

Autoantibodies to DNA-dependent Protein Kinase

Probes for the Catalytic Subunit

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Abstract

DNA-dependent protein kinase (DNA-PK) is an important nuclear enzyme which consists of a catalytic subunit known as DNA-PKcs and a regulatory component identified as the Ku autoantigen. In the present study, we surveyed 312 patients in a search for this specificity. 10 sera immunoprecipitated a large polypeptide which exactly comigrated with DNA-PKcs in SDS-PAGE. Immunoblot analysis demonstrated that this polypeptide was recognizable by a rabbit antiserum specific for DNA-PKcs. Although the patient sera did not bind to biochemically purified DNA-PKcs in immunoblots or ELISA, they were able to deplete DNA-PK catalytic activity from extracts of HeLa cells in a dose-dependent manner. We conclude that these antibodies should be useful probes for studies which aim to define the role of DNA-PK in cells. Since six sera simultaneously contained antibodies to the Ku protein, these studies suggest that relatively intact forms of DNA-PK complex act as autoantigenic particles in selected patients. (*J. Clin. Invest.* 1996, 97:1417–1421.) Key words: antinuclear antibodies • autoantibodies • autoimmunity • DNA-dependent protein kinase • Ku protein

Introduction

DNA-dependent protein kinase (DNA-PK)¹ is found in many kinds of eukaryotic cells (1–5). It consists of an ~460-kD catalytic component referred to as DNA-PKcs and a regulatory subunit known as the Ku protein (6–9). The latter is made up of 70- and 80-kD polypeptides that associate noncovalently to form a DNA binding heterodimer capable of recognizing a number of DNA structures including free ends (10–17), nicks (18), hairpins (19), and bubbles (20). Once bound to double-

stranded DNA, the Ku protein creates a binding site for DNA-PKcs and thus mediates assembly of the DNA-PK holoenzyme (6, 7). This structure disassembles progressively as salt concentrations increase beyond 0.2 M (21). Recently, it has been observed that this enzyme plays roles in transcription (4–7, 22, 23), DNA repair, and V(D)J recombination (24–29), and that it represents the causative defect in the SCID mouse (30–32). The catalytic subunit is structurally related to the phosphatidylinositol 3-kinases (9). Several reports have provided detailed information about autoantibodies to the regulatory Ku protein subunit (10, 11, 13, 33–35).

In the present study, we have identified 10 patients whose sera directly immunoprecipitate DNA-PKcs. These sera deplete the 460-kD polypeptide, as well as DNA-PK catalytic activity, from HeLa cell extracts. Six patients have associated anti-Ku antibodies indicating that autoimmune responses to DNA-PKcs and the Ku protein tend to occur simultaneously. Thus, it appears that relatively intact forms of the DNA-PK holoenzyme trigger humoral responses to this nuclear enzyme.

Methods

Antisera. Sera were obtained from 312 patients with various rheumatic diseases followed in clinics at Keio University School of Medicine, Kawasaki Municipal Hospital, Yale University School of Medicine, and University of Pittsburgh School of Medicine. Diagnostic criteria were met for SLE in 147 cases (36), for scleroderma in 69 cases (37), for polymyositis or dermatomyositis in 17 cases (38), for RA in 40 cases (39), and for Sjögrens' syndrome in 20 cases (40). 19 patients had clinical features which would meet two or more of these disease categories and we classified them as having overlap syndrome. Control sera were obtained from 45 patients with nonrheumatic diseases (idiopathic interstitial pneumonia in 10 cases, chronic hepatitis in 10 cases, multiple myeloma in 5 cases, malignant lymphoma in 5 cases, chronic thyroiditis in 5 cases, inflammatory bowel disease in 5 cases, and gastric cancer in 5 cases) and 80 healthy laboratory volunteers. Autoantibodies were identified in Ouchterlony immunodiffusion, ELISA, immunoblot, and immunoprecipitation assays.

A mAb specific for the 80-kD Ku polypeptide (mAb 111) was a gift from Dr. Westley H. Reeves (University of North Carolina). Rabbit polyclonal antibodies (9543-2) to DNA-PKcs and a normal rabbit serum were a gift from Dr. Stephen P. Jackson (Wellcome/CRC Institute, Cambridge, England).

Radioimmunoprecipitation assays. Cells were labeled with [³⁵S]methionine at 370kBq/ml of cell culture (ICN Biomedical Inc., Irvine, CA) for 14 h and immunoprecipitation steps were carried out essentially as described previously (13, 21). Briefly, HeLa cells were harvested after radiolabeling and sonicated three times for 40 s in IPP buffer (10 mM Tris Cl, pH 7.5, 0.1% (vol/vol) NP-40, 500 mM NaCl) on setting 3 of a Branson sonifier (Branson Sonic, Danbury, CT). The

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Received for publication 8 September 1994 and accepted in revised form 4 January 1995.

1. Abbreviations used in this paper: DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-PK; IPP, immunoprecipitation.

J. Clin. Invest.

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0021-9738/96/03/1417/05 \$2.00

Volume 97, Number 6, March 1996, 1417–1421

sonicates were centrifuged at 16,000 g at 4°C for 10 min and supernatants were used as cell extracts. In some experiments, cell extracts were prepared from unlabeled HeLa cells.

For immunoprecipitation, 10 µl of patient serum was generally incubated with 2 mg of protein A-Sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) in 500 µl of 0.5 M IPP buffer at 4°C for 2 h. The antibody coated beads were washed three times in the same buffer and mixed with 400 µl of [³⁵S]methionine labeled cell extracts (2 × 10⁶ cells) at 4°C for 2 h in IPP buffer at the appropriate salt concentration. After four washes with the same buffer, the beads were resuspended in SDS-sample buffer (62.5 mM Tris Cl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue) and bound proteins were extracted by boiling. Proteins were resolved in 0.1% SDS-7.5% polyacrylamide gels and detected with autoradiography. For immunoprecipitation of unlabeled cell extract, antibodies were linked covalently to protein A-Sepharose beads with dimethylpimelimidate (PIERCE, Rockford, IL).

Immunodepletion experiments. Depletion of specific antigen was performed as previously described (41). Briefly, [³⁵S]methionine-labeled HeLa cell extracts were incubated for 2 h with protein A-Sepharose beads which had been coated with IgG from variable amounts of patient serum. The beads were removed with centrifugation and the resulting extract was used as the antigen source for a subsequent immunoprecipitation assay. In some experiments, HeLa cell extracts containing DNA-PK activity were prepared from unlabeled HeLa cells as previously described (8), and immunodepleted with IgG from variable amounts of patient serum as above. Kinase assay was carried out using the immunodepleted extracts as previously described (8). In brief, the nuclear extracts were prepared in 20 mM Tris Cl (pH 7.9) buffer containing 0.2 mM EDTA, 10% glycerol, 1 mM DTT, and 0.4 M KCl, and they were applied to a column of DEAE-cellulofine A-800. The flow-through fraction was immunodepleted with protein A-Sepharose beads which had been coated with IgG from variable amounts of patient serum. The resulting extracts were dialyzed against 20 mM Tris Cl (pH 7.9) buffer containing 0.2 mM EDTA, 10% glycerol, 1 mM DTT, and 40 mM KCl. The kinase reaction was carried out at 30°C for 10 min in 100 µl of reaction mixture containing 12 mM Hepes-KOH, pH 7.9, 0.6 mM EDTA, 0.6 mM EGTA, 0.6 mM DTT, 6% glycerol, 0.012% Tween-20, 7.5 mM MgCl₂, 25 µM ATP containing 370 kBq[γ-³²P]ATP (DuPont, Boston, MA), 0.4 µg of sonicated calf thymus DNA and 5 µg of peptide 15 (EPPLSQEAFADLWKK, a specific substrate for DNA-PK). After stopping reaction with equal volume of 30% acetic acid, radioactivity bound to p81 paper was counted in a liquid scintillation counter (8).

Immunoblots. Immunoblots were performed by a modification of the procedure described by Towbin et al. (42). Chemiluminescence was used to detect enzyme-conjugated antibodies bound to specific protein bands.

Results

Patient sera immunoprecipitate an ~460-kD polypeptide. To search for autoantibodies to DNA-PKcs, we examined the ability of sera from 312 patients with rheumatic diseases, 45 patients with nonrheumatic diseases, and 80 normal controls to immunoprecipitate proteins of higher molecular weights from extracts of HeLa cells labeled with [³⁵S]methionine. Sera from 10 patients were selected for further study because they contained antibodies which bound to a candidate polypeptide that appeared to comigrate with the antigen recognized by a known rabbit serum to DNA-PKcs. Among these patients, two had SLE, two had scleroderma, two had polymyositis, and four had an overlap syndrome (1 with scleroderma-SLE-polymyositis, 1 with SLE-polymyositis, and 2 with scleroderma-polymyositis). 6 of these 10 patients had anti-Ku antibodies which were detectable in radioimmunoprecipitation. Overall, the latter speci-

ficity was found in 32 patients all of whom were from the 312 patients with rheumatic diseases.

Representative examples of these studies are shown in Fig. 1. It can be seen in lanes 3–10 that these sera identify a polypeptide compatible with DNA-PKcs (as defined by a standard antiserum, lane 1). Anti-Ku antibodies were present in sera TK, TB, CS, and NS (as well as YY and ZM which are not shown) but not YM, ND, YK, or MR. These sera also recognized the Ku protein in immunoblots and gave lines of identity with a standard Ku serum in Ouchterlony immunodiffusion (data not shown). Some sera also contained additional associated autoantibodies (see Fig. 1 legend).

Identification of DNA-PKcs in immunoprecipitates. To determine if the large polypeptide described above is DNA-PKcs, all ten sera were used to immunoprecipitate extracts of

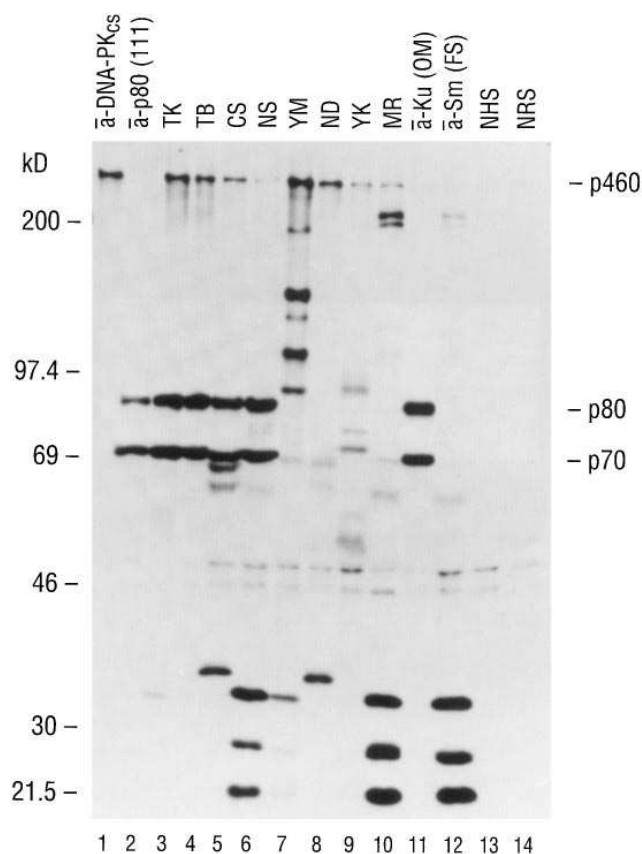


Figure 1. SDS-polyacrylamide gel analysis of immunoprecipitates prepared from [³⁵S]methionine-labeled HeLa cell extracts. Cell extracts and immunoprecipitates were prepared using buffers containing 0.5 M NaCl. Immunoprecipitates were subjected to electrophoresis in an SDS-7.5% polyacrylamide gel. Anti-DNA-PKcs (9543-2) is a rabbit antiserum to the catalytic subunit of DNA-PK; anti-p80 (111) is a mAb to the 80-kD Ku polypeptide. Sera TK, TB, CS, NS, YM, ND, YK, and MR are test patient sera. Positive control patient sera are OM and FS which contain anti-Ku and anti-Sm antibodies, respectively. NHS is normal human serum and NRS is normal rabbit serum. Left margin indicates the position of molecular weight markers. The positions of DNA-PKcs and the Ku polypeptides (p70 and p80) are marked from the migration of the known structures in lanes 1 and 11. The three smallest bands in lanes marked NS, YM (weak), MR, and FS are polypeptides of U series RNP particles. The band of ~34 kD is fibrillarin immunoprecipitated by anti-U3 RNP antibodies.

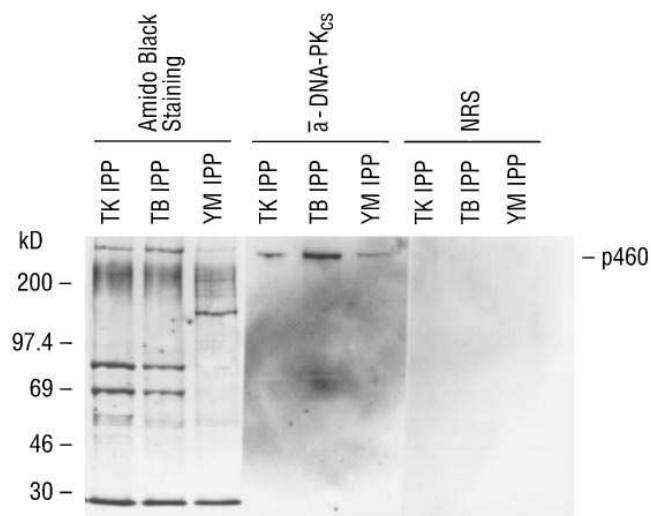


Figure 2. Immunoblot analysis of immunoprecipitates prepared with patient sera. Immunoprecipitates prepared from extracts of unlabeled HeLa cells with patient sera TK, TB and YM were dissolved in sample buffer, subjected to electrophoresis in an SDS-7.5% polyacrylamide gel, and transferred to nitrocellulose membranes electrophoretically. The membranes were either stained with amido black (*left panel*) or probed in immunoblots with rabbit antiserum to DNA-PKcs (*middle panel*) or normal rabbit serum (*right panel*) as shown. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG.

unlabeled HeLa cells using conditions that dissociate the DNA-PK holoenzyme (i.e., buffers containing 0.5 M NaCl) (21). The resulting immunoprecipitates were subsequently used as substrates in immunoblots and probed with the rabbit antiserum to DNA-PKcs.

As shown by representative examples in Fig. 2, immunoprecipitates based on sera TK and TB contained the large DNA-PKcs candidate polypeptide as well as the 70- and 80-kD subunits of the Ku protein whereas the immunoprecipitate based on serum YM contained the DNA-PKcs candidate polypeptide as well as several other unidentified components. In each case, the large polypeptide was identified as DNA-PKcs with the rabbit antiserum (*middle panel*). The ~460-kD polypeptide precipitated by the other seven sera judged to contain antibodies to DNA-PKcs was also recognized by the anti-DNA-PKcs antiserum used as a positive control (data not shown).

Immunodepletion of DNA-PKcs catalytic activity. Immunodepletion studies demonstrated that the patient sera and the rabbit antiserum to DNA-PKcs are directed against a common enzyme. HeLa cell extracts were initially immunoprecipitated (exhaustively) with serum TK (which contains anti-Ku and anti-DNA-PKcs antibodies) or serum OM (which contains only anti-Ku antibodies) using buffers with 0.5 M NaCl, and subsequently immunoprecipitated with the rabbit antiserum to DNA-PKcs. As shown in Fig. 3, the patient sera completely removed virtually all of the putative DNA-PKcs in the pre-clearing step (compare untreated extract in lane 1 with immunodepleted extracts in lanes 2–4). In contrast, anti-Ku antibodies did not deplete cell extracts of DNA-PKcs (lane 5) as expected because the Ku protein does not associate with this polypeptide in such high salt environments (21). When the

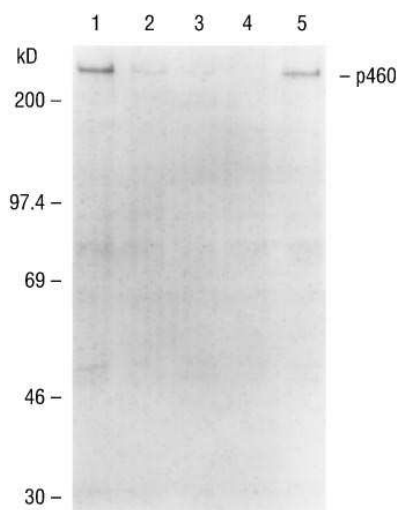


Figure 3. SDS-polyacrylamide gel analysis of immunoprecipitates prepared from immunodepleted HeLa cell extracts. An extract of HeLa cells labeled with [³⁵S]methionine was prepared at 0.5 M NaCl and preincubated with protein A–Sepharose beads coated with IgG from serum TK (contains anti-DNA-PKcs and anti-Ku antibodies) or serum OM (contains anti-Ku antibodies). These extracts were then subjected to immunoprecipitation

with the rabbit serum (9543-2) to DNA-PKcs. Immunoprecipitates were fractionated electrophoretically in an SDS-7.5% polyacrylamide gel. The preclearance step was carried out with progressive amounts of antiserum: lane 1, uncoated beads alone; lane 2, beads coated with 3 μ l of serum TK; lane 3, beads coated with 10 μ l of serum TK; lane 4, beads coated with 30 μ l of serum TK; lane 5, beads coated with 10 μ l of serum OM.

rabbit anti-DNA-PKcs serum was used for the initial depletion step, some residual DNA-PKcs remained which could be immunoprecipitated with patient antibodies (data not shown). This finding is consistent with the apparent preference of the rabbit serum for forms of DNA-PKcs which are not associated with Ku protein (21).

The patient sera were also able to remove DNA-PK catalytic activity from cell extracts. Cell extracts were subjected to passage through a DEAE-cellulofine column equilibrated with a buffer containing 0.4 M KCl to enhance DNA-dependent kinase activity. The extracts were subsequently incubated with antibodies from increasing amounts of serum YM on protein A–Sepharose beads. As shown in Fig. 4, DNA-PK activity was removed in a dose dependent manner whereas antibodies from normal human serum did not deplete the enzyme activity. These results indicate that patient antibodies recognize catalytically active DNA-PK.

Discussion

These studies demonstrate that some sera from patients with rheumatic diseases immunoprecipitate a large polypeptide of ~460-kD which can be identified as the catalytic subunit of DNA-PK in several ways. The immunoprecipitated polypeptide was recognized by a well characterized rabbit antiserum to DNA-PKcs. Moreover, the target for the latter antiserum was removed when cell extracts were pre-absorbed with antibodies from the patient sera and such cell extracts were deficient in DNA-PK catalytic activity. Therefore, we concluded that the patient sera contain reasonably high titers of high affinity autoantibodies to the catalytic subunit of DNA-PKcs.

The patient antibodies to DNA-PKcs appear to occur in linkage with antibodies to the Ku protein. Among the present group of 312 patients, 6 of 10 patients with anti-DNA-PKcs antibodies were positive for anti-Ku antibodies whereas only 26

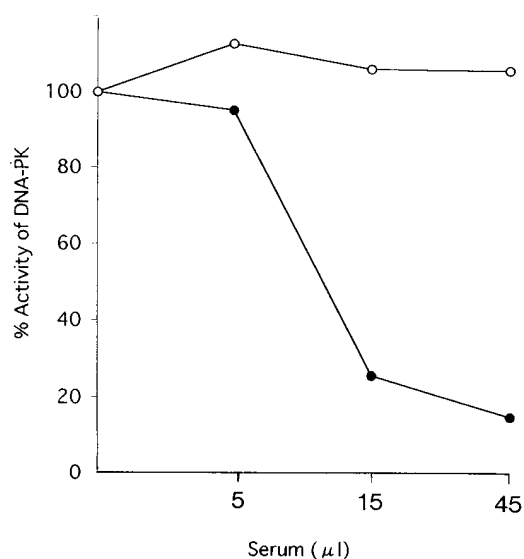


Figure 4. Depletion of DNA-PK catalytic activity with patient autoantibodies. HeLa cell extracts were preincubated with protein A-Sepharose beads coated with IgG from increasing amounts of patient serum YM (●) or normal human serum (○) as shown. Following removal of the beads, the extracts were combined with the specific substrate peptide EPPLSQEAFADLWKK and [γ - 32 P]ATP in the presence and absence of DNA to determine DNA-PK activity. In the experiment shown, extracts preincubated with uncoated beads incorporated 11,479 cpm into the peptide in the presence of DNA (percent Activity = 100) and only 1,856 cpm in the absence of DNA, confirming DNA dependency of the kinase. This experiment was repeated twice with virtually identical results.

of 302 patients without anti-DNA-PKs antibodies had this specificity ($P < 0.01$). We cannot exclude the possibility that both autoantibody specificities occur independently in the same disease subset. However, it seems more likely that the apparent linkage is related to the fact that the Ku protein and the catalytic subunit assemble into a complex which is treated by the immune system as a single autoantigenic particle. This concept is in keeping with observations which relate the architecture of several other autoantigens that appear to elicit linked sets of autoantibodies (43, 44). To rationalize these considerations with the apparent linkage of antibodies to the different components of DNA-PK, we hypothesize that relatively intact Ku protein-p460 complexes enter into intracellular pathways for antigen processing. Alternatively, generation of an antibody to one of the molecular components could set the stage for an expanding autoantibody repertoire that embraces all of the various subunits of this enzyme, as has recently been shown for expanding autoantibody responses to small nuclear RNP particles (45–47).

There are several considerations which relate to the autoantigenic epitopes on DNA-PKs. The 10 patient sera identified here immunoprecipitated the 460-kD polypeptide but did not recognize it in immunoblots or ELISA (data not shown). In contrast, the prototype rabbit serum binds to DNA-PKs strongly in immunoblots but is only partially reactive in immunoprecipitation reactions (see reference 21). In an unpublished study, we immunized mice with purified Ku protein and DNA-PKs. The resulting anti-Ku sera immunoprecipitated Ku protein and were positive in a Ku protein based ELISA. The anti-

DNA-PKs antibodies were strongly positive in immunoprecipitation but gave negative results in a corresponding ELISA in a manner comparable with that observed here for patient sera. These observations are compatible with the hypothesis that the patient sera recognize conformational epitopes which are easily denatured in this large protein whereas some denaturation may be required for binding with the prototype rabbit serum. It seems reasonable to suspect that some denaturation might occur as a protein with higher molecular weight such as DNA-PKs associates with a charged plastic surface. Denaturation of DNA-PKs on a plastic surface would also explain why the rabbit anti-DNA-PKs antiserum binds to DNA-PKs more readily in ELISA than in fluid phase immunoprecipitation assay under low salt condition (21).

Autoantibodies to DNA-PKs should be useful tools for gathering further information about the DNA-PK enzyme. These antibodies are able to deplete this enzyme from cell extracts almost completely and should prove useful in studies of its various biological activities. Potentially, this ability might be useful for identifying molecules *in vivo* which are phosphorylated by this enzyme. However, caution should be exercised because of the possibility that all autoantisera may contain unidentified specificities.

Acknowledgments

We thank Dr. Westley H. Reeves for mAb to the Ku protein and Dr. Stephen P. Jackson for rabbit polyclonal antibodies to DNA-PKs. We thank Drs. Thomas A. Medsger, Masashi Akizuki, Junichi Kaburagi, Takao Fujii, Mitsuhiro Kawagoe and Shoichiro Irimajiri for patient information.

This work was supported by National Institutes of Health Grants AR32549 (to J.A. Hardin) and GM 35866 (to W.S. Dynan), a Biomedical Research Grant from the Arthritis Foundation, funds from the Georgia Research Alliance, a grant from the Japan Rheumatism Foundation, a Grant-in Aid for Scientific Research (04670395) from the Japanese Ministry of Education, Culture and Science, a grant from Inamori Foundation, a grant from Keio University and a generous donation from the Matuzak family.

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