Autoantibody to the proliferating cell nuclear antigen neutralizes the activity of the auxiliary protein for DNA polymerase delta

Cheng-Keat Tan, Kevin Sullivan¹, Xiangyang Li, Eng M.Tan¹, Kathleen M.Downey and Antero G.So

Department of Medicine, Department of Biochemistry and Molecular Biology and Center for Blood Diseases, University of Miami School of Medicine, Miami, FL 33101 and ¹The W.M.Keck Foundation Autoimmune Disease Center, Scripps Clinic and Research Foundation, La Jolla, CA 92037, USA

Received August 14, 1987; Revised and Accepted October 9, 1987

ABSTRACT

Two murine monoclonal antibodies to the proliferating cell nuclear antigen (PCNA), a rabbit anti-N-terminal peptide antibody and human autoantibody to PCNA reacted with the auxiliary protein for DNA polymerase delta from fetal calf thymus following SDS-polyacrylamide gel electrophoresis, confirming the identity of PCNA and the auxiliary protein. Undenatured auxiliary protein was immunoprecipitated by the human autoantibody, but not by the monoclonal antibodies, which were raised to SDS-denatured PCNA, nor by the anti-N-terminal peptide antibody, suggesting that the epitopes recognized by both the monoclonal antibodies and the anti-peptide antibody are not exposed in the native protein. The human anti-PCNA autoantibody neutralized the activity of the auxiliary protein for DNA polymerase delta, but did not inhibit the activity of pol delta itself. The ability of pol delta to utilize template/primers containing long stretches of single-stranded template was inhibited by the anti-PCNA autoantibody, whereas the activity of pol alpha on such templates was not affected, confirming the specificity of the auxiliary protein for pol delta. The ability of PCNA, a cell cycle-regulated protein, to regulate the activity of pol delta suggests a central role for pol delta in cellular DNA replication.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA), a 36 kDal nuclear protein that is the target of autoantibodies in the sera of a subset of patients with the autoimmune disease systemic lupus erythematosus (SLE), is detectable in the nuclei of rapidly dividing normal and transformed cells (1,2). PCNA has been found to be identical to a cell cycle regulated protein, cyclin (3), initially identified by two-dimensional gel electrophoresis as a nuclear protein associated with proliferating cells (4,5). Immunofluorescence studies using human autoantibodies to PCNA have shown that PCNA/cyclin synthesis is induced in late G1-early S phase, immediately preceding the onset of DNA synthesis (6), and that the nuclear distribution of PCNA mimics the topographical patterns of DNA synthesis (7,8), suggesting that PCNA/cyclin may function in regulating DNA replication.

Recently, direct biochemical links between PCNA and DNA replication were established by the findings that PCNA is identical to a 36 kDal protein purified from human cells that is required for efficient replication of SV40 DNA in vitro (9) and to an auxiliary protein for DNA polymerase delta purified from calf thymus (10,11). The auxiliary protein, which stimulates both the activity and processivity of pol delta on template/primers containing long stretches of single-stranded template, e.g., primed homopolymers or single-stranded phage DNA, does not affect the activity of pol alpha (12), the enzyme usually considered to be the only DNA polymerase required for eukaryotic DNA replication. This finding has therefore raised the possibility that pol delta is also involved in cellular DNA replication.

In the studies reported here polyclonal and monoclonal antibodies to PCNA were tested for their ability to recognize both native and denatured auxiliary protein and to neutralize its activity. Neutralizing antibody was further examined for its effect on the activity of pol alpha, to determine whether the ability of pol alpha to utilize template/primers containing long stretches of single-stranded template is the result of an endogenous PCNA.

MATERIALS AND METHODS

Materials

Mouse myeloma IgG (P3x63AgB) was from Pharmacia. Mouse myeloma IgM, x(TEPC183), anti-mouse IgM (u chain specific), and Staphylococcus aureus (Cowan strain, formalin fixed) were from Sigma. Biotinylated goat anti-mouse IgG and IgM antibodies, avidin and biotinylated horseradish peroxidase were from Vector Laboratories. Anti-PCNA antibodies included two murine monoclonal antibodies: an IgM (19A2) and an IgG (19F4) prepared against SDS-denatured rabbit thymus PCNA (13), a rabbit anti-N-terminal peptide antibody (RAPAb) prepared by immunizing rabbits with a synthetic peptide whose amino acid sequence comprises the 11th to the 23rd residues from the N-terminus of PCNA (14), and human lupus serum (AK) monospecific for PCNA (2,13). Normal human serum was from a healthy volunteer. DNA polymerases alpha C and delta (Step VI) and the auxiliary protein for pol delta (Step VII) were prepared from fetal calf thymus as previously described (12,15). DNA polymerase delta was further purified before use by rechromatography on phosphocellulose and DEAE-Sephadex.

Assays

DNA Polymerase and Auxiliary Protein Assays - DNA polymerase delta was assayed with poly(dA-dT) as template/primer as previously described (15).

DNA polymerases alpha and delta were assayed with poly(dA)/oligo(dT) (20:1) as template/primer as described (16) except that Bis-Tris buffer, pH 6.5, was used. Incubation was at 37°C for 15 min. One unit of DNA polymerase is defined as the incorporation of 1 nmole dNMP per hr at 37°C. The auxiliary protein for pol delta was assayed as previously described (12) in the presence of 0.4 unit of pol delta.

Immunoprecipitation Assays - Purified immunoglobulins (IgG or IgM) were incubated with 20 ng auxiliary protein at 5°C for 1 hr, followed by the addition of 20% formalin fixed *S. aureus*, and incubation at 5°C for 30 min. After centrifugation the supernatant was assayed for auxiliary protein as described above. For IgM antibody (19A2), anti-IgM was incubated with the antigen-antibody complex prior to the addition of *S. aureus*. Sera were pre-absorbed to 20 µl of 20% formalin fixed *S. aureus*, washed twice in TKEBSND buffer (10 mM Tris-HCl, pH 7.5, 120 mM KCl, 0.5 mM EDTA, 250 µg/ml bovine serum albumin, 250 µg/ml soybean trypsin inhibitor, 0.5% Nonidet P-40, 1 mM dithiothreitol) by resuspension and centrifugation and suspended in 20 µl TKEBSND. Auxiliary protein (20 ng) was added, incubated at 5°C for 1 hr, centrifuged, and the supernatant assayed for auxiliary protein activity as described above.

Neutralization Assay - Various amounts of immunoglobulin or serum were incubated at 5°C for 30 min with either pol alpha, pol delta, or auxiliary

Fig. 1. Western blots of calf thymus auxiliary protein (lanes 1,3,5) and rabbit thymus PCNA (lanes 2,4,6) with anti-PCNA antibodies: 19F4 (lanes 1, 2), AK human sera (lanes 3,4) and RAPAb (lanes 5,6). Detection was with ^{125}I -protein A for human and rabbit antibodies and ^{125}I -goat antimouse IgG for 19F4.

protein followed by the addition of the reaction mixture for detection of the relevant activity.

SDS-Polyacrylamide Gel Electrophoresis

Samples were run on either 10% or 12.5% slab gels (4% stacking gel) according to Laemmli (17) and stained with Coomassie blue. Prestained molecular weight markers (Sigma) were calibrated with high and low molecular weight markers (Bio-Rad) on a 4-12.5% gradient SDS-polyacrylamide gel.

Immunoblotting

Samples in SDS-slab gels were electrophoretically transferred to nitrocellulose sheets according to Towbin *et al.* (18). PCNA and the auxiliary protein for DNA polymerase delta were detected using protein A and radioiodinated or biotinylated secondary antibody as previously described (19).

RESULTS

Anti-PCNA Antibodies Recognize SDS-Denatured Auxiliary Protein

Western blots of purified rabbit thymus PCNA and calf thymus pol delta auxiliary protein (Fig. 1) demonstrate that both proteins were detected by a mouse monoclonal antibody against SDS-denatured rabbit thymus PCNA (19F4), by human anti-PCNA autoantibody (AK) and by a rabbit polyclonal antibody prepared against an N-terminal peptide of PCNA (RAPAb), demonstrating the immunological identity of these two proteins. Another mouse monoclonal antibody against rabbit thymus PCNA (19A2) was used to immunoblot an SDS-gel of fractions from several steps in the purification of auxiliary protein from fetal calf thymus. These results (Fig. 2) show that the antibody detects a protein of 36 kDa in all fractions and, in crude extracts (lanes 1, 2 and 3), three high molecular weight species (70, 74 and 120 kDa) are also detected. Similar results were obtained with 19F4. The identity of the high molecular weight polypeptides which cross react with the antibody awaits further study.

Human Anti-PCNA Autoantibody Binds and Neutralizes Native Auxiliary Protein

To determine whether the anti-PCNA antibodies recognize the native auxiliary protein, immunoprecipitation studies were carried out with the

Fig. 2. SDS-PAGE profiles of samples from a preparation of auxiliary protein from fetal calf thymus as described in (12), protein bands (2A) and Western blot (2B). Proteins were detected by Coomassie Blue staining and auxiliary protein by Western blot using biotinylated anti-mouse IgM as secondary antibody as described in Materials and Methods. Lane 1, DE52 I; lane 2, phenyl-agarose; lane 3, phosphocellulose; lane 4, L-arginine-agarose; lane 5, DE52 II; lane 6, hydroxylapatite; lane 7, prestained molecular weight markers.

Fig. 3. Immunoprecipitation of auxiliary protein by human autoantibody to PCNA. 20 ng of auxiliary protein were immunoprecipitated and 20 μ l of supernatant taken for assay as described in Materials and Methods. Results with control serum ($\blacktriangle$) or AK serum ($\bullet$) are expressed as percent of activity without serum ($\blacksquare$). 100% activity was 55 pmol dTMP incorporated in 15 min. The activity of pol delta in the absence of the auxiliary protein was 2 pmol dTMP incorporated in 15 min.

Fig. 4. Effect of human autoantibody to PCNA on the activities of pol delta and pol alpha in the presence and absence of the auxiliary protein. Panel A: 0.4 units of pol delta and 20 ng of auxiliary protein (when present) were incubated with the indicated amount of serum in a total volume of 25 μ l and subsequently assayed for activity with poly(dA)/oligo(dT) as template/primer as described in Materials and Methods. Panel B: same as panel A except 0.4 units of pol alpha was substituted for pol delta. Open symbols, minus auxiliary protein; closed symbols, plus auxiliary protein; ($\square$, $\blacksquare$) without serum; (Δ , $\blacktriangle$) control serum; ($\circ$, $\bullet$) AK serum.

Fig. 5. Effect of human autoantibody to PCNA on the activity of pol delta with poly(dA-dT) as template/primer. 0.9 unit of pol delta was incubated with the indicated amount of serum in a total volume of 25 μ l and subsequently assayed for pol delta activity as described in Materials and Methods. (□) without serum; (Δ) control serum; (●) AK serum.

mouse monoclonal antibodies, the rabbit anti-N-terminal peptide antibody and the human autoantibody. In these studies the immunoprecipitates were sedimented by centrifugation and the supernatants were assayed for auxiliary protein activity under conditions where the incorporation of [3 H]dTMP into poly(dA)/oligo(dT) is linear as a function of auxiliary protein concentration. As shown in Fig. 3, 1 μ l of human antiserum completely abolished the activity of 20 ng of auxiliary protein while normal human serum had no effect. However, neither of the mouse monoclonal antibodies (19A2 and 19F4), at concentrations up to 1.5 mg/ml for 19A2 and 150 μ g/ml for 19F4 during the antigen-antibody interaction, nor 30 μ l of the rabbit anti-N-terminal peptide antibody immunoprecipitated the native protein, even when the interaction was extended to 16 hr. (data not shown). These results suggest that the epitopes recognized by both the anti-peptide antibody and the monoclonal antibodies to denatured PCNA are not exposed in the native protein, whereas the human autoantibody recognizes both SDS-denatured and native auxiliary protein. The human autoantibody was also found to directly neutralize the activity of the auxiliary protein (Fig. 4A), however the mouse monoclonal antibodies did not (data not shown).

Human Anti-PCNA Autoantibody Inhibits the Activity of Pol Delta but not Pol Alpha on Poly(dA)/Oligo(dT) Template/Primers

Previous studies have shown that pol delta has little or no activity on template/primers containing long stretches of single-stranded template, e.g., poly(dA)/oligo(dT) (20:1) in the absence of the auxiliary protein, whereas pol alpha utilizes such template/primers whether PCNA is present or not (12), suggesting the possibility that pol alpha is associated with an intrinsic PCNA. To determine whether pol alpha has an intrinsic PCNA activity we determined whether the activity of pol alpha on poly(dA)/oligo(dT) could be inhibited by a neutralizing anti-PCNA antibody. In Fig. 4B is shown the effect of increasing concentrations of AK serum (and control serum) on the activity of pol alpha in the presence and absence of 20 ng of auxiliary protein. The human autoantibody inhibited pol delta activity (panel A) but not pol alpha activity (panel B), confirming the specificity of the auxiliary protein for pol delta, and suggesting that the activity of pol alpha with poly(dA)/oligo(dT) (20:1) as template/primer is not the result of an endogenous PCNA. Western blots of pol alpha and pol delta with monoclonal antibody to PCNA (19F4) did not detect an endogenous 36 kDa PCNA nor any higher molecular weight proteins (data not shown).

That the antibody inhibits the auxiliary protein and does not affect pol delta directly is shown in Fig. 5. In a PCNA independent polymerase assay using poly(dA-dT) as template/primer, pol delta activity is unaffected by the presence of the antibody. The ability of pol delta to utilize poly(dA-dT) as template/primer has previously been shown to be independent of the presence of the auxiliary protein (12).

DISCUSSION

The studies reported here confirm the identity of PCNA and the auxiliary protein for DNA polymerase delta and allow the classification of both polyclonal and monoclonal antibodies to PCNA as neutralizing or non-neutralizing based on their ability to inhibit the activity of the auxiliary protein which is required by pol delta to utilize template/primers containing long stretches of single-stranded template, e.g., poly(dA)/oligo(dT) (20:1). The results of immunoprecipitation studies suggest that, whereas the human autoantibodies recognize both SDS-denatured and native auxiliary protein, the epitopes recognized by both the anti-peptide antibody and the monoclonal antibodies to denatured PCNA are not exposed in the native protein. Previous studies in which the epitopes recognized by the monoclonal antibodies and several human autoantibodies were analyzed by competitive

inhibition using a modified ELISA (13), suggested that the 19F4 and 19A2 monoclonal antibodies recognize epitopes that are closely related but not identical, whereas the human anti-PCNA antibodies recognize different epitopes on the PCNA molecule.

Both mouse monoclonal antibodies (19F4 and 19A2) immunoblotted high molecular weight polypeptides (70, 74 and 120 kDal) in SDS-gels of crude calf thymus extracts. The possibility that these polypeptides are precursor forms of PCNA is unlikely in view of the results of studies in which the products of in vitro translation of PCNA/cyclin mRNA were found to be identical in size to the in vivo synthesized protein (20,21). The molecular weight of unndenatured PCNA has been reported to be 100- to 160,000 (2,19), whereas the human replication protein which is required for efficient SV40 replication (9) and the calf thymus auxiliary protein for pol delta (12) have been reported to have molecular weights by gel filtration and/or velocity sedimentation of 70- to 80,000, suggesting that the native proteins are multimers of the 36 kDal subunit. Although it seems unlikely that the high molecular weight polypeptides that are immunoblotted by the anti-PCNA monoclonal antibody are multimers of the 36 kDal subunit, since the samples were heated at 95°C in the presence of 2-mercaptoethanol and SDS prior to electrophoresis, stability of multimeric structures to such treatment has been reported (22,23). It is also possible that PCNA shares epitopes with other cellular proteins, but these epitopes are only exposed following denaturation and blotting. The finding that human autoantibodies recognize an active or functional site of a protein has also been observed for autoantibody to threonyl-tRNA synthetase (27) and raises the interesting consideration that this may be a common feature of many spontaneously occurring human autoantibodies.

The ability of human anti-PCNA antibody to inhibit the activity of pol delta but not pol alpha with poly(dA)/oligo(dT) (20:1) as template/primer confirms the specificity of the auxiliary protein for pol delta and, together with the observation that none of the anti-PCNA antibodies are able to immunoblot polypeptides in the pol alpha preparation, suggests that the activity of pol alpha on template/primers containing long stretches of single-stranded template is not due to an endogenous PCNA.

The role of pol alpha in DNA replication in eukaryotes is well established (24-26). However, the ability of PCNA, a cell cycle regulated protein, to regulate the activity of pol delta but not pol alpha strongly suggests that pol delta may also play a central role in cellular DNA repli-

cation. A recent report suggests that the auxiliary protein (PCNA) may also be involved in DNA repair after ultraviolet light irradiation (28).

ACKNOWLEDGEMENTS

This work was supported by NIH grants DK26206 and AR38695 and in part by the Monica Besade Research Fund.

REFERENCES

1. Miyachi, K., Fritzler, M.J. and Tan, E.M. (1978) *J. Immunol.* **121**, 2228-2234.
2. Takasaki, Y., Fishwild, D. and Tan, E.M. (1984) *J. Exp. Med.* **159**, 981-992.
3. Mathews, M.B., Bernstein, R.M., Franza, B.R. and Garrels, J.I. (1984) *Nature* **309**, 374-376.
4. Bravo, R. and Celis, J.E. (1980) *J. Cell Biol.* **84**, 795-802.
5. Bravo, R., Fey, S.J., Bellatin, J., Larsen, M.P., Arevalo, J. and Celis, J.E. (1981) *Exp. Cell Res.* **136**, 311-319.
6. Kurki, P., Vanderlaan, M., Dolbeare, F., Gray, J. and Tan, E.M. (1986) *Exp. Cell Res.* **166**, 209-219.
7. Celis, J.E. and Celis, A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3262-3266.
8. Madsen, P. and Celis, J.E. (1985) *FEBS Lett.* **193**, 5-11.
9. Prelich, G., Kostura, M., Marshak, D.R., Mathews, M.B. and Stillman, B. (1987) *Nature* **326**, 471-475.
10. Prelich, G., Tan, C.-K., Kostura, M., Mathews, M.B., So, A.G., Downey, K.M. and Stillman, B. (1987) *Nature* **326**, 517-520.
11. Bravo, R., Frank, R., Blundell, P.A. and Macdonald-Bravo, H. (1987) *Nature* **326**, 515-517.
12. Tan, C.-K., Castillo, C., So, A.G. and Downey, K.M. (1986) *J. Biol. Chem.* **261**, 12310-12316.
13. Ogata, K., Kurki, P., Celis, J.E., Nakamura, R.M. and Tan, E.M. (1987) *Exp. Cell Res.* **168**, 475-486.
14. Ogata, K., Ogata, Y., Takasaki, Y. and Tan, E.M., submitted for publication.
15. Lee, M.Y.W.T., Tan, C.-K., Downey, K.M. and So, A.G. (1984) *Biochemistry* **23**, 1906-1913.
16. Tan, C.-K., So, M.J., Downey, K.M. and So, A.G. (1987) *Nucleic Acids Res.* **15**, 2269-2278.
17. Laemmli, U.K. (1970) *Nature* **227**, 680-685.
18. Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
19. Ogata, K., Ogata, Y., Nakamura, R.M. and Tan, E.M. (1985) *J. Immunol.* **135**, 2623-2627.
20. Sadaie, M.R. and Mathews, M.B. (1986) *Exp. Cell Res.* **163**, 423-433.
21. Almendral, J.M., Huebsch, D., Blundell, P.A., Macdonald-Bravo, H. and Bravo, R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1575-1579.
22. Vos-Scheperkeuter, G.H. and Wiltholt, B. (1984) *J. Mol. Biol.* **175**, 511-528.
23. Gazin, C., Rigolet, M., Briand, J.P., Van Regenmortel, M.H.V. and Galibert, F. (1986) *EMBO J.* **5**, 2241-2250.
24. DePamphilis, M. and Wassarman, P. (1980) *Annu. Rev. Biochem.* **49**, 627-666.
25. Fry, M. (1982) in *Enzymes of Nucleic Acid Synthesis and Modification* (Jacob, S.T., Ed.) Vol 1, CRC Press, Boca Raton, FL.
26. Hubscher, U. (1983) *Experientia* **39**, 1-25.
27. Dang, C.V., Tan, E.M. and Trough, J.A., submitted for publication.
28. Celis, J. and Madsen, P.V. (1987) *FEBS Lett.* **209**, 277-283.