Defining the Minimal Domain of Ku80 for Interaction with Ku70*

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The Ku protein has a critical function in the repair of double-strand DNA breaks induced for example by ionizing radiation or during VDJ recombination. Ku serves as the DNA-binding subunit of the DNA-dependent kinase and is a heterodimeric protein composed of 80- and 70-kDa subunits. We used the two-hybrid system to analyze the interaction domains of the Ku subunits and to identify possible additional partners for Ku. Screening a human cDNA library with the Ku heterodimer did not reveal any novel partners. Screening with the individual subunits, we detected only Ku70 clones interacting with Ku80 and only Ku80 clones interacting with Ku70, indicating that these are the primary partners for one another. Ku80 and Ku70 formed only heterodimers and did not homodimerize. Ku80 was restricted to interacting with just one Ku70 molecule at a time. The minimal functional interaction domain of Ku80 that interacted with Ku70 was defined. It consisted of a 28-amino acid region extending from amino acid 449 to 477. This region was crucial for interaction with Ku70, since mutation within this critical site at amino acids 453 and 454 abrogated the ability to interact with Ku70. We furthermore verified that the same region is crucial for interaction with Ku70 using in vitro co-translation of both subunits followed by an immunoprecipitation with anti-Ku70 antibodies. This interaction domain of Ku80 does not contain any motif previously recognized in protein-protein interactions.

The Ku protein was originally identified as a major autoantigen in patients with autoimmune diseases such as scleroderma-polymyositis (1). Ku is predominantly localized in the nucleus. It consists of two subunits (70 and 80 kDa) and binds to DNA ends in vitro. These double-stranded DNA ends can be blunt or 5′- or 3′-protruding (2, 3). The Ku heterodimer is in turn a component of a larger complex, DNA-PK, serving as the DNA binding subunit that enables the kinase subunit, DNA-PKcs, to be activated by double-stranded DNA breaks (4, 5). The DNA-PK is a crucial component of the double-stranded DNA repair pathway (6–8). Ku deficiency therefore also implicates transcription factor Sp1, or Ku itself (4, 12). However, their roles in double-stranded break repair are not yet known. Recently c-abl has been identified as a relevant in vivo substrate for DNA-PK (41).

Double-stranded breaks occur in the genome at random sites after ionizing radiation or at specific gene sequences during VDJ recombination of T cell receptor and Ig genes. During VDJ recombination, diverse gene elements that encode different parts of T cell receptor and Ig genes are recombined. Thus, the enormous repertoire of the immune system is created (for a review, see Ref. 13). Mice that have a spontaneous mutation in the DNA-PKcs are known as "severe combined immunodeficient" (SCID) mice. Mice that are deficient in Ku80 have been generated by targeted disruption of the Ku80 gene (14, 15). As expected, cells from either Ku80−/− or SCID mice are extremely sensitive to ionizing radiation and fail to rearrange T cell receptor or Ig genes. However, there are differences in the phenotype of the Ku80−/− or SCID mice (14–16). For example Ku80−/− mice show general growth retardation, and embryonic fibroblasts can only be passaged a few times in vitro, suggesting a role for Ku in senescence (14). The greater severity of the Ku-deficient phenotype could be due to other Ku functions that do not involve the DNA-PKcs or due to the fact that the SCID mouse retains some residual catalytic function, since it is not a null mutation in the DNA-PKcs.

Thus, although Ku is necessary for certain aspects of genome integrity, its precise role has not yet been defined. A number of questions are unanswered. Does the Ku dimer interact with kinases other than the DNA-PKcs? Does Ku interact with other proteins to align broken DNA ends? Does Ku anchor broken DNA ends to the nuclear skeleton, thus stabilizing them? Is there a family of Ku homologues (17)? Are there related proteins forming heterodimers with either Ku subunit via the leucine zipper described for both Ku subunits (18, 19)? Does one Ku heterodimer interact with another dimer? We hoped to address these questions with a search for Ku-associated proteins by the use of the yeast two-hybrid system. We further addressed the question of how the two Ku subunits interact with each other by identifying their minimal regions of interaction. Defining regions for dimerization would be useful in dissecting distinct functions of the Ku protein complex and in generating negative dominant mutants that would allow the study of Ku’s function in mammalian cells.

EXPERIMENTAL PROCEDURES

Plasmid Preparation—The full-length human Ku80 and Ku70 cDNAs in a pBlueBac2 baculovirus expression vector were the kind gift of Dr. J. D. Capra (20). For studying protein-protein interaction in yeast, the Matchmaker two-hybrid system from CLONTECH was used (21). The full coding sequences of human Ku80 and Ku70 cDNAs were inserted into pGBT9 and pAS2 vectors (22) and expressed as fusion proteins to the DNA binding domain of the transcription factor Gal4 (referred to as Ku80-bait and Ku70-bait). Both Ku80 and Ku70 cDNAs were also subcloned into pGAD424 and expressed as fusion proteins to the activation domain of the transcription factor Gal4 (referred to as...
For simultaneous expression of both Ku subunits from the same plasmid, we subcloned Ku70 cDNA bounded by ADH promoter and terminator sequences into the SacI site of pAS2-Ku80 as described previously for the expression cassette cyclin D1-Cdk4 (23). The resulting plasmid expresses Ku80 as a fusion protein to the GAL4 DNA-binding domain (referred to as Ku80-bait/Ku70). Junctions of all constructs were verified by sequencing. As negative and positive controls, the following vectors were used: the vectors pAS2 or pGAD424 without cDNA insert, p53-bait (encodes p53 fused to the Gal4 DNA-binding domain in pGBT9, LTA-prey (encodes SV40 large T antigen fused to the Gal4 activation domain in pGAD3P), and lamin C-prey (encodes lamin C fused to the Gal4 DNA-binding domain in pGBT9). Ku80 and Ku70 deletion mutants were obtained by subcloning of polymerase chain reaction products into pGBT9. Proofreading Pfu thermostable DNA polymerase (Stratagene) was used in accordance with the manufacturer’s instructions. All polymerase chain reaction primers were designed to have BamHI sites on the 5′-end and a SalI site on the 3′-end. Functional analysis of multiple clones was performed after junctions had been sequenced by the dideoxynucleotide chain termination method. Plasmid Ku70-(377–609) was generated by subcloning an Eco36II-SalI fragment from the full-length clone into pAS2. Several deletion mutants of Ku80 were found screening the library with full-length Ku70 as bait, the shortest being Ku80-(449–732). Deletion mutant Ku70-(194–609) was found screening the library using full-length Ku80 as bait.

Transfection and Growth Selection of Yeast Strains—For library screening and measurement of protein interaction, plasmids were transformed by lithium acetate method. ADH-driven bait plasmids were transformed into a competent lacZ-expressing in vitro fragment of Ku70 cDNA and respective Ku80 fragments were subcloned into lacZ-X-gal solution (10 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM EDTA). The media were transferred onto Whatman No. 3MM filters, permeabilized with 30 mM 3-AT, and measured for β-galactosidase activity. Positive interaction was only scored when both growth in histidine-deficient medium and β-galactosidase activity were detected.

| Proteins fused to the transcriptional activation domain (prey) | Proteins fused to the DNA-binding domain (bait) |
|---------------------------------------------------------------|-------------------------------------------------|
| Controlb | Ku80 | Ku70 | Ku80/Ku70 | p53 |
| Controlb | + | - | + | - |
| Ku80 | - | + | + | + |
| Ku70 | - | + | + | + |
| Lamin C | - | + | + | + |
| Large T antigen | - | + | + | + |

a Ku80 is fused to the DNA-binding domain. Ku70 is coexpressed. b Vector without cDNA insert: bait, pAS2; prey, pGAD424. c Weak interaction.

Interactions of Full-length Ku80 and Ku70 Subunits—To define association of the Ku subunits with each other or with other proteins, we used the yeast two-hybrid system. Full-length cDNA for human Ku80 or Ku70 was subcloned into the yeast vector pAS2 (referred to as “bait”) that expresses the transcriptional activation domain of the yeast transcription factor Gal4. The reporter gene expressed either the lacZ or the his3 gene under the control of a Gal4-dependent promoter. Reporter activity was measured as β-galactosidase activity as well as growth in histidine lacking medium. To suppress basal promoter activity, a wide range of 3-amino-triazol concentrations was used during selection. Ku80 had been reported as a transcriptional activator of a number of mammalian genes (28–31). We have expressed full-length Ku80 fused to the Gal4 DNA-binding domain and in addition full-length Ku70. Controls included vectors without inserts as well as p53, SV40 large T antigen, or lamin C as described under “Experimental Procedures.” Yeast cells were transfected with the indicated plasmid combinations and tested for growth in histidine-deficient medium. In the presence of 0.5 mM 3-amino-triazol and measured for β-galactosidase activity. Positive interaction was only scored when both growth in histidine-deficient medium and β-galactosidase activity were detected.

Site-directed Mutagenesis of Plasmids—To obtain specific substitution of amino acid residues, the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s recommendation. Single or co-translated products or immunoprecipitated products were subsequently separated on a 7.5% SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed overnight.

RESULTS

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Full-length Ku80 and Ku70 were also subcloned in the yeast vector pGAD424 (referred to as Ku80-prey or Ku70-prey) that expresses the transcriptional activation domain of Gal4. Neither plasmid showed reporter activity on their own. This indicated that Ku80 or Ku70 does not have detectable transcriptional activity on its own.
modimers or heterotrimers. Yeast two-hybrid system as heterodimers but not as homodimers. Competition Ku70-prey and Ku80-bait (Table I). This is likely to be explained by competition between Ku70 and Ku70-prey for binding to Ku80-bait, thus reducing reporter gene activation. Less reporter activity was observed than with the simple combination Ku70-prey and Ku80-bait (referred to as Ku80-bait/Ku70). This allowed interaction with full-length Ku70 (Table I). There was also no evidence for Ku80 homodimers, only heterodimers.

Hypothetical Ku-interactive proteins might bind to the heterodimer but not to the individual Ku chains. We therefore screened the library with the Ku heterodimer using the Ku80-bait/Ku70 double expression plasmid as bait. After screening about 1 million clones, the only interactive clones found were identified as full-length Ku70. The HELa library might be missing Ku-associated proteins that are involved in VDJ recombination. Since VDJ recombination is unique to lymphoid precursors, we screened a thymus library with the Ku80-bait/Ku70 heterodimer. No specific interaction products were identified. Moreover, no DNA-PKcs clones were detected in any screen. This probably indicates the technical limits of the yeast two-hybrid system, as discussed below.

Interaction of Ku80 Deletion Mutants with Full-length Ku70—A number of potential functional domains have been identified in Ku80 based on sequence homology (17–19, 36). These include leucine zipper-like domains that could promote protein-protein interaction as well as clusters of basic amino acid domains that could be involved in DNA recognition.

To delineate the regions of Ku80 that are necessary for heterodimerization, we constructed truncation mutants of Ku80. In addition we characterized the N-terminal deletion mutants obtained in screening the library with Ku70-bait. They were expressed as fusion proteins with the DNA-binding domain and tested for transcriptional activation of the reporter gene. Thus, the regions upstream of aa 477 are important for binding to the DNA recognition domain from aa 1 to 477. Deletion of the 5’-region from aa 1 to 448 was dispensable for interaction with full-length Ku70. Thus, the two minimal deletion Ku80 mutants that still show interaction with Ku70 cover the region from aa 1 to 477 or the region from aa 449 to 732. These Ku80 mutants overlap in the region from aa 449 to 732, suggesting a minimal domain of only 28 aa that is important for dimerization with Ku70. However, expression of the minimal region comprising aa 427–477 showed transcriptional activity on its own, which will be discussed below. A Ku80 mutant containing the region between aa

![FIG. 1. Interaction of Ku80 deletion mutants with full-length Ku70.](Image)

Yeast cells were transfected with Ku80-bait deletion mutants fused to the G alpha DNA-binding domain and tested for interaction with full-length Ku70-prey. Deletion mutant Ku80-(449–732) was obtained as prey by library screening and thus tested against Ku70-bait. None of the deletion mutants showed detectable reporter gene activation on their own. Positive interaction was scored as in Table I.

- **Ku80 Full length**
- **Ku80 [1-531]**
- **Ku80 [1-477]**
- **Ku80 [1-401]**
- **Ku80 [1-316]**
- **Ku80 [210-732]**
- **Ku80 [357-732]**
- **Ku80 [427-732]**
- **Ku80 [449-732]**
- **Ku80 [427-531]**

Peptide Length (amino acids) 1 732 Interaction with Ku70

- +
- +
- +
- -
- +
- +
- +
- +
- +

Only full-length Ku80 and deletional mutants of Ku80 were isolated as prey. The deletional mutants are discussed below.

Thus, it appears that only Ku70 is the predominant partner for Ku80 and vice versa. Furthermore, the screening confirms the above observation that neither Ku80 nor Ku70 form homodimers, only heterodimers.

In summary, the Ku subunits Ku80 and Ku70 interact in the yeast two-hybrid system, as discussed below.
Vitro—amino acids for ones of different charge; and 477 (Fig. 2). Each mutation site exchanged two adjacent Ku80; two were located within the minimal region of aa 449 interaction with Ku70. Four mutation sites were created to determine whether regions of importance are indeed crucial for minimal region. Thus point mutants of Ku80 were created to may be sufficient for interaction with Ku70 but not necessary.

Thus, the region of Ku80 tested that is sufficient for interaction with full-length Ku70 comprises aa 427–531. Within this region, the stretch from aa 449 to 477 of Ku80 is probably most important for heterodimerization.

Interaction of Ku80 Point Mutants with Full-length Ku70—The minimal critical region that has been identified in Ku80 may be sufficient for interaction with Ku70 but not necessary. It is possible that additional sites could substitute for the minimal region. Thus point mutants of Ku80 were created to determine whether regions of importance are indeed crucial for interaction with Ku70. Four mutation sites were created in Ku80; two were located within the minimal region of aa 449 and 477 (Fig. 2). Each mutation site exchanged two adjacent amino acids for ones of different charge; e.g., aa 453* and 454* were changed from neutral to basic, and aa 471* was changed from acid to neutral. Two other mutation sites that were adjacent to the minimal region contained proline "kinks" that were removed upon mutation. The point mutants were first tested in the deletion construct stretching from aa 427 to 732. Of all these mutants, only point mutant, aa 453*454*, became unable to induce reporter gene activation and thus lost the ability to interact with full-length Ku70 (Fig. 2). To test whether these amino acids at position 453/454 were also crucial in the full-length protein, we introduced the same mutations into the wild length protein, we introduced the same mutations into the wild type as well as Ku70 show a major translational product of the expected size and smaller truncated versions. Ku80 mut1 has point mutations at position aa 471/472. Ku80 mut2 has point mutations at aa 453/454, ctrl, the translational product of the reticulocyte lysate alone. Δ3* is a Ku80 mutant with a 3′-primed deletion from aa 532 to 731.

Interaction of Ku80 Mutants with Wild Type Ku70 in Vitro—To confirm that binding of Ku80 to Ku70 was indeed dependent on the minimal region identified in Ku80, we used an in vitro binding assay. Various mutants of Ku80 and wild type Ku80 were expressed in an in vitro reticulocyte lysate system. The Ku80 mutants were either translated alone or co-translated with Ku70. Co-translation products were then immunoprecipitated with specific antibodies against Ku70. Fig. 3A shows that Ku80 wild type and the silent point mutant aa 471*472* (Ku80 mut1) were immunoprecipitated equally well with anti-Ku70 antibody. Thus, Ku80 point mutant aa 453*454* (Ku80 mut2) was hardly detectable after immunoprecipitation with anti-Ku70 antibody (Fig. 3A). Thus, the Ku80 point mutant aa 453*454* that failed to interact with Ku70 in the yeast two-hybrid system also failed to interact sufficiently in the in vitro binding assay. The same result was obtained when the co-translation products were precipitated with anti-Ku80 antibody. Thus, Ku70 was pulled down by anti-Ku80 Ab when co-translated with wild type Ku80 but not with Ku80 point mutant aa 453*454* (not shown). In addition, the 3′-deletional Ku80 mutant aa 1–531 (delta 3′) was pulled down by anti-Ku70 Ab as well as wild type Ku80 (Fig. 3B), as expected from the yeast two-hybrid analysis. Furthermore, Ku70 was pulled down equally well by Ku80 precipitation when co-translated with either 3′-deletional mutant or wild type Ku80 (not shown).

Thus, the minimal region of Ku80 is critical for interaction with Ku70 in vivo as well as in vitro.

Interaction of Ku70 Deletional Mutants with Wild Type Ku80—To delineate the crucial minimal region of Ku70 for interaction with full-length Ku80, deletion mutants of Ku70 were created. Either 5′ or 3′ deletional mutants of Ku70 were tested. These deletion mutants had been created by suitable subcloning or had been identified during the library screen with Ku80-bait.

The carboxyl-terminal region of Ku70 from aa 530 to 609 appeared to be dispensable for interaction with full-length Ku80 (Fig. 4). Further deletion of Ku70 to aa 484 abrogated interaction with Ku80. Deletion of the N terminus of Ku70 from aa 1 to 243 still preserved interaction with Ku80, but further deletion to aa 377 abolished the interaction. This identified two
minimal functional constructs aa 1–530 and aa 243–609, which overlapped in the region aa 243–530. To test whether this core region is not only important but also sufficient, a minimal mutant of Ku70 was created consisting only of a sequence extending from aa 243 to 530. However, this truncated Ku70 protein was not able to interact with Ku80. This suggested that more than one region in Ku70 was crucial for interaction with Ku80, either because more than one region is directly involved in binding to Ku80 or because these regions in Ku70 are important for stability or folding.

To test whether the truncated Ku70 binds to Ku80 as well as wild type Ku70, we used a competition approach. Full-length Ku70 completely blocked the interaction of Ku80-bait with truncated prey (1–530 or 243–609), but not full-length prey (Table II). This result indicates that wild type Ku70 binds better than the truncations to Ku80. This is consistent with the existence of multiple sites in Ku70 having additive effects. A Ku70 construct lacking a binding site would have sufficient avidity to interact with Ku80 but would not compete for interaction with a full-length construct having all sites. However, it is also possible that the truncated Ku70 mutants bind less strongly in comparison with wild type because of differences in folding or stability.

DISCUSSION

To analyze the interaction of Ku subunits with themselves and with other proteins in the cell, we employed a yeast two-hybrid system. By screening cDNA libraries from HeLa cells or thymocytes, we observed that the major cellular partner for Ku80 is Ku70 and vice versa. Homodimerization of Ku80 or of Ku70 was not observed, only heterodimerization. Two heterodimers did not bind to one another. The interaction of Ku80 with Ku70 was controlled by a very small region of Ku80 and abrogated by a point mutation. The crucial role of this minimal region for heterodimerization was confirmed using an abrogated by a point mutation. The crucial role of this minimal region for heterodimerization was confirmed using an in vitro binding assay. In contrast to Ku80, multiple regions of Ku70 were necessary for successful interaction with Ku80.

Despite the leucine zipper-like domains of the Ku subunits, which may suggest possible interaction with other leucine zipper family members, we found no evidence that the Ku subunits heterodimerize with any protein other than each other. This is in accordance with a recent report that also used single Ku subunits in a two-hybrid screen (37). Also, a family of Ku70 homologue genes has been suggested as judged by multiple hybridization bands observed in Southern analysis (17). However, there is no evidence that the protein products of those cross-hybridizing genes can substitute for Ku70 for heterodimerization with Ku80, a point that may be relevant for interpreting the phenotype of Ku knockout mice.

A library screen with the Ku heterodimer has not yet been reported. We did not recover the expected DNA-PKcs clones from the library using the Ku heterodimer as bait, a failure for which there are several possible explanations. Perhaps the yeast cells do not express DNA-PKcs as a prey fusion protein, because the gene product is too large, not properly folded, or posttranslationally modified. It is also possible that yeast Ku homologues compete for binding to DNA-PKcs. Alternatively, the Ku heterodimer may first have to bind broken DNA before it can associate with DNA-PKcs (this is a point of controversy) (5, 38). It would be difficult to artificially introduce broken DNA ends into yeast cells, and they might be repaired or cause cell death. Thus, for whatever reason DNA-PKcs was not retrieved, the same limitations could apply to other hypothetical Ku interactive proteins.

It has been speculated that the Ku dimer could play a role in bringing broken DNA ends directly together. However, full-length Ku subunits did not form assemblies larger than single heterodimers. Also no homodimerization was detected in our experiments. Thus, if Ku were to bridge two DNA ends, it would require something not present in the yeast. Perhaps “activation” of the Ku heterodimer by broken DNA is required or possibly a third protein is necessary.

The region of Ku80 that binds to Ku70 has been defined by deletional analysis and is located between aa 449 and 477, thus spanning only 28 aa. Point mutants within this domain have verified that this region is crucial for interaction with Ku70 in vivo and in vitro. Recently, a larger Ku80 fragment was reported that was still able to interact with Ku70 in the yeast two-hybrid system (37). This deletion mutant spanned 283 amino acids, whereas our study defined the interaction region within its carboxyl-terminal region to 28 amino acids. This domain does not contain any motif previously recognized in

![Minimal Domain of Ku80](27263)
protein-protein interactions. The leucine zipper-like structure of Ku80 lies 250 aa upstream of this site and does not appear to contribute at all to heterodimerization. The interaction domain between aa 449 and 477 is highly conserved between the Ku80 sequences of different species. The yeast Ku homologue shows no homology in this region. The human point mutant Ku80–453*454* completely abrogated interaction with Ku70. The alanine at position aa 453 is conserved among human, mouse, and hamster. The high conservation between species in the interaction domain may be one reason why human and rodent Ku80s are functionally interchangeable (9, 11).

Ku70, on the other hand, appears to require multiple regions to associate with Ku80. These regions may interact directly with Ku80 or may be required for proper folding of Ku70 to expose a single interaction site for Ku80. The Ku70 N-terminal deletion mutant, aa 377 to 609, failed to interact with Ku80 as demonstrated in this study (Fig. 4). This contrasts with a recently reported Ku70 mutant, aa 439 to 609, that retained interaction with full-length Ku80 (37). It is possible that the larger deletion mutant, aa 377 to 609, has different folding or stability properties that interfere with exposure of the Ku70 interaction domain. Our study also compared the affinity of different Ku70 mutants for binding to Ku80 using a competition approach (Table II). Neither the N-terminal nor the carboxyl-terminal Ku70 mutants were able to compete with full-length Ku70 for binding to Ku80, although each of them was able to interact with Ku80 in the absence of full-length Ku70. Thus, Ku70 deletion mutants may have lower affinity for Ku80 than full-length. This may be due to improper folding or due to multiple binding sites of Ku70 involved in the interaction with Ku80.

There are numerous reports of binding of the Ku complex to a variety of different sequence-specific promoter elements that are driven by either polymerase I or polymerase II (28–31), and hamster. The high conservation between species in the minimal interactive region of Ku80 in mammalian cells are driven by either polymerase I or polymerase II (28–31), and hamster. The high conservation between species in the minimal interactive region of Ku80 in mammalian cells (9, 11).

Understanding the functional domains of the Ku complex will elucidate the role of Ku in vitro. Expression of the distinct Ku80 mutants that allow heterodimerization but no DNA end binding or, alternatively, that allow heterodimerization and DNA end binding but not association with DNA-PKcs may reveal distinct functions of the Ku complex. Thus, expression of the minimal interactive region of Ku80 in mammalian cells should lead to heterodimerization with endogenous Ku70. This should result in a non-functional dimer and a negative dominant phenotype as has been reported for other heterodimers (NF-xB, AP-1, etc.). This dominant negative Ku80 would be useful in examining the role of Ku in cell cycle control, apoptosis, and other DNA repair-deficient systems.

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