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MONOCLONAL ANTIBODY SPECIFIC FOR CHICKEN DNA POLYMERASE α ASSOCIATED WITH DNA PRIMASE

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SUMMARY: Four monoclonal antibodies against chicken DNA polymerase α were obtained from mouse hybridomas (see ref. 1). Two of them, 4-2D and 4-8H, recognized different epitopes of the DNA polymerase α-DNA primase complex as determined by a competitive enzyme-linked immunosorbent assay. Antibody 4-8H partially (about 30%) neutralized the combined activity of primase-DNA polymerase α as well as the DNA polymerase α activity. In contrast, antibody 4-2D did not neutralize DNA polymerase α activity, but neutralized the primase-DNA polymerase α activity extensively (up to 80%). Furthermore, although an immunoaffinity column made with 4-8H antibody retained virtually all of the DNA polymerase α with and without associated primase, a column made with 4-2D antibody did not bind DNA polymerase α without the primase, but retained the enzyme associated with the primase. These results indicate that 4-8H monoclonal antibody is specific for DNA polymerase α and 4-2D monoclonal antibody is specific for the primase or a special structure present in the primase-DNA polymerase α complex.

Eukaryotic DNA is replicated via Okazaki fragments, which are short DNA strands associated covalently with oligoribonucleotide "primers" (2-6). Primase, an enzyme responsible for the synthesis of primers, has been found in the form of a complex with DNA polymerase α, and the complex synthesizes oligoribonucleotide-primed DNA chains on single stranded DNA template in vitro (7-9). Therefore, the complex of the primase and DNA polymerase α occupies the core

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Abbreviations used: DNA polymerase, deoxynucleotidetriphosphate:DNA deoxynucleotidyltransferase (EC 2.7.7.7.).; ELISA, enzyme-linked immunosorbent assay.

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part of the DNA replication machinery in which the active centers of these enzymes may closely co-operate. However, the structure of the complex has not yet been fully elucidated. Previously, we prepared monoclonal antibodies to chicken DNA polymerase α (1). In the present work, we obtained results indicating that two of these antibodies are specific for the primase and DNA polymerase α, respectively. Results with these antibodies suggested that active centers of the primase and DNA polymerase α are located in different regions of the primase-DNA polymerase α complex.

MATERIALS AND METHODS

Preparation of chicken embryo DNA polymerase α A preparation after the hydroxylapatite column chromatography step was used (10). This preparation contained DNA polymerase α with and without associated primase activity as shown in Fig. 3. The specific activity of DNA polymerase α at this step was about 2,000 units (nmol dNTP polymerization/60 min) per mg protein and its purity was estimated as 4% (10).

Immunological reagents Establishment of the mouse hybridoma producing monoclonal antibodies against chicken DNA polymerase α was described previously (1). Both 4-2D and 4-8H antibodies are IgG_1. Antisera were obtained from nude mice into which these hybridoma cells had been transplanted, and control serum was obtained from nude mice without hybridomas. For biotin-labeling, monoclonal antibodies were purified from antisera on a protein A-Sepharose column (11). The IgG_1 fractions obtained were labeled with biotin as described Guesdon et al. (12). Protein A-Sepharose was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden and horseradish peroxidase conjugated avidin from E-Y Laboratories Inc., San Mateo, Ca.

Assays of DNA polymerase α and primase-DNA polymerase α activities The reaction mixture for assay of DNA polymerase α in a final volume of 25 μl consisted of 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 10 mM $MgCl_2$, 80 μg/ml activated calf thymus DNA, 20 mM KCl, 15% glycerol, 0.1 mM each of dATP, dGTP, dCTP and [^{3}H]dTTP(100 cpm/pmol) and enzyme. Mixtures were incubated at 37°C for 20 min. Primase-DNA polymerase α activity was measured as the polymerization of dATP depending on the addition of rATP and poly(dT) as a template (8, 13). The reaction mixture in a final volume of 25 μl consisted of 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 10 mM $MgCl_2$, 15% glycerol, 400 μg/ml bovine serum albumin, 20 mM KCl, 8 μg/ml poly(dT), 2 mM rATP, 25 μM [^{3}H]dATP (1000 cpm/pmol) and enzyme. Mixtures were incubated at 37°C for 60 min. After the reaction, the polymer product was collected on a disc of DEAE-cellulose paper and its radioactivity was measured.

Competitive immunobinding assay by an enzyme-linked immunosorbent assay (ELISA) Wells of a 96 well microtiter plate were coated by incubating them with enzyme solution containing 50-100 ng protein (2-4 ng with respect to DNA polymerase α) in 50 μl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) at room temperature for 2 hr. Residual protein-binding sites on the wells were blocked by incubation for 2 hr periods with two changes of 100 μl of phosphate bufferd saline (PBS) containing 0.05% Tween 20, 0.1% NaN_3, 5% fetal calf serum and 5% normal goat serum. The wells were then washed once with 100 μl of PBS containing 0.05% Tween 20 (PBS-Tween). The washing solution was removed and 50 μl of serially diluted competitor antiserum was added to the wells. After overnight incubation at 4°C, 10 μl of the diluted biotin-labeled antibody was added without removing the competitor serum and the plate was incubated at 4°C for 1 hr more. The wells were then washed with PBS-Tween and incubated with 50 μl of diluted (1:500) avidin conjugated with horseradish peroxidase at 4°C for 1 hr. Then they were washed

and color was developed by incubation with 50 μl of peroxidase substrate solution containing 0.1 M citrate buffer (pH 5.0), 0.03% hydrogen peroxide and 1 mg/ml o-phenylenediamine. After 2 min at 4°C, the reaction was terminated by adding 50 μl of 2 N HCl, and the absorbance at 492 nm was read spectrophotometrically.

Immunobinding and neutralization assays For immunobinding assay, 1.4 unit of DNA polymerase α was mixed with 10 μl of diluted serum in 50 mM Tris-HCl (pH 7.4), 0.1 M KCl, 1 mM dithiothreitol, 15% glycerol and 1 mg/ml bovine serum albumin and incubated at 37°C for 60 min. Then, the immuncomplex was removed from the supernatant by incubating the mixture with 10 μl of 10% (w/v) suspension of formalin-fixed Staphylococcus aureus Cowan I at 37°C for 30 min and centrifuging the mixture at 1,000g for 5 min. Ten microliter of the supernatant was mixed with the reaction mixture for assay of DNA polymerase α or primase-DNA polymerase α activity. For the neutralization assay, the enzyme was incubated in a volume of 10 μl with diluted antiserum at 37°C for 60 min as for the immunobinding assay and then mixed with the reaction mixture for assay of DNA polymerase α or primase-DNA polymerase α activity.

Immunoaffinity column chromatography A column of protein A-Sepharose was prepared and charged with antiserum that had been dialized against 0.1 M Na-phosphate buffer (pH 8.1) containing 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonylfluoride and 0.02% NaN_3. DNA polymerase α that had been dialized against the same buffer was applied to the column which was followed by washing with the buffer. The antibody-antigen complex was recovered by washing the column with 0.1 M Na-citrate buffer (pH 5.6) containing 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonylfluoride, 0.02% NaN_3 and 0.2 M KCl.

RESULTS AND DISCUSSION

The monoclonal antibodies used in this study were produced by the mouse hybridoma cells established as described previously (1). These hybridoma clones were selected by an immunobinding assay of DNA polymerase α activity. Therefore, the antigenic determinant of these monoclonal antibodies were located either on the DNA polymerase α molecule itself or on a polypeptide(s) associated with the DNA polymerase α. Two of these IgG_1 antibodies, 4-2D and 4-8H, were used in this work for the immunochemical analysis of the complex of primase and DNA polymerase α.

Fig. 1 shows results by ELISA on the competitive binding of the two antibodies to the enzyme molecule. Like control mouse serum, the unlabeled 4-8H antiserum at more than eightfold concentration of the 4-2D antibody did not interfere with the binding of the biotin-labeled 4-2D antibody to the DNA polymerase α preparation containing primase activity, and vice versa. Therefore, the antigenic determinant of these two antibodies are apparently located in different regions of the primase-DNA polymerase α complex molecule.

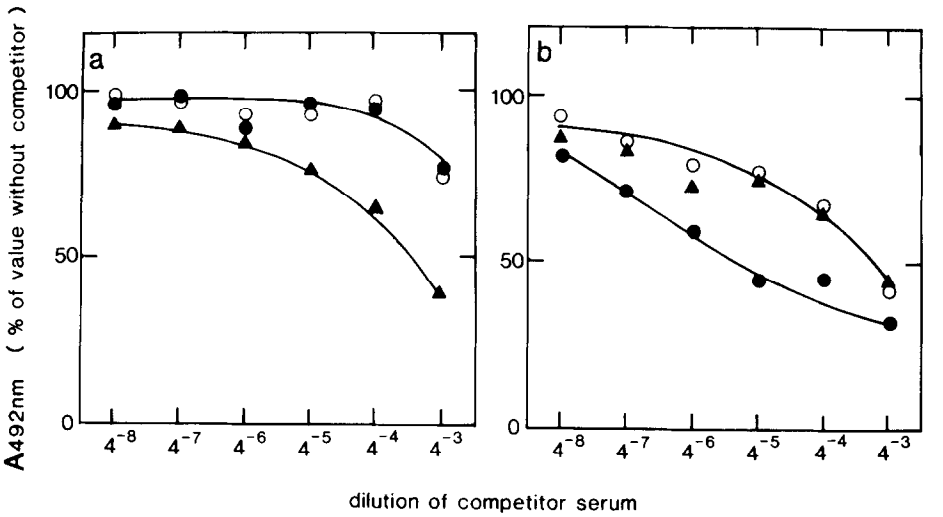

Fig. 1 Competition between 4-2D and 4-8H monoclonal antibodies in immunobinding to primase-DNA polymerase α. Competitive binding was assayed by the ELISA described under Materials and Methods. a. Competition between antisera and biotin labeled 4-2D antibody. b. Competition between antisera and biotin labeled 4-8H antibody. The competitor sera were: ○ , normal nude mouse serum; ● , 4-2D antiserum and ▲ , 4-8H antiserum. The value without competitor serum is taken as 100%.

The 4-2D and 4-8H antibodies both bound to DNA polymerase α (Fig. 2-a). However. only the 4-8H antibody neutralized DNA polymerase α activity to some extent (about 30%) (Fig. 2-b). The primase-DNA polymerase α activity was measured as the polymerization of dATP which was dependent on the presence of

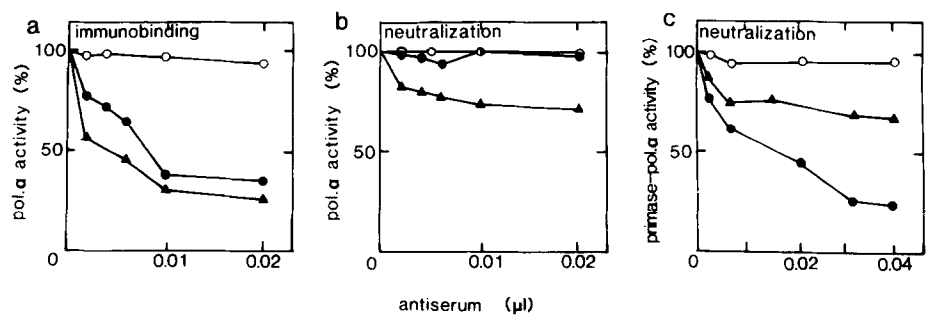

Fig. 2 Immunobinding, neutralization of DNA polymerase α activity and neutralization of primase-DNA polymerase α activity with monoclonal antibodies 4-2D and 4-8H. Immunobinding and neutralization assays were performed as described under Materials and Methods. Immunobinding assay of DNA polymerase α activity (a). The value for 100% activity was 137 pmol [^{3}H]dTTP incorporation. Neutralization assay of DNA polymerase α activity (b) and primase-DNA polymerase α activity (c). The values for 100% DNA polymerase α and primase-DNA polymerase α activities were 87 pmol [^{3}H]dTTP incorporation and 4.0 pmol [^{3}H]dATP incorporation, respectively. ○ , serum from normal nude mouse; ● , antiserum from nude mouse bearing 4-2D hybridoma cells and ▲ , antiserum from mouse bearing 4-8H hybridoma cells.

single stranded poly(dT) as a template and rATP for synthesis of oligoribonucleotide primers (Fig. 2-c). The 4-8H antibody neutralized the primase-DNA polymerase α activity to the same extent (about 30%) as the DNA polymerase α activity. This findidng is understandable because this antibody neutralized the combined primase-DNA polymerase α activity by affecting the active center of DNA polymerase α. On the other hand, the 4-2D antibody extensively neutralized the primase-DNA polymerase α activity (Fig. 2-c). Since this antibody did not affect the DNA polymerase α activity (Fig. 2-b), this result indicates that the antigenic determinant for this antibody is closely related to the primase, but not the DNA polymerase α.

The specificities of the 4-2D and 4-8H antibodies were confirmed by an immunoaffinity chromatography using these antibodies. Partially purified preparations of DNA polymerase α with and without associated primase activity were subjected to the chromatography. If the primase activity is associated with a special domain or a polypeptide that is different from that for DNA polymerase α, 4-2D antibody should bind to DNA polymerase α associated with the primase, but not to primase-free DNA polymerase α. As shown in Fig.3, the expected results were obtained; A column made of protein A-Sepharose and 4-8H antibody retained all the DNA polymerase α and primase-DNA polymerase α activities, and these activities were eluted with the buffer of pH 5.6 which dissociates IgG_1 from protein A (11), The enzyme isolated in this way synthesized the combined products of short RNA of about 10 nucleotides and DNA strands of 100-1000 nucleotide length on single stranded DNA template (data not shown), which are the products of the primase-DNA polymerase α complex reported by a number of groups (7, 14-16). In contrast to the column with the 4-8H antibody, that with 4-2D antibody did not bind a considerable amount of DNA polymerase α, the amount depending on the batch of DNA polymerase α preparation, and unbound fraction contained no primase activity. DNA polymerase α associated with primase activity was recovered in the form of complex with antibody in the fraction of the eluate obtained with pH 5.6 buffer, although the recovery of primase-DNA polymerase α was very low because it was extensively neutralized.

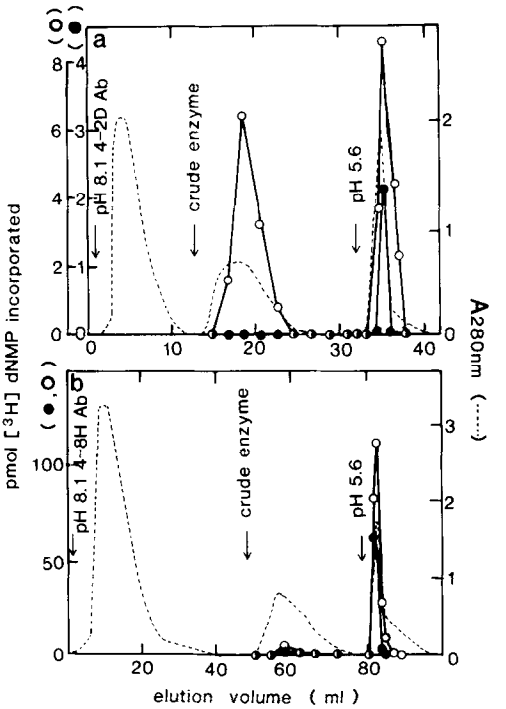

Fig. 3 Immunoaffinity column chromatographies of primase-DNA polymerase α with monoclonal antibodies 4-8H (a) and 4-2D (b). Column was made of protein A-Sepharose equilibrated with 0.1 M Na-phosphate buffer (pH 8.1) containing 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonylfluoride and 0.02% NaN_3 and antiserum was charged. Then, a DNA polymerase α preparation dialized against the same buffer was applied and the column was washed with the buffer. The DNA polymerase α-antibody complex was eluted with Na-citrate buffer (pH 5.6) containing 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonylfluoride and 0.02% NaN_3. DNA polymerase α and primase-DNA polymerase α activities were measured as described under Materials and Methods except that rGTP, [^{3}H]dGTP and poly(dC) were used instead of rATP, [^{3}H]dATP and poly(dT). ○, DNA polymerase α activity; ●, primase-DNA polymerase α activity and ------, absorbance at 280 nm.

Virtually the same result was obtained when the amount of enzyme preparation relative to the column size or to the amount of the antibody was reduced. Therefore, the lack of absorption of the primase-free DNA polymerase α was not due to a low capacity of the column. Thus, it is reasonable to conclude that 4-2D antibody is specific for the structure responsible for the primase activity, and only DNA polymerase α associated with this structure or polypeptide is retained by a column prepared with this antibody.

In a preliminary experiment by immunoblotting, we found that the antigenic dete .inant for 4-8H antibody was present on the 130-150 kDa subunit of DNA polymerase α. However, it was difficult to identify the structure that reacted

with 4-2D antibody, because the antigenicity for this antibody was lost on denaturation of the enzyme with sodium dodecyl sulfate or heat treatment. Our preliminary result demonstrated that the enzyme preparation eluted from an immunoaffinity column containing 4-2D antibody had increased amount of 34 kDa and 78 kDa polypeptides in addition to those of DNA polymerase α (130-150 kDa and 50-60 kDa). These results suggest that either 78 kDa or 34 kDa polypeptide or a complex of these polypeptides is responsible for the primase activity.

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