Site-Specific Proteolytic Cleavage of Ku Protein Bound to DNA

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ABSTRACT Ku protein, a relatively abundant nuclear protein associated with DNA of mammalian cells, is known to be a heterodimer with subunits of 85 and 72 kDa which binds in vitro to DNA ends and subsequently translocates along the molecule. The functional role played by this protein in the cell, however, remains to be elucidated. We have observed here that Ku protein, purified from cultured monkey cells, is the target of specific endoproteolysis in vitro, by which the 85 kDa subunit is cleaved at a precise site while the 72 kDa subunit remains intact. This cleavage releases an 18 kDa polypeptide and converts Ku protein into a heterodimer composed of the 72 kDa subunit associated with a 69 kDa fragment from the 85 kDa subunit. The proteolyzed form of Ku protein, denoted Ku', has DNA binding properties similar to those of Ku protein. The proteolytic mechanism, which is inhibited by leupeptin and chymostatin, is extremely sensitive to ionic conditions, in particular to pH, being very active at pH 7.0 and completely inhibited at pH 8.0. In addition, cleavage occurs only when Ku protein is bound to DNA, not free in solution. We suggest that in vivo, such proteolysis might be necessary for Ku protein function at some stage of the cell cycle. © 1993 Wiley-Liss, Inc.

Key words: DNA-binding proteins, DNA-protein interactions, nonhistone protein, site-specific proteolytic cleavage, nuclear autoantibody

INTRODUCTION

Detailed analysis of nuclear proteins has provided an effective approach to understanding the fundamental mechanisms of genome structure and function in higher organisms. Ku protein is among the nonhistone chromosomal proteins which have been well characterized physically but whose function is still unclear. Ku protein was detected initially as the autoantigen in patients with scleroderma-polymyositis overlap syndrome¹ and was first studied with the aid of autoantibodies. It was shown to be localized in nuclei and to be bound to DNA during interphase with release during metaphase.²⁻⁴ Its DNA binding properties in vitro have also been analyzed. The protein binds to the ends of DNA fragments, slides along the molecule with no specific binding sites⁵⁻⁷ and finally requires ends to be released from the DNA.⁷ Ku protein has been shown to be a dimer composed of 85 and 72 kDa subunits, and the cDNAs for both subunits have been cloned. Sequence analysis⁸⁻¹² has shown the presence of an element similar to a leucine zipper which might be responsible for dimer formation.

Here we have used polyacrylamide gel electrophoresis to study complexes formed between DNA and Ku protein purified from cultured monkey cells. Under certain conditions, Ku protein is converted into a modified form, denoted Ku', whose complexes with DNA have a higher electrophoretic mobility. Our data show that the conversion of Ku protein into Ku' is due to a proteolytic cleavage at a specific site within the large subunit of Ku protein and that this cleavage is strictly dependent upon Ku protein being bound to DNA.

MATERIALS AND METHODS Materials

The DNA fragment used for protein binding was a 224 base pairs (bp) StyI fragment from the cloned control region of Simian Virus 40, extending from the map positions 37 to 333, with one of the 72 bp repeats in the transcription enhancer deleted.¹³ Experiments performed with DNA fragments of similar size from plasmid pBR322 gave identical results. The DNA fragment was 5' end labeled with $[\gamma^{32}P]$ ATP and polynucleotide kinase. Nonradioactive competitor DNA was *E. coli* DNA, sonicated to an average size of 1,000 bp. Molecular weight markers for SDS gel electrophoresis were from Pharmacia (Milwaukee, WI). Protease inhibitors were from Boehringer (Mannheim, Germany).

DNA-Protein Interactions

Unless otherwise noted, protein was incubated with $\sim 10,000$ cpm (~ 0.1 ng) of labeled DNA plus 15 ng unlabeled competitor DNA for 15 mn at 37°C in 25 μ l of 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Triton

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X100, 100 μ g bovine serum albumin/ml. In the experiments performed to study the effect of pH on the velocity of the reaction, the Tris and NaCl in the above buffer were replaced by a buffer containing 50 mM sodium ion plus the appropriate amount of 3-(N-morpholino)propanesulfonic acid (Mops) to bring the pH to the desired value, while keeping the total

Electrophoresis of DNA-Protein Complexes

Complexes were analyzed by gel retardation on 4% polyacrylamide gels at 4°C in 6.7 mM Tris, 3.3 mM Na acetate, 1 mM Na₂EDTA, adjusted to pH 7.6 with acetic acid, using buffer recirculation.¹⁴ Preparative gel retardation, for further analysis of bound protein by SDS polyacrylamide electrophoresis, was performed as described.¹⁵

Purification of Ku Protein

ionic strength constant.

HPLC fractions containing Ku protein were prepared simultaneously with the sequence-specific single strand DNA binding protein H16, according to procedures described elsewhere,¹⁵ and were generously given to us by Claire Gaillard. Briefly, nuclei were purified from cultured monkey CV1 cells; proteins were extracted with 0.4 M NaCl, and loaded on a column of hydroxyapatite equilibrated in 10 mM K phosphate pH 7.5, 1 mM DTT, 15% glycerol. The column was first washed with 0.4 M NaCl, 10 mM K phosphate pH 7.5, 1 mM DTT, 15% glycerol, then with 10 mM K phosphate pH 7.5, 1 mM DTT, 15% glycerol, and the proteins were eluted with a linear gradient of K phosphate pH 7.5 from 10 mM to 1 M, in 1 mM DTT, 15% glycerol. Ku protein did not elute as a sharp peak but was present in all the fractions eluting between 10 and 400 mM K phosphate. Pools of fractions containing Ku protein were dialyzed against 25 mM Tris-HCl pH 7.5, 30 mM NaCl, 1 mM DTT, and further fractionated by HPLC on a mono Q column (Pharmacia) as described.¹⁵ Elution from mono Q was performed with a linear gradient of NaCl. Ku protein eluted as a sharp peak at 0.24 M NaCl. The Ku protein fraction appeared to be pure with respect to DNA binding activity but still contained many other proteins as can be judged from Figure 2A. It was observed that the fractions eluting early from the hydroxyapatite column yielded Ku protein preparations which were more stable, suggesting that a component of the proteolytic activity described here eluted at high concentration of potassium phosphate from hydroxyapatite.

Immunoblotting

After electrophoresis, proteins were transferred to nitrocellulose following standard procedures. The membranes were incubated with serum from a patient with anti-Ku autoantibodies, a kind gift of Tsuneyo Mimori. Bound antibodies were detected by incubation with secondary antibodies directed against human IgG coupled to alkaline phosphatase, followed by detection of alkaline phosphatase by appearance of dark purple color in the presence of a chromogenous substrate (ProtoBlot from Promega [Madison, WI]).

RESULTS

Ku Protein Can Give Rise to Two Forms of Complexes With DNA

When incubated with a labeled DNA fragment, Ku protein forms complexes which can be resolved by electrophoresis on polyacrylamide gels and detected by autoradiography as a series of bands. These regular ladders correspond to complexes of the DNA fragment with one or several Ku protein molecules, and form with any DNA fragment whose length is sufficient to bind Ku protein.^{6,7} During the course of our work on the mechanism of interaction of simian Ku protein with DNA, we noted, in addition to this ladder, a series of bands of lower intensities migrating slightly faster than the complexes of Ku protein, and forming a ladder which was always similar to the Ku protein ladder, as shown in Figure 1A. The intensities of these faster bands varied from one experiment to another, but their presence was accompanied remarkably by the presence of Ku protein, leading us to consider that they might arise from a protein very similar to Ku protein or even from a conformational variant of Ku protein itself. In addition, the influence of salt concentration on the appearance of this new series of bands strongly suggested that Ku protein could be converted into a different form yielding DNA complexes of higher mobility. Figure 1B shows that the usual forms of Ku protein DNA complexes are observed when the NaCl concentration is higher than 100 mM, whereas at lower NaCl concentrations this form disappears while the faster form appears. Therefore, we set out to study the interesting possibility that a change in the structure of Ku protein could occur under certain incubation conditions to give rise to a different form, denoted Ku'.

Ku' Results From Proteolytic Cleavage of Ku Protein

The subunit composition of the Ku and Ku' complexes were determined by preparing a large quantity of each by preparative retardation gel electrophoresis. They were then cut from the retardation gel and loaded onto an SDS polyacrylamide gel which was stained with silver. Figure 2A shows that the slow-migrating complex, Ku, contains the 85 and 72 kDa subunits known to make up Ku protein, whereas the fast-migrating complex, Ku', contains an apparently identical 72 kDa subunit, a polypeptide of apparent molecular mass 69 kDa present in equimolar amount, and no 85 kDa subunit. Since the initial fraction contains no 69 kDa polypeptide,



Fig. 1 (Top). Ku protein can be converted into a different form Ku'. A. Ku protein was incubated with a labeled DNA fragment in the presence of various amounts of unlabeled competitor DNA, in a buffer containing 50 mM NaCl. The complexes were analyzed by gel retardation electrophoresis and autoradiography. Competitor DNA amounts: 1, 4, 15, 60 ng in lanes a–d, respectively. Lane e: no protein added. B. Ku protein was incubated with labeled DNA plus 15 ng of unlabeled competitor DNA, keeping all parameters constant except the NaCl concentration, which was varied as indicated. The complexes were analyzed by gel retardation as in A. The positions of the one-to-one complex of Ku protein with DNA, and of its variant Ku', are indicated.

Fig. 2 (Bottom). Ku' formation results from proteolytic cleavage of Ku protein. A. SDS polyacrylamide gel analysis of the com-

this result strongly suggests that Ku' is derived from Ku by proteolytic cleavage of the 85 kDa subunit at a specific site. Figure 2B shows the result of plexes. The complexes formed by Ku protein and Ku' with approximately 100 ng of the 224 bp DNA fragment were purified by preparative gel retardation electrophoresis, cut from the retardation gel, and analyzed by SDS polyacrylamide gel electrophoresis and silver staining. Free DNA migrated to the end of the gel. Controls shown in lanes a and b were performed by loading samples with no DNA on the retardation gel, in order to detect proteins which migrate at the same position as the complexes without being bound to DNA. The Ku protein fraction used was loaded in lane F. MW: molecular mass marker LMW from Pharmacia: 94, 67, 43, 30 and 20.1 kDa. **B**. Immunoblotting analysis of the complexes. Complexes Ku and Ku' prepared as in A, and the starting Ku protein fraction (lane F), were fractionated on an SDS poly-acrylamide gel, transferred to a nitrocellulose membrane, and probed with serum from a patient with anti-Ku autoantibodies.

immunoblotting with a similar gel. Complexes Ku and Ku' were loaded onto an SDS polyacrylamide gel, with an aliquot of the starting Ku protein frac-

PROTEOLYTIC CLEAVAGE OF KU PROTEIN





Fig. 3 (Top). Ku' can be purified from Ku protein as a stable heterodimer. A pool of protein fractions containing Ku protein, obtained by hydroxyapatite chromatography as described in Materials and Methods, was loaded on a mono Q column and proteins were eluted with a NaCl concentration gradient. Fractions were assayed for DNA binding by gel retardation. Binding was performed in the presence of a large excess of competitor DNA, therefore only the first bands of the Ku and Ku' ladders are visible. The positions of the complexes of Ku and Ku' protein with DNA are indicated.

Fig. 4 (Bottom). Conditions of the proteolytic conversion of Ku into Ku'. A. Effect of pH. Complexes of Ku protein with DNA at pH 7.0 or 7.5 were incubated at 37°C for the indicated times and loaded on a retardation gel. B. Effect of protease inhibitors, analyzed by gel retardation as in A. Ku protein was incubated with DNA, in a buffer containing no NaCl, in the presence of the indicated proteinase inhibitors at the following concentrations: Chymostatin 10, 1 and 0.1 μ g/ml in lanes a–c; Leupeptin 250, 25, 2.5 and 0.25 μ g/ml in lanes d–g; Pepstatin 200, 20 and 2 μ g/ml in lanes h–j, respectively; lane k: no protease inhibitor added.

tion. After electrophoresis and transfer to nitrocellulose, the membrane was incubated with serum from a patient with anti-Ku autoantibodies, a kind gift of T. Mimori. It is observed that both polypeptide chains contained in the Ku' complex are recognized by the anti-Ku antibodies. The weakness of the signal given by the 69 kDa polypeptide may be due to the fact that the epitopes of the 85 kDa subunit are distributed nonuniformly along the molecule¹⁶ and may be largely removed by the proteolytic cleavage (see Discussion).

Ku' Is a Heterodimer of 72 and 69 kDa Subunits With DNA Binding Properties Similar to Those of Ku Protein

That the 72 and 69 kDa polypeptides are still associated as a heterodimer to form Ku' protein is strongly suggested by the fact that it is possible to purify this dimer by chromatography. Figure 3 shows that Ku' can be separated from Ku protein on mono Q. In the course of Ku protein purification as described in Materials and Methods, a pool of hy-

droxyapatite fractions containing Ku protein was loaded on a mono Q column, proteins were eluted with a NaCl concentration gradient, and the presence of Ku or Ku' proteins in the fractions was assayed by gel retardation. Ku protein eluted at 0.24 M NaCl in fractions 11-12, and a protein yielding a complex identical to Ku' eluted earlier in the gradient, at 0.19 M NaCl, in fractions 7-8. SDS polyacrylamide gel electrophoresis followed by silver staining or immunoblotting showed that the complex formed by these fractions did contain the 72 and 69 kDa subunits of Ku' protein (data not shown). It should be noted that although an~18 kDa polypeptide is released during the conversion of Ku into Ku' (see below), no such peptide could be detected in the complexes of Ku' protein with DNA (Fig. 2, and data not shown). After its partial purification, studies of the DNA binding properties of Ku' protein gave data which are not shown here since they are, in all respects, identical to those obtained previously for Ku protein.⁵⁻⁷ At high protein-DNA ratio, DNA fragments were found to bind up to one molecule of Ku' protein for every 30 bp of DNA and form ladders which extended very high on retardation gels. Ku' protein was found also to recognize the ends of DNA fragments, to be unable to bind to DNA circles, to slide along DNA molecules, and to be unable to redistribute from one fragment to another.

Conditions Necessary for Proteolytic Cleavage of Ku Protein

In addition to the effect of NaCl concentration already shown in Figure 1, which is also observed with other salts such as KCl or sodium phosphate, the pH of the incubation buffer was found to be extremely important for proteolysis of Ku protein. Figure 4A shows a comparison of the velocity of proteolytic cleavage at pH 7.0 and 7.5 in a Mops-based buffer. Under the conditions used, 50% of Ku protein is converted into Ku' in 5 mn at pH 7.0, and conversion is complete in 20 mn. At pH 7.5, on the other hand, only a small percentage of Ku protein is converted after a 24 hr incubation. Therefore, in this experiment the ratio of velocities between pH 7.0 and 7.5 is higher than 10³. In a similar experiment performed at pH 8.0, we were unable to detect any conversion of Ku into Ku' after 24 hr. The effect of protease inhibitors has also been studied, and is shown in Figure 4B. Under the conditions employed, leupeptin at 0.25 µg/ml completely blocks the proteolytic activity; chymostatin results in 50% inhibition at 0.1 μ g/ml and complete inhibition at 1 μ g/ml, and pepstatin has little effect.

Conversion of Ku Protein Into Ku' Occurs Only When Ku Protein Is Bound to DNA

An important and original characteristic of the proteolytic activity is its dependence on Ku protein being bound to DNA. Figure 5 shows an experiment



Fig. 5. Ku protein can be cleaved into Ku' only when bound to DNA. Lane a: Ku protein was mixed with DNA, incubated at 37°C for 30 min, and loaded on a retardation gel. Lane b: Ku protein was incubated at 37°C in the same buffer for the same period of time; DNA was then added and the sample loaded on the retardation gel. The positions of Ku and Ku' complexes were determined by loading markers (not shown), prepared as in Figure 1B, in adjacent lanes.

in which Ku protein was incubated either in the presence of DNA and directly loaded on a retardation gel, or in the absence of DNA, the DNA being added just before loading of the gel. It can be seen that Ku protein was converted completely into Ku' when it was bound to DNA, whereas Ku protein incubated in the absence of DNA lost a slight amount of its DNA binding activity but was not cleaved.

The Conversion of Ku Protein Into Ku' Releases an 18 kDa Polypeptide

Since, as judged by the apparent molecular masses of the polypeptide chains, the proteolytic conversion of Ku protein into Ku' removes about 16 kDa from the large subunit, and since no polypeptide of that size could be found in the complex of Ku' protein with DNA, we looked for such a peptide in the incubation buffer. Ku protein was incubated in the presence or in the absence of DNA, the samples were loaded on an 18% polyacrylamide gel containing SDS, and polypeptides were analyzed by immunoblotting. Figure 6 shows that, although not all of the Ku protein present in the sample has been proteolyzed, an 18 kDa polypeptide is clearly visible in the sample which contained DNA, and absent both from the starting Ku protein fraction and from the sample incubated in the absence of DNA. The bands around 38 kDa are present in all samples and probably correspond to different degradation products of



Fig. 6. Proteolytic cleavage of Ku protein releases an 18 kDa polypeptide. Ku protein was analyzed by immunoblotting after incubation at 37°C in the presence of DNA (lane a), or after incubation at 37°C in the absence of DNA (lane b), or without incubation (lane c). The samples were analyzed by electrophoresis on an 18% polyacrylamide gel in the presence of SDS and immunoblotting. Positions of the molecular markers were determined by coloration of the nitrocellulose membrane with Ponceau red.

Ku protein. The lack of complete proteolysis of Ku protein observed in this experiment is probably due to the fact that some of the Ku protein of this fraction had lost its DNA binding activity and therefore its capacity for being converted into Ku'.

Is Ku Protein an Autoprotease?

As fractions containing Ku protein also contained the proteolytic activity responsible for conversion of Ku into Ku', we asked whether Ku protein itself might be the protease responsible for its conversion into Ku'. At present, we cannot confirm or exclude this possibility, but several lines of evidence suggest that the proteolytic activity has a component that is different from Ku protein. First, the velocity of the cleavage reaction is sensitive to Ku protein concentration, since Ku protein is proteolyzed more slowly when diluted, while its DNA binding activity is unaffected (data not shown). Second, even though the proteolytic activity is always present, it shows large variations in specific activity from one Ku protein preparation to another. Third, Figure 7 shows that it is possible to detect an activity which stimulates the conversion of Ku into Ku' and which chromatographs independently from Ku protein. In order to search for an activity that would stimulate the conversion of Ku into Ku', aliquots from fractions of a mono Q chromatogram obtained in the course of Ku protein purification were added to the complex of Ku protein with DNA. The appearance of Ku' in lanes 6-7 is due to the presence of Ku' protein itself in the



Fig. 7. Partial purification of a factor stimulating Ku protein cleavage. A preparation of Ku protein showing little endogenous proteolytic activity was used to detect such activity in fractions obtained by mono Q chromatography during Ku protein purification. Complexes of Ku protein with DNA were incubated at 37° C with a given amount of each fraction, and loaded on a retardation gel. The Ku' complex visible in fractions, whereas fraction 19, eluting at 0.45 M NaCl, contains no Ku' protein but a factor which enhances the proteolysis of Ku into Ku'.

corresponding fractions, and Ku protein is present in fractions 10-11. Therefore, the complete conversion of Ku into Ku' by fraction 19 shows that a component of the proteolytic activity studied here is present in this fraction. To decide whether this component is a proteolytic enzyme or a cofactor will most probably require its purification.

DISCUSSION

We have shown the existence in vitro of a mechanism by which Ku protein is converted into a smaller form, Ku', by proteolytic cleavage of its large 85 kDa subunit at a specific site, yielding a 69 kDa polypeptide which remains associated as an heterodimer with the 72 kDa subunit, plus an 18 kDa polypeptide which is released in the incubation medium.

A striking characteristic of this mechanism is that it operates only when Ku protein is bound to DNA, free Ku protein being resistant to cleavage. The possible explanation that a component of the proteolytic activity binds to DNA seems excluded by the fact that addition of variable amounts of competitor DNA has no effect on the proteolysis (see e.g., Fig. 1). Therefore, it seems more likely that the site of proteolysis is not accessible on the free protein, and that a change of conformation occurs upon Ku protein binding to DNA, thus making the cleavage site accessible.

Although the exact site of cleavage within the amino acid sequence of the large subunit has not been determined, it appears very likely that this site is located in the C-terminal region for the following two reasons. First, we observe a large decrease in affinity of the anti-Ku autoantibodies for the 69 kDa cleavage product relative to the intact 85 kDa subunit (see Fig. 2B), indicating that most epitopes recognized by the autoantibodies have been removed. It has been shown that most epitopes of the 85 kDa subunit are located in its C-terminal region.¹⁶ Second, the leucine zipper, believed to be involved in Ku protein heterodimer formation, is located in the Nterminal region of the 85 kDa subunit, and hypothetical cleavage of 18 kDA from the N-terminus would remove more than half of this zipper.^{8,11} The fact that we do not observe any destabilisation of the heterodimer upon proteolysis also implies, therefore, that the cleavage site is not at the N-terminus but in the C-terminal region.

Several lines of evidence lead one to believe that this proteolytic cleavage of Ku protein might also operate in vivo. The conclusion that most epitopes for Ku protein autoantibodies are found in a region which can be released from Ku protein by proteolysis immediately suggests that this mechanism might, in fact, be the trigger for most Ku protein autoantibody formation, the cleaved and perhaps dislocated peptide serving as the principal antigen. In addition, two kinetic properties of the proteolytic cleavage must also be considered. First, the reaction is highly specific, involving a single endoproteolytic cut at a specific site in only one of the Ku protein subunits, which leaves the other subunit intact and produces a well-defined Ku' protein which is stable and resistant to further proteolytic cleavage. Second, under proper conditions, this proteolytic activity is both highly active and highly specific for Ku protein, and its extreme sensitivity to ionic conditions, particularly to pH, suggests that there could easily exist a mechanism to regulate its activity within the cell.

Since the function of Ku protein is still unknown, it seems premature to make hypotheses as to the precise functional role of the proteolytic mechanism observed here. It is interesting to note, however, that in our in vitro experiments, the DNA binding properties of Ku' were indistinguishable from those of the intact protein, suggesting that the role of the cleavage is not directly related to DNA binding. Ku protein is a large protein of 150,000 kDa whose function is certainly not limited to binding DNA. In addition, it is entirely possible that the 18 kDa polypeptide released by Ku protein cleavage might play a functional role, perhaps in another compartment of the cell.

The importance of proteases in the cell has long been recognized, and it is very likely that they are also important in nuclear function. However, few examples of proteolytic cleavage of nuclear proteins have been studied so far. This is due largely to the fact that non-specific degradation, occurring during

nuclear isolation, can be so overwhelming that it easily obscures any specific proteolysis. For example, among the many High Mobility Group proteins (HMG) originally isolated, most were eventually shown to be degradation products of histone H1. Only HMG 1, 2, 14, and 17 have emerged as genuine nuclear proteins.¹⁷ More recently, however, with improvements in nuclear isolation techniques and the availability of multiple protease inhibitors, several examples of specific proteolysis of nuclear proteins have been documented. They include topoisomerase II,¹⁸ transcription factors fos and jun,¹⁹ nuclear oncogenes,²⁰ scaffold proteins,²¹ prot-amines,²² histone H2a,^{23,24} and nuclear proteins in general.²⁵ The importance of proteolysis of specific nuclear proteins during certain viral infections has also been demonstrated for transcription factor TFIIIC,²⁶ histone H3,^{27,28} and protein p53.²⁹ In addition, the importance of proteolysis mechanisms in the cell cycle has been well documented in the past few years. The best example is provided by cyclins, components of M phase promoting factor, which accumulate during interphase to be abruptly degraded during mitosis.³⁰ It has also been shown that the level of thymidine kinase is subject to cell cycle regulation by the specific degradation of the protein during cell division,³¹ and topoisomerase II also seems to be degraded at about the time of cell division.¹⁸ In addition, some protease inhibitors have been shown to arrest cells at mitotic metaphase.^{32,33} Ku protein expression is regulated during the cell cycle,³⁴ as is probably also the case for its binding to DNA since the protein is released from metaphase chromosomes. It will therefore be interesting to have more data on the fate of Ku protein inside the cell during the cell cycle, and on the possible involvement of the proteolytic mechanism described here at certain stages of the cell cycle.

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