–28). In this report, we purify the proteins interacting with these mAbs and show them to be components of multisubunit complexes with PCNA (PCNA complex) and determine in part the cellular function of PCNA.

Materials and Methods

Sera

Anti-PCNA standard sera, PT and EB, were kindly provided by E. M. Tan (Scipri’s Clinic and Research Foundation, La Jolla, CA). Three anti-PCNA sera, AK, YO, and MI, obtained from lupus patients in Juntendo Hospital, were selected and their specificity to PCNA was confirmed in double immunodiffusion (DID) and counterimmunoelectrophoresis (CIE) assays. Serum AK that showed a single anti-PCNA precipitin line in these assays served as the source of the anti-PCNA IgG used to make an affinity chromatography column and was also used to monitor the antigenicity of PCNA in DID, CIE, immunoblotting (IB), ELISA, and immunoprecipitation (IP) conducted during the Ag purification. In addition to serum AK, other anti-PCNA sera were used to test the reactivity to purified Abs. Standard sera were then used to determine antibodies to other nuclear antigens such as U1 ribonucleoprotein (RNP), Sm, and topoisomerase (Topo) I. They were also donated by E. M. Tan. Anti-proteasome activator 28 γ (Ki) prototype serum (kindly provided by T. Tojo, Keio University, Tokyo, Japan) served as a negative control. Using DID, we confirmed the homospecificity of our own standard sera against all of the prototype sera given to us.

Monoclonal Abs to PCNA

Hybridomas TOB7, TO17, and TO30 (IgG1, IgM, and IgM, respectively) were transplanted into pristane-primed BALB/c nude mice. The mAbs were purified from the ascites fluid, as previously reported (22), and then used to prepare affinity chromatography columns for purification of the PCNA complex. The mAbs were also used as probes for the PCNA complex in IP, IB, and ELISAs.

Abs to molecules associated with cell proliferation

Murine mAbs and rabbit polyclonal Abs raised against various molecules associated with cell proliferation were used to analyze the components of the PCNA complex. These included mAbs against human p21 (BioSource International, Camarillo, CA), human cyclin B (Transduction Laboratories, Lexington, KY), malignant cyclin D (BD PharMingen, San Diego, CA), human Cdc2 (Transduction Laboratories), replication protein A (RPA; NeoMarkers, Fremont, CA), DNA ligase I (NeoMarkers), DNA helicase II (NDH II; Cosmo Bio, Tokyo, Japan), cyclin-dependent kinase (CDK) 8 (Transduction Laboratories), mouse p36 (Transduction Laboratories) and p53 (BD PharMingen), and polyclonal Abs against human cyclin A (Transduction Laboratories), DNA primase (NeoMarkers), and CDK4 and 5 (BD PharMingen). The respective Abs were used in IB according to the manufacturers’ instructions. In addition, murine mAbs against dsDNA (G2B and BW6; kindly provided by T. Shirai, Juntendo University, Tokyo, Japan) were used in ELISAs to assess the reactivity to the PCNA complex.

Immunoprecipitation

HeLa cells were grown in DMEM supplemented with 10% FBS, 2 mM l-glutamine, and antibiotics (streptomycin/penicillin G). Cell samples (2 × 10^5 cells/sample) were labeled overnight with [35S]methionine (translation grade; 10 μCi/sample; NEN, Boston, MA), after which the labeled cells were lysed in NET-2 buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.05% Nonidet P-40), the lysates cleared by centrifugation, and the supernatant was used as the source of the anti-PCNA IgG used to make an affinity chromatography column and was also used to monitor the antigenicity of PCNA in DID, CIE, immunoblotting (IB), ELISA, and immunoprecipitation (IP) conducted during the Ag purification. In addition to serum AK, other anti-PCNA sera were used to test the reactivity to purified Abs. Standard sera were then used to determine antibodies to other nuclear antigens such as U1 ribonucleoprotein (RNP), Sm, and topoisomerase (Topo) I. They were also donated by E. M. Tan. Anti-proteasome activator 28 γ (Ki) prototype serum (kindly provided by T. Tojo, Keio University, Tokyo, Japan) served as a negative control. Using DID, we confirmed the homospecificity of our own standard sera against all of the prototype sera given to us.

Purification of PCNA and Ki

Rabbit thymus extract (RTE), prepared as previously reported (2), was used as the source material for PCNA. To each 90 mg of rabbit thymus acetone powder (Pel-Freeze Biologicals, Rogers, AR), 1 ml PBS was added. Saline-soluble Ag was then extracted by stirring this mixture at 4°C for 24 h; the mixture was then cleared by centrifugation at 3000 × g for 30 min, and the supernatant was used as the source of the Ag.

An IgG fraction of serum AK, prepared by 33% ammonium sulfate fractionation and DES2 (Whatman, Clifton, NJ) ion exchange chromatography coupled to cyrogen-bromide-activated Sepharose 4B (Amersham Pharmacia, Piscataway, NJ), followed by anti-PCNA affinity chromatography using 2 mg of mAbs (TOB7, TO17, and TO30) per milliliter of Sepharose 4B gel, were conducted to purify PCNA (30). Anti-PCNA gels were packed into a Bio-Rad Econo Column (Bio-Rad, Richmond, CA), and PCNA-containing RTE was passed over the column at 5 ml/h. After washing with more than three column bed volumes of PBS and 1 M NaCl/0.01 M phosphate buffer (pH 7.4), the bound material was eluted with 3 M NaSCN and the eluate was dialyzed against PBS. Ki Ag was purified from RTE by affinity chromatography using IgG separated from anti-Ki serum UC (30) and used as a negative control in ELISA.

DID and CIE

DID assays were conducted on plates containing 0.4% agarose (Sea Kem, Rockland, ME) and 0.01% sodium azide in PBS (2, 4). Precipitation reactions were allowed to develop for 48 h at room temperature. CIE was conducted as previously described (2).

SDS-PAGE

SDS-PAGE was performed to analyze the protein profile of the purified PCNA (2). The slab gel consisted of 12.5% acrylamide and 5% acrylamide. Proteins were stained with 0.1% amido black 10B in 7% acetic acid.

Immunoblotting

Purified PCNAs were transferred electrochemically onto nitrocellulose filters (Bio-Rad) as previously reported (2, 30). For immunohistochemical detection of proteins, the filters were first blocked in 3% BSA in 0.1% Tween 20 PBS (T-PBS) for 24 h and then incubated for 2 h with anti-PCNA mAbs in T-PBS (2.5 μg/ml) or with anti-PCNA serum AK, anti-U1 RNP, and Sm-positive serum TY, or anti-top I serum YK diluted 1:200 in T-PBS. Murine mAbs and rabbit polyclonal Abs were diluted according to the manufacturer’s instructions, and filters that were being washed with T-PBS, the filters were incubated with HRP-conjugated goat anti-mouse γ-globulin (1:1000 dilution in T-PBS; Cappel, West Chester, PA) or with HRP-conjugated goat anti-human or anti-rabbit IgG (1:2000 dilution: Cappel) for 2 h. After a final wash, the bound conjugate was detected by incubation with substrate solution (250 μg of 3,3-diaminobenzidine-4 HCl/ml, 0.5 μl of 30% H2O2/ml, and 0.05 M Tris-HCl buffer (pH 7.6)), and the resultant bands were stained with 0.1% amido black 10B in 7% acetic acid.

ELISAs

ELISAs were performed to detect the activity of PCNA in HPLC fractions and to test the reactivity of the purified PCNAs to anti-PCNA sera AK, PT, EB, YO, and MI and mAbs (30). Fifty microilters of each fraction was added to the wells of Immunoplate II plates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. The coating solutions were removed, and the plates were washed three times with T-PBS (2.5 μg/ml anti-PCNA) or with anti-PCNA serum AK, anti-U1 RNP, and Sm-positive serum TY, or anti-top I serum YK diluted 1:200 in T-PBS. Murine mAbs and rabbit polyclonal Abs were diluted according to the manufacturer’s instructions, and filters that were being washed with T-PBS, the filters were incubated with HRP-conjugated goat anti-mouse γ-globulin (1:1000 dilution in T-PBS; Cappel, West Chester, PA) or with HRP-conjugated goat anti-human or anti-rabbit IgG (1:2000 dilution: Cappel) for 2 h. After a final wash, the bound conjugate was detected by incubation with substrate solution (250 μg of 3,3-diaminobenzidine-4 HCl/ml, 0.5 μl of 30% H2O2/ml, and 0.05 M Tris-HCl buffer (pH 7.6)), and the resultant bands were stained with 0.1% amido black 10B in 7% acetic acid.

Downloaded from http://www.jimmunol.org/ by guest on August 31, 2017
reactivities of anti-PCNA sera AK, PT, EB, YO, and MI diluted as described above, and mAbs were assessed. Reactivity of anti-dsDNA mAbs was also tested in the same manner.

In addition, reactivity of serially diluted anti-PCNA sera AK, PT, EB, YO, and MI and TOB7, TO17, and TO30 (protein concentration; 1 mg/ml) to the PCNAs purified by serum AK and TOB7 was tested by ELISA to confirm the results obtained by the above experiments.

**HPLC gel filtration chromatography**

HPLC gel filtration was used to fractionate RTE and to analyze the purified PCNAs (30). Gel filtration was conducted at room temperature with a Gilson 302 model equipped with a variable wavelength UV monitor (Gilson, Middleton, WI). Proteins were detected as a function of OD 280 nm. A TSK-G3000SW column (7.5 x 600 mm inner dimension; Toyo Soda, Tokyo, Japan) was used. With PBS as the solvent, HPLC was conducted at a pump speed of 1 ml/min (injection volume, 400 μl). Blue dextran (m.w., 2,000,000), β-amylase (m.w., 200,000), alcohol dehydrogenase (m.w., 150,000), BSA (m.w., 66,200), carbonic anhydrase (m.w., 29,000), and cytochrome C (m.w., 12,400) were used as standards for the determination of the calibration curve. The immunoreactivity of PCNAs was detected by ELISA, and IB using anti-PCNA sera, AK and YO, and TOB7 as described above.

**Other methods**

Protein concentrations were estimated by the method of Lowry et al. (31).

**Results**

**Immunoprecipitation of proteins reacting with serum AK and TOB7 in HeLa cell extract**

Using HeLa cell extract as an Ag source, proteins binding to anti-PCNA mAb (TOB7) and serum AK were analyzed by IP. As shown in Fig. 1, serum AK (lane 3) immunoprecipitated only the 34-kDa PCNA polypeptide; 90-kDa and 45-kDa polypeptides were also precipitated by normal human control serum (lane 2). In contrast, TOB7 precipitated a variety of proteins in addition to the 34-kDa PCNA (lane 1). To determine which proteins in the HeLa cell extract were reacting with the TOB7, these proteins were purified and characterized further.

**Purification of PCNA and immunoblot analysis using mAbs and autoantibodies to PCNA**

Anti-PCNA affinity chromatography was conducted, and the polypeptide components of the purified PCNAs were analyzed by SDS-PAGE (Fig. 2). Purification using serum AK yielded only a single 34-kDa band (lane A). Consistent with the IP results, purification using TOB7 yielded a number of additional bands (lane B), and similar results were obtained using TO17 (lane C) or TO30 (data not shown). IB assays showed serum AK, TOB7, and TO17 to react with the 34-kDa PCNA polypeptide (lanes 1, 3, and 4, respectively), whereas neither anti-U1 RNP- nor Sm-Abs in serum TY, serving as negative controls, reacted with any of the purified polypeptides (lane 2). Although purification using TOB7 and TO17 yielded many polypeptides, only 34-kDa PCNA reacted with the mAbs used to construct the anti-PCNA affinity columns, suggesting that the other proteins were copurified. Alternatively, the mAbs may have reacted with other polypeptides within the affinity columns but then lost their affinity when transferred to nitrocellulose filters. HPLC gel filtration was therefore conducted to determine whether the 34-kDa PCNA did indeed interact with the copurified proteins.

**Analysis of the purified PCNAs by HPLC gel filtration**

Using selected proteins as molecular mass markers, the two peaks produced by PCNA purified using serum AK (AK PCNA) were shown to have molecular mass of 34 kDa (monomeric PCNA) and 110 kDa (trimeric PCNA) (Fig. 3, bottom trace). In contrast, the PCNAs purified using TOB7, TO17, or TO30 were excluded by the column and were, thus, in excess of 2000 kDa (blue dextran). The fact that the resolution of this column was high enough to separate the monomer-PCNA from the trimer-PCNA in AK PCNA suggested that the proteins copurified with the 34-kDa PCNA in TOB7 PCNA, TO17 PCNA, and TO30 PCNA comprised multi- protein complexes in which the 34-kDa PCNA was one component. Moreover, the data show that, whereas serum AK reacted...
only with monomeric and trimeric PCNA, the mAbs reacted with PCNA complexes as well. To confirm these observations, the reactivities of the purified PCNAs were assessed by ELISA.

Reactivity of the purified PCNAs tested by ELISA

Using the PCNAs purified with serum AK and mAbs as Ags, the reactivity of each Ag to anti-PCNA sera or the mAbs was assessed by ELISA. TOB7, TO17, and TO30 reacted with PCNAs purified using either mAbs (TOB7 PCNA and TO17 PCNA) or serum AK (AK PCNA). Autoantibodies in lupus sera, AK, PT, EB, MI, and YO, reacted very weakly to TOB7 and TO17 PCNAs, but reacted strongly to AK PCNA.

Anti-PCNA sera, AK, PT, EB, MI, and YO, also showed weak reactivities to the TOB7 PCNA, even in the low dilution. These results suggest that, unlike TOB7, TO17, and TO30, autoantibodies to PCNA in lupus sera cannot strongly react with the 34-kDa PCNA polypeptide when it is integrated into a PCNA complex.
34-kDa PCNA polypeptide when it is integrated into a PCNA complex.

**Reactivity of the fractions of RTE separated by HPLC to anti-PCNA sera and mAb**

Because autoantibodies to PCNA in lupus sera appeared unable to react with PCNA complexes, the reactivity of anti-PCNA sera (AK and YO) and mAb (TOB7) to PCNAs fractionated by HPLC gel filtration was tested using CIE, IB, and ELISA (Fig. 6, A and B). ELISA and IB showed that TOB7 reacted with PCNAs in fractions 30–35, which were excluded by the column, and to PCNAs in fractions 39–61 (Fig. 6A). Because mAbs cannot generally obtain precipitin lines in DID and CIE, none of the fractions of Ags could react with TOB7 in CIE (Fig. 6A). In contrast, anti-PCNA serum YO reacted with PCNAs in fractions 39–44, 49–61 in CIE, and in fractions through 39–61 in ELISA, but not with PCNAs in the excluded fractions in both assays (Fig. 6A). The other anti-PCNA serum, AK, showed the same reactivity as that of serum YO, although it weakly reacted with the excluded fractions in ELISA. The PCNAs in the included fractions were monomer- and trimer-PCNAs and were separated by this column as shown by the analysis using CIE. In contrast, the ELISA detected PCNAs in fractions through 39–61 because its sensitivity was higher than CIE to react with the low amounts of PCNAs between the peak tops of monomer- and trimer-PCNAs. Interestingly, IB assays showed that anti-PCNA sera, AK and YO, could react with the 34-kDa PCNA polypeptide in the excluded fractions (Fig. 6B, lanes 2 and 3, respectively) in the same manner as TOB7 (Fig. 6B, lane 1) if it was isolated from proteins in the PCNA complexes by SDS-PAGE. These results confirm that anti-PCNA mAbs react with both free and complexed PCNA polypeptide, whereas autoantibodies in anti-PCNA sera such as AK and YO can strongly react with only the free forms of PCNA.

**Analysis of molecules composing the PCNA complex**

Because PCNA apparently binds to proteins associated with DNA replication and repair and with cell cycle regulation, we used IB assays to determine which of the proteins known to interact with PCNA were components of PCNA complexes isolated in the present study. As shown in Fig. 7 and Table I, we found that p21, RPA, NDH II, CDK4, CDK5, and Top I were all detected, whereas cyclin A, cyclin B, DNA primase, DNA ligase I, CDK2, CDK8, Cdc2, p36, and p53 were not. This finding suggests that the PCNA complexes purified using our anti-PCNA mAbs comprise the “protein machinery” associated with DNA replication and repair. We therefore tested whether DNA interacted with these PCNA complexes and found that G2B and BW6, two murine mAbs against dsDNA, reacted with the PCNA complexes purified using TOB7 (TOB7 PCNA) or TO17 (TO17 PCNA), but did not react with monomeric or trimeric PCNA purified using serum AK (AK PCNA) or with control Ki Ag purified using anti-Ki serum UC (Fig. 8).

**Discussion**

Anti-PCNA mAbs (e.g., TOB7, TO17, and TO30) have been shown to selectively react with PCNA and to be useful for the diagnosis and the evaluation of prognosis of patients suffering from a variety of malignant tumor disease (23–28). The epitopes that react with these mAbs have been studied using recombinant proteins and overlapping 15-mer synthetic peptides encoding the entire PCNA amino acid sequence (32, 33), and it is now known
that these mAbs recognize linear epitopes on PCNA; more specifically, that TOB7 reacts with amino acid residues 181–195, whereas TO17 and TO30 react with the sequence from 111 to 125. Because there have been no reports of cross-reactivity with these mAbs (22–28), it was very difficult to understand the observation that, unlike PCNA autoantibodies isolated from the sera of lupus patients, these mAbs immunoprecipitated numerous proteins in addition to the 34-kDa PCNA polypeptide. A scenario that accounts for these findings is one in which PCNA mAbs are able to react with the PCNA protein, which in turn interacts with other proteins.

When first purified and characterized using autoantibodies to PCNA in sera obtained from lupus patients, PT and EB, that were the same sera used in this study, PCNA was found to exist in monomeric and homotrimeric forms, but no interaction between PCNA and nucleic acids or other protein was observed (2). In view of our present findings that autoantibodies to PCNA in lupus sera selectively react with the monomeric and trimeric forms of PCNA, but not with PCNA complexes, those earlier findings would seem reasonable. Further analysis of the structure and function of PCNA revealed it to be an auxiliary protein of Pol-δ that forms a toroidal-shaped trimer that binds to RFC and Pol-δ during the synthesis of the DNA leading strand (5, 6, 10, 11). Moreover, in fibroblasts, PCNA also appears to exist within a quaternary PCNA/p21/CDK/CAF1 complex that is reorganized during the mitotic phase of the cell cycle (5, 12, 14, 41), as does human Mcm7, which is associated with DNA methylation (15), and CAF1, which is identified as an essential factor for the coupling of chromatin assembly (16). Thus, by forming multiprotein complexes with a variety of proteins mediating a variety of processes, PCNA plays key roles in the regulation of DNA synthesis, in the reorganization of replicated DNA at replication forks, and in the regulation of the cell cycle.

Using a PCNA-Sepharose 4B affinity chromatography with calf thymus extract as an Ag source, Loor et al. analyzed the proteins interacting with PCNA and found them to include Pol-δ and Pol-ε, RFC, RPA, nuclear NDH II, and Top I (42). Of these, only the Pols and RFC directly interact with PCNA. In addition, CDK4 and CDK5, most likely bound to cyclin D, were also detected, although cyclin D itself was not reactive in IB. Our present findings are consistent with these earlier results and demonstrate that proteins interacting with PCNA can be readily purified from tissue extract, even when they do not bind directly to PCNA.

Our observations are also in agreement with those of Applegren et al. (43), who purified a multiprotein form of Pol from HeLa cell extract using a series of centrifugations, polyethylene glycol precipitation, Q-Sepharose column chromatography, and sucrose gradient centrifugation. They showed the DNA replication complex to consist of Pol-δ, Pol-α, DNA primase, RFC, PCNA, RNase H, Top I and II, DNA ligase I, DNA helicase, RPA, and DNA-dependent ATPase in addition to the DNA itself. The proteins comprising the DNA replication complex thus form a multiprotein complex within the nucleoplasm that can be purified from the cell extract.

We did not test whether the PCNA complexes purified using anti-PCNA mAbs contained all of the proteins associated with DNA replication. It is nonetheless likely that a fraction of the isolated complexes did represent the DNA replication fork, because it reacted with murine mAbs to DNA, RPA, NDH II, and autoantibodies to Top I. In addition, the fact that p21, CDK4, and CDK5 were also detected suggests some of the isolated complexes were involved in cell cycle regulation.

PCNA complexes could not be isolated using anti-PCNA autoantibodies, which is explained by the difference in the epitopes recognized by anti-PCNA mAbs and autoantibodies. Fine epitope
mapping performed using recombinant proteins and synthetic peptides showed that mAbs recognize linear epitopes on PCNA, whereas anti-PCNA sera including AK, PT, EB, MI, and YO that have been used in our studies do not (32, 33), indicating that PCNA autoepitopes are strongly dependent on the conformation of the PCNA polypeptide. Brand et al. have also studied the epitopes on PCNA recognized by lupus sera and found that autoantibodies to PCNA bind highly conformation-dependent epitopes, and some autoantibodies specifically react to the native three-dimensional structure of monomer- and/or trimer-PCNAs is altered when other proteins bind to the PCNA, and, then, autoantibodies no longer react with the PCNA in the PCNA complexes.

In addition, it is well known that autoantibodies often recognize biologically functional sites on the autoantigen (45). For instance, anti-PCNA in the serum AK used to purify PCNA in this study recognizes the same binding sites recognized by Pol-δ and RFC, as well as sites affecting DNA synthesis, and can inhibit DNA synthesis in isolated frog egg nuclei (17), in vitro DNA synthesis using poly(dA)/oligo(dT) as template (18), and plasmid DNA replication in living cells (19). In contrast, anti-PCNA mAbs such as 19F4 and 19A2, which recognize the same epitopes as TO17 and TO30, do not inhibit DNA synthesis (17–19, 32, 33). Functional sites on PCNA have been mapped with mutant PCNAs, and it has been shown that most of them are located in loop structures protruding on the C-side of PCNA, and the sequence from 254 to 256 (C-terminal tail) is responsible for interaction with RFC, Pol-δ, p21, Gadd 45, and MCMT (46). In addition, center loop, the sequence from 41 to 44, on the N side is also known to be the site necessary for the specific interaction with RFC, Pol-δ, p21, and MCMT (46). It is possible that the epitopes recognized by autoantibodies in anti-PCNA sera such as PT, EB, MI, and YO are hidden when the PCNA polypeptide binds to the proteins associated with cell proliferation, because it has been reported that many anti-PCNA sera react with epitopes near either both ends of the molecule (44, 47). The linear epitopes recognized by mAbs are apparently not associated with biologically functional sites, enabling the mAbs to bind to all forms of PCNA.

Analysis of the reactivities to the PCNAs fractionated by HPLC gel filtration strongly support the aforementioned scenario. Autoantibodies to PCNA in sera obtained from lupus patients, including YO and AK, did not react or weakly reacted with the complexed PCNA excluded by the column, but did react with the 34-kDa PCNA polypeptide once it was dissociated from the multiprotein complex by SDS-PAGE. This confirms that PCNA polypeptide was itself reactive to the anti-PCNA sera, but the reactivity was lost when PCNA was complexed with other proteins.

It is also possible that autoantibodies reactive with the PCNA and other proteins in the PCNA complexes were already bound to the PCNA complexes in the sera and could not react with the PCNA complexes purified by mAbs because elevated levels of serum PCNA have been reported in lupus patients with anti-PCNA using a sandwich-type ELISA (22, 23, 48). The amount of PCNAs in the PCNA complexes may be larger than that of monomer- or trimer-PCNAs in proliferating and activated cells, and, therefore, in lupus sera, free autoantibodies to PCNA reactive only with monomer- or trimer-PCNA can still exist in the sera although Abs to the components in the PCNA complex are all adsorbed by the complexes.

Using anti-PCNA mAb affinity chromatography, we were able to copurify more proteins than have been mentioned so far. In that regard, Hannan et al. identified a preassembled RNA polymerase I holoenzyme complex that contains all the factors necessary to initiate transcription of ribosomal DNA. PCNA was detected along with other proteins associated with DNA replication/repair, including Ku 70/80, Top I, TFIIH, SL-1, and ribosomal DNA transcription terminator factor (49). This supports our earlier finding that PCNA first appears in nucleoli in early G1 phase, soon after mitogenic stimulation (4). Other candidates that may interact with PCNA are proteins associated with the ubiquitin-proteasome pathway (50). Proteasome was originally described as a holoenzyme playing an important role in the production of peptides presented by MHC class I molecules (50), but recent studies have revealed proteasome to be involved in cell cycle regulation and to bind to cyclin B, cyclin D, p21, CDK 5, and p27, all of which interact with PCNA (50–53).

Finally, our findings indicate that anti-PCNA mAbs, which bind to PCNA complexes with other proteins, are useful tools with which to identify interacting proteins and to examine the nature of the protein-protein interactions within the complexes associated with cell proliferation. Furthermore, analysis of autoimmune responses to proteins interacting with PCNA may shed light on the mechanisms of autoantibody production in lupus patients.

References


