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Physical association of the human base-excision repair enzyme uracil DNA glycosylase with the 70,000-dalton catalytic subunit of DNA polymerase α

(multienzyme complex/monoclonal antibodies/gene regulation/cell proliferation/human genetic disorders)

GITA SEAL AND MICHAEL A. SIROVER

Fels Research Institute and the Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA 19140

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ABSTRACT **A monoclonal antibody prepared against a partially purified human uracil DNA glycosylase was found, on further purification of the enzyme, to be inactive against the glycosylase. However, immunoreactivity was observed in other protein fractions that contained DNA polymerase activity. The immunoreactive protein was purified to homogeneity and identified as a catalytic subunit of DNA polymerase α by molecular mass, by aphidicolin sensitivity, and by recognition by a monoclonal antibody against human KB cell DNA polymerase α. Our monoclonal antibody had no effect on homogeneous human uracil DNA glycosylase activity but severely inhibited the activity of the homogeneous human DNA polymerase α catalytic subunit. The suspicion that the two proteins were physically associated was confirmed by finding that, on mixing the DNA polymerase α subunit with the glycosylase, the latter was strongly inhibited by our monoclonal antibody. These results demonstrate that this monoclonal antibody recognizes not only the DNA polymerase α subunit but also the uracil DNA glycosylase when it is physically attached to the polymerase subunit. These results contribute to the definition of relationships between those proteins that may comprise the human base-excision repair multienzyme complex.**

Recent studies have characterized the *in vitro* individual enzymatic reactions involved in human DNA repair. Similar studies have examined cellular DNA repair synthesis and the excision of DNA adducts *in vivo* (1–4). However, specific structural interrelationships between these individual proteins within multienzyme repair complexes may be an *a priori* requirement for the proper cellular function of individual components of excision repair pathways. In addition, specific alterations of such physical relationships within distinct DNA repair complexes may provide a molecular mechanism for the individual cellular hypersensitivity in human genetic syndromes characterized by high rates of neoplasia (3).

To examine the structural associations required for the activity of multienzyme DNA repair pathways, we prepared a series of monoclonal antibodies using partially purified human placental uracil DNA glycosylase as the antigen (5). The uracil DNA glycosylase removes uracil residues from DNA as an initial step of base-excision repair (1, 2). Uracil may be formed in DNA by the mutagenic deamination of cytidine (6, 7) or by the utilization of dUTP during DNA replication (8, 9). The resultant apyrimidinic site would be the substrate for subsequent endonuclease incision. The number of individual proteins required for base-excision repair is unknown and the identity of the DNA polymerase that participates in this excision repair pathway is unclear. Four mouse monoclonal antibodies were chosen for detailed analysis based upon their anti-uracil DNA glycosylase activity. Such activity was defined either by inhibition after enzyme immunoprecipitation or by glycerol gradient centrifugation of the glycosylase-monoclonal antibody immune complex. Surprisingly, further purification of the glycosylase by DNA cellulose chromatography resulted in a loss of anti-uracil DNA glycosylase activity by one of the monoclonal antibodies, whereas other protein fractions were immunoreactive. A reactive protein was purified to homogeneity and identified as a catalytic subunit of DNA polymerase α by *in vitro* biochemical analysis. Addition of this purified protein to homogeneous human placental uracil DNA glycosylase restored the inhibition of DNA glycosylase activity by the monoclonal antibody. These results demonstrate that there exists a structural relationship *in vitro* between these two enzymes and suggests that DNA polymerase α may be the DNA polymerase involved in human base-excision repair initiated by uracil DNA glycosylase.

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MATERIALS AND METHODS

Preparation of Monoclonal Antibodies. Human placental uracil DNA glycosylase was partially purified through DEAE-cellulose, phosphocellulose, and hydroxylapatite column chromatography as described (5). The monoclonal antibodies 16.11.08, 40.10.09, and 42.08.07 were prepared against this uracil DNA glycosylase. Each antibody was analyzed in detail for its anti-uracil DNA glycosylase activity in an enzyme immunoprecipitation assay with partially purified human placental uracil DNA glycosylase. The control monoclonal antibody 1.05 was prepared from a previous fusion using mice that were not immunized with any specific antigen. Monoclonal antibodies were purified from spent culture fluid by ammonium sulfate precipitation at 50% saturation and purified by DEAE-cellulose chromatography (5). Protein concentrations were determined by the method of Lowry *et al.* (10). Monoclonal antibody to human KB cell DNA polymerase α was a generous gift of Lawrence A. Loeb (Univ. of Washington School of Medicine).

ELISA Assay. Column fractions of 25–50 μl were added to 96-well polyvinyl chloride microtiter plates. To ensure optimal protein absorption, each plate was incubated for 2 hr at 37°C followed by a further incubation at 4°C for 48–72 hr. To saturate all other binding sites, each well was washed twice with Dulbecco's phosphate-buffered saline (PBS) and then incubated with 200 μl of 1% bovine serum albumin in PBS for 30 min at 37°C. After removal of the albumin solution, each plate was rinsed twice with PBS. Plates were stored at 4°C until used; each well contained 100 μl of PBS. Each monoclonal antibody was added in 50-μl aliquots at a concentration of 1 ng/μl. After incubation for 2 hr at 37°C, the first antibody was removed and each plate was rinsed with washing buffer [New England Nuclear; 10 mM Tris·HCl (pH 8.0) plus 0.05% Tween 20]. Alkaline phosphatase-conjugated F(ab') sheep anti-mouse IgG serum (New England Nuclear) (50 μl of a

1:250 dilution) was used as second antibody. After incubation for 2 hr at 37°C, the second antibody was removed and each plate was washed once with washing buffer and then twice with deionized distilled water. Each well was tested for second antibody binding with 0.05 M *p*-nitrophenyl phosphate as substrate, followed by incubation for 16 hr at 25°C. The reaction was terminated by addition of 50 μl of 1 M NaOH. Spectrophotometric analysis at 405 nm was performed using a Uniskan plate reader.

Enzyme Assays. Uracil DNA glycosylase activity was assayed in a reaction mixture (final volume, 100 μl) that contained 100 mM Tris·HCl (pH 8.0), 10 mM K_2EDTA, 5 mM dithiothreitol, 1 μg of poly(dA)·poly([^{3}H]dU) (specific activity, 5,000–15,000 dpm/pmol), and 25–50 μl of column fraction. Uracil-containing DNA was prepared as described (5). The mixture was incubated for 60 min at 37°C, and the assay was terminated by the addition of 300 μl of ethanol at −20°C, 0.1 mg of heat-denatured calf thymus DNA in 100 μl, and 60 μl of 2 M NaCl. After a minimum of 60 min at −20°C, ethanol-precipitable material was collected by centrifugation at 2300 × *g* for 10 min at 4°C. Radioactivity in a 200-μl aliquot of the ethanol-soluble supernatant was used as a measure of glycosylase activity.

DNA polymerase activity was determined in a reaction mixture of 100 μl that contained 100 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, 100 μM dGTP, 100 μM dATP, 100 μM dCTP, 40 μM [α-^{32}P]dTTP (specific activity, 700–1000 cpm/pmol), 15 μg of DNase-activated calf thymus DNA, and 25 μl of column fraction. The mixture was incubated for 60 min at 37°C and the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid and 0.1 mg of heat-denatured calf thymus DNA in 100 μl. The precipitate was collected by centrifugation at 2300 × *g* for 10 min at 4°C. The precipitate was redissolved and reprecipitated twice using 1 ml of 0.2 mM NaOH and 1 ml of 10% trichloroacetic acid, sequentially, followed by centrifugation as described above. Acid-insoluble precipitates were then collected on a glass fiber filter and radioactivity was determined.

RESULTS

Specificity of Monoclonal Antibodies. Using partially purified human placental uracil DNA glycosylase as antigen, we demonstrated previously that all of the monoclonal antibodies exhibited anti-glycosylase activity. A typical result is shown in Fig. 1*A*. As measured by ELISA analysis, partially purified glycosylase was recognized in a concentration-dependent manner by each of three monoclonal antibodies—16.11.08, 40.10.09, and 42.08.07. However, further purification of the glycosylase through DNA cellulose resulted in a complete loss of recognition by antibody 16.11.08 (Fig. 1*B*). No absorbance was detected in the ELISA assay at all of the concentrations of 16.11.08 monoclonal antibody. In contrast, at equivalent protein concentrations, antibodies 40.10.09 and 42.08.07 retained their immunoreactivity and did so using homogeneous glycosylase (unpublished data).

To identify the unknown protein within the impure glycosylase preparation, 16.11.08-immunoreactive protein from the DNA cellulose column eluants was purified further. As shown in Fig. 2*A*, molecular sieving through Sephadex G-100 gel demonstrated that this protein eluted at a molecular size comparable to that of bovine serum albumin. No uracil DNA glycosylase activity was observed in any column fraction. In contrast, DNA polymerase activity was readily detected and eluted at a position approximate to that observed for the 16.11.08-immunoreactive reacting material. No DNA polymerase activity or any ELISA-reactive protein was observed at a molecular mass of >100,000 daltons.

The ELISA-reacting protein from the G-100 column was then chromatographed on a 16.11.08 Sepharose 4B immunoaffinity column. As shown in Fig. 2*B*, all of the 16.11.08-reacting protein was retained by the antibody column. However, the protein could be recovered by a salt gradient and eluted at a concentration of 0.125 M NaCl. DNA polymerase activity could also be recovered from the antibody affinity column. Further, all DNA polymerase activity was bound to the affinity column and coeluted with the ELISA-reactive protein in the gradient. As before, no uracil DNA glycosylase activity was observed using any of the column wash or salt gradient fractions. In contrast, uracil DNA glycosylase activity was readily eluted from antibody affinity columns that were prepared with either 40.10.09 or 42.08.07 monoclonal antibody bound to Sepharose 4B (unpublished data).

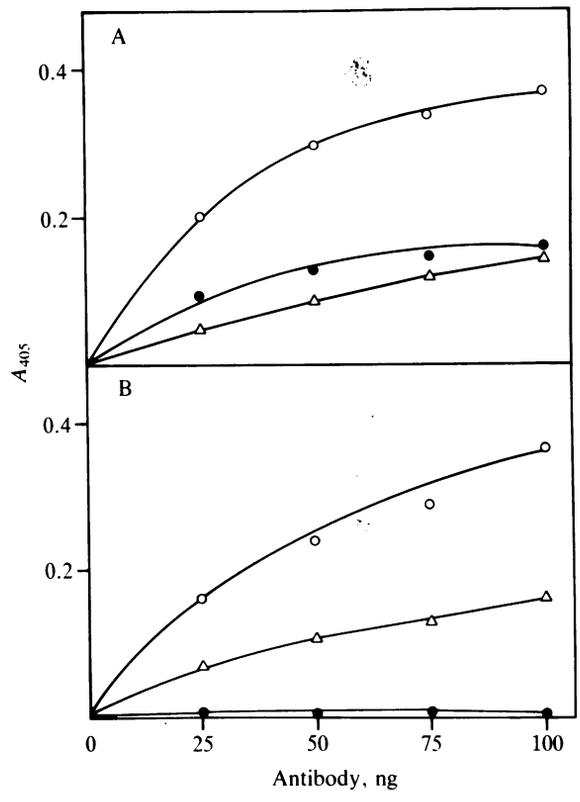

FIG. 1. ELISA analysis of purified human placental uracil DNA glycosylase. ELISA analysis with the indicated anti-human placental uracil DNA glycosylase monoclonal antibodies was performed in triplicate. Equivalent protein concentrations were used in each set of ELISA determinations. (*A*) Human uracil DNA glycosylase purified through DEAE-cellulose and phosphocellulose. (*B*) Human uracil DNA glycosylase further purified through DNA cellulose. △, 40.10.09 monoclonal antibody; ○, 42.08.07 monoclonal antibody; ●, 16.11.08 monoclonal antibody.

Characterization of ELISA-Reactive Protein as a Catalytic Subunit of DNA Polymerase α. To identify the DNA polymerase activity that was purified as the 16.11.08-immunoreactive protein, the molecular mass of the purified protein was first determined. As shown by $NaDodSO_4$/polyacrylamide gel electrophoresis, one major protein band with a molecular mass corresponding to ≈70,000 daltons was observed after the final 16.11.08 antibody affinity chromatography step (results not shown). Further, the virtual absence of other detectable proteins suggested that the protein was purified to homogeneity.

The aphidicolin sensitivity of the DNA polymerase activity was then examined. The DNA polymerase was inhibited significantly with increasing concentrations of aphidicolin (Fig. 3). Calf thymus DNA polymerase α displayed a similar aphidicolin sensitivity. In contrast, human placental DNA polymerase β and Novikoff hepatoma DNA polymerase β

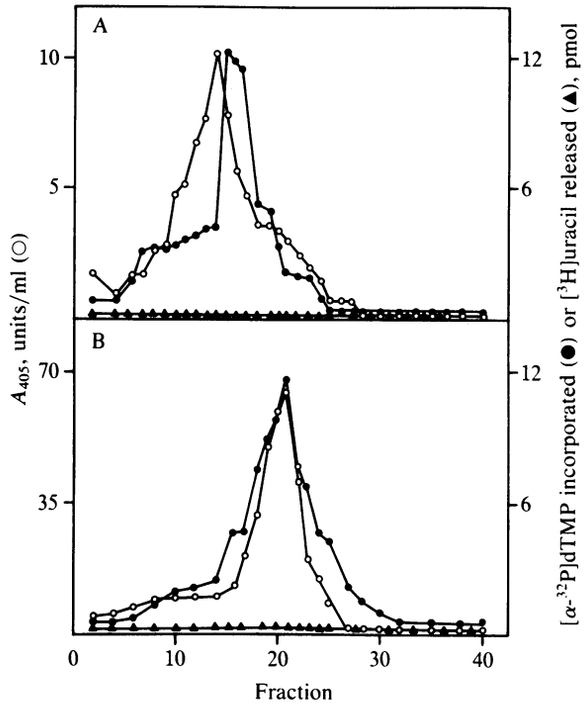

FIG. 2. Purification of 16.11.08-immunoreactive protein. Immunoreactive material detected after DNA cellulose chromatography was pooled, dialyzed, concentrated 1:10 by ultrafiltration, and purified by gel filtration through Sephadex G-100 (*A*) in buffer I (20 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/20% glycerol) containing 100 mM NaCl. Uracil DNA glycosylase, DNA polymerase, and ELISA-reactive material were examined. Fractions containing reactive material were pooled and absorbed to a 16.11.08 antibody affinity column (*B*). The column was washed with 10 vol of buffer I. ELISA-reactive material was eluted using a linear gradient of 0.05–1.0 M NaCl in buffer I (90 ml each limit). Uracil DNA glycosylase, DNA polymerase enzyme activities, and ELISA-reactive protein were then determined.

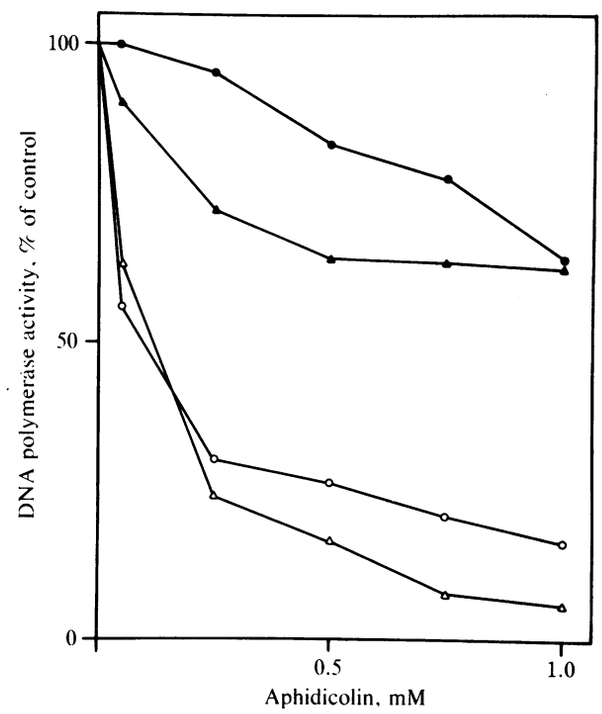

FIG. 3. Aphidicolin sensitivity of 16.11.08-immunoreactive DNA polymerase. Aphidicolin sensitivity with the indicated DNA polymerase was tested by using the DNA polymerase assay. Equivalent DNA polymerase protein concentrations were used. Human placental DNA β was prepared as described (11). Novikoff hepatoma DNA polymerase β and calf thymus DNA polymerase α were kindly provided by Lawrence A. Loeb. ○, Human placental 16.11.08-immunoreactive DNA polymerase; △, calf thymus DNA polymerase α; ▲, human placental DNA polymerase β; ●, Novikoff hepatoma DNA polymerase β.

were relatively insensitive to aphidicolin. The DNA polymerase was also sensitive to *N*-ethylmaleimide. Each of these criteria is characteristic of eukaryotic DNA polymerase α (12). The human core DNA polymerase α contains two subunits with molecular sizes of *ca.* 67,000 and 57,000 daltons (13). Thus, it appears that the 16.11.08-immunoreactive protein is the large subunit that retains catalytic activity.

The antigenic specificity of the DNA polymerase α subunit was then examined. The catalytic subunit of human placental DNA polymerase α and calf thymus DNA polymerase α were recognized by 16.11.08 antibody (Fig. 4*A*). In contrast, Novikoff hepatoma DNA polymerase β and human placental DNA polymerase β were not detected by the antibody except for some reaction with placental polymerase β at higher protein concentrations. Similarly, a monoclonal antibody to human KB cell DNA polymerase α recognized the calf thymus DNA polymerase α and the human DNA polymerase α catalytic subunit (Fig. 4*B*). As expected, both DNA polymerase β enzymes were not immunoreactive.

Restoration of Anti-Uracil DNA Glycosylase Activity by 16.11.08 Monoclonal Antibody. Glycerol gradient analysis was used to determine whether the anti-uracil DNA glycosylase activity of the 16.11.08 monoclonal antibody could be restored. First, homogeneous human placental uracil DNA glycosylase, by itself, was preincubated with 16.11.08 monoclonal antibody. The glycosylase sedimented at the top of the gradient (Fig. 5*A*) at an identical position with comparable activity after preincubation with 1.05 antibody. These results demonstrate that homogeneous human placental uracil DNA glycosylase was not recognized by antibody 16.11.08 despite its reactivity toward partially purified human placental uracil DNA glycosylase.

The effect of the DNA polymerase α subunit on these sedimentation patterns was examined by the addition of homogeneous polymerase α subunit to the glycosylase/16.11.08 preincubation mixture. In this instance, a distinctly different pattern of glycosylase sedimentation was observed (Fig. 5*A*). The initial peak of glycosylase activity was severely diminished and an additional peak of glycosylase activity was observed sedimenting further into the gradient. The extent of glycosylase activity present in the second peak is approximately comparable to the amount of glycosylase activity lost from the first peak. This result was observed in two separate experiments. Further, this pattern is identical to that reported for the sedimentation of partially purified human placental uracil DNA glycosylase after preincubation with monoclonal antibody 16.11.08 (5). Sedimentation of the residual polymerase toward the bottom of the gradient to a position identical to that of the glycosylase demonstrates directly that the addition of the homogeneous DNA polymerase α subunit to the glycosylase restored the ability of the 16.11.08 monoclonal antibody to complex with the uracil DNA glycosylase.

To determine the specificity of the human uracil DNA glycosylase–polymerase subunit interaction, three separate control experiments were performed. (*i*) The association of the subunit with highly purified *B. subtilis* uracil DNA glycosylase was examined (Fig. 5*B*). This glycosylase sedimented at the top of the gradient after preincubation of this enzyme with 16.11.08 antibody by itself. This profile was not altered by the addition of the polymerase to the preincubation mixture. Further, in that experiment, the polymerase subunit sedimented at a similar position when it, by itself, was

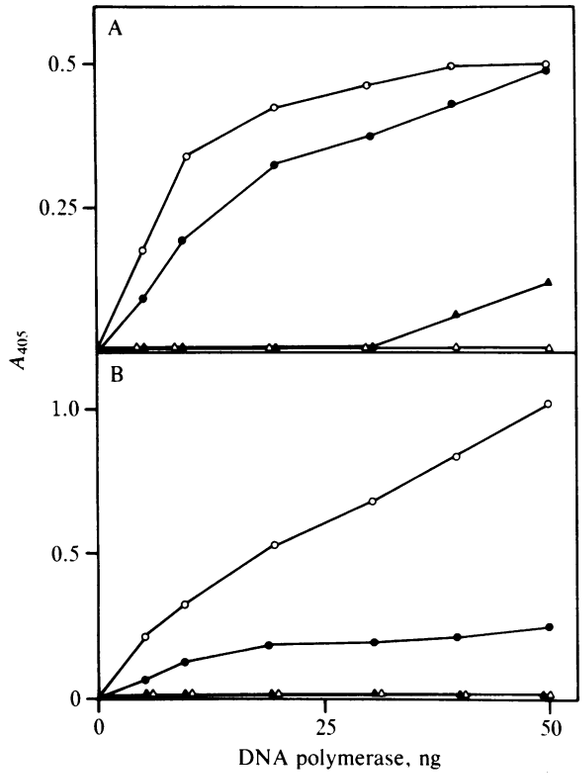

FIG. 4. ELISA analysis of 16.11.08-immunoreactive DNA polymerase. ELISA analysis with the indicated monoclonal antibody was performed and DNA polymerase activity was determined. Each ELISA quantitation contained equal DNA polymerase concentrations. (*A*) Immunoreactivity with 16.11.08 monoclonal antibody. (*B*) Immunoreactivity with anti-human KB cell DNA polymerase α monoclonal antibody. ●, 16.11.08-immunoreactive DNA polymerase; ○, calf thymus DNA polymerase α; ▲, human placental DNA polymerase β; △, Novikoff hepatoma DNA polymerase β.

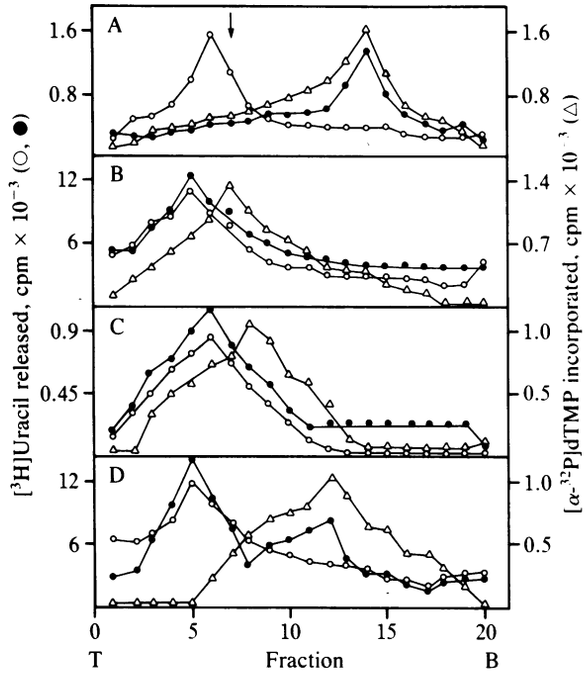

FIG. 5. Glycerol gradient analysis of anti-uracil DNA glycosylase activity of monoclonal antibody 16.11.08. Uracil DNA glycosylase (130 μg) with or without homogeneous human placental DNA polymerase α subunit (150 μg) was incubated with monoclonal antibody 16.11.08 (95 μg) for 120 min at 4°C. The enzyme/antibody mixture was then sedimented through a 5-ml 10–35% glycerol gradient as described (5). Highly purified *Bacillus subtilis* uracil DNA glycosylase was a generous gift of Nahum Duker. Crude yeast and baby hamster kidney uracil DNA glycosylases were prepared as described (5). Human uracil placental DNA glycosylase was purified 3700-fold (unpublished data). Fractions were collected from the top of the gradient (T = top; B = bottom). DNA polymerase α and uracil DNA glycosylase activities were then determined. The arrow in *A* refers to the sedimentation of the polymerase subunit when it, by itself, was preincubated with monoclonal antibody 16.11.08. ○, Uracil DNA glycosylase activity after incubation with antibody 16.11.08; ●, uracil DNA glycosylase activity after incubation with DNA polymerase α and antibody 16.11.08; △, DNA polymerase α activity after incubation with uracil DNA glycosylase and antibody 16.11.08. (*A*) Human placental glycosylase. (*B*) *B. subtilis* glycosylase. (*C*) Yeast glycosylase. (*D*) Baby hamster kidney glycosylase.

preincubated with 16.11.08 antibody. (*ii*) The association of the polymerase subunit with partially purified yeast uracil DNA glycosylase was examined (Fig. 5*C*). In this instance, identical sedimentation patterns were observed when the crude glycosylase preparation was preincubated with 16.11.08 antibody in the presence or absence of the polymerase subunit. These results would suggest that nonspecific protein interactions could not account for the association of the human glycosylase and the polymerase subunit. (*iii*) The association of the polymerase subunit with the baby hamster kidney uracil DNA glycosylase was examined (Fig. 5*D*). In this instance, the glycosylase profile showed that a portion of the enzyme activity now sedimented further into the gradient. The position of the polymerase subunit was also altered to sediment similarly to the glycosylase. The partial association of this glycosylase with the polymerase subunit conforms with the partial cross-reactivity of that glycosylase, as determined by enzyme immunoprecipitation (5).

DISCUSSION

This present report provides evidence to begin to identify physical relationships within a DNA repair pathway. In particular, we have identified a defined structural relationship of the human base-excision repair enzyme uracil DNA glycosylase with a catalytic subunit of DNA polymerase α. As defined by glycerol gradient analysis, inhibition of uracil DNA glycosylase activity by an anti-DNA polymerase α monoclonal antibody was dependent on the physical association of homogeneous DNA polymerase α with homogeneous uracil DNA glycosylase. Several eukaryotic DNA polymerases have been identified and biochemically characterized. However, it remains unclear which polymerase(s) may be involved in DNA repair. Further, different DNA polymerases might be utilized in nucleotide-excision repair and in base-excision repair. Indirect evidence has been used to postulate a primary role for DNA polymerase β as the DNA repair enzyme (14, 15). However, inhibitor data, primarily using aphidicolin, demonstrated a role for DNA polymerase α in repair synthesis as well (16, 17).

It may be argued that these results suggest that the glycosylase–polymerase subunit interaction may be due to nonspecific, adventitious binding between these two proteins. However, the following should be noted. (*i*) In the glycerol gradient sedimentation analysis, in which the polymerase subunit was preincubated with 16.11.08 antibody and with *B. subtilis* uracil DNA glycosylase, no change was observed in the sedimentation pattern of this highly purified glycosylase. Further, there was no alteration in the polymerase-16.11.08 antibody sedimentation pattern. (*ii*) If nonspecific interactions were the basis for the glycosylase–polymerase interactions, preincubation of the polymerase 16.11.08 antibody complex with the glycosylase added as part of a crude enzyme preparation should affect glycosylase and polymerase sedimentation. In the case of the crude yeast

glycosylase, this was not observed. (*iii*) The specificity of the human glycosylase–polymerase association is reflected in the equivalent cross-reactivity of the monoclonal antibody, as determined either by enzyme immunoprecipitation or by glycerol gradient sedimentation analysis. In particular, we previously determined a slight degree of cross-reactivity with the crude baby hamster kidney glycosylase, as defined by enzyme immunoprecipitation. Similarly, as defined by glycerol gradient sedimentation, an equivalent slight degree of cross-reactivity was observed. Further, the crude mammalian enzyme demonstrated that the negative results using the crude yeast enzyme were not due to species dissimilarity.

Apurinic or apyrimidinic sites can occur in DNA as a result of spontaneous base loss or as a result of the action of DNA glycosylases. The presence of apurinic (AP) sites in DNA increases the infidelity of DNA replication, resulting in the miscopying of cellular DNA and the transfer of incorrect genetic information to progeny (18–20). However, cleavage of the AP site prevented the copying of the damaged DNA by DNA polymerases, thereby preventing the mutagenic event (18). Our results demonstrate a physical association of a DNA polymerase α subunit, the putative third or fourth enzyme within the base-excision repair pathway, with a first enzyme of that pathway. It remains unknown what relationships may exist with other repair enzymes that comprise the intermediate nuclease steps of base-excision repair. However, it seems reasonable to speculate that such defined physical structures may exist within the base-excision repair multienzyme complex for each individual enzyme. This structure would be designed to ensure the immediate, efficient cleavage of the apyrimidinic or apurinic site after DNA glycosylase action. This would prevent miscopying during DNA repair or during DNA replication by the DNA replitase (21) if the repair complex were to be dissociated from damaged DNA prior to the completion of base-excision repair. Previous studies in prokaryotes demonstrated a defined multiprotein complex for nucleotide-excision repair (22). The results presented in this report present an opportunity to examine such structural associations in normal human cells. Further, these results suggest a mechanism to probe potential alterations in DNA repair multienzyme complexes in hypermutable cells from cancer-prone individuals.

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