FEBS Letters 351 (1994) 219–224

FEBS 14476

Similar DNA binding properties of free P70 (KU) subunit and P70/P80 heterodimer

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Received 20 July 1994

Abstract The Ku antigen consists of 70 and 80 kDa protein subunits (p70 and p80, respectively) that form the DNA binding component of a DNA-dependent protein kinase (DNA-PK). It is controversial whether the interaction of Ku with DNA is mediated by p70 alone or requires formation of p70/p80 dimers. In the present studies, the DNA binding properties of p70/p80 heterodimers and full-length human p70 expressed in the absence of p80 were investigated. The binding of free p70 and p70/p80 heterodimers to DNA showed similar sensitivity to high ionic strength buffers. Competitive DNA binding studies revealed that free p70, like the p70/p80 heterodimer, bound preferentially to linear double stranded DNA fragments, whereas tRNA and closed circular DNA molecules competed poorly with the radiolabeled linear DNA for binding to Ku. These studies suggest that free p70 and p70/p80 heterodimers have similar DNA binding properties, and that the interaction of Ku with DNA may depend primarily on the p70 subunit, possibly with implications for the assembly and function of DNA-PK.

Key words: Ku antigen; DNA-dependent protein kinase; DNA binding protein

1. Introduction

The Ku antigen is a dimer of 70 and ~80 kDa protein subunits (p70 and p80, respectively) recognized by autoantibodies from sera of certain patients with scleroderma-polymyositis overlap syndrome, SLE, and related disorders [1–3]. Although the role of Ku as a DNA binding factor is generally accepted [2–6], the precise cellular function of Ku is uncertain. Possible roles in DNA repair/transposition [4], cell cycle regulation [7], or regulation of the transcriptional activity of cellular genes [8,9] or endogenous retroviral sequences [10], have been suggested. More recently, it has been reported that Ku is the DNA binding component of a DNA-dependent protein kinase (DNA-PK), the catalytic activity of which is carried by a 350 kDa protein (p350) [11–14]. The hallmark of DNA binding by Ku is its strong preference for 'non-specific' interactions with the termini of double stranded (ds) DNA, nicks, gaps, or single to double strand transitions [4,6,15–17]. Once bound, Ku is capable of translocating along the DNA strand until blocked by other DNA binding proteins [5]. It is controversial whether the binding of Ku to DNA is mediated by the p70 subunit alone [4,18,19] or by the p70/p80 heterodimer [15,20]. In vitro translated p70 appears incapable of binding a linear dsDNA probe in gel shift assays [15]. However, amino acids 536–609 of p70 bind to DNA in vitro by southwestern blot and DNA immunoprecipitation assays [19], and the free p70 protein expressed in eukaryotic cells using a recombinant vaccinia virus is released from the nucleus by deoxyribonuclease treatment [21]. Since the possibility has not been excluded that free p70 interacts with DNA by a different mechanism than the p70/p80 dimer, we examined the DNA binding properties of free p70. We show here that, like the p70/p80 dimer, the free p70 subunit binds preferentially to linear dsDNA and that this binding has similar salt sensitivity to that of the p70/p80 dimer.

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Abbreviations: DNA-PK, DNA-dependent protein kinase; NP-40, Nonidet P-40; dsDNA, double stranded DNA.

2. Materials and methods

2.1. Antibodies

MAbs N3H10 (IgG2b anti-p70, amino acids 506–541), 111 (IgG1 anti-p80, amino acids 610–705) and 162 (IgG2a anti-p70/p80 heterodimer) have been described previously [2,21–23], and were purified by ammonium sulfate precipitation from ascitic fluid. A murine IgG1 anti-trpE mAb was obtained from PharMingen (San Diego, CA).

2.2. p70 fusion proteins

Construction of the plasmid pATH 11–70.77 and expression of fusion protein trpE-70.77 (carrying residues 419–609 of p70 fused to trpE) were described previously [19,24]. *Bam*HI sites were added by PCR to the both ends of the large *Eco*RI fragment of p70 5 (70.5-1), coding for amino acids 116–609 of human p70 [24]. The fragment was cloned into the *Bam*HI site of the prokaryotic expression vector pGEX-3X [25], and expressed in *E. coli* RR1 as a fusion protein with the *Schistosoma japonicum* glutathione *S*-transferase (gst) protein. Fusion protein production was induced by adding 0.1 mM IPTG to the cultures for 4 h at 37°C. The bacterial pellet was solubilized in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.3% Nonidet P-40 (NP-40), before affinity purifying the fusion proteins on 25 μl of either protein A-Sepharose beads (Pharmacia, Piscataway, NJ) coated with N3H10 or the anti-trpE mAb [19], or glutathione agarose beads (Sigma Chemical Company, St. Louis, MO) [25].

2.3. Recombinant vaccinia viruses

The full-length human p70 and p80 proteins were expressed and assembled into dimers in rabbit kidney (RK13) cells infected with recombinant vaccinia viruses p70-vacc and p80-vacc as described [21]. Briefly, RK13 cells were infected with p70-vacc or wild type (strain WR) vaccinia virus at a multiplicity of infection (m.o.i.) of 3, or co-infected with p70-vacc plus p80-vacc, each at a m.o.i. of 3. The cells were harvested 20 h later and lysed in 50 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 0.3% NP-40, and the extract was cleared twice by centrifugation at 10,000 × *g*.

2.4. DNA immunoprecipitation assay

The binding of radiolabeled DNA to affinity purified Ku antigens on protein A-Sepharose beads was measured as described [19] with modifications. Briefly, the 564 bp *Hin*dIII fragment of bacteriophage λ DNA was purified from a low melting point agarose gel and end labeled with

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[^{32}P]dATP (3,000 Ci/mmol, DuPont-New England Nuclear, Boston, MA) using Klenow fragment (Boehringer Mannheim Corporation, Indianapolis, IN). Ku (p70/p80 dimer) from 2×10^5 K562 (human erythroleukemia) cells, or 5×10^5 RK13 cells co-infected with the recombinant vaccinia viruses p70-vacc plus p80-vacc [21], or free p70 from RK13 cells infected with p70-vacc, or trpE-70.77 fusion protein were immunoaffinity purified on protein A-Sepharose beads coated with mAb 162, 111, N3H10, or anti-trpE mAbs [19]. As shown previously [19,21,24], Ku purified in this manner was highly purified (Fig. 1). The amount of each antigen incubated with the beads was determined in preliminary experiments so that beads bound approximately the same amount of radiolabeled probe. Unless stated otherwise, the beads carrying affinity purified proteins were prewashed 3 times with 50 mM Tris-HCl pH 7.5/2 mM EDTA/0.3% NP-40 containing 1.5 M NaCl, and once with the same buffer containing 50 mM instead of 1.5 M NaCl, and incubated for 1 h with 25 ng of labeled DNA at 22°C in 100 μl of binding buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 50 μg/ml methylated bovine serum albumin). The beads were then washed 3 times with 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.3% NP-40 containing 50, 100, 150, 300, or 500 mM NaCl, resuspended in 100 μl binding buffer (minus methylated bovine serum albumin) containing 100 μg/ml proteinase K (International Biotechnologies Inc., New Haven, CT) for 1 h at 50°C, and phenol extracted. Carrier RNA (0.8 mg of yeast tRNA) and NaCl (250 mM final concentration) were added, and the nucleic acids were ethanol precipitated and resuspended in 30 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Aliquots of 10 μl were then used for scintillation counting. In some experiments, the gst-70.5-I fusion protein was affinity purified for the DNA binding assays on glutathione-agarose beads instead of mAb coated beads.

2.5. Competitive DNA binding assay

The specificity of DNA binding by free p70 and p70/p80 heterodimers was examined by competitive binding assay using sonicated salmon sperm DNA (Sigma), closed circular ΦX174 DNA (> 90% RF I form by agarose gel electrophoresis, from New England Biolabs, Beverly, MA), or yeast tRNA (Boehringer Mannheim) as cold competitors [4]. Ku antigens were affinity purified on mAb coated protein A-Sepharose beads as described above, except that half the amount of antigen was used, and incubated with 50 or 500 ng competitor DNA or RNA for 10 min before adding 25 ng of end-labeled probe (564 bp λ *Hin*dIII fragment). After incubating an additional 1 h at 22°C, the beads were washed 3 times with 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.3% NP-40 buffer. The beads were then treated with proteinase K and phenol extracted, and the bound probe quantitated by scintillation counting as above.

2.6. Binding of Ku to dsDNA cellulose

RK13 cells were infected with recombiant vaccinia viruses as above, and labeled with [^{35}S]methionine/cysteine (Translabel, ICN Biomedical Inc., Costa Mesa, CA; 25 μCi/ml) as described previously [21]. Briefly, the cells were lysed in 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.3% NP40 and the extract was cleared by centrifugation. Cleared extracts from 5×10^6 cells infected with either p70-vacc alone or p70-vacc + p80-vacc were incubated for 1 h at 4°C with dsDNA-cellulose (Sigma) with gentle mixing. The DNA cellulose was washed extensively with the starting buffer, and step eluted with the same buffer containing 0.15, 0.3, 0.4, or 0.5 M NaCl. The eluates were centrifuged at 10,000 $\times$ *g* for 10 min at 4°C and the supernatants were immunoprecipitated with N3H10 or 162 coated on protein A-Sepharose beads essentially as described for the DNA immunoprecipitation assay. After washing the beads, bound proteins were eluted by boiling in SDS sample buffer, and analyzed by SDS-PAGE and autoradiography [21].

3. Results

It is controversial whether the interaction of Ku with DNA is mediated by p70 only or requires dimerization of p70 with p80. We therefore compared DNA binding by free p70 with that of the p70/p80 heterodimer by DNA immunoprecipitation assay [19]. The same type of assay has been used previously to investigate DNA binding by SV40 large T antigen [26], RNA polymerase [27,28], and yeast repressor proteins [29]. The DNA

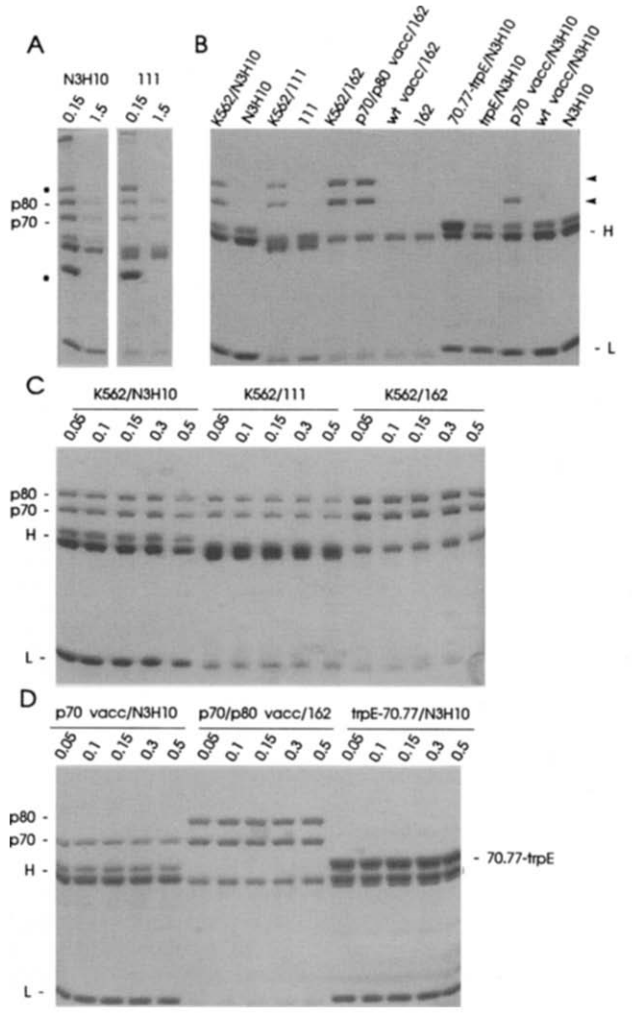

Fig. 1. Purification of Ku antigens. Ku antigens were immunoprecipitated using mAbs, and analyzed by SDS-PAGE and Coomassie blue staining. (A) Ku from K562 cell extract was immunoprecipitated with N3H10 (anti-p70) or 111 (anti-p80). Immunoprecipitates were then washed with buffer containing 0.15 or 1.5 M NaCl. Positions of p70 and p80 are indicated. Proteins of 350 kDa (the p350 component of DNA-PK), and ~100 kDa/40 kDa (unidentified), that co-purified with Ku are indicated by (*). Note that 350, 100, and 40 kDa proteins were dissociated from Ku by 1.5 M NaCl washing. Prewashing with 1.5 M NaCl was used in all subsequent immunoprecipitations (panels B-D). (B) Immunoprecipitation of Ku antigens from K562 cells, RK13 cells infected with p70-vacc, p70-vacc + p80-vacc (p70/p80 vacc) or wild type vaccinia virus (wt vacc), or *E. coli* expressing 70.77-trpE fusion protein or trpE alone using mAbs N3H10, 111, or 162. Positions of p70 and p80 are indicated by arrowheads. H, immunoglobulin heavy chain (two bands are seen for N3H10 and 111, and a single heavy chain band is seen for 162); L, immunoglobulin light chain. Note that fusion protein 70.77 migrates just behind immunoglobulin heavy chain. (C) Effect of washing N3H10, 111, or 162 immunoprecipitates with buffers containing 0.05, 0.1, 0.15, 0.3, or 0.5 M NaCl. K562 cell extract was source of p70/p80 dimer. H, immunoglobulin heavy chain; L, immunoglobulin light chain. Note that salt washing had little effect on amount of Ku associated with immunoprecipitates. (D) Effect of washing N3H10 or 162 immunoprecipitates of recombinant Ku antigens with buffers containing 0.05, 0.1, 0.15, 0.3, or 0.5 M NaCl. Sources of recombinant antigens: p70 vacc, extract of p70-vacc infected RK13 cells; p70/p80 vacc, extract of p70-vacc + p80-vacc infected RK13 cells; trpE-70.77, extract of *E. coli* expressing 70.77-trpE fusion protein. Positions of p70, p80, and 70.77-trpE are indicated. H, heavy chain; L, light chain. 70.77-trpE migrates near heavy chain. Note that salt washing had little effect on amount of recombinant Ku antigens bound to the beads.

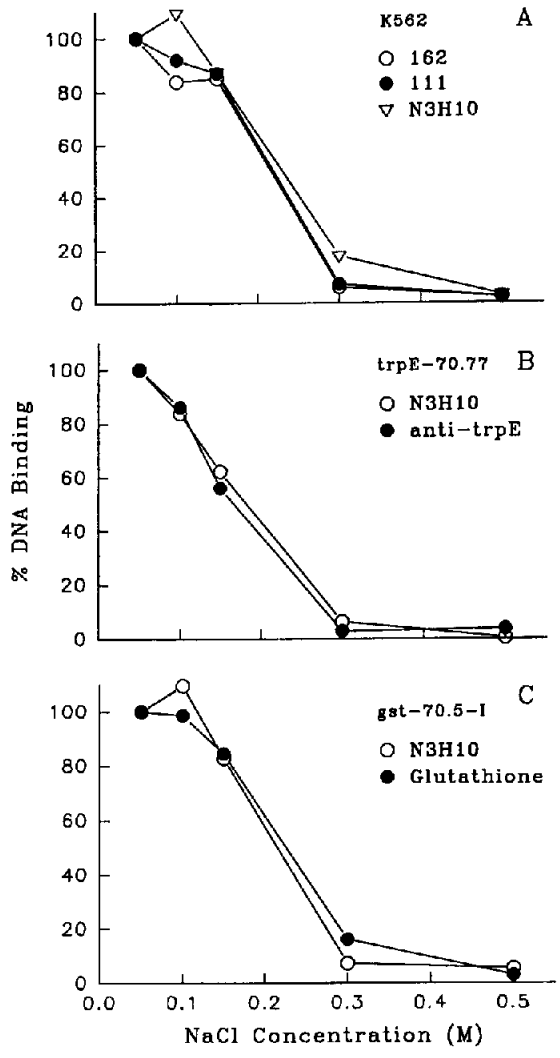

Fig. 2. Salt sensitivity of DNA binding to the p70/p80 heterodimer and p70 fusion proteins. Binding of ^{32}P-labeled linear DNA probe to affinity purified Ku antigens prewashed with 1.5 M NaCl buffer was determined after washing with 0.05, 0.1 M, 0.15 M, 0.3 M, or 0.5 M NaCl buffer. Radioactivity remaining on the beads was quantitiated by scintillation counting and plotted as% bound compared to beads washed with the 50 mM NaCl buffer. (A) p70/p80 heterodimer from 2×10^5 K562 cells was affinity purified onto protein A Sepharose beads coated with N3H10 (anti-p70), 111 (anti-p80), or 162 (anti-p70/p80 heterodimer). Values represent total cpm minus cpm using beads coated with mAb but no antigen. (B) trpE-70.77 fusion protein (p70, amino acids 416–609) was affinity purified onto protein A Sepharose beads coated with N3H10 or anti-trpE mAbs. Values represent total cpm minus cpm using beads coated with mAbs and incubated with the trpE protein alone. (C) gst-70.5-I fusion protein (p70, amino acids 115–609) was affinity purified onto protein A Sepharose beads coated with N3H10, or glutathione agarose beads. Values represent total cpm minus cpm using N3H10 or glutathione beads incubated with the gst protein alone.

immunoprecipitation assay was advantageous because other proteins remaining associated with Ku antigen at 0.15 M NaCl (Fig. 1A, (*)) could be removed by washing the affinity purified antigen with 1.5 M (Fig. 1A). Thus, a 1.5 M NaCl prewashing step was used for all DNA binding assays in order to eliminate DNA binding proteins, such as Oct-1 [30], that co-purify with Ku and might potentially complicate interpretation of the DNA binding assays. The only proteins remaining on the beads after high-salt washing were the p70 and p80 Ku proteins and the immunoglobulin heavy (H) and light (L) chain of the mAbs (Figs. 1B–D). In agreement with previous observations [21], the Ku antigens purified from RK13 cells infected with recombinant vaccinia viruses p70-vacc or p80-vacc were primarily of human origin (Fig. 1B and 1D) due to the extremely low level of endogenous Ku in RK13 cells and the poor crossreactivity of the mAbs with rabbit Ku [23]. Additional washing of the affinity purified cellular or recombinant Ku antigens with 0.05–0.5 M NaCl caused little or no reduction in the amount of antigen associated with the beads (Figs. 1C–D).

3.1. *Effect of ionic strength on DNA binding*

Since N3H10 (anti-p70, amino acids 506–541) binds immediately adjacent to, or overlapping, the proposed p70 DNA binding site, the possibility that this mAb might interfere with, or otherwise alter, DNA binding was considered. The effect of N3H10 was examined by incubating radiolabeled DNA with K562 cell p70/p80 heterodimers affinity purified using three different mAbs followed by washing with buffers containing NaCl at 0.05–0.5 M (Fig. 2A). These buffers had little or no effect on the amount of Ku bound to the beads (Fig. 1C,D). The binding of DNA to p70/p80 heterodimers affinity purified with mAbs N3H10 (anti-p70), 111 (anti-p80) or 162 (anti-p70/p80 dimer) fell from 100% at 0.1 M NaCl to approximately 10% at 0.3 M NaCl, consistent with previous observations [4,6]. The similar effect of ionic strength on the DNA binding of Ku purified with mAbs N3H10, 111, or 162 suggests that binding of the mAbs did not alter DNA binding affinity. The salt sensitivity of DNA binding by trpE-70.77 fusion protein was very

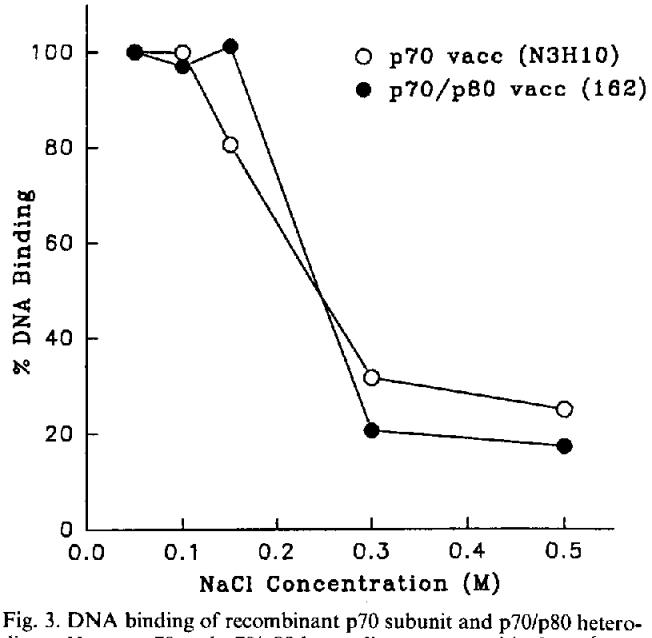

Fig. 3. DNA binding of recombinant p70 subunit and p70/p80 heterodimer. Human p70 and p70/p80 heterodimer expressed in 5×10^5 vaccinia-infected RK13 cells were affinity purified onto N3H10 or 162-protein A Sepharose beads, respectively . The beads were prewashed with 1.5 M NaCl buffer followed by 50 mM NaCl buffer before adding the radiolabeled probe. The beads were then washed with 0.05 to 0.5 M NaCl buffer as in Fig. 2. Radioactivity remaining on the beads was quantitiated by scintillation counting and plotted as% bound compared to beads washed with the 50 mM NaCl buffer. Values represent total cpm minus cpm using beads coated with mAb and extract from the same number of RK13 cells infected with wild type vaccinia virus.

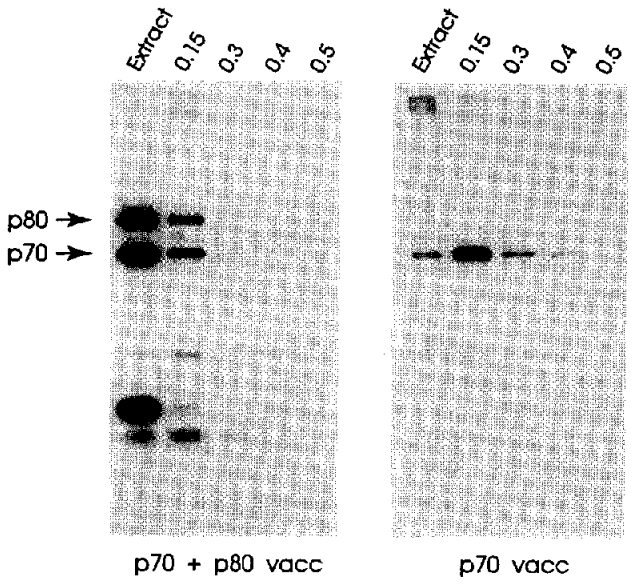

Fig. 4. Binding of p70/p80 dimer and free p70 to DNA-cellulose. Human Ku antigens were expressed in RK13 cells using recombinant vaccinia viruses p70-vacc + p80 vacc (left panel) or p70-vacc alone (right panel). ^{35}S-labeled cell extract was incubated with dsDNA cellulose, and bound proteins were eluted with 0.15, 0.3, 0.4, or 0.5 M NaCl followed by immunoprecipitation of crude cell extract (extract) or eluted fractions with mAb 162 (left panel) or N3H10 (right panel). Proteins were analyzed by SDS-PAGE and autoradiography. Positions of p70 and p80 are indicated.

similar to that of p70/p80 dimers from K562 cells (compare Fig. 2 sections A and B), and was not significantly different whether the fusion protein was purified using mAb N3H10 or anti-trpE (Fig. 2B). To exclude the possibility that fusion of p70 (amino acids 419–609) to trpE affected DNA binding, DNA binding by a longer p70 fragment (amino acids 116–609) fused to the glutathione *S*-transferase protein (gst-70.5-I) was tested. The salt sensitivity of DNA binding by this fusion protein was also similar to that of trpE-70.77 and K562 p70/p80, and was not significantly different whether the the fusion protein was purified using mAb N3H10 coated protein A Sepharose beads or glutathione-agarose beads (Fig. 2C). Taken together, these data indicated that the salt sensitivity of DNA binding by free p70 was similar to that of p70/p80 heterodimers, and that the use of mAbs to affinity purify the antigens had little effect on DNA binding. To further compare the strength of DNA binding by these two forms of Ku, the salt sensitivity of DNA binding by free p70 and p70/p80 heterodimers expressed in RK13 cells infected by recombinant vaccinia viruses was evaluated. As shown in Fig. 3, DNA binding by recombinant free p70 and p70/p80 heterodimers affinity purified with N3H10 or 162, respectively, from extracts of p70-vacc or p70-vacc + p80-vacc infected RK13 cells had comparable salt sensitivity, and closely resembled that of the cellular Ku antigen from K562 cells (compare Figs. 3 and 2A).

3.2. *Binding of free p70 to DNA-cellulose*

To exclude the possibility that the binding of DNA to free p70 was a consequence of immobilizing the protein on protein A-Sepharose or glutathione-agarose beads, the binding of solution phase p70 and p70/p80 heterodimers expressed in RK-13 cells infected with p70-vacc or p70-vacc + p80-vacc to DNA cellulose was investigated. As shown in Fig. 4A, p70/p80 heterodimers bound to DNA cellulose at 50 mM NaCl, and were eluted by raising the salt concentration. A portion of the dimer remained bound at 0.4 M NaCl, and was eluted at 0.5 M. A similar elution profile was seen with free p70 (Fig. 4B), although the binding may have been slightly more sensitive to salt, since nearly all of the p70 protein was eluted at 0.4 M NaCl, compared with 0.5 M for the dimer. The similar binding of p70 and p70/p80 dimer in solution to DNA cellulose strongly suggested that the DNA binding of free p70 in the DNA immunoprecipitation assay was not due to immobilizing p70 on the affinity beads.

3.3. *DNA binding preferences of p70/p80 heterodimer and free p70*

Competitive DNA binding assays were performed to further compare the DNA binding of free p70 and p70/p80 heterodimers. Previous studies have shown that the p70/p80 heterodimer preferentially binds linear fragments of dsDNA, and that closed circular DNA and tRNA are poor competitors, whereas sonicated salmon sperm DNA is a good competitor [4,6]. Ku heterodimers from K562 cells affinity purified onto beads using

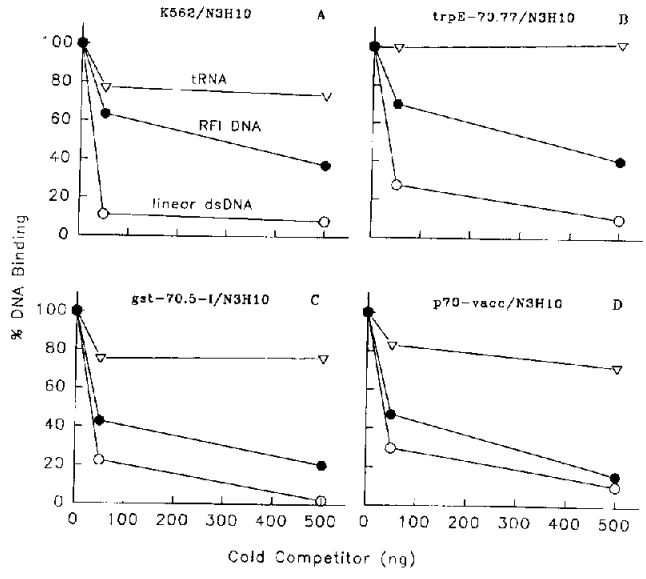

Fig. 5. DNA binding preference of p70/p80 heterodimer versus free p70. Ku antigens were purified onto N3H10 coated protein A-Sepharose beads, and prewashed with 1.5 M NaCl followed by 50 mM NaCl. Binding of ^{32}P-labeled probe (25 ng) was determined in the presence of 0, 50, or 500 ng of cold competitor (sonicated salmon sperm DNA (linear dsDNA), closed circular ΦX174 (RFI DNA), or yeast tRNA (tRNA), respectively). Radioactivity remaining on the beads was quantitiated by scintillation counting and plotted as % of DNA bound without cold competitor. (A) Binding of radiolabeled DNA by cellular p70/p80 heterodimer from 10^5 K562 cells. Non-specific binding to N3H10 coated beads was subtracted from all values. (B) Binding of radiolabeled DNA by trpE-70.77 fusion protein. Non-specific binding to N3H10 coated beads incubated with trpE alone was subtracted from all values. (C) Binding of radiolabeled DNA by gst-70.5-I fusion protein. Non-specific binding to N3H10 coated beads incubated with gst alone was subtracted from all values. (D) Binding of radiolabeled DNA by free human p70 expressed in 2.5×10^5 RK13 cells infected with p70-vacc. Non-specific binding to N3H10 coated beads incubated with extract from the same number of RK13 cells infected with wild type vaccinia virus was subtracted from all values.

mAb N3H10 showed preferential binding to linear DNA fragments (Fig. 5A), as expected from the results of previous studies. Sonicated salmon sperm dsDNA (50 ng) competed effectively with the radiolabeled probe for binding to p70/p80 heterodimers, whereas tRNA competed poorly, even at 500 ng. ΦX174 DNA, which was >90% RFI form, but contained a small amount of the nicked (RFII) form, also competed inefficiently, but was a better competitor than tRNA. Linear dsDNA fragments also competed efficiently for binding of the radiolabeled probe to fusion proteins trpE-70.77 and gst-70.5-I, but tRNA and ΦX174 DNA competed poorly (Fig. 5, sections B and C, respectively). The affinity purified full-length recombinant human p70 protein expressed in p70-vacc infected RK13 cells also displayed a similar pattern (Fig. 5D).

4. Discussion

Although DNA binding by the p70 subunit has been reported by several groups [4,18,19], free p70 appears completely unable to bind to DNA in gel shift assays, and it has been suggested that dimerization with p80 is necessary for DNA binding [15,20]. However, free p70 undergoes nuclear transport, and can be released from the nucleus by deoxyribonuclease treatment [21]. In view of the potential role of free p70 as a DNA binding factor, the present studies compared the DNA binding properties of free p70 and p70/p80 heterodimers.

Several characteristics features of the Ku–DNA interaction have been defined previously. First, Ku binds preferentially to the termini of dsDNA fragments, and closed circular DNA and RNA compete poorly for binding with linear dsDNA [4–6]. Second, the binding of linear dsDNA to Ku is sensitive to moderately high ionic strength, with dissociation at 0.35 M NaCl [4,6,31]. However, if a linear target DNA is circularized after Ku binds, the binding becomes considerably less salt sensitive, suggesting that termini facilitate dissociation as well as binding [6]. Third, Ku can translocate freely from the termini of dsDNA fragments toward the central portion of the molecule, forming regular multimeric complexes, with one heterodimer bound per 30 bp of DNA [6]. We examined the DNA binding of free p70 with respect to the first two of these characteristics, i.e. preferential binding to dsDNA termini and salt sensitivity. The binding of DNA to free p70 or p70/p80 heterodimers exhibited comparable similar salt sensitivity (Figs. 2 and 3). Moreover, free p70 and p70/p80 heterodimers both bound preferentially to linear dsDNA fragments (Fig. 5). In both cases, linear DNA was bound preferentially over closed circular (RFI) DNA or tRNA. RFI DNA competed more effectively than tRNA, possibly due to a small degree of contamination with the RFII form, which is expected to bind to Ku [17].

It is unlikely that an association of recombinant human p70 with endogenous RK13 cell p80 was responsible for the DNA binding of p70 expressed using p70-vacc, in view of the extremely low level of Ku in this cell line (Fig. 1 and [21]), and the fact that most of the recombinant p70 migrated at ~4S by density gradient centrifugation (J. Wang, unpublished data), rather than 10S, as expected for p70/p80 heterodimers [2]. However, because we could not completely exclude the possibility that a small amount of human p70/rabbit p80 dimers were formed, the salt sensitivity and DNA binding preference were confirmed using p70 fusion proteins expressed in bacteria. Although a Ku-like factor has been reported in yeast [32], a bacterial form of Ku has not been described, and bacterial proteins that bind to human p70 or p80 fusion proteins were not seen by SDS-PAGE (Fig. 1 and [19]). Little difference was found between the DNA binding properties of p70/p80 dimers and free p70 expressed in the vaccinia virus system or the truncated p70 fusion proteins carrying amino acids 116–609 or 419–609 expressed in bacteria.

Since our DNA binding assay makes use of anti-Ku mAbs, we also considered the possibility that the binding of certain mAbs might either block DNA binding, or else mimic the binding of p80 to p70. In particular, mAb N3H10 binds immediately adjacent to the proposed DNA binding site of p70 [19,23]. However, the DNA binding properties were similar whether Ku was purified by N3H10 (anti-p70), 111 (anti-p80), or 162 (anti-p70/p80 dimer) (Figs. 2A and 5). Moreover, the same DNA binding pattern was apparent when trpE fusion proteins were affinity purified using an anti-trpE mAb (Fig. 2B), and when gst fusion proteins were affinity purified on glutathione agarose beads (Fig. 2C). Finally, free p70 and p70/p80 dimers in solution both bound to dsDNA cellulose and could be eluted at similar salt concentrations (Fig. 4). Thus, it is unlikely that the binding of free p70 to DNA was influenced either positively or negatively as an artifact of the DNA immunoprecipitation assay. It should be noted that although the leucine zipper-like sequence of p70 is not necessary for DNA binding in vitro [19], p70 constructs in which this sequence was deleted were not evaluated, and we cannot exclude the possibility that interactions mediated by the leucine zipper-like sequence (e.g. p70-p70 homodimerization) play a role in DNA binding specificity. In addition, we cannot exclude the possibility that Ku contains more than one DNA binding site. The present studies also have not addressed the question of whether free p70 can translocate along the DNA strand. Although it seems likely that the initial binding of Ku to DNA is mediated by p70, it remains possible that dimerization with p80 is necessary for translocation. Alternatively, p80 may have little or no role in DNA binding, and may mediate some other function of Ku, such as the interaction with p350 or other nuclear components [33,34]. Further studies are needed to answer this question.

The present data stand in contrast with previous observations suggesting that the p70/p80 heterodimer, but not free p70, binds DNA in gel shift assays [15]. We have also noted the lack of binding of DNA to free p70 by gel shift assay [21]. There are several possible explanations for this discrepancy between the DNA immunoprecipitation and gel shift assays. First, unlabeled DNA fragments in the cell extract might compete for binding with the radiolabeled probe, and this competition may be reduced by high-salt prewashing. However, to affect the binding of free p70 to a greater degree than binding to p70/p80, it would be necessary to assume that DNA dissociates from the dimer more rapidly than from p70. It is also possible that high-salt washing enhances weak p70 binding to DNA by inducing a conformational change. A third possibility is that a cellular inhibitor is washed away by high-salt prewashing, thereby exposing the p70 DNA binding site. The presence of additional factors that might either enhance or block the Ku–DNA interaction is a potential drawback of gel shift assays. Indeed, Fig. 1A suggests that unidentified proteins of ~100 and 40 kDa are associated with the p70/p80 dimer under salt conditions typically used for gel shift assays, and that these proteins

are dissociated by the high-salt prewashing step included in the DNA immunoprecipitation assay. It is not known for certain whether these or other factors can also interact with free p70 under low-salt conditions, but studies in p70-vacc infected cells suggest that this may be the case (J. Wang, unpublished data). Finally, technical differences between the two assays, such as temperature (4°C in the gel shift assay vs. 22°C in the DNA immunoprecipitation assay) might influence DNA binding.

Regardless of explanation for the discrepancy between the gel shift and DNA immunoprecipitation assays, the nuclear transport and chromatin association of free p70 expressed in the vaccinia virus system [21] suggest that p70 may interact with DNA in vivo. We have recently identified a pool of free p70 in human K562 cells, raising the possibility that newly synthesized p70 undergoes nuclear transport and DNA binding prior to its assembly with p80 and p350 (M. Satoh et al., submitted). It is tempting to speculate that the sequential binding of p80, followed by p350, to free p70 prepositioned on DNA might prevent premature activation of DNA-PK prior to its association with chromatin.

Acknowledgements: This work was supported by Grants R01-AR40391, P50-AR42573, and P60-AR30701 from the United States Public Health Service. Dr. Wang is the recipient of a postdoctoral fellowship from the Arthritis Foundation. We thank Dr. Mark Knuth (Promega Corporation, Madison, WI) for providing mAb N3H10. Wild type vaccinia virus (strain WR) was provided by the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH.

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