Reduced sulphhydril groups are required for DNA binding of Ku protein

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The Ku protein, a DNA-binding complex that is composed of two subunits of 70 kDa and of 86 kDa, has been suggested to play a role in gene transcription. The dependence of the in vitro DNA-binding activity of affinity-purified Ku protein on reduced cysteine residues has been studied using sulphhydril-modifying agents. Inhibition of the DNA-binding activity was caused by alkylation with N-ethylmaleimide and by crosslinking with azadicarboxylic acid bis(dimethylamide). Treatment of the protein with a large excess of N-ethylmaleimide after it had bound to DNA did not completely dissociate the complex from the DNA, suggesting that some cysteines may be in direct contact with DNA. Pre-incubation of the protein at 37°C or above caused rapid inactivation of DNA binding. The elevated temperature and azadicarboxylic acid bis(dimethylamide) treatments resulted in the formation of a crosslinked product, which was detected by Western blotting. The effects of azadicarboxylic acid bis(dimethylamide) and heat were completely reversible by treatment with a reducing agent, such as dithiothreitol. These results demonstrate that in vitro DNA-binding activity of the Ku protein requires reduced sulphhydril groups. Interestingly, the DNA-binding activity of Ku protein was protected from heat inactivation by the presence of a HeLa cell nuclear extract, suggesting that a nucleic factor or factors may be responsible for the maintenance of the reduced cysteines of the Ku protein in vivo. Thus, the biochemical function of the Ku protein may be regulated through oxidation-reduction of its cysteine residues.

INTRODUCTION

The Ku protein is a heterodimer that is composed of two subunits of 70 kDa and of 86 kDa, which was originally discovered as an autoantigen that reacts with antibodies from patients with rheumatic diseases (Mimori et al., 1981). Anti-Ku positive sera from autoimmune patients reacted selectively with a subset of transcriptionally active sites on the insect polytene chromosomes (Amabis et al., 1990). The cDNA-derived sequences of both subunits revealed that the Ku protein has characteristics that are typical of some trans-acting factors, including leucine-zipper motifs with adjacent basic regions and an upstream-activating motif (Reeves and Sthoefer, 1989; Yaneva et al., 1989; Mimori et al., 1990). These features led to the speculation that the protein may function as a transcription factor (Reeves and Sthoefer, 1989).

Treatment of isolated HeLa nuclei with DNAase I or with micrococcal nuclease released the Ku protein complex as a part of a 10 S chromatin fragment that lacked histone H1, suggesting that the Ku protein selectively associates with DNA in regions of active gene expression in vivo (Yaneva and Busch, 1986). It has been demonstrated that the Ku protein is involved in specific binding to the promoter region of the human transferrin receptor gene (Roberts et al., 1989) and to the proximal and distal regulatory DNA sequences of the U1 small nuclear (sn) RNA gene (Knuth et al., 1990). In addition, the in vitro transcription of the U1 snRNA gene depended to a large extent on the presence of the Ku protein (Gunderson et al., 1990; Knuth et al., 1990). Ku2, a protein with high sequence similarity to the Ku protein, was found to bind specifically to the octamer DNA motif of the human immunoglobulin genes (May et al., 1991). Thus, the Ku protein seems to be involved in the process of gene transcription, perhaps through the regulation of the activity of the recently discovered DNA-dependent protein kinase that phosphorylates the C-terminal domain of RNA polymerase II in vitro (Dvir et al., 1992) and transcription factor SP1 (Gottlieb and Jackson, 1993).

It has been demonstrated that the Ku protein binds to free DNA ends in vitro, possibly through the 70 kDa subunit (Mimori and Hardin, 1986; De Vries et al., 1989; Zhang and Yaneva, 1992). In the course of the purification and biochemical characterization of Ku protein we noticed that the DNA-binding activity of the protein strongly depends on the presence of reducing agents. Since it has been demonstrated that the activity of some transcription factors can be controlled by a redox system operating in the cell nucleus (Abate et al., 1990a), we examined the role of the reduced sulphhydril groups of the Ku protein in its DNA-binding activity. In this report we present data demonstrating that Ku protein DNA-binding activity requires reduced cysteine residues, which could be a potential mechanism for the regulation of the biological function of the Ku protein.

MATERIALS AND METHODS

Purification of Ku protein

The Ku protein was extracted from HeLa cells and was purified by DEAE-cellulose and phosphocellulose chromatography, as previously described (Yaneva et al., 1985). The final step of purification involved either immunoaffinity (Yaneva et al., 1985) or DNA-affinity chromatography. A DNA-affinity column was constructed using concatenated 45 bp oligonucleotides from the 5' upstream region of the p120 nucleolar protein gene (Zhang et al., 1991). All solutions used in the purifications contained 5 mM dithiothreitol (DTT).

DNA fragments and labelling

A 125 bp DNA fragment was prepared from a pTZ18R subclone of the 5' flanking region (−1430 to −1327) of the p120 gene by digestion with EcoRI (Zhang et al., 1991). The DNA fragment was purified from agarose-gel slices and was labelled by filling in the ends with DNA polymerase (Klenow fragment) in the presence of [α-32P]dATP (Sambrook et al., 1989).

Abbreviations used: diamide, azadicarboxylic acid bis(dimethylamide); DTT, dithiothreitol; NEM, N-ethylmaleimide; snRNA, small nuclear RNA.
Electrophoretic-mobility-shift assay

DNA binding was allowed to occur by mixing $^{32}$P-labelled DNA fragment (1 ng) with various amounts of purified Ku protein, in 20 µl reactions, in a buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM MgCl$_2$, 0.1 mM DTT, 200 mM KCl and 7% (v/v) glycerol (DNA-binding buffer). After incubation at room temperature for 15 min, the protein–DNA complexes were analysed in 5% PAGE, under non-denaturing conditions, using the method of Prywes and Roeder (1989).

Preparation of the nuclear extract

Extracts from HeLa cell nuclei were prepared with 0.4 M NaCl, using the method of Dignam et al. (1983). The final protein extract was dialysed extensively against buffer containing 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM phenylmethanesulphonyl fluoride, 1 mM DTT, 0.2 mM EDTA and 20% (v/v) glycerol. The protein concentration of the final preparation, determined by the Coomassie method (Bradford, 1976), was usually around 10 mg/ml. Before incubating with purified Ku protein, the extract was diluted 1:1000 with DNA-binding buffer.

Buffers for pH studies

The following buffers were used for adjusting the pH of the DNA-binding buffer: homo-Pipes, pH 4.5 and 5.0; Mes, pH 5.5 and 6.0; Pipes, pH 6.5; Mops, pH 7.0; Hepes, pH 7.5; Tris/HCl, pH 8.0 and 8.5; 2-(N-cyclohexylamino)ethanesulphonic acid, pH 9.0 and 9.5; 3-(N-cyclohexylamino)-1-propanesulphonic acid, 10.0. In addition, every buffer contained 2 mM MgCl$_2$, 0.1 mM DTT, 200 mM KCl and 7% (v/v) glycerol.

Western-blot analysis

Ku protein was separated by SDS/8%–PAGE and was subsequently transferred electrophoretically to nitrocellulose. The proteins were allowed to bind with monoclonal antibodies that were specific for each subunit, as previously described (Yaneva et al., 1985).

RESULTS

Effect of sulphhydryl-modifying agents on the DNA-binding activity of the Ku protein

Affinity-purified Ku protein (immunoaffinity column or DNA-affinity column) bound to the DNA fragment in an electrophoretic-mobility shift assay (Figure 1, lane 12). The number of retarded bands depended on the protein concentration and on the size of the DNA fragment, as previously described (DeVries et al., 1989; Zhang and Yaneva, 1992). To examine the role of the sulphhydryl groups in binding to DNA, purified active Ku protein was treated with N-ethylmaleimide (NEM), an agent that is known to alkylate -SH groups irreversibly. The formation of Ku–DNA complexes was inhibited after the pre-incubation of the protein with NEM (Figure 1, lanes 2–5). When the protein was first incubated with DNA and then was treated with increasing concentrations of NEM, the inhibition was reduced (Figure 1, lanes 7–11). In both cases the major bands disappeared and a band with changed mobility, perhaps due to the protein alkylation, was observed. This band remained resistant to alkylation with 5 mM and with 10 mM NEM, only when the protein was bound to DNA. The alkylation of the DNA-bound protein occurred at a reduced rate. These results indicate that some -SH groups become less sensitive to alkylation after the protein is bound to DNA: either they are in direct contact with DNA or binding to DNA changes their accessibility for modification by NEM as a result of a change in the protein conformation.

The inhibition of the DNA-binding activity was also observed after pre-incubation of the Ku protein with azadicarboxylic acid bis(dimethylamide) (diamide), an oxidizing agent that causes reversible crosslinking of the sulphhydryl groups (Figure 2a). There was no change in the mobilities of the DNA–protein complexes on this treatment, as was observed on treatment with NEM. This difference in the effects of the diamide and of NEM may be due to the different chemical modifications (alkylation as against crosslinking with diamide). The presence of DTT in a molar excess over diamide protected the DNA-binding activity of the protein, strongly suggesting that reduced -SH groups are required for DNA-binding activity. The diamide treatment resulted in the formation of disulphide-bonded products that were detected as faint bands in Western-blot analysis (Figure 2b).

In this analysis, the 86 kDa subunit migrated as a broad band (Figure 2b, lane 1), apparently due to oxidation, since it was converted into homogeneous polypeptides on reduction with DTT (Figure 2b, lane 5). These multiple non-reduced forms of the 86 kDa polypeptide did not affect the DNA-binding activity of the protein complex (Figure 2a, lane 2). Thus, it is probable that the reduced -SH groups of the 70 kDa subunit are more important for DNA binding than are those of the 86 kDa subunit.

**In vitro DNA-binding activity of the Ku protein is temperature-sensitive**

The Ku protein was pre-incubated at different temperatures under conditions for DNA binding, before binding to the DNA.
probe. The results of such experiments showed that the DNA-binding activity of the protein was strongly inhibited at temperatures of 37 °C or above (Figure 3a). This inhibition was not due to proteolysis, as was demonstrated by Western-blot analysis (Figure 3b). Even at 42 °C, the protein was intact, indicating that the primary structure of the protein was not sensitive to heat treatment. The heat inactivation of DNA-binding occurred within the first 5–10 min of incubation at 37 °C in a time-course study (Figure 4). These results indicate that the conformation of the DNA-binding site of Ku protein requires additional stabilization at physiological temperature in vivo. We hypothesize that the maintenance of the sulphhydryl groups in a reduced state could be a part of this stabilization.

**Effect of pH on the DNA-binding activity of the Ku protein**

Purified Ku protein was active for binding to DNA at room temperature over a wide pH range (5–10) (Figure 5). A similar stability of the antigenicity of the Ku protein, as measured by immunodiffusion, has been reported previously (Mimori et al., 1981). On heat treatment, the residual activity was maximal at pH 7.5 (Figure 5). The activity of the heat-treated protein at pH 8.0 to 9.0 was greatly reduced, in contrast with the activity at room temperature at these pH values. In this range of pH, -SH groups become more sensitive to oxidation, since their pKₐ is at pH 8.5. The inhibition was significant at pH > 8, indicating that the sulphhydryl groups that are active at room temperature become oxidized at 37 °C. Thus, the inhibition of the DNA-binding activity in the range pH 8–9, at least in part, could be due to the oxidation of sulphhydryl groups.

**Restoration and protection of the Ku protein DNA-binding activity**

The DNA-binding activity of the Ku protein that had been inactivated either by crosslinking with diamide or by incubation at 37 °C could be restored by incubation with excess DTT (Figure 2a, lane 8) or could be protected by the presence of DTT (Figure 6, lanes 4–8). Western-blot analysis showed that both methods of inactivation caused the formation of disulphide crosslinked products, which were converted into single polypeptides on reduction with the sulphhydryl agent 2-mercapto-
The Ku protein (10 ng per sample) was incubated with the 32P-labelled DNA fragment for 15 min at room temperature. The DNA–protein complexes were resolved in 5% PAGE. After drying, the gel was exposed to film and the bands of the autoradiograph were scanned on an LKB densitometer. The values on the ordinate represent the relative areas under each peak.

Figure 5 Effect of pH on the DNA-binding activity of the Ku protein

The Ku protein (10 ng per sample) was incubated with the 32P-labelled DNA fragment for 15 min at room temperature in binding buffers with different pHs as indicated on the abscissa. The DNA–protein complexes were resolved in 5% PAGE. The gel was dried, exposed to film and scanned as in the legend to Figure 4. (●) Probes incubated at room temperature; (O) probes incubated at 37 °C.

ethanol (Figure 7). These results demonstrate that reduced sulphhydryl groups are necessary for the DNA-binding activity of Ku protein.

We attempted to prevent the inhibition of Ku protein DNA-binding activity, using a factor or factors derived from the cell nucleus. For this purpose, Ku protein was mixed with diluted nuclear extracts, incubated at 37 °C for 5 min and was subsequently bound to DNA. This treatment protected the DNA-binding activity (Figure 6, compare lanes 4 and 10). No DNA-binding activity of the nuclear extracts alone was detected under these conditions (Figure 6, lane 9). These experiments show that the reduced state of the Ku protein sulphhydryl groups can be maintained by a factor that is present in the nuclear extract.

DISCUSSION

The experiments that are described in this paper demonstrate that the Ku protein requires reduced sulphhydryl groups for binding to DNA. Treatment of the protein with two sulphhydryl-
modifying agents that introduced different modifications (alkylation by NEM and crosslinking by diamide) led to the inactivation of the DNA binding. Under these conditions some DNA-binding activity was protected from the alkylation treatment by pre-incubation with DNA. If -SH groups were modified (which is the primary effect of NEM), these results would mean that cysteine residues are localized at or near the DNA-binding site of the protein. However, the effect of NEM could be more complex, since it is not known precisely which cysteines are modified and whether some α- and ε-amino groups (Smyth et al. 1964) are modified as well: modification of groups that are not involved in DNA binding directly can cause conformational changes that result in the destabilization of the protein-DNA complex. Future experiments with site-specific mutations of Ku protein cysteines will distinguish between these possibilities.

The inhibition of the DNA binding with diamide was fully reversible with the reducing agent DTT. Similarly, the inhibition observed after incubation at 37 °C was reversed by the same agent. In both cases, Western-blot analysis showed that disulphide bonds were formed, suggesting that there are one or more functionally critical sulphydryls that are available to be oxidized and that this oxidative inactivation is readily reversible. These results demonstrate that the formation of disulphide bonds is sufficient to prevent DNA binding. At present, it is not clear if this crosslinking occurs between or within the two subunits, or both. The DNA-binding inhibition was always accompanied by the formation of oxidized bands of lower and of higher molecular mass. The faster-migrating bands in Figure 2(b), lanes 2-4 and in Figure 7, lanes 2 and 4 (indicated by an arrow) may represent intramolecular disulphide-bond formation that causes additional folding of the polypeptide and so faster migration through the gel (Silva and Cidlowski, 1989). Slow-migrating bands may be the result of intermolecular disulphide crosslinking. The high-molecular-mass crosslinked products may represent dimers and tetramers, as we have noticed previously in gel-filtration studies (Yaneva et al., 1985).

Examination of the cDNA-derived amino-acid sequences of both Ku protein subunits (Reeves and Sthoeger, 1989; Yaneva et al., 1989) reveals that nine of a total of fourteen cysteine residues are located on the 86-kDa subunit and five on the 70-kDa subunit. It is possible that the 86-kDa subunit cysteines are not involved in DNA binding, since their oxidation did not inhibit DNA binding. In addition it has been demonstrated that the 70-kDa subunit is the one that is primarily involved in contacts with DNA (Mimori and Hardin, 1986; Abu-Elheiga and Yaneva, 1992; Zhang and Yaneva, 1992). Thus, the cysteine residues of the 70-kDa subunit, in particular those located downstream of the basic region that is implicated in binding DNA (Cys398 and Cys498), may play an important role in the function of the Ku protein. However, the role of the 86-kDa subunit in the formation of the DNA-binding site needs further investigation. The presence of numerous cysteines in the 86-kDa subunit that are not involved in DNA binding may explain the requirement for a large excess of modifying agents to achieve significant DNA-binding inhibition.

Chemical modifications of cysteine residues, as well as elevated temperatures, have complex effects on the biochemical function of a protein. These factors could cause inactivation either by crosslinking of cysteines or by conformational alterations at the binding site. Our results definitely point to the formation of crosslinked products; however, the possibility for reversible conformational changes that result from chemical modifications or from elevated temperatures cannot be excluded. The effect of systematic site-specific mutations of the cysteine residues, especially those of the 70-kDa subunit, on the DNA-binding activity of Ku protein will differentiate between these possibilities and will establish the role of defined cysteines.

As well as the environmental factors such as temperature, pH and ionic strength that alter protein conformation and affect the oxidation equilibrium constant ($K_{ox}$), the ratio of reduced to oxidized glutathione in the cell nucleus is an important factor that determines the redox state of a protein in vivo. A vast excess of reduced over oxidized glutathione, which can change significantly under certain physiological conditions, is largely responsible for the cellular reducing potential. Thioredox proteins such as thioredoxin and glutathione S-transferase, which have been detected in the nucleus (Bennett et al., 1986), may play important roles in this delicate balance that affects reduction and oxidation of sulphydryl groups of proteins under physiological conditions (Cappel and Gilbert, 1988). A nuclear protein has been identified that causes the reduction of cysteines in the Fos and Jun transcription factors and thus stimulated their in vitro DNA-binding activity (Abate et al., 1990a,b). We found that HeLa cell nuclear extracts that are active in in vitro transcription assays (Dignam et al., 1983) contain a similar factor or factors that stimulated the in vitro DNA-binding activity of the Ku protein. It is unlikely that this factor is free glutathione itself, because the nuclear extracts were dialysed extensively. Whether it is the same as the factor that stimulates Fos and Jun or it is a part of the whole nuclear system that regulates the DNA-binding activities of transcription factors through oxidation–reduction remains to be determined. In any case, the results presented here suggest that the biochemical function of the Ku protein can be subject to regulation by oxidation–reduction of important cysteine residues.

This work was supported by a grant from the NIH, number AR 39308 and in part by PHS grant CA-10893 from the National Cancer Institute.

REFERENCES

Received 21 October 1992/19 February 1993; accepted 3 March 1993