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Abstract

Proliferating cell nuclear antigen (PCNA) has been shown to interact with a variety of DNA polymerases (pol) such as pol delta, pol epsilon, pol iota, pol kappa, pol eta, and pol beta. Here we show that PCNA directly interacts with the newly discovered pol lambda cloned from human cells. This interaction stabilizes the binding of pol lambda to the primer template, thus increasing its affinity for the hydroxyl primer and its processivity in DNA synthesis. However, no effect of PCNA was detected on the rate of nucleotide incorporation or discrimination efficiency by pol lambda. PCNA was found to stimulate efficient synthesis by pol lambda across an abasic (AP) site. When compared with pol delta, human pol lambda showed the ability to incorporate a nucleotide in front of the lesion. Addition of PCNA led to efficient elongation past the AP site by pol lambda but not by pol delta. However, when tested on a template containing a bulky DNA lesion, such as the major cisplatin Pt-d(GpG) adduct, PCNA could not allow translesion synthesis by pol lambda. Our results suggest that the complex between PCNA and pol lambda may play an important role in the bypass of abasic sites in human cells.
Human DNA Polymerase λ Functionally and Physically Interacts with Proliferating Cell Nuclear Antigen in Normal and Translesion DNA Synthesis*

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Proliferating cell nuclear antigen (PCNA) has been shown to interact with a variety of DNA polymerases (pol) such as pol δ, pol ε, pol ι, pol ρ, pol η, and pol β. Here we show that PCNA directly interacts with the newly discovered pol λ cloned from human cells. This interaction stabilizes the binding of pol λ to the primer template, thus increasing its affinity for the hydroxyl primer and its processivity in DNA synthesis. However, no effect of PCNA was detected on the rate of nucleotide incorporation or discrimination efficiency by pol λ. PCNA was found to stimulate efficient synthesis by pol λ across an abasic (AP) site. When compared with pol δ, human pol λ showed the ability to incorporate a nucleotide in front of the lesion. Addition of PCNA led to efficient elongation past the AP site by pol λ but not by pol δ. However, when tested on a template containing a bulky DNA lesion, such as the major cisplatin Pt-d(GpG) adduct, PCNA could not allow translesion synthesis by pol λ. Our results suggest that the complex between PCNA and pol λ may play an important role in the bypass of abasic sites in human cells.

In the last few years the number of known eukaryotic DNA polymerases (pols),1 including terminal transferase and telomerase, has increased to at least 19 (1). A particular pol might have more than one functional task in a cell, and a particular DNA transaction may require more than one pol, suggesting that nature has provided various safety mechanisms. This multifunctional feature is especially valid for the variety of novel pols identified in the last 5 years. These are the lesion-repli-

cating enzymes pol ζ, pol η, pol ε, pol κ, and Rev1, and a group of pols called pol θ, pol λ, pol μ, pol σ, and pol φ that fulfill a variety of other tasks. The gene encoding the novel pol λ was cloned and mapped to mouse chromosome 19 and to human chromosome 10 (2, 3). pol λ contains all the critical residues involved in DNA binding, nucleotide binding, nucleotide selection, and catalysis of DNA polymerization and has been assigned to family X based on sequence homology with pol β, pol μ, and terminal deoxynucleotidyltransferase. pol λ has been suggested to play a role in meiotic recombination and DNA repair, and the recent demonstration of an intrinsic 5′-deoxyribose-5-phosphate lyase activity in pol λ supports a function of this enzyme in base excision repair (4, 5). Cloned and purified human (h) pol λ inserts nucleotides in a DNA template-dependent manner and is processive in small gaps containing a 5′-phosphate group. These properties, together with its nucleotide insertion fidelity parameters and lack of proofreading activity, indicate that hpol λ is a novel pol β-like enzyme (5). Recently, pol λ was purified from calf thymus tissue, and the biochemical properties of the native calf thymus pol λ were shown to be similar to the recombinant hpol λ protein (6). In particular, it has been shown that the native calf thymus enzyme was able to synthesize DNA on a template containing abasic (AP) sites with the same efficiency as on undamaged DNA, thus suggesting a potential role of pol λ in translesion synthesis. The in vivo function of pol λ has also been addressed by using transgenic null mice but with conflicting results (7, 8).

PCNA is a highly conserved eukaryotic protein essential for DNA replication and repair (9, 10). In DNA replication PCNA acts as the processivity factor for pol δ and pol ε and plays an important role in coordinating leading and lagging strand synthesis (11). Kinetic analysis showed that the mechanism of PCNA interaction with pol δ and pol ε is different. PCNA increased the stability of the pol δ-DNA primer complex (thus reducing the dissociation rate of the enzyme from the DNA template) without affecting the nucleotide incorporation step (12), whereas for pol ε PCNA increased the association rate of the enzyme to the DNA primer and also the nucleotide incorporation rate without affecting the dissociation step (13, 14). The amount of PCNA in the cell is more than 10-fold that of pol δ, suggesting that this protein might have roles in addition to that of a polymerase accessory factor. Indeed, several PCNA-binding proteins have been discovered with various functions such as Okazaki fragment processing, DNA repair, DNA meth-

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† The abbreviations used are: pol, DNA polymerase; AP, abasic; PCNA, proliferating cell nuclear antigen; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; hpol, human DNA polymerase.

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ylation, chromatin remodeling, and cell cycle control. Most of these proteins share a consensus sequence for PCNA binding designated as the PCNA interacting proteins box (15–17). Among its recently discovered partners are some of the novel eukaryotic pols, namely hpol, hpol κ, and hpol λ (18–21). PCNA has been shown to stimulate their DNA synthetic activity on both undamaged and damaged DNA, but the processivity of these enzymes was not significantly increased. Steady-state kinetic studies indicated a novel mechanism for this stimulation that was caused by an increase in the efficiency of nucleotide insertion and resulted from a reduction in the apparent $K_m$ for the incoming nucleotide. As a result, the efficiency of correct versus incorrect nucleotide incorporation and the incorporation in front of a variety of DNA lesions was increased.

Recently a direct interaction of PCNA with pol λ was shown that might have potential implications in the mechanism of short-patch base excision repair (22). In light of the similarity between pol β and pol λ, in the present work we wanted to investigate the effect of PCNA on the activity of hpol λ. We show that PCNA directly binds to hpol λ and stimulates its activity. However, this stimulation appears to take place by a mechanism that is different from the cases of hpol η, hpol κ, and hpol λ, closely resembling the interaction of PCNA with pol δ. Indeed PCNA stabilizes the binding of pol λ to the primer/template, thus increasing its processivity without affecting the affinity for the nucleotide substrate or the misincorporation efficiency. Furthermore, PCNA increases the efficiency of DNA synthesis by pol λ through an AP site but not past a cisplatin adduct.

**MATERIALS AND METHODS**

**Chemicals**

$[^3]H$IdTTP (40 Ci/mmol) and $[^3]P$IdCTP (3000 Ci/mmol) were from Amersham Biosciences; unlabeled dNTPs, poly(dA), and oligo(dT)$_{12,18}$ were from Roche Molecular Biochemicals. The tetrahydrofuran (dSpacer) was from Glen Research. Activated calf thymus DNA was prepared as described (23). Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and were purchased unless otherwise stated, and the DNA was precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described (27). For sequencing gel analysis the reaction mixture included 50 mM Tris-HCl (pH 7.6), 0.25 mg/ml bovine serum albumin, 1 mM DTT, 0.5 mM MnCl$_2$, 5 µM each of unlabeled dNTPs, and $[^3]P$-labeled end-labeled DNA template as indicated in the figure legends. pol activity was assayed in the absence or in the presence of 1 µM of either dTTP, dATP, dGTP, or dCTP. All reactions were incubated for 15 min at 37°C unless otherwise stated, stopped by addition of sequencing gel loading buffer, and heated for 3 min at 95°C. Reaction products were resolved on a 20% polyacrylamide, 7 M urea gel.

**Pull-down Assay**

Pull-down experiments were performed by incubating 20 pmol (as trimer) of $[^3]P$-labeled human/PCNA with nickel-nitriolractic acid-agarose beads in the absence or in the presence of 10 pmol of histidine-tagged hpol λ in buffer A (50 mM Tris-HCl (pH 7.6), 1 mM DTT, 0.1 mM NaCl, 0.005% Nonidet P-40). Incubation of 1 µM DTT was found to increase the specificity of binding and reduce the background. After extensive washing in buffer A containing 20 mM imidazole, proteins bound to the beads were eluted with buffer A containing 1 mM imidazole and analyzed by SDS-PAGE.

**Steady-state Kinetic Data Analysis**

For kinetic analysis, time-dependent nucleotide incorporation was measured in the presence of increasing concentrations of DNA or nucleotide substrate. The slopes of the curves obtained by linear interpolation of the data points were taken as the initial velocities of the reaction at any given substrate concentration. The $K_m$ and $V_{max}$ values were calculated by plotting the initial velocities in dependence of the substrate concentrations and fitting the data according to the Michaelis-Menten equation in the following form,

$$v = \frac{k_{cat}E_0}{K_m + [S]}$$

where $k_{cat}E_0 = V_{max}$. The steady-state rates ($k_{cat}$) of synthesis values for time-dependent nucleotide incorporation by pol λ on the undamaged template were determined according to the exponential equation,

$$(\text{products}) = A(1 - e^{-kt}) + k_{cat}$$

where $A$ is the burst amplitude and $t$ is time. The products were calculated from the values of integrated gel band intensities, $P + T + C + A$, where $P$ is the target site, the template position of interest and $A$ is the sum of the integrated intensities at positions $T, T + 1, \ldots, T + m$.

**RESULTS**

Human PCNA Stimulates the Processivity of Human DNA Polymerase λ—Fig. 1 shows a time course of nucleotide incorporation by pol λ on a sp17:72-mer oligonucleotide primer template in the absence (panels A and C) or in the presence (panels B and D) of PCNA. Under distributive conditions the comparison of the reaction products at the different time points revealed an apparent slower DNA synthesis by pol λ in the

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presence of PCNA, with the accumulation of 30 nucleotide products after 8 min and few full-length products appearing after only 15 min (compare Fig. 1, A with B). The same experiment was then repeated under processive conditions (Fig. 1, C and D), i.e., in the presence of an excess of cold poly(dA)/oligo(dT) as a trapping reagent. In this case, a 5–6-fold processivity increase was evident in the presence of PCNA. In fact, without PCNA pol λ showed a processivity of about 5 nucleotides (Fig. 1C), which was increased to 25 nucleotides in the presence of PCNA (Fig. 1D). The apparent rate of DNA synthesis was 0.012 (± 0.002) s⁻¹ without PCNA and 0.06 (± 0.01) s⁻¹ in its presence. These results clearly indicated that pol λ is a highly distributive enzyme whose processivity is enhanced by PCNA and suggested that the apparently slower DNA synthesis observed in Fig. 1B could be caused by a reduced dissociation rate of the pol from the DNA template. The experiment shown in Fig. 1A shows that after 5 min of incubation DNA products longer than 72 nucleotides appeared. These could be caused by template slippage of pol λ at the GC-rich sequences of the template strand. Apparently, pol λ was able to add 12–13 extra nucleotides at the last time point analyzed. Taking into account the calculated rate of DNA synthesis and the length of the template to be replicated, this was compatible with one extra nucleotide added every five to six incorporation events. This latter value corresponds to the processivity observed with pol λ for a single binding event (Fig. 1C), further supporting the hypothesis of a template-slippage mechanism. Significantly,

fewer extra nucleotides were added in the presence of PCNA under distributive conditions (Fig. 1B).

PCNA Increases the Affinity of DNA Polymerase λ for the 3′-OH Primer—To further investigate the mechanism of interaction of PCNA with human pol λ, the variation of the initial velocities of the reaction catalyzed by pol λ from the PCNA concentration. Kan and kcat values were determined as described under “Materials and Methods” in the presence of 0.5 pmol of pol λ and 5 pmol of poly(dA)/oligo(dT) (as 3′-OH ends), B, dependence of the catalytic efficiency kcat/Km of pol λ from the PCNA concentration. Km and kcat values were determined as described under “Materials and Methods” in the presence of 0.5 pmol of pol λ and 5 pmol of poly(dA)/oligo(dT) (as 3′-OH ends). C, dependence of the increase (Incr) for the 3′-OH primer from the PCNA concentration. Data points were fitted to the equation, Incr = Incr max/[1 + Kc(1/[PCNA])], where Incr = 1 – (Kcat/Km) pcNA, D, dependence of the increase of kcat/Km of pol λ for the 3′-OH primer from the PCNA concentration. Data were fitted to the equation, Δkcat/Km = Δmax/[1 + (Kc/[PCNA])].

PCNA Does Not Influence the Affinity or the Incorporation/Misincorporation Efficiency of DNA Polymerase λ for the Nu-
pol λ Interacts with PCNA in Translesion Synthesis

Fig. 3. PCNA does not influence the nucleotide misincorporation/incorporation ability of pol λ. A, PCNA does not change the apparent affinity of pol λ for the nucleotide substrate. The dependence of the reaction velocity of pol λ from the dTTP concentration was measured in the presence of 0.5 pmol of enzyme and 5 pmol of poly(dA)/oligo(dT) (in terms of 3′-OH primer ends) and in the absence (circles) or in the presence of 10 nM (triangles), 20 nM (squares), and 50 nM (diamonds) PCNA. B, DNA synthesis catalyzed by human pol λ on a d17:d73 oligonucleotide. Reactions were performed as described under "Materials and Methods" in the presence of 0.2 pmol of enzyme and 0.5 pmol of DNA substrate and in the absence (lane 6) or in the presence of 1 μM dGTP (lane 1), 1 μM dTTP (lane 2), 1 μM dCTP (lane 3), 1 μM dATP (lane 4), or the mixture of all four dNTPs (1 μM each) (lane 5). C, the same experiment as described in panel B but in the presence of 50 nM PCNA, nt, nucleotide.

cleotide Substrate—Previous reports have shown that PCNA increased the affinity for the nucleotide substrate of pol η, λ, and κ. To verify whether PCNA had the same effect on pol λ, the dependence of the reaction velocity from the dTTP concentration was tested in the absence or in the presence of increasing amounts of PCNA. As shown in Fig. 3A, PCNA did not significantly change the affinity of pol λ for the substrate dTTP, which showed a K_m value of 3.6 (± 0.1) μM in its absence and 3.3 (± 0.2) μM in its presence. These results indicated that the effect of PCNA was specifically to increase the stability of the complex between pol λ and the primer template. Next, we tested whether PCNA affected the incorporation efficiency of correct versus wrong nucleotides by pol λ. As shown in Fig. 3B, lane 2, addition of the first encoded nucleotide (dTTP) resulted in termination of synthesis at position +1, as expected, whereas in the presence of all four nucleotides full-length synthesis was achieved (lane 5). Limited misincorporation was observed at position +1 when only dATP (lane 4) was added but not with dGTP (lane 1). Interestingly, when only dCTP was added to the reaction mixture (lane 3) a product appeared at position +3. When the same experiment was repeated in the presence of PCNA (Fig. 3C), there was an increase in the +1 product synthesized in the presence of dTTP (lane 2) as well as in the full-length product synthesized in the presence of all four dNTPs (lane 5), according to the fact that PCNA increases the efficiency of primer utilization. However, no significant changes could be detected in the misincorporation ability of pol λ. The product appearing in Fig. 3B, lane 3, can be explained as a combination of limited misincorporation and template slippage; after addition of the first cytosine residues (encoded by the two guanosine residues in the template), reannealing of the template allowed insertion of an additional cytosine. Therefore, a mismatch dA/dCMP is introduced followed by two correct mismatches. This results in a template templating. All together these results suggested that PCNA did not influence the nucleotide discrimination efficiency of pol λ.

A Direct Physical Association of PCNA with pol λ Stabilizes the Binding of the Enzyme to the Primer/Template by Reducing Its Dissociation Rate—The results presented above suggested that PCNA increases the processivity of pol λ and its apparent affinity for the 3′-OH primer end, possibly by increasing the residence time of pol λ at the primer/template junction. To directly test this hypothesis, an EMSA was performed with 5′-labeled 17:72-mer oligonucleotide in which preformed pol λ-DNA complexes were challenged at different time points with an excess of poly(dA)/oligo(dT) trap and 1% formaldehyde. At any given time point the fraction of unbound enzyme should be trapped by the cold DNA, whereas formaldehyde should allow fixation of the fraction of pol λ bound to labeled DNA. As shown in Fig. 4A, in the presence of PCNA slower dissociation of pol λ.
was observed as indicated by the higher amount of enzyme-DNA complex present at any time point (compare lanes 1–4 with lanes 5–8). Quantification of the bound fraction allowed the fitting of the curves to a simple exponential with dissociation rates of 0.0086 (±2 × 10⁻³) s⁻¹ in the absence of PCNA and 0.0032 (±8 × 10⁻⁵) s⁻¹ in its presence (Fig. 4B). In addition, to verify whether pol λ and PCNA directly interacted a pull-down assay was performed with radiolabeled PCNA and His-tagged pol λ. Nickel-nitrilotriacetic acid-agarose beads were incubated with C-terminal-labeled PCNA alone or in combination with His-tagged pol λ. After extensive washing, beads were eluted with 1 M imidazole, and the eluted proteins were loaded onto a SDS-polyacrylamide gel. As shown in Fig. 4C, nickel-nitrilotriacetic acid-agarose beads incubated with PCNA alone resulted only in a background level of bound PCNA. However, inclusion of pol λ resulted in significant binding of PCNA. These results indicated that PCNA interacted functionally and physically with pol λ.

PCNA Promotes Translesion DNA Synthesis by DNA Polymerase λ at an Abasic Site but Not at a Cis-Pt Adduct—The results presented so far indicated that PCNA interacts with pol λ and increases its residence time on the DNA template. Besides providing pol λ with higher processivity, this interaction could influence the ability of the enzyme to bypass sites along the template sequence where it normally would dissociate (e.g. at DNA lesions). To address this point, the effect of PCNA was evaluated on the ability of pol λ to replicate across two different lesions: first, an AP site and second, a cis-Pt (d(GpG)) adduct. AP sites are very common non-instructional lesions that can arise spontaneously in the DNA as a consequence of normal cellular metabolism. On the other hand, cisplatin, a clinically used anticancer drug, causes bulky DNA adducts, the prominent one being formed between two adjacent guanines on the same DNA strand (Pt-(d(GpG))). Both lesions are able to block DNA synthesis in vitro by the replicative pols α, δ, and ε. As shown in Fig. 5A, a d17:d73-mer primer template containing a single synthetic AP site (tetrahydrofuran moiety) at a defined position on the template strand was used as the substrate for DNA synthesis by pol λ in the absence or in the presence of PCNA. Without added PCNA pol λ mainly incorporated a nucleotide at the position corresponding to the AP site and stopped, although a limited elongation from the nucleotide incorporated in front of the lesion was also observed (Fig. 5A, lane 3). When increasing amounts of PCNA were added, however, pol λ was able to efficiently bypass the AP lesion synthesizing full-length 73-nucleotide products in a PCNA dose-dependent manner (Fig. 5A, lanes 4 and 5). E. coli pol I was used as a positive control for lesion bypass (Fig. 5A, lane 2). When the major cellular PCNA-dependent replicative enzyme pol δ was tested under similar conditions a different result was obtained. As shown in Fig. 5B, in the presence of PCNA pol δ was stopped at the position immediately before the AP site (Fig. 5B, lane 1). When increasing amounts of PCNA were added, significant incorporation in front of the lesion was achieved, but no subsequent elongation was detected (Fig. 5B, lanes 2–5) even in the presence of saturating amounts of PCNA. Thus, pol λ showed the ability to catalyze both incorporation in front of the abasic lesion and subsequent elongation of the nucleotide opposite the AP site in a PCNA-stimulated manner. When the ability of pol λ to bypass an AP site was tested in the presence of increasing dNTP concentrations (Fig. 5C), PCNA was again found to promote translesion synthesis as revealed by the increase in the full-length products at low dNTP concentrations (compare lanes 1–3 to lanes 6–8), but the overall nucleotide incorporation efficiency of pol λ was not affected (kcat/Km values were 2.7 (±0.3) × 10⁵ M⁻¹ s⁻¹ in the absence of PCNA and 3.5 (±0.3) × 10⁵ M⁻¹ s⁻¹ in its presence). It is known that the replicative pols α, δ, and ε cannot bypass the Pt-(dGpG) adduct, whereas pol β is able to make translesion synthesis opposite to it, a capacity that it shares with E. coli pol I (24). Because pol λ shares significant homology to pol β, we were interested in verifying its ability to bypass this cisplatin lesion. As shown in Fig. 5D, pol λ could not synthesize across the lesion placed on a d17:60-mer primer template, showing a strong stop at the position immediately before the guanosine implicated in the adduct exactly the same as pol α (compare lane 3 to lanes 5–7) and pol δ (data not shown). When increasing amounts of PCNA were
added there was an increase in the products synthesized up to the stop site, but no translesion synthesis was observed. Thus, despite its similarity to pol β, human pol λ showed a different profile of DNA lesion bypass when dealing with a bulky DNA lesion such as the major cisplatin adduct. All of the experiments were carried out in the presence of MnCl₂, because this was the optimal ion cofactor for recombinant hpol λ (5). It has been reported that for some pols (pol α, pol β, T4 pol, herpes simplex virus pol), Mn²⁺ ions can increase the lesion bypass efficiency (28). However, the concentrations required to see such effects were much higher than the one used in our assays (0.5 mM); thus it seems unlikely that MnCl₂ significantly influenced the ability of pol λ to bypass an AP site under these conditions (29). In addition, when similar experiments were performed with native calf thymus pol λ in the presence of 10 mM MgCl₂, efficient AP site bypass was detected, whereas no differences were detected by replacing Mn²⁺ with 10 mM MgCl₂ for pol I, pol α, and pol δ under these conditions (data not shown).

**DISCUSSION**

Recently pol λ was purified from calf thymus tissue, and this native calf thymus pol λ was able to synthesise DNA on a template containing abasic sites with the same efficiency as on undamaged DNA, thus suggesting a potential role of pol λ in translesion synthesis (6). Interestingly, pol λ mRNA expression is apparently cell cycle-dependent, being higher in quiescent and S to M phase cycling cells (3). Thus, pol λ could be specialized in DNA repair taking place during specific stages of the cell cycle required in nonproliferative cell types or during differentiation processes. Such a specialized DNA repair function of pol λ might be related to the ability of the enzyme to establish distinct interactions that could regulate its cellular function(s). To investigate the specific pathway(s) recruiting this enzyme as a first step in understanding its contribution to the maintenance of genetic stability, we have investigated its interaction with PCNA. The rationale of such an approach was based on the recent observations that PCNA, besides acting as a processivity factor for the replicative pols δ and ε, was able to interact with the repair enzyme pol β and with a variety of novel translesion pols such as pol ι, pol κ, and pol η, suggesting a potential role of this protein in targeting the translesion pols to the replication machinery. These pols have been shown to contain a sequence closely resembling the consensus sequence for PCNA interaction, SRGVLSSF for pol ι and SMSVTRFT for pol κ (the conserved PCNA-interacting protein box amino acids are in bold) (18).2 Interestingly, inspection of the hpol λ sequence revealed the presence of a very similar box, SVPVELELF (amino acids 331–338). We found that PCNA directly interacts with pol λ, and this interaction stabilizes the binding of the enzyme to the template/primer as reflected by the reduction of the dissociation rate of pol λ and the 5-fold increase in its processivity (Fig. 1). However, PCNA did not influence the affinity or the incorporation efficiency of the nucleotides by pol λ (Fig. 3); instead it increased the affinity of pol λ for the 3'-hydroxyl primer (Fig. 2). This was different from what has been observed with pol ι, pol κ, and pol η where the stimulation was caused by an increased affinity for the nucleotides (18, 19, 21). Rather, it closely resembled the mechanism of interaction between PCNA and pol δ, whose processivity is stimulated as a consequence of the reduced enzyme/DNA dissociation rate (12).

The lack of effects of PCNA on nucleotide incorporation by pol λ was also reflected by the fact that PCNA did not alter the ability of pol λ to discriminate between correct and wrong nucleotides; pol λ showed a very limited misincorporation abil-

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2 G. Maga, unpublished data.

**REFERENCES**


