

Absence of Autoantigen Ku in Mature Human Neutrophils and Human Promyelocytic Leukemia Line (HL-60) Cells and Lymphocytes Undergoing Apoptosis

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Summary

The Ku autoantigen is a heterodimer of 70- and 80-kD proteins recognized by autoantibodies from patients with systemic lupus erythematosus and related diseases that is the DNA-binding component of a DNA-dependent protein kinase. The catalytic activity of DNA-dependent protein kinase is carried by a 350-kD subunit (p350). In light of the recently described role of Ku in repairing double-strand DNA breaks, we investigated the regulation of Ku and p350 levels in neutrophils, a terminally differentiated cell type destined to undergo apoptosis. Since the appearance of double-strand DNA breaks is characteristic of apoptosis, we were interested in the possibility that Ku might oppose programmed cell death. Analysis of peripheral blood cells by flow cytometry using anti-Ku and anti-p350 monoclonal antibodies revealed that neutrophils were unstained, whereas resting (G0) lymphocytes were positive. The absence of Ku in mature neutrophils was confirmed by Western blotting and enzyme-linked immunosorbent assay for Ku antigen. In contrast, the human promyelocytic leukemia line, HL-60, which undergoes differentiation toward neutrophils after dimethylsulfoxide treatment, was positive for Ku and p350. In view of the short lifespan of neutrophils and the prolonged half-life of Ku and p350 (>5 d), these data suggested that Ku was actively degraded during myeloid differentiation. Analysis of HL-60 cells by flow cytometry revealed that Ku staining was bimodal. Cells in G1/G0, S, or G2/M were all stained positively, whereas cells with a subdiploid DNA content characteristic of apoptosis were Ku negative. Similar results were obtained with phytohemagglutinin-stimulated human lymphocytes. These data suggest that the Ku antigen is actively degraded in both myeloid cells destined to undergo apoptosis and apoptotic lymphocytes, raising the possibility that degradation of Ku may help to prevent the inappropriate repair of fragmented nuclear DNA during apoptosis.

The Ku autoantigen is a heterodimer of 70- and 80-kD proteins (p70 and p80, respectively) recognized by autoantibodies from sera of certain patients with scleroderma-polymyositis overlap syndrome, SLE, and related diseases (1, 2). Ku is the DNA-binding component of a DNA-dependent protein kinase, the catalytic activity of which is carried by a 350-kD protein (p350) (3-5). DNA-dependent protein kinase phosphorylates a variety of DNA-binding proteins *in vitro* (6, 7), although its substrates *in vivo* are not yet well defined. Eukaryotic cells with a mutant p80 subunit have been shown recently to be defective in double-strand DNA break repair and V(D)J recombination (8-11).

In addition to its role in repairing double-strand DNA breaks, the Ku antigen may play a role in regulating cell proliferation (12-14). Charge variants of the p70 and p80 proteins have been described, and the levels of these variants depend on the cell's proliferative state (12, 13). Moreover, p70

and p80 gene expression is increased during mitogen-induced activation of quiescent (G0) human lymphocytes to a proliferative state, and it is inactivated by phorbol ester-induced monocytic differentiation of HL-60 cells (15). However, despite dramatic changes in Ku mRNA expression, the total level of Ku has been reported to be similar in quiescent and proliferating cells, possibly because the antigen is highly stable (12-15). In the present study, we report that, although the promyelocytic leukemia cell line HL-60 and PBLs are positive, mature peripheral blood neutrophils and apoptotic lymphocytes contain no detectable Ku antigen. The absence of Ku in these cells may reflect active degradation before or during apoptosis.

Materials and Methods

Preparation of PBLs and Neutrophils. Blood samples were obtained from healthy volunteers, and lymphocytes and neutrophils

were separated on Ficoll-Hypaque gradients (16, 17). In some experiments, monocytes were depleted by incubating the cell suspension in plastic dishes for 1 h at 37°C in RPMI 1640/10% fetal bovine serum and collecting the nonadherent cells (17). In other experiments, peripheral blood nucleated cells were separated by dextran sedimentation followed by hypotonic RBC lysis and Ficoll-Hypaque gradient centrifugation (17). Purity of the cell populations was determined by staining with antibodies specific for CD16 (neutrophils and NK cells) or CD3 (T cells) (see below). Cell viability was >90% by trypan blue exclusion.

Cell Lines and mAbs. The HL-60 (human promyelocytic leukemia) and K562 (human erythroleukemia) cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640/10% fetal bovine serum supplemented with L-glutamine and penicillin/streptomycin. Murine anti-Ku mAbs 162 (IgG2a anti-p70/p80 dimer [2, 18]), 111 (IgG1 anti-p80 [2, 19]), and N3H10 (IgG2b anti-p70 [19, 20]) were described previously. Murine anti-p350 mAb 25-4 was kindly provided by Dr. Timothy Carter (St. John's University, Jamaica, NY) (4). Polyclonal rabbit anti-p350 serum was a gift from Dr. Carl Anderson (Brookhaven National Laboratory, Upton, NY) (3). Purified murine anti-human CD3 (SK7, IgG1) and CD16 (GO22, IgM) mAbs were purchased from Becton Dickinson and Co. (Mountain View, CA). Murine mAb BM6.5 is an IgG2a antihistone H1 (Rapoport, R. G., B. Kotzin, and P. L. Cohen, unpublished data). Murine mAb Y2 (IgG2a anti-Sm B'/B and D) was provided by Dr. Joan Steitz (Yale University, New Haven, CT) (21). mAb PAb 101 (TIB 117, murine IgG2a anti-SV40 large T antigen; American Type Culture Collection) was used as an isotype control.

Determination of Ku Half-life. K562 cells were pulse labeled with [³⁵S]methionine and cysteine (DuPont/New England Nuclear, Boston, MA) as described previously (15). Briefly, 1.4×10^7 cells were washed twice with PBS, preincubated for 30 min in 2 ml of methionine-free RPMI 1640 containing 10% PBS-dialyzed fetal bovine serum, and labeled for 15 min at 37°C by adding 1.5 mCi of [³⁵S]methionine and cysteine. After pulse labeling, 45 ml of ice-cold RPMI 1640 was added, and the cells were collected by centrifugation and washed with PBS. 1 aliquot of pulse-labeled cells (2×10^6 cells) was harvested immediately, and the remaining aliquots of cells were cultured at 37°C in five tissue culture flasks containing 5 ml of complete medium. One flask (2×10^6 cells) was harvested after 1, 2, 3, 4, and 5 d of chase. The cells were washed once with PBS and frozen. The cells were later thawed and sonicated in 0.15 M NaCl NET/NP-40 (0.5 M NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.3% NP-40). Extracts were cleared by centrifugation, and lysates derived from 5×10^5 starting cells (i.e., at the time of pulse labeling) were immunoprecipitated with 5 μ l of mAbs 162, 111, or N3H10 ascitic fluid, or with 30 μ l of 25-4 culture supernatant as described (15, 18). Immunoprecipitates were washed three times with 0.15 M NaCl NET/NP-40 buffer and once with NET buffer alone, and analyzed by SDS-PAGE. Gels were fluorographed and bands were quantified using a PhosphorImager with Image Quant software (Molecular Dynamics, Inc., Sunnyvale, CA).

PHA Stimulation of PBLs. PBLs were separated on Ficoll-Hypaque gradients (17) and washed twice with PBS. The cells were depleted of plastic adherent cells as above and resuspended in RPMI 1640/10% fetal bovine serum with 10 μ g/ml PHA (Gibco BRL, Gaithersburg, MD) in RPMI 1640. Cells were harvested 4 d later and analyzed by flow cytometry for DNA content and Ku antigen (see below).

Immunoblotting. Immunoblot analysis of p70 and p80 was performed using anti-Ku mAbs N3H10 and 111 (19) and cell extracts

derived from equal numbers of neutrophils or PBLs. Briefly, the cells were sonicated in lysis buffer (2.5% SDS, 0.1 M dithiothreitol, 50 mM Tris-HCl, pH 7.5) or in 0.01 M sodium phosphate, pH 7.2, 0.1 M dithiothreitol, 1% SDS, and 6 M urea containing protease inhibitors (0.5 mM PMSF, 0.5 μ g/ml leupeptin, and 0.3 trypsin inhibitor units/ml aprotinin, all from Sigma Chemical Co., St. Louis, MO), followed by SDS-PAGE and transfer of the proteins to nitrocellulose membrane. The blots were probed with N3H10 or 111 ascitic fluid (1:500), followed by washing and incubation with 1:1,000 alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Tago, Inc., Burlingame, CA) and substrate.

Sandwich ELISA for Ku Antigen. The ELISA for quantitating Ku antigen was described previously (2). Briefly, wells of 96-well polystyrene plates (MaxiSorp; Nunc, Inc., Naperville, IL) were coated with 50 μ l of ~ 10 μ g/ml purified mAb 162 in 20 mM Tris buffer at pH 8.0 and incubated at 4°C for 16 h. After blocking the wells, serial dilutions of cell lysates were added to the wells for 90 min, followed by washing and incubation for 90 min with mAb 111 (1:500). The wells were again washed, followed by incubation with 1:1,000 alkaline phosphatase-conjugated goat anti-mouse IgG1 (γ chain-specific) antibodies (Southern Biotechnology Associates, Birmingham, AL). Substrate was added and absorbance was determined 45 min later at 405 nm using an ELISA reader (Molecular Dynamics, Inc.).

Analysis of DNA Content and Ku Antigen Level by Flow Cytometry. Double staining for intracellular protein and DNA was performed as described (22, 23). Briefly, cells were washed twice with PBS and fixed with 70% methanol at -20°C for 5 min, and then washed three more times with PBS, resuspended in staining buffer (HBSS containing 3% fetal bovine serum, 15 mM Hepes, pH 7.3, and 0.1% sodium azide), and incubated for 15 min. The cells were then incubated 45 min with anti-Ku antibodies (1:100 ascitic fluid or 1:1 culture supernatant) and washed with HBSS, 0.5% fetal bovine serum, and 0.1% sodium azide, followed by 45 min of incubation with appropriate isotype-specific FITC-labeled second antibodies (Southern Biotechnology Associates). The cells were then washed again and fixed with 1% (wt/vol) paraformaldehyde in PBS. DNA content was determined by propidium iodide (PI)¹ staining after treating the cells with ribonuclease A (Sigma Chemical Co.) (22, 23). FITC intensity in gates placed on G1, S, and G2/M regions of the DNA histogram was determined by flow cytometry using a FACSCAN[®] (Becton Dickinson and Co.) with data acquisition software (Cicero; Cytomation, Fort Collins, CO). In some experiments, HL-60 cells were double stained with PI plus mAb 162, BM6.5, Y2, or PAb101 in the same manner. FITC gates were placed on the subdiploid region of the DNA histogram (apoptotic cells), or on the G1, S, plus G2/M regions (nonapoptotic cells), and FITC fluorescence was plotted on a 4-decade logarithmic scale.

Analysis of Apoptotic Cells. Apoptotic cells appeared in the <2N DNA peak (24) and were distinguished from necrotic cells by analyzing the light scatter profile. Necrotic cell death resulted in a large drop in forward light scatter (fcs) and side scatter (ssc), whereas cell death by apoptosis resulted in a smaller decline in fsc and an initial increase in ssc (25). Samples were collected in list mode so that, when phenotypic markers were used, the percentage of cells undergoing apoptosis could be determined by gating on FITC-stained cells and subsequently analyzing DNA staining. Data were plotted on a 4-decade logarithmic scale, except for DNA

¹ Abbreviations used in this paper: fsc, forward light scatter; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; ssc, side light scatter.

staining, which was plotted linearly. At least 25,000 events were collected per sample.

Double Staining for Surface Markers and Ku Antigen. Cells were first stained for the surface markers CD3 or CD16 (1:100 for 45 min on ice) using PE-conjugated isotype-specific second antibodies and then fixed with 1% paraformaldehyde as above. The fixed cells were permeabilized by incubating for 30 min in 45% ethanol (22), stained for Ku as above using FITC-conjugated isotype-specific antibodies, and fixed for an additional 15 min in 1% paraformaldehyde. The cells were then analyzed by flow cytometry as above. The specificities of the isotype-specific second antibodies were confirmed in all experiments.

Results

While examining the intracellular staining of human peripheral blood nucleated cells with mAbs specific for the Ku antigen, we unexpectedly found that different cell populations exhibited wide variations in the intensity of Ku staining. Of particular interest was an apparent lack of Ku in some cells. To identify this cell population, double staining experiments were performed.

Lack of Ku Staining in Mature Human Neutrophils. Preliminary analysis of fsc and ssc of the Ku-positive and -negative cell populations suggested that these might correspond to lymphocytes and neutrophils, respectively (data not shown). That possibility was confirmed by double immunofluorescent staining of purified neutrophils and lymphocytes (Fig. 1). The neutrophil fraction after Ficoll-Hypaque gradient centrifugation was 96% CD16 positive. Double staining with mAb 162 revealed that the CD16-positive cells did not stain for p70/p80 heterodimer (Fig. 1, *left*). Similar results were obtained with N3H10 (anti-p70) and 111 (anti-p80) (not

shown). Staining of PBLs with anti-CD3 (Fig. 1, *right*) revealed both positive and negative populations, as expected, consistent with the presence of T cells and non-T cells (B lymphocytes plus monocytes and NK cells), respectively. Both the CD3⁺ (T) and CD3⁻ (non-T) cells stained positively with mAb 162, but the staining of CD3⁻ cells (primarily B lymphocytes and monocytes) was weaker than that of the CD3⁺ T cells (Fig. 1, *right*). The somewhat more heterogeneous staining of non-T than T cells by 162 may reflect the heterogeneity of the non-T cell population.

These results indicated that the Ku-positive and -negative cell populations corresponded to lymphocytes (possibly along with monocytes and NK cells) and neutrophils, respectively. The lack of neutrophil Ku staining might be explained by a true absence of the antigen, neutrophil-specific masking of Ku due to the binding of other nuclear antigens, or the loss of the Ku antigen from neutrophils, but not lymphocytes, during fixation. To distinguish between these possibilities, neutrophils were purified and extracts of the cells were analyzed for the presence of Ku by Western blot and ELISA.

Absence of Ku Antigen in Neutrophils. For Western blot analysis, cell lysates from neutrophils or PBLs were fractionated by SDS-PAGE, blotted onto nitrocellulose membrane, and probed with a mixture of anti-Ku mAbs N3H10 (anti-p70) and 111 (anti-p80). The p70 and p80 proteins were undetectable in extracts derived from 0.5, 1.0, or 2.0 × 10⁶ neutrophils (Fig. 2, lanes 2, 4, 6), but were readily detectable in extracts derived from the same numbers of PBLs (Fig. 2, lanes 1, 3, 5). Since the cells were lysed directly in buffer containing strong denaturants, the Western blot studies suggested that masking by other antigens was not responsible for the absence of Ku in neutrophils seen by flow cytometry (Fig. 1). However, although the cell extracts used for Western blot-

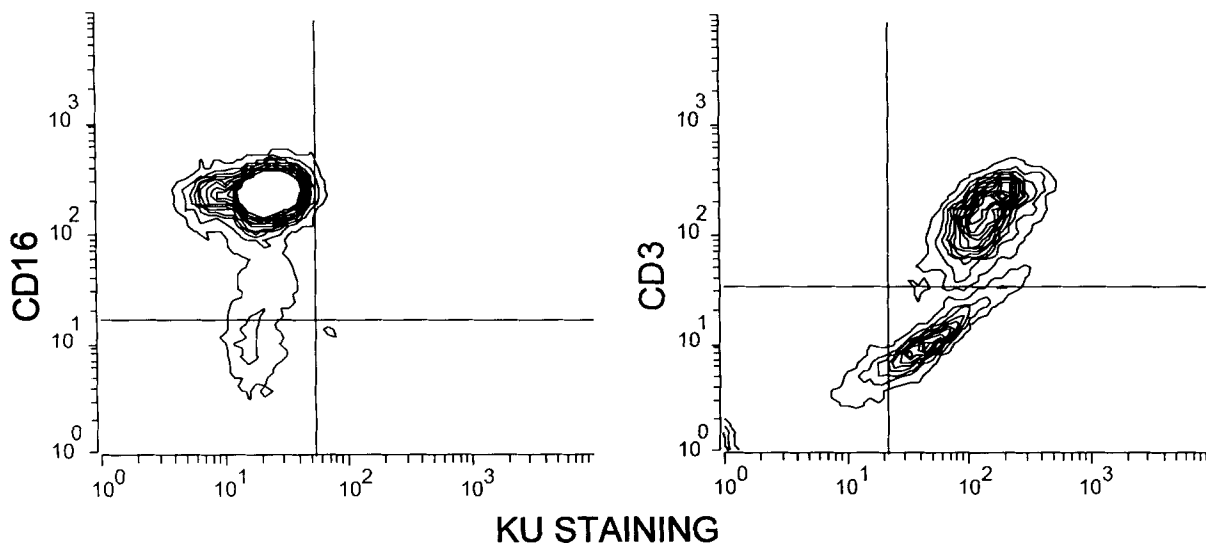


Figure 1. Staining of neutrophils and lymphocytes with anti-Ku mAb. Human neutrophils were stained for surface expression of CD16 (mAb GO22, *left*), and lymphocytes were stained for surface expression of CD3 (mAb SK7, *right*) followed by fixation, permeabilization, and staining for intracellular expression of Ku with mAb 162. Surface staining was detected using PE-conjugated goat anti-mouse IgG1 (to detect SK7) or IgM (to detect GO22) antibodies (*y*-axis). Intracellular Ku staining was detected with FITC-conjugated goat anti-mouse IgG2a antibodies (*x*-axis). Staining was analyzed by two-color flow cytometry.

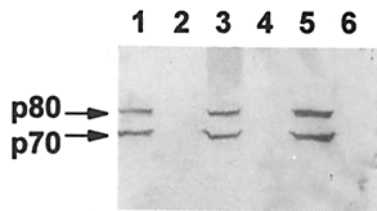


Figure 2. Western blot analysis of Ku antigen in lymphocytes and neutrophils. Extracts from 0.5 (lanes 1 and 2), 1.0 (lanes 3 and 4), or 2.0 (lanes 5 and 6) $\times 10^6$ cells were analyzed by SDS-PAGE and transferred to nitrocellulose. Blots were probed with a mixture of mAbs N3H10 (anti-p70) and 111 (anti-p80), both at 1:500, followed by alkaline phosphatase-conjugated goat anti-mouse IgG antibodies. Lanes 1, 3, and 5, human lymphocyte extract; lanes 2, 4, and 6, human neutrophil extract. Positions of p70 and p80 are indicated.

ting were prepared by immediately boiling cell pellets in buffer containing 1% SDS, 6 M urea, and a cocktail of protease inhibitors, the possibility remained that proteases in the neutrophil extract might have degraded Ku. That possibility was examined by mixing studies using a sandwich ELISA to quantitate Ku antigen. As shown in Fig. 3, K562 cells contained high levels of Ku antigen (*solid squares*). A slight decrease in the Ku activity exhibited by K562 cell lysate was observed when non-denatured K562 and neutrophil cell lysates were mixed at a ratio of 1:1 in the presence of the same protease inhibitors used for Western blotting (*open squares*). However, neutrophil lysate by itself contained no detectable Ku antigen at all dilutions tested (*open circles*). Resting PBLs (*solid tri-*

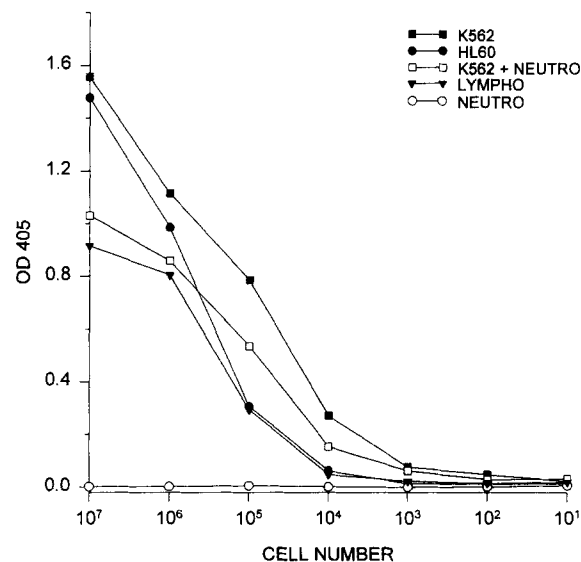


Figure 3. Quantitation of Ku antigen by sandwich ELISA. ELISA was performed as described in Materials and Methods. Human cell extracts derived from 10^7 - 10^1 K562 cells (*solid squares*), HL-60 cells (*solid circles*), lymphocytes (*solid triangles*), or neutrophils (*open circles*) were tested. Extract from K562 cells was also mixed 1:1 with neutrophil extract (*open squares*) to evaluate whether proteases in the neutrophil extract degraded Ku in K562 lysate. In that case, cell number (*x-axis*) represents the number of K562 cell equivalents added per well.

angles) contained somewhat less Ku on a per cell basis than proliferating K562 cells. The human promyelocytic leukemia cell line HL-60, which can be induced to differentiate toward neutrophils by DMSO treatment (26, 27), had a lower level of Ku than K562 cells (*solid circles*). However, the level of Ku in HL-60 was higher than that in resting PBLs, and considerably higher than that in neutrophils. Taken together, the flow cytometry, Western blot, and ELISA data all indicated that Ku antigen was not present in mature neutrophils.

Relationship of Ku Staining to DNA Content. Because of the presence of Ku antigen in resting PBLs and its absence in mature neutrophils, it seemed unlikely that the loss of Ku antigen was an attribute of G0 cells in general. The relationship between the level of Ku or p350 and position in the cell cycle was examined further by double staining PBLs with PI and mAbs 162 or 25-4, respectively. Staining of freshly isolated resting PBLs with PI revealed a single population of cells with G1/G0 DNA content (Fig. 4, *top*). When PBLs were cultured for 4 d in RPMI 1640 + 10% fetal bovine serum (Fig. 4, *middle*), the majority of the cells remained in G1/G0, although small numbers of cells had S (8.9%) or G2/M (9.5%) DNA content, and 9.3% of the cells had a subdiploid DNA content typical of cells undergoing apoptosis (24). Activation of the cells with PHA for 4 d increased

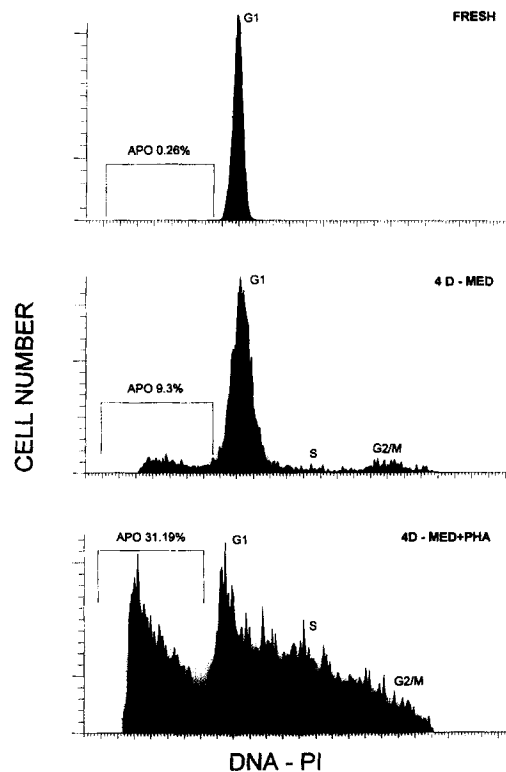


Figure 4. DNA content of PBLs. PI staining of freshly isolated PBLs (*top*), or PBLs cultured for 4 d in medium alone (*4D-MED*) or in medium containing PHA (*4D-MED+PHA*). Cells in G1, S, and G2/M are indicated. The percentages of apoptotic cells in resting PBLs, PBLs cultured 4 d in medium, and PBLs cultured 4 d in medium with PHA are shown. APO, cells with subdiploid DNA content characteristic of apoptosis.

the numbers of cells in S and G2/M, as expected (Fig. 4, *bottom*). Mitogen activation also increased the number of apoptotic cells dramatically, as reported previously (28). Less than 1% of fresh PBLs exhibited a subdiploid DNA content, compared with 9.3% of PBLs cultured for 4 d in medium alone and 31% of cells cultured 4 d in medium plus PHA.

Double staining of fresh PBLs, PBLs cultured for 4 d in medium alone, and PBLs grown in medium containing PHA is presented in Fig. 5 (A-C, D-F, and G-I, respectively). Fresh PBLs all exhibited G1/G0 PI staining (Fig. 5, A-C) and were stained with mAbs 162 (anti-p70/p80 dimer, B) and 25-4 (anti-p350, C). After 4 d of in vitro culture in RPMI 1640 + 10% fetal bovine serum, nearly all PBLs remained positive for Ku (162 staining, compare E and D) and p350 (25-4 staining, compare F and D). Small numbers of cells with S or G2/M DNA content appeared after 4 d in culture, consistent with the weak mitogenic effect of fetal bovine serum. In addition, the intensity of both Ku and p350 staining in the G1/G0 population increased after 4 d in culture, suggesting that the levels of both Ku and p350 are higher in G1 lymphocytes than in resting (G0) lymphocytes (B-E and C-F, respectively).

PBLs activated for 4 d with PHA showed a strikingly different staining pattern. As shown in Fig. 5 G, PI staining revealed increased numbers of cells with S and G2/M DNA content, consistent with the data shown in Fig. 4. In addition, a prominent population of cells with subdiploid DNA content was apparent after staining the mitogen-activated PBLs with PI (Fig. 5 G). Double staining of the PHA-activated cells with mAb 162 plus PI (Fig. 5 H) showed that the cells with G1, S, and G2/M DNA content were strongly positive for p70/p80 dimer, whereas the cells with a subdiploid DNA

content failed to stain above background. Similarly, PHA-activated PBLs in G1, S, and G2/M stained positively with anti-p350 mAb 25-4, whereas the cells with subdiploid DNA content were negative (Fig. 5 I). These data indicated that both Ku antigen (p70/p80 heterodimer) and p350 were lost over a period of 4 d or less during apoptosis. Since mature neutrophils are destined to undergo programmed cell death (29, 30), the lymphocyte staining data raised the possibility that the absence of Ku antigen in neutrophils might reflect their entry into an apoptosis pathway rather than terminal differentiation. That possibility was addressed by double staining of HL-60 cells, a Ku antigen-positive (Fig. 3) human promyelocytic leukemia cell line that undergoes a low level of spontaneous myeloid differentiation and apoptosis (27).

Absence of Ku in Apoptotic HL-60 Cells. As seen in the case of PHA-activated PBLs, analysis of the staining of HL-60 cells by anti-Ku mAb 162 in relationship to DNA content by flow cytometry revealed the presence of Ku-positive and Ku-negative cell populations. Analysis of fsc and ssc suggested the presence of two populations of HL-60 cells (Fig. 6, *top*), one consisting of cells in G1, S, and G2/M (*top right*, population I) and showing greater relative fsc (*top left*, population I), and the other consisting largely of apoptotic cells with a subdiploid DNA content and exhibiting relatively less fsc (Fig. 6, *top*, population II). Staining of HL-60 with both anti-Ku (162) and PI showed a Ku-positive population corresponding to cycling (G1, S, and G2/M) cells (Fig. 6, *bottom right*, population I), and a Ku-negative population with subdiploid DNA content characteristic of apoptotic cells (24) (Fig. 6, *bottom*, compare population II with control). Fixation with paraformaldehyde revealed a similar pattern of staining, suggesting that the absence of Ku in apoptotic cells

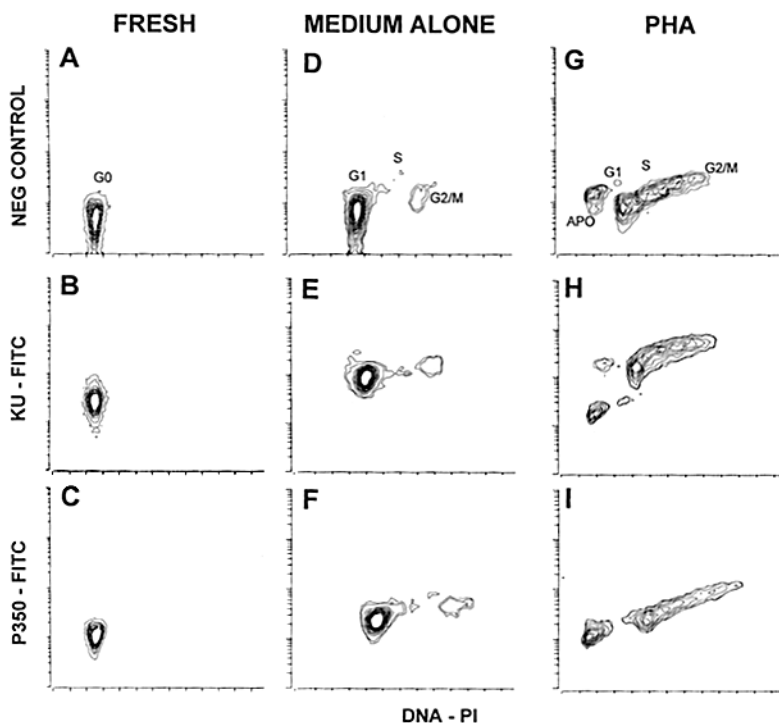


Figure 5. Level of Ku and p350 in PBLs in relationship to DNA content. Freshly isolated PBLs (A-C), or PBLs grown in culture medium without (D-F) or with (G-I) PHA were stained for DNA content with PI (*x-axis*). The *y-axis* of A, D, and G shows costaining of the cells with FITC-conjugated goat anti-mouse IgG second antibody alone. The *y-axis* of B, E, and H shows costaining with mAb 162 (anti-Ku, p70/p80 dimer) followed by the FITC-conjugated second antibody. The *y-axis* of C, F, and I shows costaining with mAb 25-4 (anti-p350) followed by the FITC-conjugated second antibody. Cells in G0, G1, S, and G2/M as well as cells with a subdiploid DNA content characteristic of apoptosis (APO) are indicated in A, D, and G.

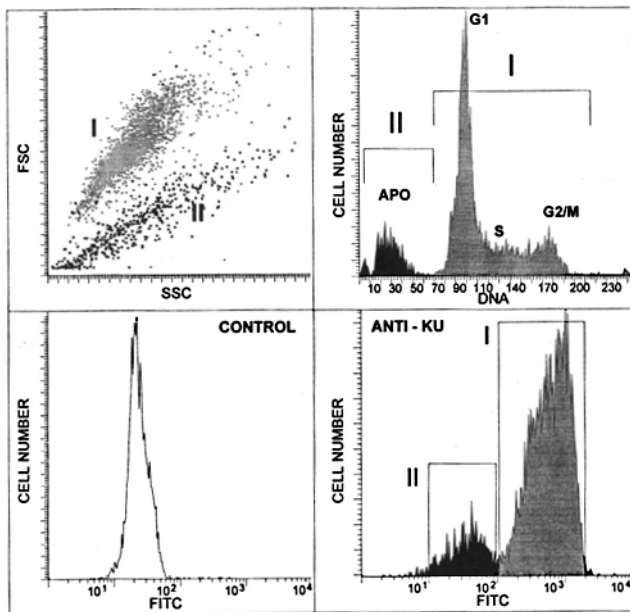


Figure 6. Staining of cycling and apoptotic HL-60 cells for Ku. (Top) Analysis of fsc and ssc vs DNA content. (Left) Analysis of fsc and ssc in HL-60 cells. (Right) DNA content of the same cells was determined by PI staining. Cells with G1, S, and G2/M DNA content (population I) are shaded gray, and cells with subdiploid DNA content characteristic of apoptosis (APO, population II) are shaded black. (Bottom) Staining with anti-Ku mAb. HL-60 cells were stained with second antibody alone (left, Control) or with PI plus anti-Ku mAb 162 (right). Ku staining of cell population I (G1, S, and G2/M DNA content) is indicated by gray shading. Ku staining of cell population II (subdiploid DNA content) is indicated by black shading. Note that Ku staining of population II is the same as seen with second antibody alone, whereas Ku staining of population I is higher than that of control.

was not due to looser binding to some nuclear structure and subsequent loss during fixation, as seen in the case of proliferating cell nuclear antigen (PCNA) (31).

To evaluate further the possibility that the decreased staining of Ku in apoptotic cells did not merely reflect leakage of nuclear proteins from the permeabilized apoptotic cells, staining of HL-60 cells for histone H1 and for components of U small nuclear ribonucleoproteins (the Sm B'/B and D proteins) (32), was performed (Fig. 7). By gating on the subdiploid (apoptotic) cells, it was confirmed that staining with anti-Ku mAb 162 was no different than that of an isotype control (Fig. 7, top left), whereas positive staining was found for both histone H1 (Fig. 7, middle left) and Sm antigen (Fig. 7, bottom left). Nonapoptotic cells stained brightly for Ku (top right) as well as histone H1 (middle right) and Sm (bottom right). The intensity of H1 and Sm staining was somewhat reduced in the apoptotic compared with nonapoptotic cells (compare Fig. 7, left vs right, middle, and bottom), a finding which is consistent with the loss of some nuclear material in the form of apoptotic blebs (33). However, the complete loss of Ku, in contrast to the modest reduction of antihistone and anti-Sm staining, suggests that loss of Ku staining did not reflect a general "leakiness" of apoptotic cells. This interpretation was further supported by the staining of necrotic K562 cells

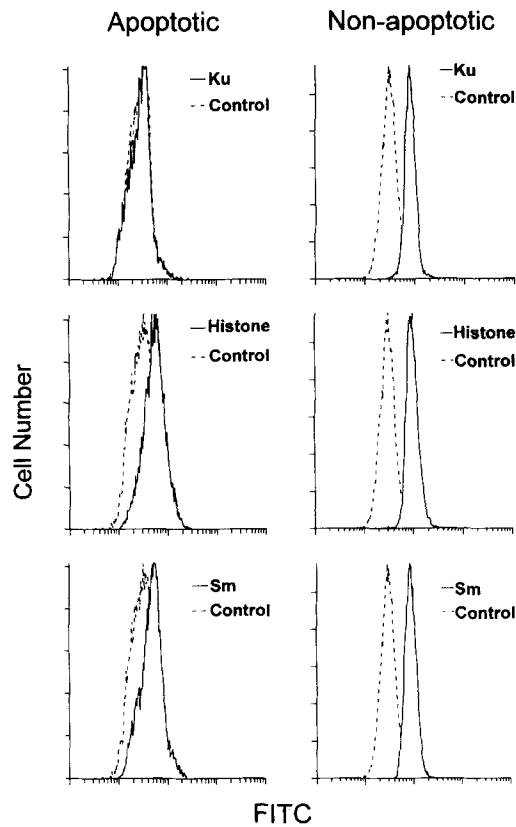


Figure 7. Staining of apoptotic HL-60 cells with anti-Ku, histone, and Sm mAbs. HL-60 cells were double stained with PI (DNA content) and the IgG2a mAbs 162 (anti-Ku), BM6.5 (anti-histone H1), Y2 (anti-Sm B'/B and D), or PAb101 (isotype control, anti-SV40 large T antigen), followed by FITC-conjugated goat anti-mouse IgG2a. Samples were analyzed by FACSCAN® (Becton Dickinson and Co.), placing FITC gates on the subdiploid (apoptotic) region of the DNA histogram (left) or on nonapoptotic cells having G1, S, or G2/M DNA content (right). (Top) FITC fluorescence histogram of anti-Ku (162, solid line) vs control (PAb101, dotted line) staining of apoptotic (left) and nonapoptotic (right) cells. (Middle) FITC fluorescence histogram of anti-histone (BM6.5, solid line) vs control (dotted line) staining of apoptotic vs nonapoptotic cells. (Bottom) FITC fluorescence histogram of anti-Sm (Y2, solid line) vs control (dotted line) staining of apoptotic vs nonapoptotic cells.

with anti-Ku mAbs. After treatment with 0.5% sodium azide, necrotic cells were identified by PI uptake followed by methanol fixation and were found to stain positively with mAb 162 (data not shown), suggesting that the loss of Ku staining was not due to nuclear leakage or proteolysis as a nonspecific consequence of cell death.

Half-life of Ku and p350. The loss of Ku in apoptotic cells might reflect either a shutdown of de novo synthesis of proteins with a relatively short half-life or else active degradation of proteins with a long half-life. To distinguish between these possibilities, the turnover of Ku and p350 was examined in K562 (human erythroleukemia) cells by pulse labeling and immunoprecipitating cell extracts derived from aliquots of cells obtained after 1, 2, 3, 4, or 5 d of chase (Fig. 8) using an excess of mAb 162 (anti-Ku) or 25-4 (anti-p350). It should be noted that each extract was derived from an equal number

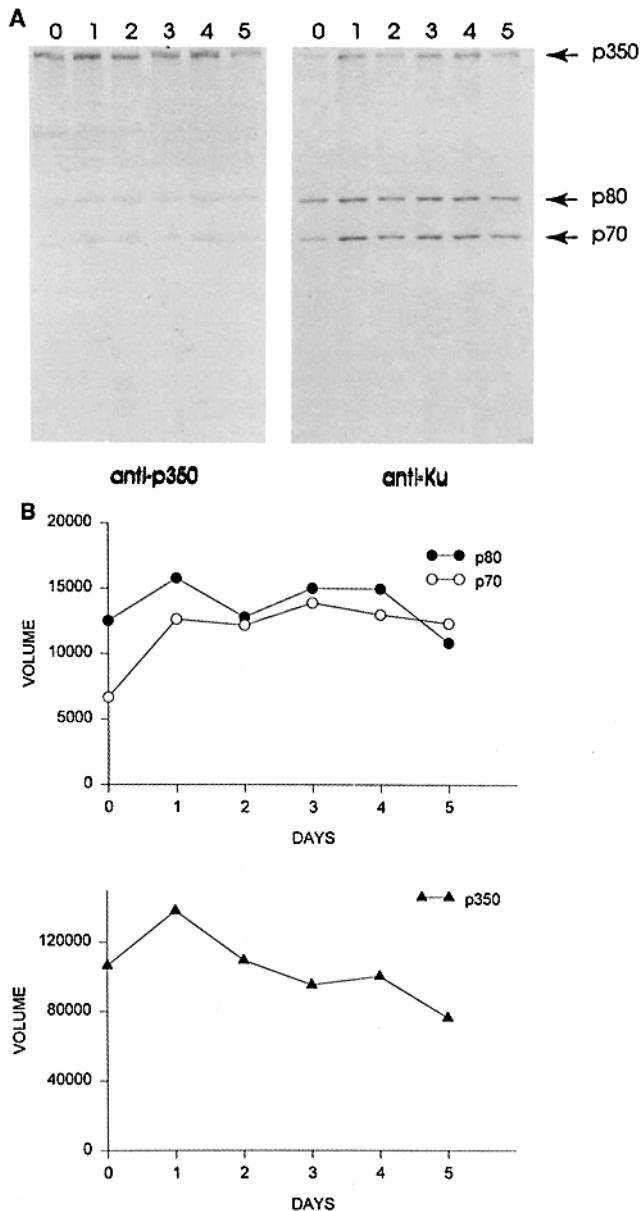


Figure 8. Turnover of Ku and p350 antigens. K562 cells were pulse labeled with ^{35}S and divided into equal aliquots. Cells were harvested at 0, 1, 2, 3, 4, and 5 d after pulse labeling, and extracts were analyzed for Ku or p350 by immunoprecipitation with mAbs 162 or 25-4, respectively. (A) Autoradiographs showing turnover p70 and p80 bands immunoprecipitated by mAb 162 (right) or p350 band immunoprecipitated by 25-4 (left). Delayed association of pulse-labeled p350 with p70/p80 heterodimer and lower intensity of p70 than of p80 after pulse labeling (right, lane 0) due to an endogenous pool of free p70 subunits have been reported previously (15). (B, top) Quantitation of radioactivity remaining associated with p70 (open circles) and p80 (closed circles) over time. (Bottom) Quantitation of p350 radioactivity over time.

of cells pulse labeled at day 0, and that the total number of cells per flask increased with time because of continued cell proliferation during chase. Immunoprecipitations of cell extracts obtained 0–5 d after pulse labeling revealed that the turnover of both Ku and p350 is slow (Fig. 8 A). Quantitation of the amount of pulse-labeled p70 and p80 remaining

at different time points during chase revealed little turnover of the newly synthesized Ku up to 5 d after its synthesis (Fig. 8 B), findings which were consistent with our previous observations (15). Likewise, little turnover of newly synthesized p350 was seen until the fifth day of chase (Fig. 8 B). Studies of the turnover of Ku and p350 in resting and PHA-stimulated PBLs indicated that the antigens were not synthesized at detectable levels in resting PBLs, whereas all three proteins were synthesized in mitogen-activated PBLs. The turnover of the three proteins was more rapid in PHA-activated PBLs than in K562, but considerable amounts of Ku and p350 antigen remained at 5 d (data not shown). The interpretation of Ku turnover in PHA-activated PBLs was complicated, however, by Ku loss related to the high level of programmed cell death in these cells. Taken together, these data indicated that the rate of turnover of Ku and p350 was too slow to account for the disappearance of Ku during apoptosis.

Discussion

The present studies indicate that mature peripheral blood neutrophils do not express the p70 Ku, p80 Ku, or p350 antigens, as determined by both the lack of fluorescent staining and the absence of the protein subunits by Western blot analysis and ELISA. In contrast, the promyelocytic leukemia line HL-60 and PBLs both expressed Ku antigen but lost it during programmed cell death.

Mechanism of Ku Disappearance in Neutrophils. Stability of the Ku antigen has been noted previously in human epidermal basal cells, which contain a significant amount of Ku but do not synthesize it (12). The present studies suggest that the half-life of Ku antigen in K562 cells is >5 d (Fig. 5). Since the postmitotic life span of neutrophils is ~ 6.5 d and their half-life in the blood is ~ 10 h (34), it is unlikely that the absence of Ku in neutrophils was the consequence of normal Ku turnover in G0 cells. Moreover, the life span of neutrophils is shorter than that of Ku-positive PBLs. The long half-life of Ku and the relatively high level of Ku in resting PBLs, in contrast to its absence in short-lived neutrophils, argue that, although the levels of both p70 and p80 mRNA are reduced dramatically in HL-60 cells undergoing DMSO-induced differentiation toward neutrophils (14), the disappearance of Ku protein during myeloid differentiation is a consequence of enhanced degradation. In contrast, the disappearance of *c-myc* protein during myeloid differentiation of HL-60 (27) is mediated largely at the level of transcription, because of its extremely short half-life (35, 36).

The presence of Ku antigen in HL-60 cells and its loss during terminal differentiation or apoptosis are somewhat reminiscent of the staining pattern of HL-60 for the *c-myc* protein, which has been implicated in some forms of apoptosis (37, 38). Moreover, resting lymphocytes have low but detectable levels of both *c-myc* (37, 39) and Ku, and the expression of both proteins is increased in PHA-stimulated PBLs (reference 39 and Fig. 5). Expression of *bcl-2* prevents apoptosis induced by *c-myc* (40) and is restricted to early myeloid cells (41, 42). The absence of *bcl-2* in mature neutrophils is also

reminiscent of the expression of Ku reported here. It is interesting to note that, like Ku antigen and *c-myc*, *bcl-2* is normally expressed in quiescent circulating lymphocytes, which is consistent with a role of *bcl-2* in maintaining lymphocyte survival (43).

Association of Ku Degradation with Apoptosis. The similar patterns of Ku and *bcl-2* protein expression in lymphocytes and myeloid cells raise the possibility that, like *bcl-2*, Ku antigen might play a role in some forms of apoptosis. The absence of both Ku (Fig. 6) and p350 in apoptotic HL-60 cells and PBLs (Fig. 5) is consistent with the possibility that Ku is involved in regulating programmed cell death, possibly acting as an inhibitor. However, since apoptosis generally accompanies terminal granulocytic differentiation (29, 30), and, in culture, ~5% of HL-60 cells exhibit spontaneous differentiation into myelocytes, metamyelocytes, or banded or segmented neutrophils (27), it is difficult to completely exclude the possibility that the loss of Ku was a consequence of differentiation. However, terminally differentiated epithelial cells and other types of cells contain high levels of Ku (references 12 and 44 and Reeves, W. H., unpublished data), suggesting that, if loss of Ku is a differentiation marker, it must be relatively specific for the myeloid lineage.

The absence of Ku in apoptotic PBLs provides strong evidence that disappearance of Ku is a marker of apoptosis rather than of terminal differentiation, and it suggests that the degradation of Ku is not a unique feature of apoptosis in myeloid cells. The absence of Ku in the apoptotic cell population resulting from activation with the T cell mitogen PHA suggests that the Ku-deficient lymphocytes may have been predominantly T cells. However, further studies will be needed to determine whether Ku loss is associated with apoptosis in T lymphocytes, B lymphocytes, or both. It will also be of interest to determine whether the kinetics of Ku disappearance in lymphocytes and neutrophils undergoing apoptosis is similar.

There are several potential explanations for the loss of Ku from apoptotic cells. Loss of Ku from apoptotic cells due to nonspecific nuclear leakage or proteolysis is unlikely because other nuclear DNA-binding (histone H1) and non-DNA-binding (Sm B'/B and D) proteins were detected readily in cells undergoing apoptosis (Fig. 7). Moreover, necrotic cells stained positively for Ku despite their permeability to PI. It is also possible that Ku is redistributed within apoptotic cells, such that it is more readily extracted from the cell. For ex-

ample, cell cycle-regulated redistribution of PCNA is responsible for the apparent lack of that antigen in resting cells (31). Ku antigen dissociates from condensing chromosomes during early prophase (45), raising the possibility that the chromatin condensation characteristic of apoptosis likewise causes Ku to dissociate from chromosomes, making it more readily extracted during fixation. Although this possibility cannot be excluded completely, Ku staining was absent in apoptotic cells fixed with paraformaldehyde, whereas PCNA is retained in resting cells fixed in this manner (31). In addition, immunofluorescence studies indicate that Ku is not extracted from the cytoplasm of mitotic HeLa cells during methanol fixation (reference 45 and Ajmani, A.K., unpublished observations). The most likely explanation for the loss of Ku in apoptotic HL-60 cells and lymphocytes is that enhanced Ku degradation accompanies and/or plays a role in some forms of apoptosis. In view of the enhanced susceptibility of DNA-bound p80 Ku to site-specific proteolysis (46), it is possible that DNA fragmentation accompanying apoptosis increases the fraction of Ku bound to DNA, thereby accelerating its degradation by proteases involved in programmed cell death, such as IL-1 β -converting enzyme (47, 48) or calpain (49). However, it should be noted that nearly all peripheral blood neutrophils in the present study had a G1/G0 DNA content, suggesting that they had not yet begun to undergo apoptosis, a finding which is in agreement with previous observations that apoptotic neutrophils are removed rapidly from the circulation (50). Thus, the absence of Ku and p350 in neutrophils suggests that these antigens are degraded either before onset of apoptosis or at an early stage before DNA fragmentation and intracellular proteolysis. Further studies will be necessary to determine whether the loss of Ku precedes apoptosis in all cell types or if the early loss of Ku is an event unique to the differentiation of neutrophils, rendering these cells more susceptible to the initiation of apoptosis. In view of the recently described role of Ku antigen in double-strand DNA break repair (8-11), degradation of Ku during apoptosis may prevent the repair of the double-strand DNA breaks that are a central feature of programmed cell death (51). The inability of mature neutrophils, unlike resting PBLs, to repair DNA strand breaks induced by γ irradiation (52) may be related, in part, to the absence of Ku. Thus, the loss of Ku antigen and/or p350 may be an important feature of certain forms of apoptosis that allows nucleases to cleave nuclear DNA unopposed by DNA repair mechanisms.

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