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# AUTOANTIBODIES AGAINST DNA DOUBLE-STRAND BREAK REPAIR PROTEINS

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## 1. ABSTRACT

Autoantibodies against cellular components are commonly present in sera from patients with systemic rheumatic diseases and may play an important role in pathogenesis. The Ku protein was recognized 20 years ago as a major target of autoantibodies in a subset of Japanese patients with scleroderma-polymyositis overlap syndrome, and anti-Ku antibodies have since been shown to occur in 10-20% of patients with these and other systemic rheumatic diseases, including systemic lupus erythematosus. Ku functions physiologically in the repair of DNA double-strand breaks, where it carries out the initial recognition of damaged DNA ends. The three dimensional structure of the Ku-DNA complex has recently been solved, and helps illuminate the relationship between the autoimmune epitopes and other features of the protein. In addition to Ku, three other polypeptides in the same DNA repair pathway have more recently been identified as autoantigens: the DNA-dependent protein kinase catalytic subunit, DNA ligase IV, and XRCC4. Two hypotheses have been invoked to explain the ability of these proteins to elicit an autoimmune response in susceptible individuals. One is that DNA damage induces formation of nucleoprotein complexes that present novel composite or conformational epitopes. The other is that cleavage of these proteins by caspases or Granzyme B leads to presentation of immunocryptic peptides capable of stimulating autoreactive T lymphocytes. In the case of DNA double-strand break repair proteins, there is evidence that both of these mechanisms may be at work. Because of their role in the maintenance of genome stability, DNA double-strand break repair proteins have been the subject of intense study, and a wealth of new structural, biochemical and functional information makes them excellent models for investigation of the humoral autoimmune response.

## 2. INTRODUCTION

The production of autoantibodies against cellular components is one of the distinctive characteristics of patients with systemic rheumatic diseases (1). The amounts and specificities of autoantibodies that are present in a particular patient are useful diagnostic and prognostic indicators, which suggests that the autoantibodies themselves are involved in pathogenesis (1). Systematic characterization of autoantibodies and their target molecules may contribute to development of more refined diagnostic and prognostic indicators and new therapeutic approaches, as well as providing clues to the underlying causes of the autoimmune response.

One of the principal enigmas with regard to autoimmune diseases is that the target antigens do not share common structural and functional characteristics and do not have a unique subcellular localization. However, one

observation that provides an important clue to the etiology of the autoimmune response is that autoantibodies tend to occur in linked sets. That is, within a single patient, autoantibodies may be present that recognize different components in the same supramolecular complex, such as the nucleosome, spliceosome, or hY RNA-protein complex (Ro/La) (2, 3). This finding suggests that production of autoantibodies is at least in part antigen-driven, and that the antigen may be the assembled complex itself, rather than the individual components.

Another clue as to the etiology of the autoimmune response is that target proteins are often substrates for proteases that are associated with stress and cell death. Many autoantigens are substrates for caspases, which carry out selective protein degradation during apoptosis (4-9). Caspase cleavage sites seem to occur more commonly among proteins that become autoantigens than among cellular proteins as a whole. More recently, an even stronger correlation has been noted between autoantigenic potential and the presence of cleavage sites for Granzyme B, a protease that is produced by cytotoxic T lymphocytes and natural killer cells (10). These observations have led to the hypothesis that peptides generated from products cleaved by caspases and Granzyme B contain cryptic epitopes capable of stimulating an autoreactive T-cell response (5, 8).

In this review, we discuss the characteristics of autoantibodies against DNA double-strand break (DSB) repair proteins. These antibodies are moderately common, being present in 10-20% of patients, and may reach extremely high titers. The first of these autoantigens to be identified was the Ku protein (11). Ku binds tightly to DNA ends and other structural discontinuities, and was later revealed to play an important role in repair of DSBs (12). Recently, autoantibodies against other components in the Ku-dependent DSB repair pathway have been identified (13, 14). Components of the DSB repair machinery are thought to assemble into a functional complex bound to broken DNA ends. The occurrence of linked sets of antibodies against Ku and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) suggests that they form one of the "autoantigenic complexes" that are targeted by the immune system of autoimmune patients. In addition, several of the DSB repair proteins are substrates for caspases, Granzyme B, or both (5, 8, 10, 15, 16). Thus, the DSB repair proteins provide illustrations of two of the major mechanisms that have been proposed to elicit the autoimmune response in susceptible individuals.

## 3. KU AUTOANTIGEN AND THE DSB REPAIR PATHWAY

The Ku protein was first described some 20 years ago as a target for autoantibodies in patients with scleroderma-polymyositis overlap syndrome (11). Molecular characterization revealed that Ku is a heterodimer of 70 kDa and 83 kDa subunits, referred to as Ku70 and Ku80, respectively (17-19). Ku binds to ends (20), nicks (21), bubbles (22) and hairpins (23) in double-strand DNA (dsDNA) in a sequence-independent manner. Biochemical studies have shown that the Ku protein is a component of the DNA-dependent protein kinase, DNA-PK (24-27). Ku associates with the catalytic subunit of the enzyme, DNA-PKcs, to form an enzymatically active complex capable of phosphorylating a number of different nuclear proteins (24-27). DNA-PKcs is a 470 kDa polypeptide (28), and its carboxyl-terminal 500 residues comprise a catalytic domain that falls into the phosphatidylinositol 3-kinase family of serine/threonine kinases (28-30).

The biological function of Ku and DNA-PKcs remained obscure until 1994, when a series of reports showed that these proteins were defective or absent in ionizing radiation-sensitive rodent cell lines (31-38). These cell lines are deficient in the repair of DSBs, which are induced by exposure to ionizing radiation or to certain genotoxic drugs, such as bleomycin. DSBs also occur naturally during the process of V(D)J recombination, in which site-specific DNA cleavage in B and T lymphocytes is followed by the joining of free DNA ends to generate diverse antigen receptors (39). DSBs are among the most lethal forms of DNA damage (40-43). Even a single DSB can lead to chromosomal fragmentation, resulting in either cell death or genomic instability and a consequent risk of neoplastic transformation. In eukaryotic cells, DSBs are repaired by at least two different pathways, homologous recombination and nonhomologous end joining (NHEJ) (40-43). NHEJ is the predominant pathway in most human cells (44), and it is in this pathway that Ku and DNA-PKcs have been shown to function.

A number of ionizing radiation-sensitive cell lines exist, and they can be classified into several complementation groups. Cell lines in the IR4, IR5, and IR7 groups have specific defects in NHEJ, and the human genes that rescue the radiation-resistant phenotype are known as X-ray cross-complementing (XRCC) genes 4, 5, and 7, respectively (45, 46). Genetic and biochemical studies showed that the XRCC5 gene encodes Ku80 (31-35), and the XRCC7 gene encodes DNA-PKcs (36-38). Although Ku70 is not encoded by one of the classical XRCC genes, knockout mouse experiments show that loss of this protein gives a similar radiation-sensitive phenotype (47).

At least two other proteins are now known to be essential for the NHEJ pathway (48-50). One of these, XRCC4, was cloned by a complementation strategy and proved to be a novel protein (51). The other, DNA ligase IV (DNL IV), is an ATP-dependent DNA ligase that interacts with XRCC4 in a mixed tetramer (52-54).

A proposed sequence of events that occur during NHEJ is shown in Figure 1. By binding to DNA, Ku protein recruits DNA-PKcs to the site of damage (55). Photocross-linking studies showed that the Ku dimer is oriented, with Ku70 located proximal and Ku80 distal to the free DNA end (56). When DNA-PKcs interacts with Ku heterodimer, Ku translocates inward and DNA-PKcs comes into contact directly with the DNA end (57). Binding to Ku heterodimer on broken DNA ends stimulates

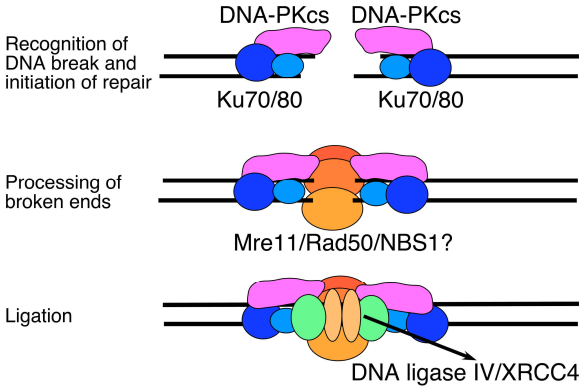


**Figure 1.** Nonhomologous end-joining pathway of DNA double-strand break repair. Step 1: Broken DNA ends are recognized by Ku70/Ku80 heterodimer. Ku binds to DNA ends and recruits DNA-PKcs. DNA-PK (Ku/DNA-PKcs complex) phosphorylates other proteins involved in DNA repair or DNA damage signaling. Step 2: Broken DNA ends are processed to facilitate the ligation reaction. The complete sets of proteins that participate in this step have not yet been determined, although the Mre11/Rad50/NSB1 complex may play a role. Step 3: the DNA ligase IV/XRCC4 complex catalyzes the final ligation step of the nonhomologous end joining. XRCC4 is required for stabilization and for maximum activity of DNA ligase IV. The DNA ligase IV/XRCC4 complex contains two copies of each polypeptide.

the serine/threonine kinase activities of DNA-PKcs by 5-50 fold (24). The *in vivo* substrates of DNA-PK have not been well characterized, but these substrates are likely to be components of the DNA repair machinery or signaling molecules that respond to DNA damage. For example, there are *in vitro* phosphorylation sites in Ku, XRCC4, and DNA-PKcs itself (58-61). The subsequent steps in the pathway are less clear, although a complex of three proteins, Mre11, Rad50, and NBS1, has a proposed role in the processing of mismatched or damaged DNA ends (62-65). The final step in NHEJ is the covalent rejoining of the DNA strands (54). The dyad-symmetric structure of the DNL IV/XRCC4 complex may allow it to bridge the two ends of the broken DNA and carry out this final step (54, 66). As described in more detail below, both XRCC4 and DNA-PKcs have been found to be cleaved during apoptosis (5, 8, 10, 15, 16). One of the hallmarks of apoptosis is the fragmentation of cellular DNA. Blocking the DSB repair pathway during apoptosis may be required in order to forestall a futile cycle of DNA cleavage and repair.

## 4. AUTOANTIBODIES AGAINST KU PROTEIN

### 4.1. Frequency and clinical correlation

Autoantibodies against Ku protein were first identified by immunodiffusion tests (11). Out of 330 Japanese patients with systemic rheumatic diseases, 9 patients were found to have autoantibodies against Ku. Among these, 6 were diagnosed as having scleroderma-polymyositis overlap syndrome. Subsequent studies in the U.S. suggest a broader distribution of anti-Ku antibodies among patients with a variety of systemic rheumatic diseases. In the first such study, sera were tested for the presence of anti-Ku autoantibodies by the "capture ELISA" method, which uses anti-Ku monoclonal antibody to capture the Ku antigen on the plate (18). The plate is then incubated with the test sera, and bound autoantibodies are detected with an anti-human IgG secondary antibody. In this assay, 7 patients out of 51 with systemic lupus erythematosus (SLE), 2 out of 11 with mixed connective tissue disease (MCTD), and 2 out of 15 with scleroderma showed a high titer (between 1:250 and 1:4,000,000) of anti-Ku antibodies. Several other U.S. studies support these findings. An immunoblotting study using biochemically purified Ku protein found anti-Ku autoantibodies in 13 of 69 patients with SLE, 9 of 57 patients with scleroderma, 2 of 23 with myositis, and 2 of 10 with Sjögren's syndrome (67). Francoeur *et al.* analyzed SLE patient sera by immunodiffusion and immunoprecipitation and found immunoreactivity to Ku in approximately 10 % of SLE sera (68). Olhoffer *et al.* found anti-Ku antibodies by immunoprecipitation in about 4% of SLE patient sera (69). The apparently broader distribution of anti-Ku antibodies in the U.S., as compared to Japan, may be due to differences in the patient population or to the use of more sensitive methods of detection.

### 4.2. Epitopes recognized by anti-Ku antibodies

The epitopes recognized by anti-Ku antibodies can be divided operationally into two classes. In the first class are epitopes formed autonomously by individual subunits or protein domains. Although these may be conformation-dependent, they can nevertheless be studied and precisely mapped using recombinant protein approaches. In the second class are epitopes reflective of the structure of the assembled Ku-DNA particle as a whole. These may include epitopes formed at subunit interfaces or that are dependent on DNA binding. These "particle-specific" epitopes are much more difficult to study experimentally, but may be of considerable importance in generating and maintaining the humoral autoimmune response. Although particle-specific epitopes are difficult to map, the presence of antibodies that recognize them can be inferred from indirect evidence, as described in a subsequent section.

Autoimmune epitopes of the first class, which are formed autonomously by the individual subunits, have been described for both Ku70 and Ku80 (17, 70, 71). Affinity-purified antibodies against Ku70 do not recognize Ku80 and vice versa (70, 71). The two subunits of Ku appear to have evolved from the same ancestral gene (12) and share a common three-dimensional folding pattern (72). Because they have diverged extensively in primary sequence, the lack of antigenic cross-reactivity may not be surprising. Interestingly, however, a different result has been obtained in the anti-Sm autoantibody system. Seven Sm proteins bind to U snRNAs to make up the common structural core that is found in all of the spliceosomal U snRNPs. The Sm system is analogous to Ku, in the sense that the Sm proteins share a common tertiary folding pattern, but have diverged extensively in primary sequence (73). Despite this divergence, antibodies directed against the U snRNP B'/B and D polypeptides show extensive cross-reactivity (74).

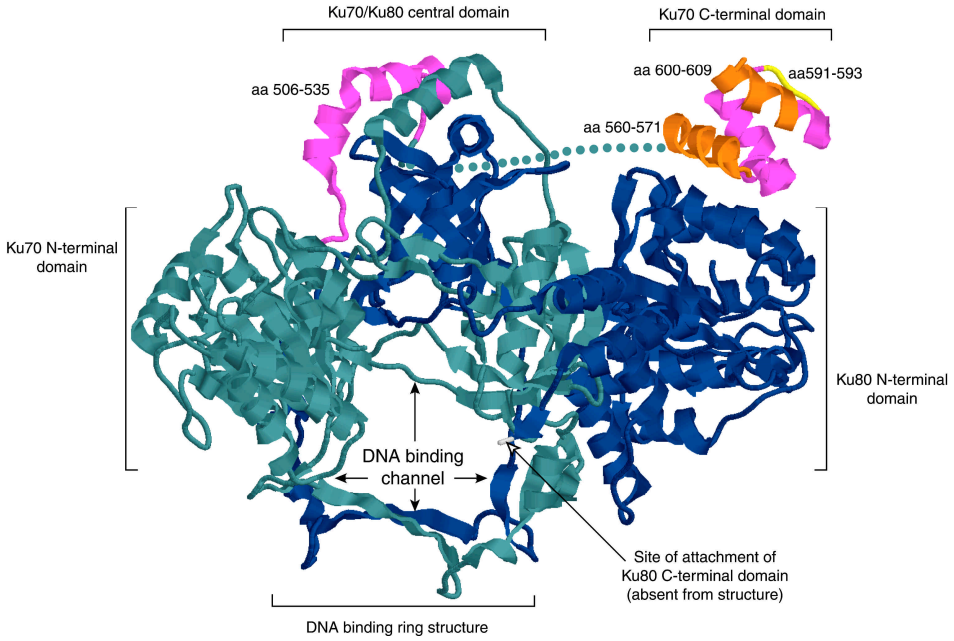


**Figure 2.** Relationships of autoimmune epitopes to the three dimensional Ku structure. The two Ku polypeptides have similar tertiary folding patterns (light blue, Ku70, dark blue, Ku80). Each is composed of several domains, as indicated. The N-terminal domains are independent of one another. The central domain forms the Ku70/Ku80 dimer interface and the DNA binding ring structure. A short, helical, C-terminal "arm" folds back on the N-terminal domain. The C-terminal domain of Ku70 forms a SAP domain separated from the central domain by a disordered loop (indicated by dots). The C-terminal domain of Ku80 is absent from the structure; the site of attachment is marked. Two of the Ku70 autoimmune epitopes are highlighted (magenta, with putative DNA and autoantibody binding sequences in the SAP domain indicated in orange and yellow; see text). An additional, poorly delimited epitope in the Ku70 N-terminal domain is not shown. Ku80 epitopes are not shown. One poorly delimited epitope is present in the N-terminal domain, and the other two lie in the extreme C-terminal domain, which is absent from the structure.

The epitopes present in each Ku subunit have been further analyzed by immunoblotting using fusion proteins representing different parts of the sequence (71). Ku70 has at least three distinct epitopes, encoded by amino acids (aa) 115-467, 506-535, and 560-609, respectively. Of these epitopes, the one formed by aa 560-609 is the most commonly recognized by autoantibodies in human sera. More detailed analysis suggests that this region contains a discontinuous epitope, which requires both aa 560-571 and 600-609 for its formation (71).

Antibodies directed against the Ku70 C-terminal epitope (aa 560-609) have been shown to be species-specific, recognizing human but not mouse Ku. When selected residues in the human protein were mutated to match the mouse sequence, autoantibody recognition was abolished (75). Again, this situation is different than in the anti-Sm system, where autoantibodies show very broad cross-reactivity against U snRNP particles from other species (76, 77).

Three distinct epitopes in Ku80 have also been defined (71). As in Ku70, the most commonly recognized epitopes are near the carboxyl terminus (71, 78). Within the C-terminal region, epitopes formed by aa 558-681 and aa 682-732 appear to be recognized independently (71). The third Ku80 epitope is formed by sequences 1-374, near the N-terminus (71).

### 4.3. Relationship of epitopes to the three dimensional Ku structure

The three-dimensional structure of Ku protein has recently been solved in the presence and absence of DNA (72). The results illuminate the spatial relationship between the autoimmune epitopes and other features of the Ku protein. The structure of the non-DNA bound form of Ku is shown in Figure 2. Each subunit has a similar folding pattern, and the molecule as a whole is therefore quasi-symmetrical. An N-terminal domain in each subunit forms an alpha/beta structure; the N-terminal domains are not in contact with each other. A central domain in each subunit forms a beta-barrel structure that makes up the Ku70/Ku80 dimer interface. The central domain of each subunit also contributes to a ring structure, which completely encircles a DNA binding channel. The central domain leads into a short C-terminal "arm" that folds back on the N-terminal domain.

In contrast to the conserved folding patterns of the rest of the protein, the extreme C-terminal domains of

the two subunits are divergent. In Ku70, there is a so-called SAP domain, which is also present in various scaffold-attachment factors (79). The SAP domain is separated from the remainder of the protein by a disordered loop (indicated by dots in Figure 2). In Ku80, the C-terminal region is also believed to form a discrete domain, the structure of which has not yet been reported.

As discussed in the previous section, Ku70 has at least three autoimmune epitopes. There is a relatively poorly delimited epitope in the N-terminal domains (for clarity, this has not been highlighted in Figure 2). A second epitope is found in the C-terminal "arm", and corresponds to a solvent-exposed alpha helix (Figure 2, colored in magenta). The third epitope, which is the most commonly recognized by autoantibodies, almost exactly coincides with the extreme C-terminal SAP domain. As predicted, two sequences that have been shown to contribute to the epitope, located at aa 560-571 and aa 600-609, lie adjacent to each other within the folded structure, allowing them to contribute to formation of the proposed discontinuous epitope.

One of the surprising aspects of the crystal structure is that it is inconsistent with an apparently well-founded conjecture about the role of the major Ku70 autoimmune epitope in DNA binding. Early studies showed that a region encompassing Ku70 aa 536-609 has a DNA binding capability that is independent of Ku80 (80). Deletion of aa 601-609 abolished both DNA binding and autoantibody binding (80). Similarly, substitution of ala-ala-ala for lys-ser-gly at aa 591-593 abolished both DNA binding and autoantibody binding (80). These data show that DNA binding and autoantibody binding are tightly linked, and suggest that the same surface in Ku is responsible for both activities. This hypothesis was further supported by the finding that autoantibodies inhibit Ku-DNA interaction (20, 80).

The crystal structure forces a reinterpretation of these data. In the non-DNA bound form of Ku, the SAP domain of Ku70, which coincides with the major autoimmune epitope, forms a well-ordered interface with Ku80 in a position that is remote from the high-affinity, functional DNA-binding channel formed by the ring structure. In the presence of DNA, the domain appears to undergo a large scale motion and possible unfolding, but nevertheless does not participate in formation of the major protein-DNA interface (72). Thus, the crystal structure is incompatible with the major C-terminal autoimmune epitope contributing to the primary binding site for DNA ends.

It is possible that this C-terminal domain has some secondary role in DNA interaction, perhaps regulating motion along the DNA or mediating sequence-specific DNA binding, and that this activity is responsible for the results obtained in earlier biochemical studies (81). The inhibition of DNA binding by autoantibodies could reflect their effect on these secondary protein-DNA interactions. Alternatively, the inhibition may arise because autoantibody binding blocks a conformational change that is necessary for occupancy of a DNA binding site located elsewhere in the Ku molecule.

Unfortunately, the crystal structure is less informative with respect to the Ku80 epitopes. As with Ku70, there are at least three epitopes in Ku80. One of these corresponds to a poorly delimited sequence in the N-terminal domain. The other two are localized in the extreme C-terminal domain, for which structural information is not yet available.

### 4.4. Particle-specific autoantibodies directed against the Ku heterodimer and the Ku-DNA complex

By definition, "particle-specific" autoantibodies do not recognize isolated subunits or protein domains, making it difficult to carry out experiments to characterize their properties or localize their binding sites within the Ku-DNA complex. The difficulty is particularly acute when particle-specific antibodies occur in a complex mixture with many other specificities, as is often the case in autoimmune patients. However, there are experimental approaches that can be used to investigate the presence of particle-specific antibodies in such mixtures.

One of these approaches is to carry out competitive binding studies, measuring the ability to interfere with binding of a monoclonal antibody of known specificity. Monoclonal antibody (mAb 162) has been shown to recognize a conformational epitope that is present only in the Ku70/Ku80 heterodimer, and not in the individual components (18). In competitive ELISAs, most anti-Ku autoimmune sera inhibit the binding of mAb 162 to the Ku protein (18, 71). This result suggests the presence of autoantibodies that recognize a similar or overlapping epitope that is dependent on the assembled heterodimer.
Another, more general, approach is to test sera for the ability to stabilize the Ku dimer or the Ku-DNA complex against dissociation. In general, if an antibody is directed against epitopes unique to an assembled particle, binding of the antibody would be expected to stabilize the particle. This is because of the favorable free energy obtained from the binding of the antibody to the particle-specific epitopes. Dissociation of the particle necessitates concurrent dissociation of the antibody, and the free energy requirement for this process is higher than for dissociation of the particle alone.

This stabilizing effect can be demonstrated experimentally. Wang *et al.* captured the Ku heterodimer on agarose beads using an anti-Ku80 monoclonal antibody and showed that the Ku subunits could be dissociated by sequential washing with buffers containing high salt and detergent (82). Using these conditions, they found stabilizing antibodies that prevented dissociation of the Ku70/Ku80 dimer in 9 of 12 anti-Ku-positive sera (83). Of these, one sample recognized only the heterodimer and not the individual Ku70 or Ku80 subunits, confirming that it contained antibodies that recognize a conformational epitope formed by the dimer. In the other eight cases, the sera appeared to have a mixed specificity, containing particle-specific antibodies as well as antibodies capable of recognizing the individual subunits.

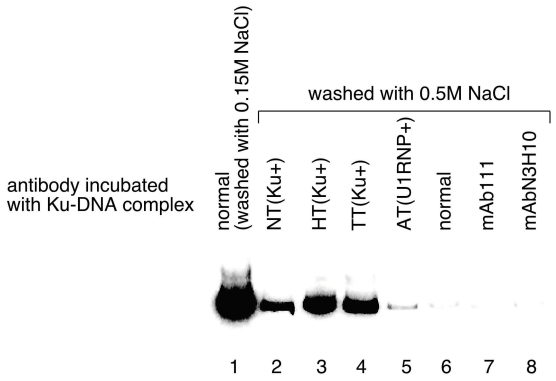


**Figure 3**. Autoantibodies that stabilize the Ku-DNA complex. Anti-Ku80 monoclonal antibody (mAb 111) was adsorbed to Protein-A Sepharose beads, which were washed and incubated with HeLa cell extracts, then with a $^{32}$P-labeled DNA fragment to allow formation of a Ku-DNA complex. The complex was incubated with various sera or mAbs. After incubation, the complex was washed with buffer containing 0.5 M NaCl to test complex stability. When Ku-DNA complexes were incubated with normal serum, they are sensitive to 0.5 M NaCl (compare lanes 1, 6). Incubation with anti-U1 RNP serum or with anti-Ku monoclonal antibodies does not result in significant stabilization (lanes 5, 7, 8). By contrast, incubation with any of three different human anti-Ku autoimmune sera markedly stabilized the complexes (lanes 2-4).

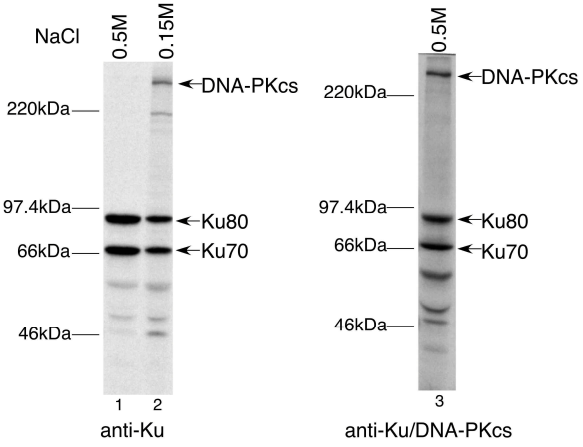


**Figure 4.** Immunoprecipitation using anti-Ku serum (left) or serum containing a mixture of anti-Ku and anti-DNA-PKcs autoantibodies (right). In both cases, autoantibodies were adsorbed to Protein A-Sepharose beads, which were washed and incubated with $^{35}$S-methionine-labeled HeLa cell extracts under physiological salt conditions (0.15 M) or high salt conditions (0.5 M) as indicated. Note that anti-Ku antibodies co-precipitate DNA-PKcs under physiological salt conditions (lane 2), but not when high salt is used to disrupt protein-DNA interactions (lane 1). By contrast, serum containing a mixture of anti-Ku and anti-DNA-PKcs antibodies is able to precipitate Ku and DNA-PKcs regardless of the salt concentration that was present (lane 3).

We have also found antibodies that stabilize the interaction of Ku with DNA (Figure 3). In these experiments, Ku protein was captured on agarose beads with monoclonal antibody, and $^{32}$P-labeled DNA fragments were allowed to bind. Ordinarily, this DNA can be released by treatment with 0.5 M NaCl (Figure 3, compare lanes 1 and 6). We screened anti-Ku-positive patient sera for the ability to stabilize the Ku-DNA complex against dissociation under these conditions. As shown in Figure 3, lanes 2-4, three different anti-Ku-positive sera prevented dissociation, whereas control antibodies had no effect. This result is suggestive of the presence of antibodies that stabilize the Ku-DNA complex by binding to conformational epitopes that are characteristic of the DNA-bound form of Ku protein.

Autoantibodies that stabilize the structure of an autoantigen have previously been reported for U snRNP autoantigen (84). The occurrence of such antibodies in the Ku system attests to the generality of this phenomenon.

### 4.5 . Cleavage by caspases and Granzyme B

As discussed previously, there is evidence that susceptibility to cleavage by certain proteases correlates with the ability of proteins to act as autoantigens. Recent work has shown that Ku70 is cleaved by Granzyme B at the sequence ISSD$^{79}\downarrow$R$^{80}$ (10), which lies near to, but not within, one of the epitope-containing regions. Neither of the Ku subunits has been shown to be susceptible to site-specific caspase cleavage, although there has been a report that the overall amount of Ku protein decreases in apoptotic cells (85).

## 5. AUTOANTIBODIES AGAINST DNA-PKCS

### 5.1. Distinguishing anti-Ku and anti-DNA-PKcs antibodies

Many anti-Ku antibodies have been found to co-precipitate the 470 kDa DNA-PKcs polypeptide, particularly when immunoprecipitation is carried out in crude cell extracts under physiological (0.15 M) salt conditions (86). When immunoprecipitation is carried out with the same antibodies in 0.5 M NaCl, a condition that disrupts protein-DNA interactions, co-precipitation of DNA-PKcs is no longer observed (Figure 4, compare lanes 1 and 2). This phenomenon is seen with both autoantibodies and anti-Ku monoclonal antibodies, and is apparently attributable to the presence of Ku/DNA-PKcs complexes assembled on endogenous DNA fragments (86). When procedures are used to remove all traces of DNA from the cell extract, co-precipitation of DNA-PKcs by anti-Ku antibodies no longer occurs (86).

Although the pattern of salt-sensitive co-precipitation shown in Figure 4 is typical of many anti-Ku autoimmune sera, there are some sera that precipitate DNA-PKcs even in the presence of 0.5 M NaCl, suggesting the presence of antibodies that recognize the DNA-PKcs polypeptide directly (Figure 4, lane 3) (13, 14). These autoantibodies are probably directed against

conformational epitopes, because they show little or no binding to DNA-PKcs in an immunoblot.

### 5.2. Frequency and clinical correlation

The occurrence of anti-DNA-PKcs autoantibodies was investigated in a study of sera from 312 patients with systemic rheumatic disease (13). In this population, 10 patients had antibodies that recognized DNA-PKcs, including 2 patients with SLE, 2 with scleroderma, 2 with polymyositis, and 4 with an overlap syndrome. Because of their extremely high titer and avidity, autoimmune sera directed against DNA-PKcs have proven useful as experimental tools, for example in the inhibition and immunodepletion of end-joining activity in cell-free DNA repair assays (54, 87).

Interestingly, anti-DNA-PKcs and anti-Ku antibodies tended to occur together in the same sera. In the study mentioned above, 6 of 10 (60%) of anti-DNA-PKcs sera contained anti-Ku specificity, whereas only 26 of 302 (9%) of anti-DNA-PKcs negative sera had anti-Ku specificity (13). This linkage is highly significant ($P<0.01$). The presence of anti-DNA-PKcs and anti-Ku antibodies in the same sera suggests that the autoimmune response may be driven by interaction of the assembled Ku/DNA-PKcs particle with the immune system. Further support for this hypothesis is provided by the observation that some autoimmune sera stabilize the association between Ku and DNA-PKcs (14). As discussed in the preceding section, stabilizing antibodies are indicative of a binding interaction with conformational epitopes unique to the assembled state.

### 5.3. Cleavage by caspases and Granzyme B

DNA-PKcs was one of the first autoantigens that was demonstrated to be a caspase substrate (5). Caspase 3 cleaves at the sequence, $DEVD^{2712\downarrow}N^{2713}$ (6). Recent work has also shown that DNA-PKcs is a substrate for Granzyme B, which cleaves at a nearby sequence, $VGPD^{2698\downarrow}F^{2699}$ (10).

## 6. AUTOANTIBODIES AGAINST XRCC4 AND DNA LIGASE IV

The DNL IV/XRCC4 complex is involved in the final ligation step of the Ku-dependent DNA repair pathway (48-50, 52-54) and has been reported to associate with Ku antigen (88). In addition, XRCC4 has been reported to be cleaved by Caspase 3 during radiation-induced apoptosis (16). These findings prompted us to screen for autoantibodies directed XRCC4 and its partner, DNL IV. Among approximately 170 sera from patients with systemic rheumatic diseases, we identified 22 sera with significant reactivities against the DNL IV/XRCC4 complex. Immunoblotting shows that some of these sera are specific for the DNL IV, some for XRCC4, and some had a significant ability to recognize both polypeptides. Further characterization of these sera, epitope mapping, and protease susceptibility studies are in progress (Lee, Dong, Takeda, Wang, and Dynan, manuscript in preparation).

## 7. HOW DO DSB REPAIR PROTEINS ELICIT AN AUTOIMMUNE RESPONSE?

The factors that lead to development of systemic rheumatic diseases are not understood. Almost certainly, a combination of genetic and environmental factors is involved. Whatever the factors that lead to initial susceptibility, there is strong evidence that the maintenance and progression of the autoimmune response is antigen-driven. That is, the response is highly specific, there are only a limited number of targets in a given individual, and the autoantibodies often occur in linked sets that are directed against multiple epitopes in the same protein or particle.

The Ku/DNA-PKcs antigen system provides a particularly good example of autoantibodies that are directed against an assembled particle. The frequency with which antibodies against both proteins are observed in the same patient, as well as the presence of stabilizing antibodies that recognize conformational epitopes characteristic of various assembled complexes, strongly suggest that some form of assembled particle is the antigen that drives the sustained autoimmune response. Biochemical studies show that Ku and DNA-PKcs do not associate except in the presence of broken DNA ends (24, 86). This raises a question of how Ku/DNA-PKcs complexes arise and are presented to B-cell antigen receptors. One interesting possibility is that they are formed extracellularly in the context of infection or inflammation. For example, if nuclear contents were released from a virally infected cell, Ku and DNA-PKcs might assemble on host or viral fragments, perhaps in combination with pathogen-encoded DNA binding proteins. Interaction with B-cell antigen receptors in this context might lead to a powerful immune response against self components that could be sustained even after the original infection was cleared.

Patients who produce anti-Ku antibodies tend to do so over long periods of time. Reeves and coworkers carried out a longitudinal study of anti-Ku antibody titers, which showed that antibody levels against Ku70, Ku80, and the native Ku70/80 particle fluctuate in parallel (89). The level of anti-Ku antibodies to some degree correlated with the level of anti-dsDNA antibodies, but did not correlate with the level of antibodies against exogenous antigens, such as bovine cytochrome c, bovine serum albumin, and tetanus toxoid (89). The presence of antibodies against multiple epitopes in the same individual, together with the persistence of the anti-Ku response over time, are consistent with the hypothesis that the production of anti-Ku antibodies is an antigen-driven process and reflects a response to the native Ku heterodimer.

An alternative mechanism, which is not mutually exclusive, invokes an aberrant pattern of antigen processing and presentation, leading to presentation of immunocryptic epitopes in the context of major histocompatibility complex (MHC). For example, Ku/DNA-PKcs/DNA complexes created in the context of infection or inflammation might be phagocytosed and processed to yield peptides different than

those generated from the non-assembled components present in normal cells. Alternatively, cleavage by caspases or Granzyme B in apoptotic cells could alter the pattern of processing. A certain dendritic cells have been shown to internalize apoptotic cells, and efficiently process and present proteins inside the apoptotic cells on MHC class I and II molecules (90). In each of these cases, the outcome might be to present immunocryptic epitopes to T-cell receptors (91). The idea that Granzyme B cleavage is related to autoantigenic potential is particularly appealing. Sites for Granzyme B are relatively rare among cellular proteins as a whole, but were present in 21 of 29 well-defined autoantigens that were characterized in a recent study (10). Because Granzyme B is secreted by cytotoxic T lymphocytes and natural killer cells, which operate only in the periphery, and not in the thymus, there should be no opportunity for negative selection of autoreactive T lymphocytes that are specific for Granzyme B-dependent immunocryptic epitopes.

It is possible that stabilizing antibodies are also able to bring about changes in the pattern of antigen presentation. If they prevent or delay the dissociation of protein components inside antigen presenting cells, they may cause an alteration of the antigen processing pattern, which in turn might lead to presentation of immunocryptic epitopes to autoreactive T lymphocytes.

Double-strand break repair proteins have been the subject of intense interest because of their role in the maintenance of genome stability. Structural, biochemical, and genetic studies have yielded a wealth of information about the functions of these proteins in the nonhomologous end-joining pathway. For this reason, DSB repair proteins are excellent models for characterization of the humoral autoimmune response. The recent availability of a high resolution structure for Ku protein has allowed us to reconsider classical epitope mapping studies in a structural context. It should be possible to extend this approach to other DSB repair proteins as additional structural information becomes available. This will allow further insights into the nature of the conformational epitopes that appear to drive the particle-specific autoimmune response. Sites of caspase and Granzyme B cleavage have recently been mapped in Ku and DNA-PKcs, and similar studies are in progress with more recently recognized autoantigens, DNL IV and XRCC4. These studies, in combination with more detailed epitope mapping, should cast new light on the relationship between autoantigenic potential and susceptibility to cleavage by proteases that are activated in the context of inflammation and cell death.

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