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An inducible Ku86-degrading serine protease in human cells

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Abstract

The Ku autoantigen has been implicated in a number of cellular functions including growth control, immunoglobulin gene rearrangement and DNA repair. A variant truncated form of Ku86, with an apparent molecular weight of 70 kDa, has been reported to be present in many human cell types. We have previously shown that the amount of variant Ku86 is strongly increased in human peripheral blood mononuclear cells (PBMC) by storage of blood prior to isolation of the PBMC. In this study we report that formation of variant Ku86 in protein extracts is mediated by an inducible trypsin-like serine protease with a higher concentration in the nuclear compartment, as compared with the cytoplasm. However, experiments with SDS-PAGE assay of whole cells yielded no evidence of truncated Ku86, suggesting that the protease is not active in intact cells, but is exerting a marked activity during the protein extraction procedure. Interestingly, the protease level became markedly reduced upon transfer of the cells to growth medium. Protease induction did not correlate with apoptosis, necrotic cell death or with signs of general proteolysis or cytotoxicity. Our findings have methodological implications for the interpretation of experimental Ku86 data, and suggest that this protease may play a role for cellular regulation of Ku function.
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Keywords: Ku autoantigen; Proteolysis; Human lymphocyte

1. Introduction

Ku is a heterodimeric protein consisting of the Ku70 and Ku86 subunits [1]. It is evolutionary well conserved from yeast to mammals, fitting with the growing list of fundamental cellular processes reported to include a role of Ku. Initially its influence on functions mediated by the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) such as DNA double-strand break repair and V(D)J recombination was documented [1–3]. More recently, vital roles of Ku have been reported in signal transduction [4], in DNA replication [5], in cell cycle control [6,7] and cell growth [7,8], and in telomere stability and cellular senescence [9–11].

In most cell types studied, Ku has been interpreted to be strictly composed of the two 70 kDa (Ku70) and 86 kDa (Ku86) subunits, without any sign of alternative molecular weight forms. However, in many cell types and tissues [12–16] a fraction of Ku86 has been reported to appear as a variant form with an apparent molecular weight of approximately 70 kDa (designated in the present work as Ku86v). In multiple myeloma bone marrow and HL-60 cells, the presence of Ku86v was shown to correlate with an increased sensitivity to gamma radiation [13,16].

Cellular control of Ku protein quantity and function has been reported to occur at different levels. Proliferation was shown to associate with intracellular redistribution and with a marked increase in Ku mRNA [17,18], and the Ku protein amount has been shown to increase in response to gamma radiation [17,19,20]. Posttranslatory epitope modifications and usage of a second promoter generating an extended mRNA transcript have also been reported [15,18,21].

The present study was undertaken to document whether the finding of Ku86v in peripheral blood mononuclear cells (PBMC) [22] should be viewed as part of a general protein disintegration in severely damaged cells, or if it rather suggests an adaptive response in human cells responding to certain environmental stimuli. The results indicate that most, if not all, of the Ku86v appearing in PBMC protein extracts are due to a nuclear serine protease with no signs of

Abbreviations: DNA-PK_{cs}, DNA-dependent protein kinase catalytic subunit; EMSA, electrophoretic mobility shift assay; PARP, poly(ADP)ribose polymerase; PBMC, peripheral blood mononuclear cells; PMSF, phenylmethylsulfonyl fluoride; Ku86v, variant form of Ku86

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activity in intact cells, but causing rapid Ku86 degradation during a protein extraction procedure. The activity of this protease increases in PBMC during in vitro blood incubation and becomes clearly reduced upon transfer of the cells to optimal growth conditions. The induction of this protease is not associated with any signs of general proteolysis, cytotoxicity, apoptosis or necrotic cell death. These findings have implications for the interpretation of experimental Ku86 data and consequently also for our understanding of Ku function in human cells.

2. Materials and methods

2.1. Antibodies and protease inhibitors

Antibodies used were polyclonal goat anti-Ku-70 (SC-1486) (reactive with a C-terminal epitope corresponding to amino acids 590-608), anti-Ku-86 (SC-1484) (reactive with a C-terminal epitope corresponding to amino acids 710–729) and anti-DNA-PK_{cs} (SC-1552) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonals anti-Ku70 (K91620) (reactive with an N-terminal epitope corresponding to amino acids 57–174) from BD Biosciences (San Jose, CA), anti-Ku86 (P80, Ab-3) (clone S10B1) (reactive with an N-terminal epitope corresponding to amino acids 8–221) and anti-tubulin (Ab-1, CP06) from Oncogene (Cambridge, MA); anti-PARP (clone 4C10-5) was a monoclonal from PharMingen (San Diego, CA); polyclonal rabbit anti-caspase-3 (AHP476) from Serotec (UK); human anti-SSA/SSB serum from Immunoconcept (cat no. 7002) (Sacramento, CA). Secondary HRP-conjugated antibodies were from DAKO (Denmark). Phenylmethylsulfonyl fluoride (PMSF), leupeptin, chicken trypsin-like protease inhibitor and the soybean trypsin-like inhibitor were all from Sigma (St. Louis, MO).

2.2. Cells and culture conditions

PBMC and HEp-2 cells were used as described [22]. The long-term T cell cultures were used after 14–21 days of growth in the presence of added IL-2 (Roche-Boehringer-Mannheim, Germany). Flow cytometry characterization at the end of the culture time, using monoclonal antibodies, demonstrated a homogeneous population of >99% $CD3^+$ (T lymphocytes), <1% $CD19^+$ (B lymphocytes) and <1% $CD14^+$ (monocytes) cells. Phytohaemagglutinine (PHA) was from Sigma and used at 1 μg/ml. The number of viable (dye-excluding) and dead cells was counted manually using trypan blue solution (Sigma) and light microscopy. Assay of mitochondria respiratory capacity was by a colorimetric tetrazolium-based MTT (Sigma) test [23]. Cells were then cultivated in a 96-well microplate for 4 h in the presence of 500 μg/ml MTT, and the amount of formazan salt produced was then determined spectrophotometrically by its absorbance at 570–590 nm.

2.3. Western blot

Crude extraction of cytoplasmic and nuclear proteins, determination of protein concentration and immunoblotting were performed using standard protocols as described by us [22]. A mixture of protease inhibitors (Complete, Roche-Boehringer-Mannheim; at 10%, v/v) (including serine, cysteine and metalloproteases according to the manufacturer) was used for the protein extraction, unless otherwise indicated. In brief, the cytoplasmic and nuclear protein extracts (10 and 5 μg, respectively) were pre-treated for 10 min at 70 °C in gel loading buffer containing detergent and reducing agent before loading onto NuPAGE 7 or 10% pre-cast gels. After electroblotting, the membranes were incubated with the indicated primary antibodies in a blocking buffer and with a secondary HRP-conjugated antibody. The blots were developed using the enhanced chemiluminescence method (ECL from Amersham-Pharmacia, UK). The relative intensity of the truncated Ku86 band was determined by examination of X-ray film in a BioRad Personal Molecular Imager FX and with the Quantity One software. For the whole-cell lysis experiments, 5×10^5 cells were lysed at 95 °C for 10 min in gel loading buffer prior to the electrophoresis.

2.4. Flow cytometry

A Coulter Epics XL-MCL flow cytometer (Coulter, FL) was used, with filters detecting emission at 515–545 nm (for FITC) and 565–595 nm (for propidium iodide). Background autoflourescence was monitored for each sample by analysing unlabelled cells. Apoptosis was measured as described [24]. At least 3000 events were scored. FITC-labelled Annexin V was from Alexis (Switzerland) and PharMingen.

2.5. Electrophoretic mobility shift assay (EMSA)

Preparation of the radiolabelled DNA probe was as follows: A double stranded 25-mer DNA was prepared by two complementary oligonucleotides (5′-ATGGGCAGGTCACGTGGTTCCAGGC-3′ and 5′-GCCTGGAACCACGTGACCTGCCCAT-3′). The two oligonucleotides (100 ng) were end-labelled with 1.2-μl T4 polynucleotide kinase (8 U/μl) in the presence of 3-μl [$\gamma - {}^{32}$P] ATP 5000 Ci/mmol (Amersham) for 10 min at 37 °C. The reaction was then stopped by incubation at 95 °C for 5 min and subsequently annealed at room temperature for 2 h. A Pharmacia G25 column was used to purify the probe from free isotope. The activity was adjusted to 50 000 cpm/μl. A master-mix was prepared by adding 10-μl 2× binding buffer (40 mM HEPES pH 7.3, 100 mM KCl, 6 mM $MgCl_2$, 2 mM EDTA, 16% glycerine, 10% BM Complete protease inhibitor and 5 μl of 2% Na-azide), 2-μl 100 mM DTT and 1 μl of radiolabelled DNA (50–100 ng, 50 000 cpm/μl). The master-mix was incubated on ice for 20 min with 2 μg of nuclear extract in the presence of 0.3-μg closed circular DNA of a pGEM plasmid (Promega, WI) as a nonspecific competitor (yield-

ing 10 × molar excess and 1000 × weight excess). In some experiments cold probe was used as specific competitor (at 50 × molar excess). All gel shift bands were lost in the presence of 50 × molar excess unlabelled linear DNA fragment probe. A 5% native polyacrylamide gel was pre-run for 30 min before loading the samples. After electrophoresis the gel was dried between two clear cellophane paper sheets overnight and exposed to X-ray film at − 70 °C for 3 days. In the super-shift experiments, the indicated antibody was added to the binding mixture followed by another incubation on ice for 30 min prior to the gel electrophoresis.

2.6. Combined EMSA and immunoblot

EMSA was performed as in Section 2.5, with some modifications. Nuclear protein, 5 μg, was loaded in duplicate onto a 6% DNA retardation pre-cast gel (Novex). One part of the gel was dried between two clear cellophane papers as in Section 2.5. The other part of the gel was blotted onto a PVDF membrane using a denaturing pH 8.0 transfer buffer containing 0.5% SDS, 20% methanol, 20 mM Tris and 150 mM glycine. Incubation of the membrane with the indicated antibodies was then done as in Section 2.3.

3. Results

3.1. Induction of a Ku86-degrading protease in human PBMC, with its highest concentration in the cell nucleus

In order to document the general occurrence of the Ku86 degradation in human PBMC previously reported by us [22], initial experiments were made with an unspecified period of time (e.g. less than 2 days) for the blood incubation before isolation of PBMC and protein extraction. Results from five representative individuals (P1–P5) are presented in Fig. 1A and B, demonstrating a great variation between blood donors in Ku86 degradation. The relative intensity (i.e. compared to the sum of full-length Ku86 plus Ku86v, but without consideration of the lower molecular weight bands observed in some samples indicating highly degraded Ku86) of the Ku86v band was determined using

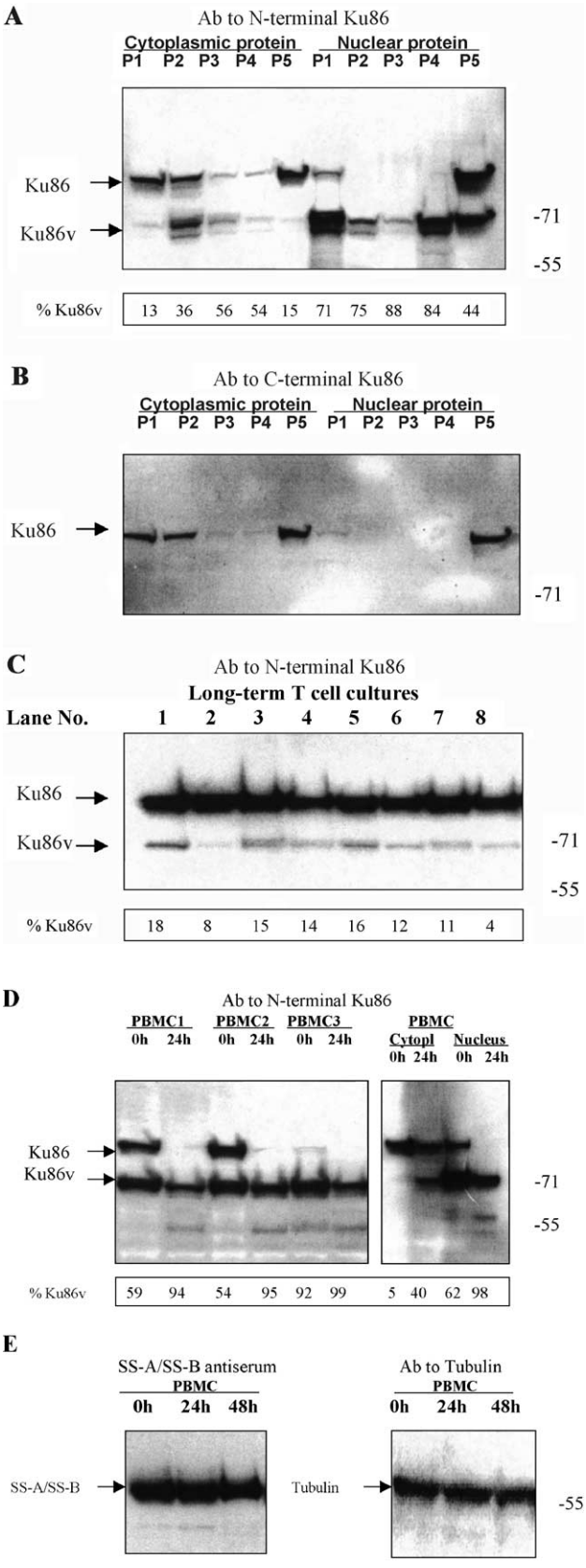

Fig. 1. Formation of Ku86 variant form (Ku86v) in human PBMC. Blood was incubated at room temperature for an unspecified time period (A, B) or for the indicated times (0–48 h) (D, E) before PBMC were isolated. Protein extracts were then separated by SDS-PAGE and immunoblotted with an antibody to an N-terminal Ku86 epitope (A, C, D), C-terminal Ku86 (B), tubulin (E, right panel) or with a human anti-SSA/SSB serum (E, left panel). P1–P5 (A, B), lanes 1–8 for T cells (i.e. long-term IL-2 dependent T lymphocyte cultures) (C) and PBMC1–3 (D) represent different healthy blood donors. Positions of molecular weight standards are shown on the right. The arrows indicate the positions of full-length Ku86 and Ku86v. The relative intensity of the Ku86v band (compared to full-length Ku86 plus the Ku86v) was determined using imaging equipment.

imaging equipment, and is indicated as percentage in the figures. A higher fraction of Ku86v is found in the nuclear extract, as compared with the cytoplasm, in all cell types studied by us, including those shown in Fig. 1A. Moreover, the Ku86v in all cases seems to result from a C-terminal cleavage, since the C-terminal anti-Ku86 antibody was found to be able to detect only intact nontruncated Ku86 protein, as judged by the close correlation between the C-

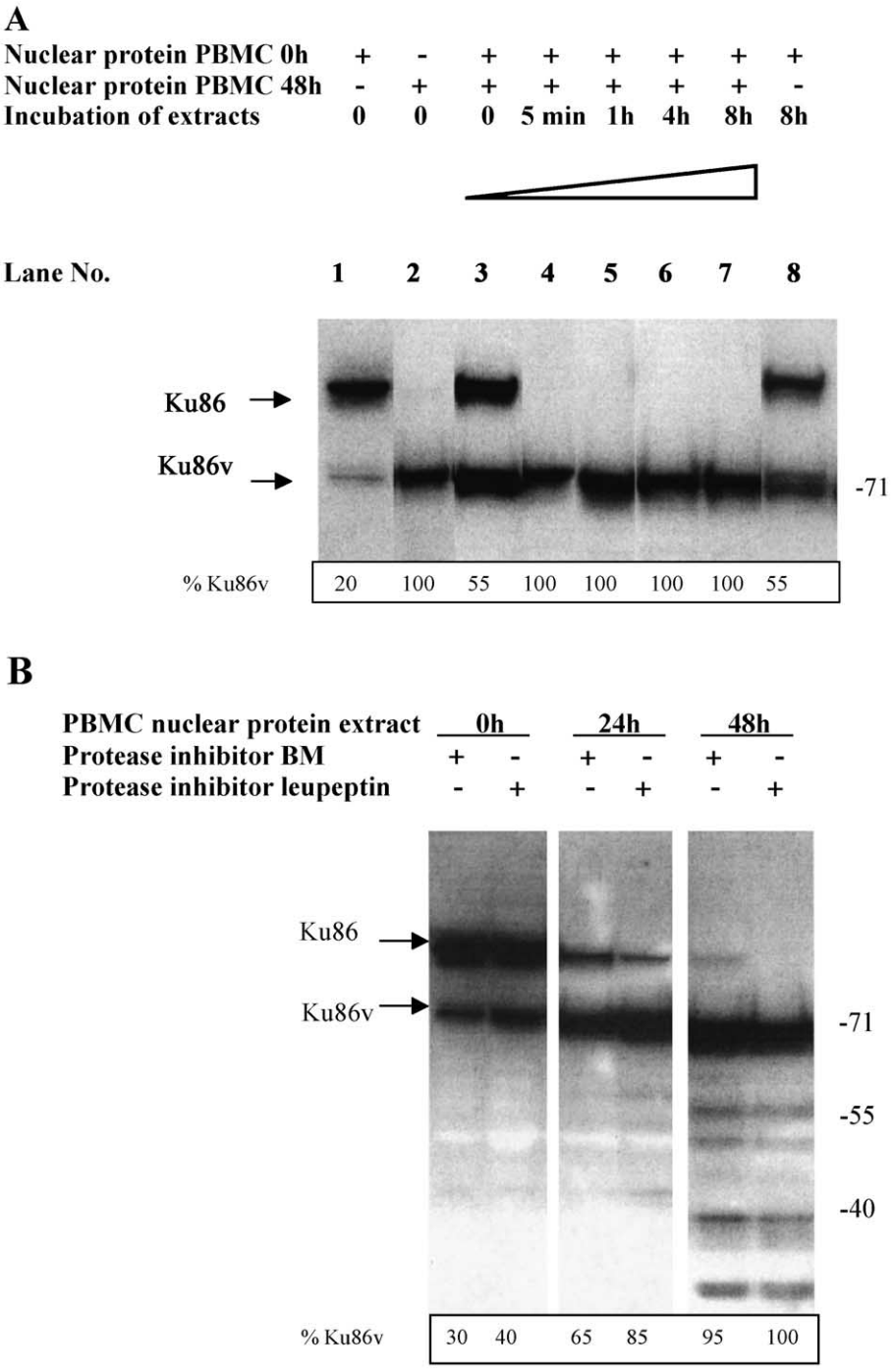

Fig. 2. Ku86 degradation is mediated by an inducible trypsin-like serine protease. PBMC were isolated after blood incubation for the indicated times (0–48 h). Equal amounts of two different nuclear protein extracts (using the standard protocol with 10% BM protease inhibitors) were then mixed, incubated at 37 °C for the indicated times (5 min–8 h), and then analysed by immunoblotting using the ab to N-terminal Ku86 (A). Additional protease inhibitors were added to the extract mixes during a 30-min coincubation: no addition (i.e. BM at 10%) or leupeptine (100 μM) (B); BM (25%, indicated by the arrow), PMSF (3 mM), chicken eggwhite trypsin-like protease inhibitor (chick, 100 μM), soybean trypsin-like protease inhibitor (soy, 100 μM) (C). Co-incubation with a higher-activity protease extract was also made to test the inhibitory effect of the soybean inhibitor (at 100 μM) (D). For the immunoblotting the antibody to N-terminal Ku86 was used. Results are from single gel experiments; the gel has been cut to improve data presentation by reordering the lanes.

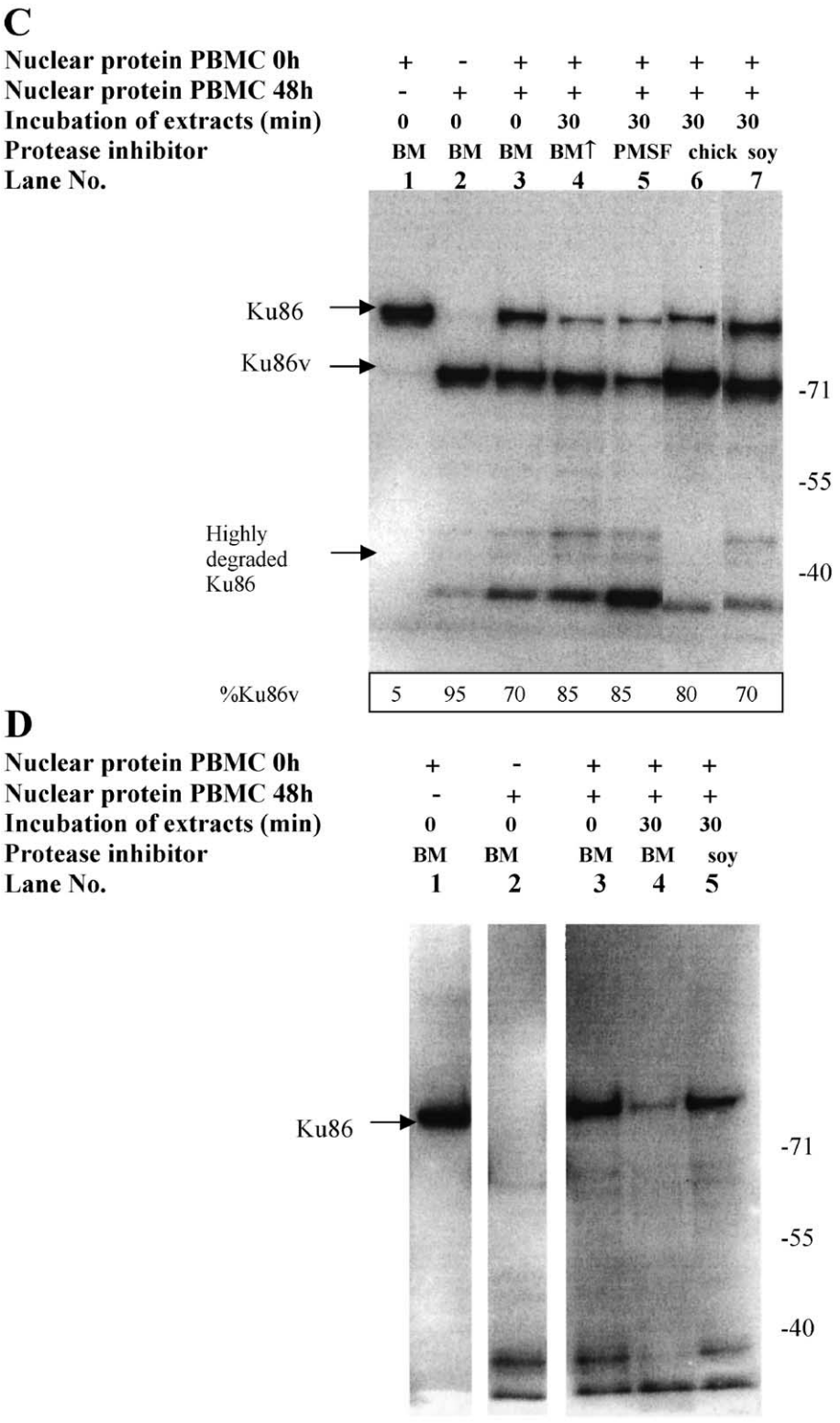

Fig. 2 (*continued*).

terminal antibody single band (Fig. 1B) and the N-terminal antibody band corresponding to full-length Ku86 (upper band in Fig. 1A). With the resolution of the present methodology, the Ku86v shows up as either a single band (Fig. 1C and D), or as a doublet band (Fig. 1A), as previously found by other workers [16,25]. A fraction of the total cellular content of Ku86 was present as Ku86v in all of the cell types studied by us, including PBMC from more

than 30 healthy blood donors, IL2-dependent long-term T lymphocyte cultures derived from eight blood donors (Fig. 1C), and in lymphoid and epithelial lines (data not shown).

The inter-individual variation in Ku86 degradation was found to be strongly influenced by the time of blood incubation, although Ku86v could be demonstrated for most of the blood donors also in unincubated blood samples (Fig. 1D). Enhancement of Ku86 cleavage due to blood incubation was found to be a general phenomenon in PBMC, occurring with all blood donors tested by us. Furthermore, this inducibility could be documented in both cytoplasmic and nuclear protein extracts (Fig. 1D, right panel).

To determine whether a general protein degradation accompanies the formation of Ku86v, immunoblotting analysis of the RNA-associated multiprotein SS-A/SS-B autoantigen and tubulin was performed, providing no evidence of alteration in band configuration in nuclear protein extracts (Fig. 1E, left panel, SS-A/SS-B), or in the cytoplasmic extracts (Fig. 1E, right panel, tubulin). Thus, the protease activity appears to be relatively specific for Ku86 protein. Tubulin immunoreactivity was usually completely lacking in nuclear extracts, verifying that the protein extraction protocol yields a nuclear preparation containing only nuclear components. No Ku70 degradation products were observed with the presently employed antibody in any of the cell types studied by us, including PBMC and cell lines, although a decrease in the intensity of the Ku70 band was seen with PBMC from some of the incubated blood samples (data not shown).

3.2. Characterization of a protease responsible for Ku86 degradation

To verify the existence of a Ku86-degrading protease activity, PBMC 0-h containing predominantly full-length Ku86 (Fig. 2A, lane 1) and 48-h nuclear protein extracts with Ku86v (Fig. 2A, lane 2) were mixed (lane 3). Within as little as 5 min of 37 °C coincubation time, truncation of Ku86 could be demonstrated (lane 4). The additional coincubation time points (1–8 h) served to indicate that no further degradation of the Ku86v into highly degraded Ku86 could be seen in these experiments (lanes 5–7). A parallel 8-h incubation of the PBMC 0-h extract caused only minor truncation of Ku86 (lane 8).

In order to characterize the protease responsible for the degradation of Ku86, the effect of various protease inhibitors was examined including leupeptin and PMSF (both being inhibitors of some serine and cysteine proteases), a commercially available cocktail of protease inhibitors (here called BM, containing unspecified inhibitors of serine, cysteine and metalloproteases), and two trypsin-like serine protease inhibitors (from chicken eggwhite and soybean). Nuclear protein was extracted from PBMC 0-, 24- and 48-h in the presence of either 10% BM (i.e. our standard protocol for protein extraction) or leupeptin as protease inhibitor. Leupeptin was first tested, due to its reported capacity to fully inhibit (at 5 μM) the Ku86 truncation occurring in senescing human fibroblasts [14]. However, extraction in the presence of a high concentration (100 μM) of leupeptin was found to lead to even slightly more truncation of the full-length Ku86, as compared with the BM standard protocol (Fig. 2B). Therefore, in contrast to senescing human fibroblasts, the Ku86 truncating protease of PBMC is not inhibited by leupeptin. Besides the band corresponding to Ku86v, bands <70 kDa indicating highly degraded Ku86 were observed with a slightly increased intensity in the 48-h extract, indicating proteolysis activity beyond a single C-terminal cleavage site (Fig. 2B).

Another extract mixing experiment was then set up in the presence of a panel of protease inhibitors. Full-length Ku86 was degraded in the presence of the PBMC 48-h extract, even when supplemented with additional BM (25%) (Fig. 2C, lane 4), PMSF (lane 5) or a chicken eggwhite trypsin inhibitor (lane 6). Both the Ku86v band and a <40 kDa band (most pronounced for PMSF) provided evidence for this proteolysis. However, the chicken egg white inhibitor protected a fraction of the Ku86 protein from degradation (lane 6). In contrast, in the presence of soybean trypsin inhibitor no protease activity was detected; the banding pattern remained the same as for the non-incubated extract mix (lanes 3 and 7). In order to verify this inhibitory influence of the soybean inhibitor, a PBMC 48-h nuclear

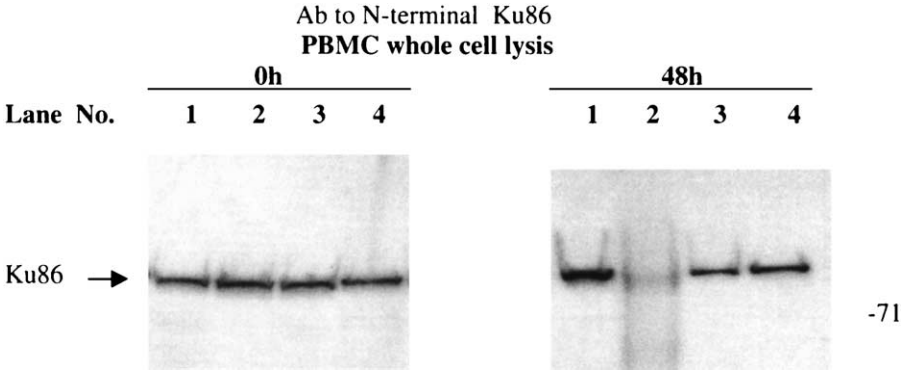

Fig. 3. SDS-PAGE assay of whole cells suggests lack of Ku86 degradation in intact cells. PBMC were isolated from blood incubated for 0 and 48 h, lysed in the denaturing and reducing gel loading buffer for 10 min at 95 °C, and then immediately subjected to SDS-PAGE. Immunoblotting was made with N-terminal anti-Ku86.

protein extract showing no intact Ku86 and no Ku86v, suggesting a very pronounced protease activity, was tested (Fig. 2D). In the absence of soybean protease inhibitor a 30-min coincubation period led to degradation of most full-length Ku86, even beyond the Ku86v form and presenting a <40-kDa band (some reactive degradation products probably were electrophoresed out of the gel) (Fig. 2D, lane 4). An unusual banding pattern was seen in lane 4, with some remaining intact Ku86 but no sign of Ku86v, probably reflecting an unusually high content of Ku86-degrading

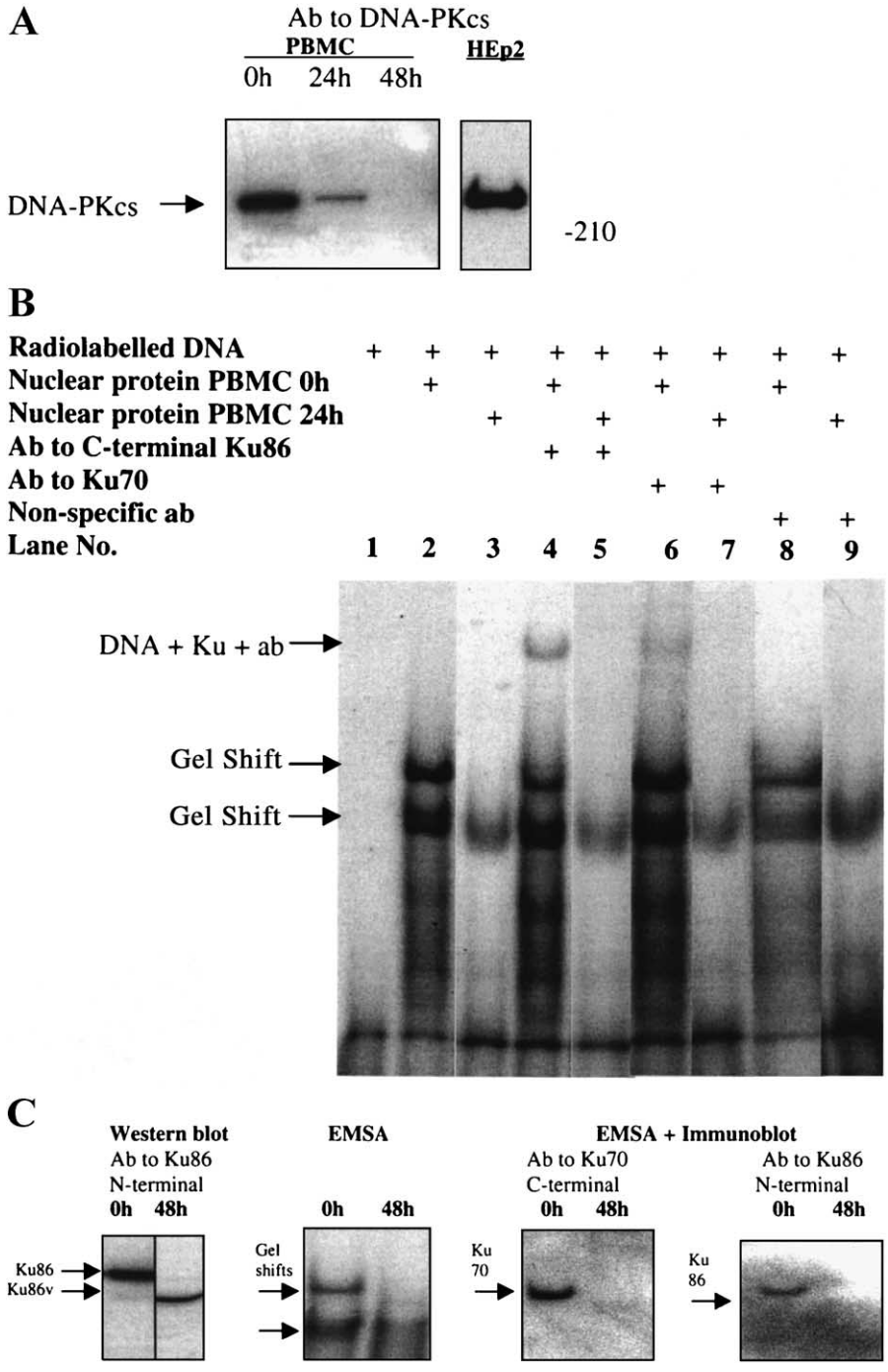

Fig. 4. Ku function analysis. Nuclear protein was extracted from PBMC isolated from blood incubated for the indicated times (0–48 h). Immunoblotting was made with anti-DNA-PK_{cs} (A). In order to determine the binding of Ku86 to free DNA ends, a ^{32}P-labelled linear DNA fragment was incubated with PBMC nuclear protein extract, followed by addition of an antibody as indicated, and subjected to native PAGE. The free probe is located just below the depicted gel. The result is from a single gel experiment; the gel has been cut to improve data presentation by reordering the lanes (B). In a separate experiment, a duplicate EMSA gel was used for gel shift analysis and immunoblotting, to determine the presence of Ku in the gel shift bands (C). The left panel shows a conventional Western blot using PBMC nuclear extracts and N-terminal anti-Ku86. The second panel from left shows EMSA, and the two right panels the immunoreactivity of the blotted duplicate EMSA gel using C-terminal anti-Ku70 and N-terminal anti-Ku86.

protease in this PBMC 48-h nuclear extract. However, the presence of the soybean trypsin inhibitor blocked the degradation of full-length Ku86 in a concentration-dependent manner (result not shown) with complete inhibition of proteolysis apparent at a concentration of 100 μM (Fig. 2D, lane 5). Taken together, our data suggest that a trypsin-like serine protease responsible for Ku86 degradation is being localized mainly to the nucleus and degrades Ku86 sequentially into 70 and <40 kDa fragments.

3.3. SDS-PAGE assay of whole cells suggests lack of Ku86 degradation in intact cells

The documentation of a strong protease activity in PBMC 48-h extracts, leading to significant Ku86 degradation within as little as 5 min in the coincubation experiments (Fig. 2A, lane 4), raised the question whether the observations of Ku86v in protein extracts reflect the action of this protease only upon experimental procedures or in intact cells. In order to minimize opportunity for this protease to exert its function, experiments were performed omitting the protein extraction procedure (Fig. 3). PBMC were lysed at 95 °C for 10 min in SDS-PAGE gel loading denaturing buffer, immediately followed by loading onto the gel. For most of the blood donors full-length Ku86 was seen irrespective of blood incubation, with no band located at the expected position of the Ku86v form (Fig. 3). Our collected data suggest that this experimental protocol with whole-cell lysis is associated with a large variation in band intensity, as may be expected from interference with protein mobility during an electrophoresis of a sample containing all cellular components. Therefore, the smear pattern seen in the 48-h lane 2 of Fig. 3 was not considered to reflect intracellular Ku degradation. This result indicates that the Ku86-degrading protease is kept in an inactive state within cells, and triggered to become active during a protein extraction procedure.

3.4. Functional analysis of Ku86v

C-terminally truncated Ku86 has been shown by others [26] to lack DNA-PK_{cs}-activating capacity. Accordingly, we observed a strong DNA-dependent phosphorylation of a p53 peptide with PBMC 0-h extracts, indicating the presence of Ku protein leading to DNA-PK_{cs} activity, whereas no such phosphorylating function could be found in PBMC 24-h and 48-h extracts (results not shown). Interestingly, in parallel with the appearance of Ku86v, there is a loss of immunoreactive DNA-PK_{cs} during blood incubation (Fig. 4A). Therefore, it remains an open-ended question whether the Ku86v found in PBMC can mediate DNA-dependent phosphorylating activity.

The DNA end-binding activity of Ku protein was also analysed. PBMC nuclear extracts were incubated with a ^{32}P-end-labelled 25-bp double-stranded linear DNA fragment assumed to permit the binding of one Ku molecule [16]. This reaction mixture was subjected to native polyacrylamide gel electrophoresis. A clear gel shift was observed with a PBMC 0-h protein extract (Fig. 4B, lane 2). The upper gel shift band is super-shifted by C-terminal anti-Ku86 (Fig. 4B, lane 4), indicating the presence of full-length Ku86. C-terminal anti-Ku70 caused a relatively weak super-shift with the PBMC 0-h extract (lane 6). PBMC 24-h protein extract produced only the lower gel shift band (lane 3), with no sign of super-shift using these antibodies (lanes 5 and 7). There was no super-shift with N-terminal anti-Ku86, with any of the extracts (results not shown), providing no data on whether Ku86v is capable of binding to DNA.

To get more detailed information on Ku binding to DNA, the EMSA (native polyacrylamide) gel was immunoblotted. Nuclear extracts were analysed with Western blot showing intact Ku86 (PBMC 0-h) or predominantly Ku86v (PBMC 48-h) (left panel, Fig. 4C), and also with EMSA showing two prominent gel shift bands (PBMC 0-h) or a weaker lower band (PBMC 48-h) (second panel from left). Western blot performed with anti-Ku70 (both N- and C-terminal, respectively) showed full-length Ku70 at both 0 and 48 h, with no indication of Ku70 degradation (not shown). A duplicate EMSA gel was blotted under denaturing conditions onto a PVDF membrane, and then analysed for immunoreactivity. Both anti-Ku70 (N- and C-terminal, respectively) and anti-Ku86 (N-terminal, detecting the Ku86v form) formed a single band located at a position corresponding to the upper gel shift band, but gave no signs of Ku protein present in the PBMC 0-h lower gel shift band or at any PBMC 48-h location (two right panels).

In summary, our DNA-binding results are in line with a prevailing view that heterodimeric Ku70/Ku86 binds to DNA ends, but that neither subunit alone can bind DNA effectively [1,3]. Furthermore, the lack of Ku-related gel shift reactivity with the PBMC 48-h extract suggests that the truncated Ku86v form does not bind to DNA.

3.5. Characterization of cell viability associated with formation of the Ku86-degrading protease

A number of experiments were performed in search of a correlation between signs of toxicity and formation of the Ku86-degrading protease, including a general cytotoxic influence, caspase activation and membrane reorganisation

Table 1
Lack of cytotoxicity signs during the induction of Ku86-truncating protease activity[a]

	Blood incubation	
	0 h	24 h
Ku86v in cytoplasmic extract	<5	80
Ku86v in nuclear extract	40	>95
Trypan blue dye excluding PBMC (%)	>95	>95
Mitochondrial respiratory activity in PBMC (MTT assay)	pos	pos

[a] Results from representative experiments are shown.

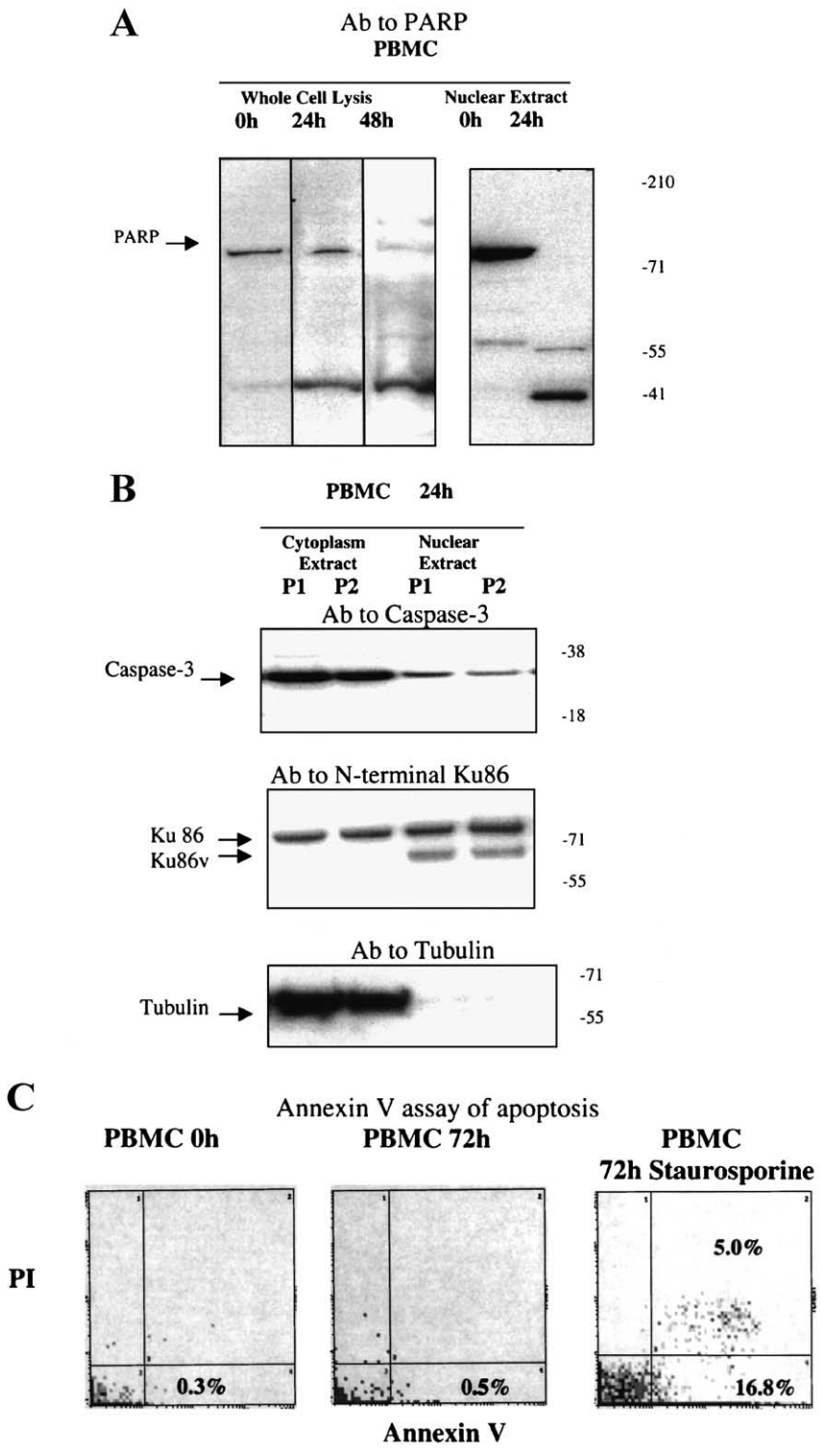

Fig. 5. No sign of apoptosis during induction of the Ku86-degrading protease. PBMC were isolated after blood incubation for the indicated times (0–72 h). SDS-PAGE assay of whole cells lysed in the gel loading buffer (A, left panel) and nuclear protein extract (A, right panel) was followed by immunoblotting with anti-PARP (the arrow indicates the position of full-length PARP). Procaspase-3 and its protease-active caspase-3 derivative were also analysed by immunoblotting of PBMC nuclear and cytoplasmic protein extracts obtained from two blood donors, P1 and P2 (B). Isolated PBMC were also subjected to annexin V analysis using flow cytometry (C). Annexin V-positive but propidium iodide (PI)-negative cells are considered to be in apoptosis, whereas double-positive cells are regarded as necrotic (or late apoptotic). Staurosporine (0.5 μM for 72 h) was used as a positive control for apoptosis induction.

characteristic of apoptosis. PBMC from incubated blood displaying an enhanced fraction of Ku86v did not present any strong signs of decreased viability, as assessed by trypan blue dye exclusion and by mitochondria respiratory capacity (Table 1). The caspase-mediated site-specific cleavage of poly(ADP)ribose polymerase (PARP) is often used as a marker of caspase activation and early apoptosis. Whole cells as well as nuclear protein extracts from 0 and 24 h were subjected to immunoblotting with anti-PARP. The 113-kDa full-length form was found to be cleaved in the incubated samples, however, without any evidence of caspase activity (expected to yield specifically PARP 89- and 24-kDa fragments) [27,28] (Fig. 5A). The similar banding pattern was seen in both extracted protein and in whole cells, indicating that the PARP degradation occurs in intact cells, and possibly also during the protein extraction procedure. In agreement with the PARP results, analysis of caspase-3 in protein extracts from incubated PBMC (24 h) showed a single band corresponding to the 32-kDa procaspase form in both cytoplasm and nucleus (10 μg), without signs of activated caspase-3 (17 kDa) (Fig. 5B). The same membrane showed the presence of Ku86v in the nuclear extract and also of tubulin in the cytoplasmic extract (Fig. 5B).

The influence of blood incubation on another marker of PBMC apoptosis, the binding of Annexin V to cell membrane phosphatidylserine, was also investigated. Flow cytometry showed a minor fraction of Annexin V-positive PBMC, with no apparent difference between cells isolated from freshly collected blood (0.3% apoptosis, Fig. 5C, PBMC 0 h, lower right quadrant) or after 72 h of blood incubation (0.5% apoptosis, Fig. 5C, PBMC 72-h). In addition, there was no increase of necrosis in PBMC 72-h, as measured by binding of both propidium iodide and Annexin V (Fig. 5C, PBMC 0-h and 72-h, upper right quadrants). The ability of the assay to detect apoptosis and necrosis was verified by the incubation of PBMC 0-h in the presence of staurosporine for 72 h (0.5 μM), causing a marked apoptosis reaction (16.8% Annexin V-positive), and also necrosis in a smaller fraction (5.0% of PBMC were positive for both Annexin V and propidium iodide) (Fig. 5C, PBMC 72-h staurosporine). Thus, our collected data indicate no correlation between cell death and the formation of the Ku86-degrading protease.

3.6. The induction of Ku86-degrading protease can be reversed

Ku86 protein participates in a number of cellular processes and its function is likely to be regulated in response to various physiological conditions. In order to collect evidence compatible with a physiological role of the Ku86-degrading protease, reversibility of the protease induction was investigated. Blood was incubated in vitro for 0–48 h, PBMC were isolated, mitogen-stimulated with PHA and then cultivated in growth medium. After 3 days of optimal growth conditions, cytoplasmic and nuclear protein was extracted and subjected to SDS-PAGE. As expected, the fraction of Ku86v was seen to increase in the nuclear extracts of uncultured PBMC from 0 to 48 h (Fig. 6, lanes 1, 4 and 7). A clearly higher fraction of full-length Ku86 was observed in extracts from cells kept in culture medium for 3 days, as compared with uncultured cells (lanes 2, 3, 5, 6, 8 and 9). This effect was slightly enhanced in the presence of PHA (lanes 3, 6, and 9). These results indicate that transferring PBMC to growth medium decreases the level of this Ku86-degrading protease. The same pattern of reversed protease concentration was found in cytoplasmic extracts, although with less pronounced Ku86 degradation (data not shown).

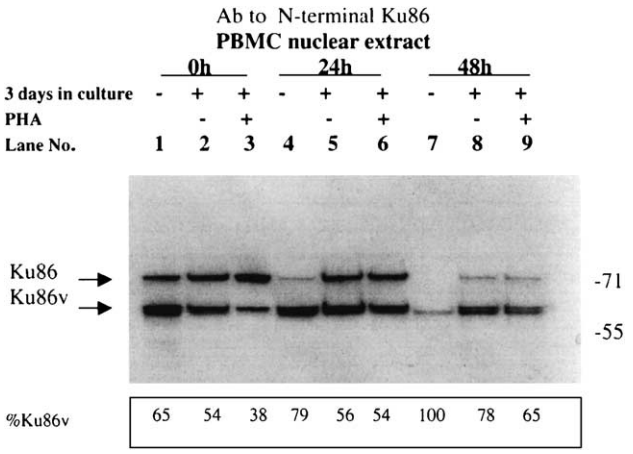

Fig. 6. The induction of the Ku86-degrading protease in PBMC can be reversed. PBMC were isolated from blood incubated for 0–48 h, and then cultivated for 72 h in the presence or absence of PHA, followed by protein extraction, SDS-PAGE and immunoblotting using N-terminal anti-Ku86.

4. Discussion

A truncated and functionally restricted Ku86v form has been reported to be present, at varying levels, in many human cell types. Our present findings suggest this Ku86v to be formed by an inducible and relatively selective trypsin-like serine protease, localized mainly to the nucleus and using the C-terminal region of Ku86 as a substrate. Although strong protease activity was observed in cellular protein extracts, leading to formation of Ku86v, we could not document any protease activity leading to Ku86 degradation in intact cells. Then, what significance can be ascribed to our findings?

There are a number of observations suggesting that Ku86v does indeed originate from the proteolytic degradation of full-length Ku86. First, although a posttranslatory epitope modification might block access to Ku86 of a C-terminal-binding antibody, this would not be expected to yield the observed increase in electrophoretic mobility of Ku86v reflecting physical shortening of Ku86. Second, there were no signs of newly synthesized Ku86v molecules being added to the pre-existing pool of full-length Ku86,

since in many experiments the appearance of Ku86v is proportionally accompanied by a loss of intact Ku86. Interestingly, the protease causing formation of Ku86v seems to be different from that of ageing human fibroblasts reported to be inhibited by leupeptin [14]. However, the soybean trypsin inhibitor was not included in that study, and the divergent results may therefore possibly be due to different conditions of the employed protease assay.

Analysis of trypan blue dye exclusion, Annexin V binding, mitochondrial respiration and caspase function showed that the Ku86-degrading protease does not reflect a general breakdown of cellular homeostasis. On the contrary, the collected data indicate a relatively limited proteolysis: (i) immunoblotting showed several proteins including cytoskeleton and ribonucleoprotein particle (RNP) components to be unaffected, whereas some large proteins including DNA-PK_{cs} and PARP were degraded; (ii) total cell protein Coomassie blue staining revealed most proteins to be kept intact, whereas some protein bands in the high molecular weight range were lost, indicating action on a minor subset of the cellular proteins (data not shown).

Reports by others have suggested a physiological role of Ku86 mutants [29–31], and some of our findings are suggestive of a potential for this protease to regulate Ku function in human cells: (i) protease induction is seen in fully viable and non-apoptotic cells; (ii) protease activity is decreasing upon transfer of cells to optimal growth conditions; (iii) the C-terminal shortening of Ku86 is most probably associated with a loss of DNA-binding capacity. The plethora of seemingly unrelated functions assigned to Ku makes it tempting to speculate that there might be a basic cellular condition including some demand for DNA modification under the influence of full-length Ku86, whereas under specific growth or signalling conditions the cell favours the presence of Ku86v.

The present findings have a general relevance for the interpretation of experimental results obtained in Ku protein studies. By paying strict attention to minimize the extent of in vitro incubation, it has been possible to document that in human B lymphocytes full-length Ku86 retains its DNA-PK_{cs}-activating function [22,32]. These findings are in contrast with published data on human B lymphocytes showing variant Ku86 [25] and an enhanced sensitivity to gamma radiation [33]; in the light of the present observations, these conflicting data may be due to experimental conditions favouring the induction of a Ku86-degrading protease. In addition, discrepancies among a number of studies on the intracellular distribution of Ku protein [6,18,34] may at least in part be explained by induction of a Ku86-degrading protease, together with the common usage of antibodies unable to bind to Ku86v epitopes. As an example, in one of these studies [18], fixation-dependent results were described with an antibody recognizing the 610–705 C-terminal segment unlikely to detect Ku86v. Regarding a reported Ku86 epitope modification during viral infection [15], it seems to be an open-ended question whether the observed change in Ku immunoreactivity may have been caused by an inducible proteolytic activity.

In conclusion, the present work emphasizes the need to pay attention to significant methodological problems and pitfalls in assessing Ku86. In addition, it describes an inducible protease with a potential to play a role in the regulation of Ku function in human cells.

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References

[1] C.W. Anderson, T.H. Carter, The DNA-activated protein kinase-DNA-PK, Curr. Top. Microbiol. Immunol. 217 (1996) 91–111.
[2] U. Grawunder, R.B. West, Antigen receptor gene rearrangement, Curr. Opin. Immunol. 10 (1998) 172–180.
[3] G.C.M. Smith, S.P. Jackson, The DNA-dependent protein kinase, Genes Dev. 13 (1999) 916–934.
[4] T. Morio, S.H. Hanissian, L.B. Bacharier, H. Teraoka, S. Nonoyama, M. Seki, J. Kondo, H. Nakano, S.-K. Lee, R.S. Geha, J.-I. Yata, Ku in the cytoplasm associates with CD40 in human B cells and translocates into the nucleus following incubation with IL-4 and anti-CD40 mAb, Immunity 11 (2000) 339–348.
[5] M.T. Ruiz, D. Matheos, G.D. Price, M. Zannis-Hadjopoulos, OBA/Ku86: DNA binding specificity and involvement in mammalian DNA replication, Mol. Biol. Cell 10 (1999) 567–580.
[6] M. Koike, T. Awaji, K. Kataoka, G. Tsujimoto, T. Kartasova, A. Koike, T. Shiomi, Differential subcellular localization of DNA-dependent protein kinase components Ku and DNA-PKcs during mitosis, J. Cell Sci. 112 (1999) 4031–4039.
[7] A. Nussenzweig, C. Chen, V. da Costa Soares, M. Sanchez, K. Sokol, M.C. Nussenzweig, G.C. Li, Requirement for Ku80 in growth and V(D)J recombination, Nature 382 (1996) 551–555.
[8] Y. Gao, J. Chaudhuri, C. Zhu, L. Davidson, D.T. Weaver, F.W. Alt, A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for Ku in V(D)J recombination, Immunity 9 (1998) 367–376.
[9] S.J. Boulton, S.P. Jackson, Identification of a *Sacharomyces cerevisiae* Ku80 homologue: roles in DNA double strand break rejoining and telomeric maintenance, Nucleic Acids Res. 24 (1996) 4639–4648.
[10] S.E. Porter, P.E. Greenwell, K.B. Ritchie, T.D. Petes, The DNA-binding protein Hdf1p (a putative Ku80 homologue) is required for maintaining normal telomere length in *Sacharomyces cerevisiae*, Nucleic Acids Res. 24 (1996) 582–585.
[11] H. Vogel, D.-S. Lim, G. Karsenty, M. Finegold, P. Hasty, Deletion of Ku86 causes early onset of senescence in mice, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 10770–10775.
[12] M.W. Knuth, S.I. Gunderson, N.E. Thompson, L.A. Strasheim, R.R. Burgess, Purification and characterization of proximal sequence element-binding protein 1, a transcription activating protein related to Ku and TREF that binds the proximal sequence element of human U1 promoter, J. Biol. Chem. 265 (1990) 17911–17920.
[13] Y.-T. Tai, G. Teoh, B. Lin, F.E. Davies, D. Chauhan, S.P. Treon, N. Raje, T. Hideshima, Y. Shima, K. Podar, K.C. Anderson, Ku86 variant expression and function in multiple myeloma cells is associated with increased sensitivity to DNA damage, J. Immunol. 165 (2000) 6347–6355.

[14] Y.-W. Jeng, H.-C. Chao, C.-F. Chiu, W.-G. Chou, Senesecent human fibroblasts have elevated Ku86 proteolytic cleavage activity, Mutat. Res. 435 (1999) 225–232.
[15] J.P. Quinn, J. Simpson, A.R. Farina, The Ku complex is modulated in response to viral infection and other cellular changes, Biochim. Biophys. Acta 1131 (1992) 181–187.
[16] Z. Han, C. Johnston, W.H. Reeves, T. Carter, J.H. Wyche, E.A. Hendrickson, Characterization of a Ku86 variant protein that results in altered DNA binding and diminished DNA-dependent protein kinase activity, J. Biol. Chem. 271 (1996) 14098–14104.
[17] Q.-Q. Cai, A. Plet, J. Imbert, M. Lafage-Pochitaloff, C. Cerdan, J.-M. Blanchard, Chromosomal location and expression of the genes coding for Ku p70 and p80 in human cell lines and normal tissues, Cytogenet. Cell Genet. 65 (1994) 221–227.
[18] J.W. Fewell, E.L. Kuff, Intracellular redistribution of Ku immunoreactivity in response to cell–cell contact and growth modulating components in the medium, J. Cell Sci. 109 (1996) 1937–1946.
[19] K.D. Brown, T.A. Lataxes, S. Shangary, J.L. Mannino, J.F. Giardina, J. Chen, R. Baskaran, Ionizing radiation exposure results in up-regulation of Ku70 via a p53/ataxia telangiectasia-mutated protein-dependent mechanism, J. Biol. Chem. 275 (2000) 6651–6656.
[20] T.S. Kumaravel, K. Bharathy, S. Kudoh, K. Tanaka, N. Kamade, Expression, localization and functional interactions of Ku70 subunit of DNA-PK in peripheral lymphocytes and Nalm-19 cells after irradiation, Int. J. Radiat. Biol. 74 (1998) 481–489.
[21] K. Myung, D.M. He, S.E. Lee, E.A. Hendrickson, KARP-1: a novel leucine zipper protein expressed from the Ku86 autoantigen locus is implicated in the control of DNA-dependent protein kinase activity, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7664–7669.
[22] A. Sallmyr, G. Henriksson, S. Fukushima, A. Bredberg, Ku protein in human T and B lymphocytes: full length functional form and signs of degradation, Biochim. Biophys. Acta 1538 (2001) 305–312.
[23] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchel, Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of che mosensitivity testing, Cancer Res. 47 (1987) 936–942.
[24] I. Vermes, C. Haanen, H. Steffens-Nakken, C. Reutelingsberger, A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, J. Immunol. Methods 184 (1995) 39–51.
[25] C. Muller, C. Dusseau, P. Calsou, B. Salles, Human normal peripheral blood B-lymphocytes are deficient in DNA-dependent protein kinase activity due to the expression of a variant form of the Ku86 protein, Oncogene 16 (1998) 1553–1560.
[26] B.K. Singleton, M.I. Torres-Arzayus, S.T. Rottinghaus, G.E. Taccioli, P.A. Jeggo, The C terminus of Ku80 activates the DNA-dependent protein kinase catalytic subunit, Mol. Cell. Biol. 19 (1999) 3267–3277.
[27] P. Decker, D. Isenberg, S. Muller, Inhibition of caspase-3-mediated poly(ADP-ribose) polymerase (PARP) apoptotic cleavage by human PARP autoantibodies and effect on cells undergoing apoptosis, J. Biol. Chem. 275 (2000) 9043–9046.
[28] Y.A. Lazebnik, S.H. Kaufmann, S. Desnoyers, G.G. Poirier, W.C. Earnshaw, Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE, Nature 371 (1994) 346–347.
[29] E. Marangoni, N. Foray, M. O'Driscoll, S. Douc-Rasy, J. Bernier, J. Bourhis, P. Jeggo, A Ku80 fragment with dominant negative activity imparts a radiosensitive phenotype to CHO-K1 cells, Nucleic Acids Res. 28 (2000) 4778–4782.
[30] R.L. Woodard, K.-J. Lee, J. Huang, W.S. Dynan, Distinct roles for Ku protein in transcriptional reinitiation and DNA repair, J. Biol. Chem. 276 (2001) 15423–15433.
[31] H.J. Beamish, R. Jessberger, E. Riballo, A. Priestley, T. Blunt, B. Kysela, P.A. Jeggo, The C-terminal conserved domain of DNA-PKcs, missing in the SCID mouse, is required for kinase activity, Nucleic Acids Res. 28 (2000) 1506–1513.
[32] A. Vral, H. Thierens, P. Bryant, L. De Ridder, A higher micronucleus yield in B-versus T-cells after low-dose gamma-irradiation is not linked with defective Ku86 protein, Int. J. Radiat. Biol. 77 (2001) 329–339.
[33] P.J. Mayer, M.O. Bradley, W.W. Nichols, Incomplete rejoining of DNA double-strand breaks in unstimulated normal human lymphocytes, Mutat. Res. 166 (1986) 275–285.
[34] G. Bakalkin, T. Yakovleva, Y.L. Hurd, A. Nussenzweig, G.C. Li, L. Terenius, Autoantigen Ku in the brain. Developmentally regulated expression and subcellular localization, NeuroReport 9 (1998) 2147–2151.