

The membrane form of the DNA repair protein Ku interacts at the cell surface with metalloproteinase 9

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The Ku heterodimer (Ku70/Ku80) plays a central role in DNA double-strand breaks repair. Ku is also expressed on the cell surface of different types of cells where its function remains poorly understood. From a yeast two-hybrid screen, we have identified a specific interaction between the core region of Ku80 and the hemopexin domain of metalloproteinase 9 (MMP-9), a key enzyme involved in the degradation of extracellular matrix (ECM) components. Ku associates with MMP-9 on the surface of leukemic cells as demonstrated by co-immunoprecipitation experiments in membrane extracts and double-label immunofluorescence studies. In normal and tumoral migratory cells, Ku80 and MMP-9 colocalize at the periphery of leading edge of cells and cellular invasion of collagen IV matrices was blocked by antibodies directed against Ku70 or Ku80 subunits as well as by Ku80-specific antisense oligonucleotides. Our results indicate that Ku and MMP-9 interact at the cell membrane of highly invasive hematopoietic cells of normal and tumoral origin and document the unexpected importance of the membrane-associated form of Ku in the regulation of ECM remodelling.

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Introduction

The Ku protein is a heterodimer of two subunits called Ku70 and Ku80. In the nucleus, Ku plays a central role in the repair of DNA double-strand breaks (DSBs) created by agents such as ionizing radiation or during several cellular recombination processes including antigen receptor gene rearrangement (V(D)J recombination) (Jackson, 2002). One unequivocal role for Ku is as a DNA-binding subunit of the DNA-dependent protein kinase (DNA-PK) complex, which is composed of the Ku heterodimer and a large catalytic subunit, DNA-PKcs, a member of the phosphatidylinositol 3-kinase super-

family (Hartley *et al*, 1995). Extensive genetic and molecular studies have identified the DNA-PK complex as an integral component of mammalian DNA DSB repair pathway (Jackson, 2002). Accordingly, mice with deletions of Ku genes are hypersensitive to ionizing radiation and lack mature lymphocytes (for review, see Jackson, 2002). Although Ku appears to be predominantly nuclear, several reports point out the presence of both Ku subunits in the cytoplasm (Bakalkin *et al*, 1998) and on the cell surface in a variety of tumor cells (Prabhakar *et al*, 1990; Dalziel *et al*, 1992; Ginis *et al*, 1995; Ginis and Faller, 2000; Lynch *et al*, 2001). Furthermore, the localization of Ku in membrane lipid rafts of mammalian cells has been recently shown (Lucero *et al*, 2003). The expression of Ku outside of the nucleus highly suggests that this protein is serving additional roles besides its main function in the DNA DSB repair. In fact, the membrane form of Ku has been shown to be involved in the regulation of cell–cell interaction (Teoh *et al*, 1998; Lynch *et al*, 2001) and in tumor cells when exposed to hypoxia to participate to a more migratory phenotype (Ginis and Faller, 2000). At present, the molecular mechanisms that contribute to these new functions of Ku have not been determined.

In this study, we describe a novel function of cell surface Ku in the regulation of extracellular matrix (ECM) proteolytic processes through its specific interaction with the matrix metalloproteinase 9 (MMP-9). MMP-9 is a member of the MMP family composed of zinc-dependent endopeptidases that have been shown to play a central role in many normal and pathological conditions including wound healing, angiogenesis, embryogenesis, arthritis and tumor metastasis (Nagase and Woessner, 1999). MMP-9 and MMP-2 are subclassified as type IV collagenases/gelatinases. They degrade type IV basement membrane collagen as well as denatured interstitial collagen, type V collagen, fibronectin and laminin (Nagase and Woessner, 1999). As most of the MMPs, MMP-9 is secreted as an inactive zymogen (proform) that is mainly activated in the extracellular milieu by a proteolytic processing that removes an 80-aa propeptide (Nagase and Woessner, 1999). Once activated, the MMPs are further regulated in the extracellular environment by tight-binding protein inhibitors, the TIMPs (Gomez *et al*, 1997). It has long been suggested that MMPs function at the vicinity of the cell surface. Accumulating evidence now suggests that secreted MMP can preferentially localize at the cell surface via their interaction with ‘docking’ molecules (for review, see Stamenkovic, 2003). The presence of these MMP-binding proteins allows cells to concentrate and activate MMPs at the close interface between their plasma membrane and ECM and, in cancer cells, may be a key event in promoting tumor invasion and angiogenesis (Toth *et al*, 1997; Bourguignon *et al*, 1998; Olson *et al*, 1998; Yu and Stamenkovic, 1999, 2000). Our present results indicate that Ku and MMP-9 interact at the cell membrane of mammalian cells and document the unexpected importance of the membrane-associated form of Ku in the regulation of ECM remodelling.

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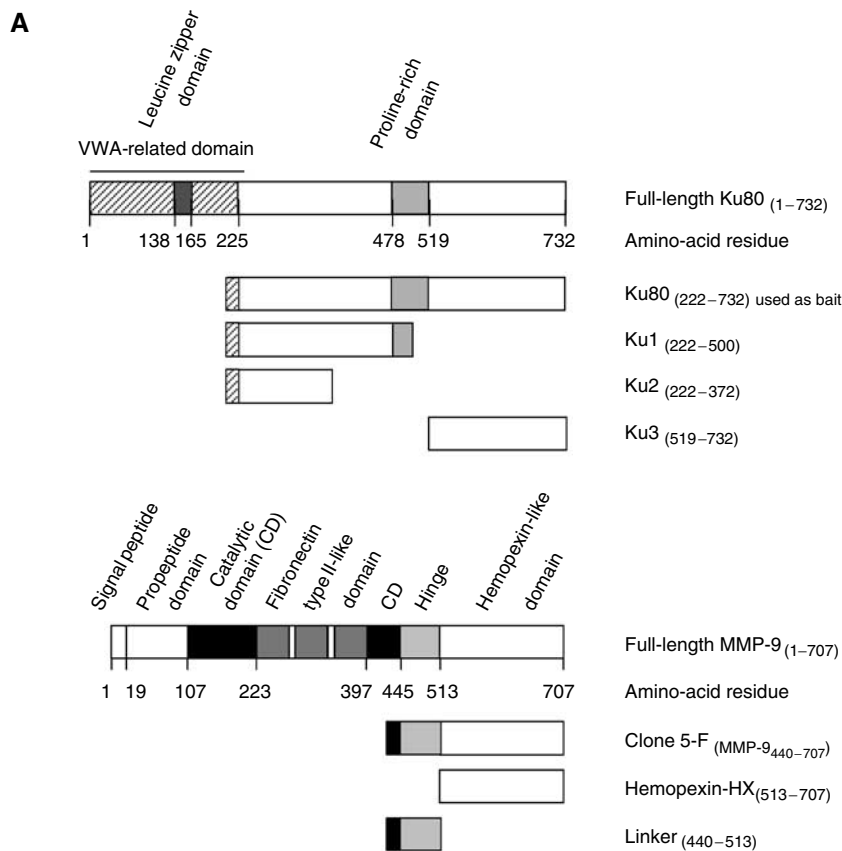
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Results

Ku80 interacts physically with MMP-9 in yeast two-hybrid experiments

In order to identify new proteins interacting with Ku, we used the yeast two-hybrid system. Using Ku80 (aa 222–732) as bait (DBDKu80; Figure 1A), we screened a human bone marrow cDNA library. Plasmid DNA was isolated from the 19 yeast positive clones obtained. Sequencing analyses of the inserts showed, as expected, that one clone encoded Ku70 (aa 23–

609). One positive clone (clone 5F) corresponded to the aa residues 440–707 of the human MMP-9 sequence, which comprised the carboxyl-terminal side of the catalytic domain connected to the hemopexin-like domain by the hinge region (Figure 1A). Specificity testing using yeast two-hybrid analyses was then performed and, as shown in Figure 1B, the isolated MMP-9 domain interacted with DBDKu80 but not with partner vector without insert nor with lamin B. Specific interaction between full-length Ku80 and MMP-9/5F was further confirmed by co-immunoprecipitation experiments



B

GAL4 DNA-binding domain (BD)	GAL4 activation domain (AD)	HIS3 ADE2
–	pAD-MMP-9 _{440–707}	–
pGBKT7	pAD-MMP-9 _{440–707}	–
pBD-Ku80 _{222–732}	pAD-MMP-9 _{440–707}	+
pBD-Lam	pAD-MMP-9 _{440–707}	–
pBD-Ku80 _{222–732}	–	–

Figure 1 Ku80 and MMP-9 interaction in yeast cells. (A) Schematic domain structure of full-length Ku80 and MMP-9 and schematic representation of deletion constructs. Ku80 (aa 222–732) fused to GAL4 DNA-binding domain was used as bait in a two-hybrid screen of cDNA human bone marrow library. (B) In the yeast two-hybrid assay, only the yeast transformants MMP-9 (aa 440–707)/Ku80 (aa 222–732) grew on medium lacking histidine and adenine. (C) Ku80 (aa 1–732) cDNA or Ku70 (aa 23–609) cDNA was inserted into pGBKT7 vector and expressed as a GAL4 fusion protein with c-myc epitope tag in AH109 yeast strain. MMP-9 cDNA (aa 440–707) was inserted into pGADT7 and expressed as a GAL4 fusion protein with HA epitope tag in Y187 yeast strain. The two transformed strains were mated and protein extracts of diploid yeasts were immunoprecipitated (IP) with control antibody (c) or antibodies that recognize either epitope tags (HA or c-myc), Ku80 (mAb111 for Ku80₁, mAbS10B1 for Ku80₂) or Ku70 (mAbN3H10) protein. (D) Deletion mapping: AH109 cells expressing the indicated deletion mutants of Ku80 were mated with Y187 expressing HA-MMP-9/5-F. Immunoprecipitation of protein extracts of diploid yeasts with control (c) or anti-HA antibodies is shown. IgGH corresponds to the heavy chain of the immunoglobulins. (E) Deletion mapping: AH109 cells expressing c-myc-Ku80 (full length) were mated with Y187 expressing the indicated HA deletion mutants of MMP-9. Immunoprecipitation of protein extracts of diploid yeasts with control (c) or anti-Ku80 (mAb111) is shown. Immunoprecipitation products were analyzed by Western blotting using antibodies that recognize either the HA or c-myc epitope tags. (F) Deletion mapping: AH109 cells expressing the c-myc-Ku2 were mated with Y187 expressing HA-HX. Immunoprecipitation products of protein extracts of diploid yeasts were analyzed by Western blotting using antibodies that recognize the epitope tags (HA or c-myc).

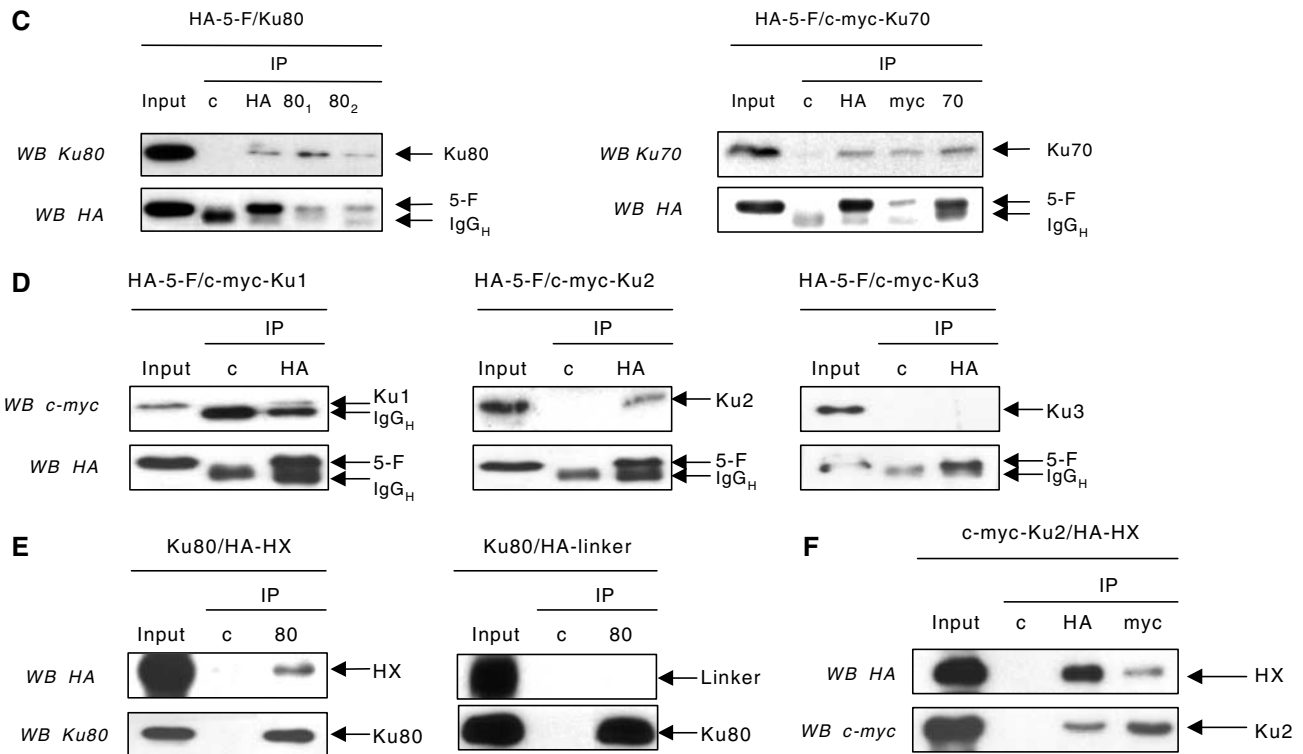


Figure 1 Continued

of epitope-tagged proteins expressed in yeasts (Figure 1C). Full-length Ku80 also interacted with both full-length and active forms of MMP-9 in similar co-immunoprecipitation experiments (data not shown). Interestingly, Ku70, which shares with Ku80 both sequence and marked structural homology (Walker *et al*, 2001), is also able to interact with MMP-9/5F (Figure 1C). These results suggest that the region of Ku80 responsible for its binding to MMP-9 is a region that exhibits Ku70 homology and therefore excludes the C-terminal domain of Ku80 (Walker *et al*, 2001). To delineate the regions of Ku80 that are necessary for MMP-9 interaction, we constructed truncation mutants of DBDKu80 (Figure 1A). Deletion mutants were expressed as c-myc-tagged proteins in yeast and interaction with MMP-9/5F (expressed as HA-tagged protein) was evaluated by immunoprecipitation experiments. Deletion of the 3' end of DBDKu80 between aa 500 and 732 allowed interaction with MMP-9/5F and this interaction was still present when aa 372–500 were further deleted. On the contrary, the deletion of aa 222–519 abrogated interaction with MMP-9/5F (Figure 1D). Thus the region of Ku80 tested that is sufficient for interaction with the C-terminal part of MMP-9 is a core region that comprises aa 222–372. To delineate the crucial minimal region of MMP-9 for interaction with full-length Ku80, deletion mutants of MMP-9/5F were produced. Deletion mutant that corresponded to the hemopexin domain (aa 513–713) of MMP-9 interacted with Ku80, whereas the interaction was abolished when a deletion mutant that corresponded to the linker domain was used in immunoprecipitation experiments (Figure 1E). Finally, co-immunoprecipitation experiments were performed using yeast extracts containing the minimal interaction domain of MMP-9 (HA-HX) and the minimal interaction domain of Ku (c-myc-Ku2). These experiments

confirmed that a central domain of Ku (aa 222–372) and the MMP-9 hemopexin domain are involved in Ku80/MMP-9 binding (Figure 1F). This region of the Ku80 protein does not contain any characterized domains involved in protein–protein interactions, but a partially overlapping region of the Ku80 protein (aa 215–276) has been previously found to be involved in interaction with the Werner's syndrome protein (Li and Comai, 2001).

Co-immunoprecipitation of Ku and MMP-9 in extracts obtained from human cell lines

On the basis of the results obtained with yeast two-hybrid analyses, the interaction between Ku80 and MMP-9 was then investigated in human cells. In order to use a relevant model, Ku/MMP-9 interaction was investigated in HL-60 cells exposed to the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In these promyelocytic cells, TPA exposure results in maturation along the monocyte/macrophage lineage characterized by the induction of cell adhesion and spreading, phagocytic activity as well as overproduction of MMP-9 (Ries *et al*, 1994; Xie *et al*, 1998). *In vivo*, MMP-9 is the major MMP produced by human macrophages and its proteolytic activity is thought to be necessary for various functions such as extravasation, migration and tissue remodelling during chronic inflammatory conditions (Opdenakker *et al*, 2001). In addition, it has been previously demonstrated that, in HL-60 cells, Ku is expressed on the cell surface along with its intracellular pool within the nucleus and the cytoplasm (Ginis and Faller, 2000; Muller *et al*, 2001). As shown in Figure 2A, both Ku70 and Ku80 subunits were expressed in HL-60 whole-cell extracts (WCEs) and the expression of the two proteins was unaffected upon TPA treatment (50 or 150 nM) for up to 48 h. As previously described (Ries *et al*,

1994; Xie *et al*, 1998), treatment of the cells with TPA induced the expression and secretion of high amounts of MMP-9 proform (92 kDa) as determined by gelatin zymography and immunoblot analysis of culture fluids (Figure 2A and B). In extracts prepared from adherent cells, three bands of ~92, ~85 and ~83 kDa with gelatinolytic activity (Figure 2B) were recognized by specific antibodies to human MMP-9 (Ab19047 and Ab805 (Figure 2A) and data not shown, respectively) after 24 h exposure to TPA. The ~85 kDa form of MMP-9 was predominantly expressed especially after 48 h treatment. According to the literature, both the ~92 and ~85 kDa immunoreactive forms are likely to correspond to proforms of MMP-9, whereas the ~83 kDa corresponds to an active form of MMP-9 (Shapiro *et al*, 1995; Mazziari *et al*, 1997; Toth *et al*, 1997). In our hands, only the ~92 and ~85 kDa immunoreactive forms were recognized by

the polyclonal antibody (Ab19092) directed against the amino-terminal prodomain of MMP-9 (data not shown). Interestingly, it has been previously suggested that the ~85 kDa form of MMP-9 is a cell surface-associated proform of MMP-9 (Mazziari *et al*, 1997) devoid of complex carbohydrates (Toth *et al*, 1997). The ~85 kDa form of MMP-9, Ku70 and Ku80 co-immunoprecipitated from extracts of TPA-treated HL-60 cells using anti-Ku80 or anti-Ku70 antibodies (Figure 2C). Interaction of Ku heterodimer with MMP-9 was further demonstrated by the presence of both Ku subunits along with the ~85 kDa form of MMP in the anti-MMP-9 but not control immunoprecipitates (Figure 2C). Structurally and functionally, MMP-9 is closely related to the other type IV collagenase, MMP-2 (Murphy and Crabbe, 1995). MMP-2 was expressed in HL-60 cells, but no induction of its expression and/or secretion was observed during TPA-induced differen-

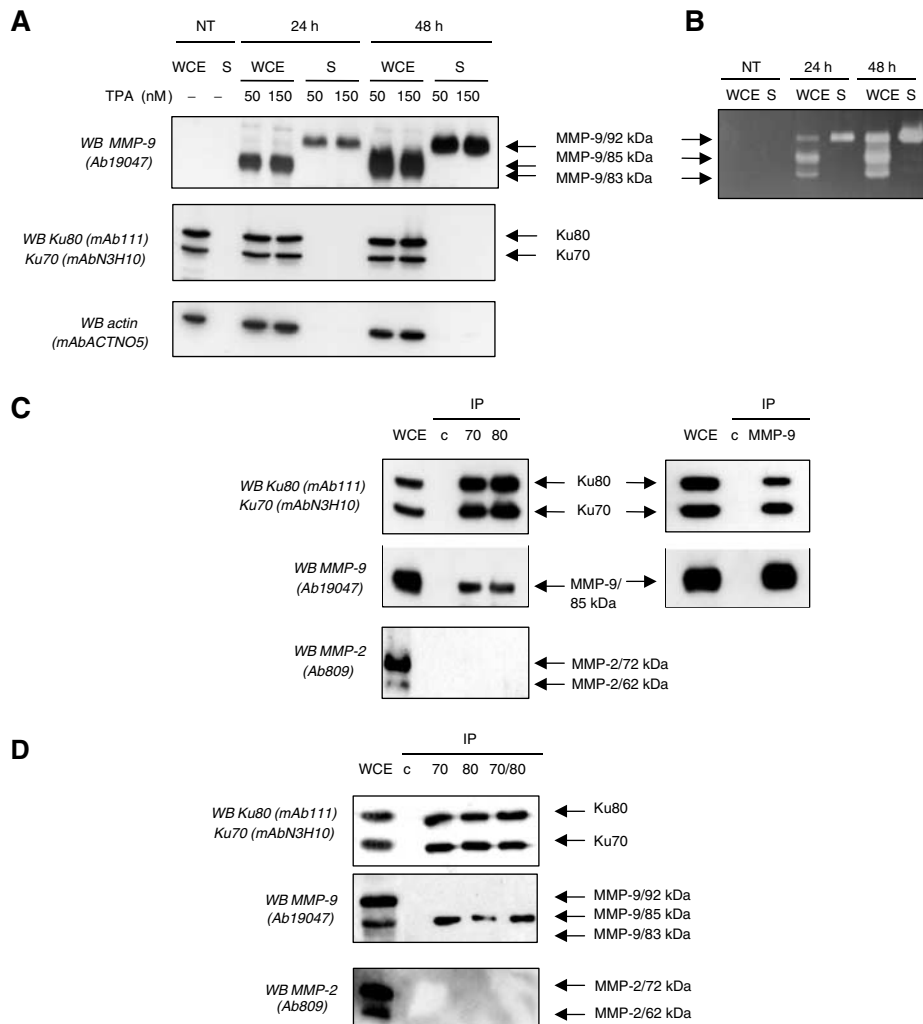


Figure 2 Ku interacts with MMP-9 in HL-60 cells treated or not with TPA. **(A)** HL-60 cells were treated or not (NT) with TPA at the indicated concentrations for either 24 or 48 h. Immunoblot analyses were carried out with samples of culture media (S, 10 μ l) or WCEs obtained from control or differentiated adherent cells (WCE, 50 μ g). Note that in these experimental conditions (50 μ g of WCE, 10 s of autoradiography), MMP-9 expression is not detected in the extracts obtained from untreated cells. **(B)** Gelatin zymography of WCEs or culture media obtained from HL-60 cells treated or not (NT) with 50 nM TPA for the indicated times. **(C)** A 50 μ g portion of WCE obtained from TPA-treated HL-60 cells was immunoprecipitated (IP) with control antibody (c) or antibodies that recognize either the Ku70 (mAbN3H10), Ku80 (mAb111) or MMP-9 (mAb13415) protein. Immunoprecipitation products were analyzed by Western blotting with the indicated antibodies. **(D)** A 200 μ g portion of WCE obtained from exponentially growing control HL-60 cells was immunoprecipitated with control antibody (c) or antibodies that recognize either the Ku70, Ku80 subunit or both Ku proteins (Ku70/80) in a dimerized state. Immunoprecipitation products were then analyzed by Western blotting using the indicated antibodies (at least, 5 min of autoradiography for MMP-9 Western blot).

tiation (data not shown). As shown in Figure 2C, neither the latent (~72 kDa) nor the active (~62 kDa) form of MMP-2 was present in the precipitates obtained with antibodies directed against Ku80 or Ku70. Interaction of Ku heterodimer with an ~85 kDa form of MMP-9 was also observed in two human monocytic cell lines, U-937 and THP-1, treated with TPA (data not shown). In these two cell lines, monocytes differentiation into macrophages upon TPA treatment was accompanied by a sharp increase of MMP-9 expression, with a highly predominant expression of the ~85 kDa form (data not shown). We then investigated if Ku/MMP-9 interaction was also observed in untreated cells. In extracts obtained from HL-60 cells, the two proforms of MMP-9 were detected by Western blot with a predominant expression of the 92 kDa form (Figure 2D). Again, the 85 kDa form of MMP-9, but not MMP-2, was found to be present in the anti-Ku70 or anti-Ku80 immunoprecipitates of HL-60 lysates (Figure 2D). Similar results were obtained with U-937 or THP-1 extracts obtained from untreated cells (data not shown). Taken together, our data demonstrate that MMP-9, but not the other gelatinase MMP-2, associates with the Ku70/Ku80 heterodimer in different leukemic cell lines, treated or not with TPA.

Ku/MMP-9 complex is present at the cell surface of macrophage-differentiated cells

Because the biochemical experiments performed in WCEs strongly suggested that MMP-9 is in a complex with Ku subunits, we carried out a series of experiments to identify

how the proteins were distributed and to determine where this complex was localized. As shown in fractionated cell extracts, Ku70 and Ku80 are expressed in the postnuclear compartment of HL-60 cells with a similar level of expression between the cytosolic and membrane fractions (Figure 3A). In contrast, MMP-9 expression is almost exclusively detected in the cell detergent-soluble membrane fraction as described previously (Toth *et al*, 1997; Yu and Stamenkovic, 1999). As shown in the zymogram, the membrane fractions contained ~92, ~85 and ~83 kDa gelatinolytic forms, which were identified as MMP-9 by immunoblot analysis (Figure 3A), with a predominant expression of the 85 kDa form. Since both Ku and MMP-9 were expressed in the membrane fraction of the extracts, immunoprecipitation experiments were carried out to evaluate the presence of a Ku/MMP-9 complex in this compartment of the cells. As shown in Figure 3B, MMP-9 was found to be present as a doublet in the anti-Ku70 or Ku80 immunoprecipitates, consistent with the presence of the ~85 and ~83 kDa forms. These results were further confirmed by the presence of a doublet in the zymogram analysis of the immunoprecipitation products (Figure 3B). The presence of both Ku subunits along with MMP-9 when the immunoprecipitations were performed with an anti-MMP-9 antibody confirmed the Ku/MMP-9 interaction. Thus, in macrophage-differentiated HL-60 cells, Ku is expressed in membrane extracts and is able to interact with two forms of MMP-9. Similar results were obtained with membrane extracts of TPA-treated THP-1 cells (data not

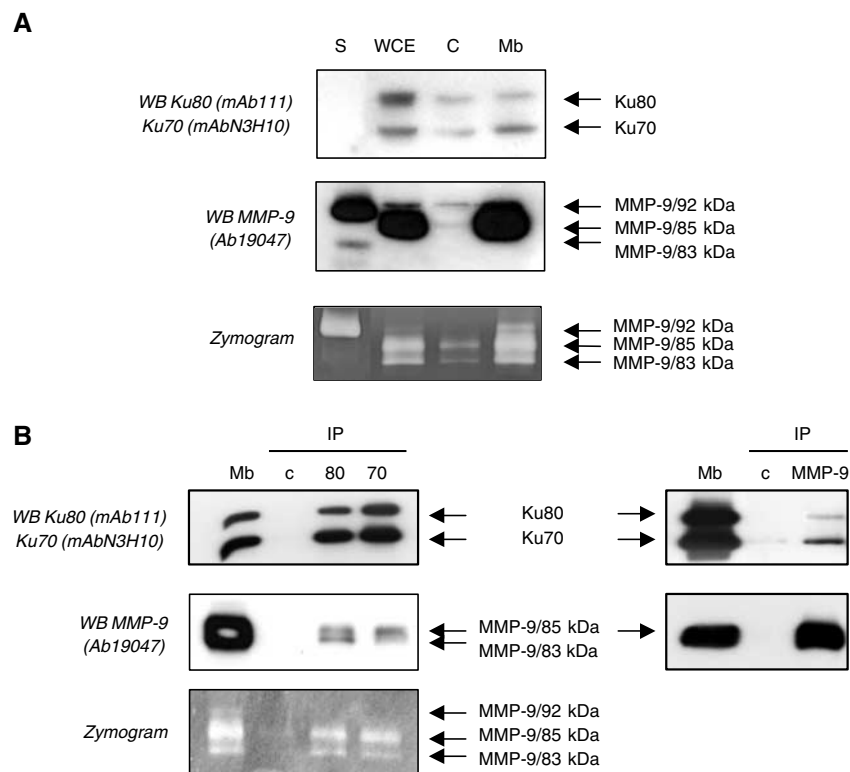


Figure 3 Ku interacts with both latent and active forms of MMP-9 in the membrane fraction of cell extracts. (A) Serum-free supernatants (S), WCEs, cytosolic extracts (C) or 0.5% Triton-soluble crude membrane extracts (Mb) of TPA-treated HL-60 cells (50 nM, 48 h) were analyzed for Ku and MMP-9 expression by Western blotting or for MMP-9 activity by zymography. (B) Left panel: 100 µg of Mb extracts were immunoprecipitated with immunomagnetic beads coupled with control antibody (c) or with antibodies that recognize either the Ku80, Ku70 or MMP-9 protein. Immunoprecipitation products were then analyzed by Western blotting using the indicated antibody or by zymography.

shown). The existence of a Ku/MMP-9 complex in the membrane fraction of the extracts raises the possibility that the two proteins associate on the cell surface. We first evaluated if the two proteins were accessible to cell surface biotinylation. The integral transmembrane protein CD44 was used as a positive control of cell surface biotinylation, whereas the DNA-PKcs protein was used as a negative control to assess that the intracellular proteins were not biotinylated (Figure 4A). Both the Ku heterodimer and MMP-9 protein were accessible to cell surface biotinylation. In MMP-9 immunoprecipitates, a ~63 kDa band that corresponded to the molecular weight of Ku70 was detected. Unfortunately, the presence of biotinylated Ku80 protein in these immune complexes could not be clearly affirmed since both MMP-9 and Ku80 migrated at about the same apparent molecular weight (Figure 4A). The cell surface localization of both Ku subunits and MMP-9 was further confirmed by positive staining of the two proteins in flow cytometric analysis of nonpermeabilized cells (Figure 4B). Detection of Ku on the cell surface was also observed with two additional monoclonal antibodies against Ku70 or Ku80 (see Supplementary Figure 1). Similar results for Ku cell surface staining were obtained for the THP-1 cell line (see Supplementary Figures 1 and 2). In order to demonstrate that the presence of Ku on the cell surface is through a physiological pathway and not by means of secondary association to the cell membrane by the nonspecific release of nuclear Ku from dying cells, additional experiments were performed using a mixture of cell surface Ku-positive and -negative cell lines. In all cases, the Ku-negative cells remain

unstained on the cell surface by Ku antibodies despite coculture with increasing relative amounts of cell surface Ku-positive cells (data not shown). Double-label immunofluorescence experiments were then performed to evaluate if the two proteins colocalize to the same structures at the cell membrane. As shown in Figure 5A–C, differentiated HL-60 or THP-1 cells were flattened and exhibited lamellipodia and membrane protrusions (so-called ‘pseudopodia’) at the cellular margin. In some cases, cells exhibited typical morphology of migrating macrophages, the lamellipodium with associated ruffles being protruded at the leading edge of the cells (Figure 5B and C). Other cells exhibited multiple adhesion contacts and cells with radial tubular protrusions still attached to adhesion points were observed (Figure 5A). As shown in pictures of representative experiments, in migratory macrophage-like cells, Ku80 and MMP-9 colocalize at the periphery of both the leading and trailing edge of cells (Figure 5B and C). Similar colocalization of Ku and MMP-9 was also observed in pseudopodia (Figure 5A). Colocalization of both Ku70, Ku80 (using mAb162 that recognizes a conformational epitope of Ku70/Ku80 heterodimer) and MMP-9 was also observed in TPA-treated HL-60 cells (see Supplementary Figure 3). In accordance with the results obtained with leukemic cells with morphological aspect of migrating macrophages, we then investigated if the two proteins colocalize at the cell membrane of monocyte-derived macrophages obtained from healthy donors. As shown in Figure 5D and E, colocalization of the two proteins was detected in the ruffled-leading edge of migrating macrophages exhibiting typical fan-like shape.

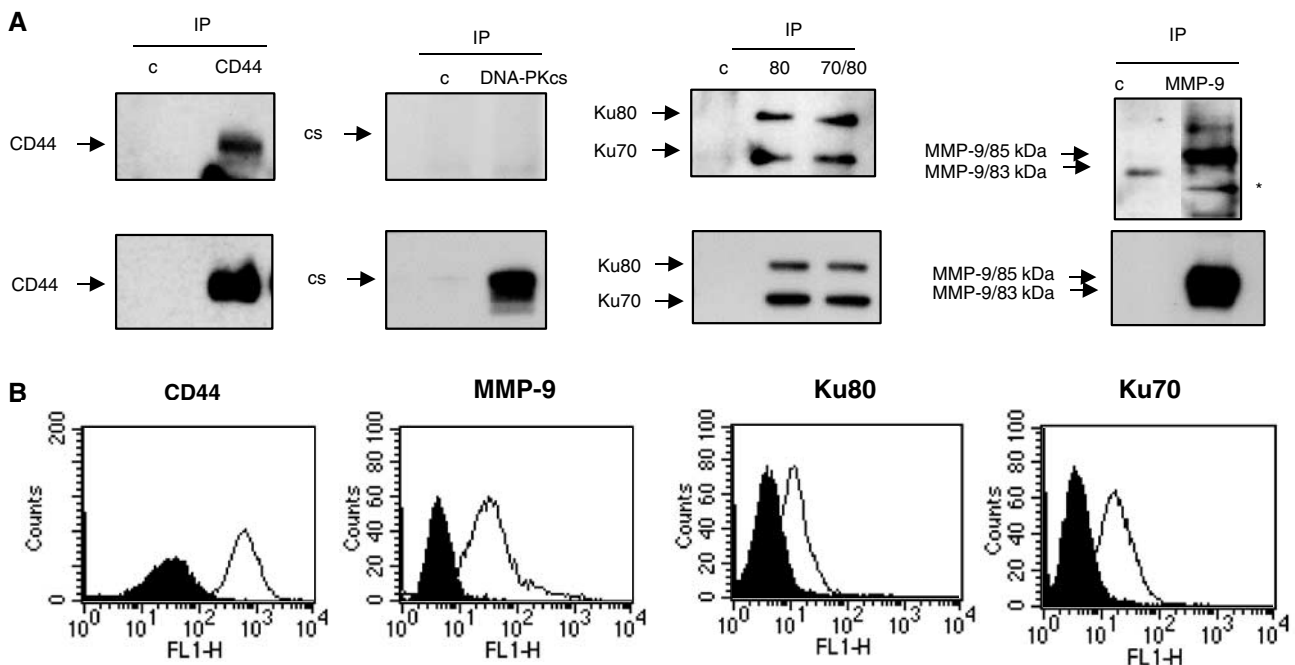


Figure 4 Ku and MMP-9 are expressed on the cell surface of HL-60 cells. (A) HL-60 cells, treated with TPA (50 nM, 48 h), were biotinylated in suspension and CD44, DNA-PKcs, Ku heterodimer and MMP-9 were immunoprecipitated from RIPA lysates of surface-biotinylated cells. Antimouse IgG was used in control immunoprecipitation experiments (c). Biotinylated proteins were visualized on blots by extravidin peroxidase (upper panel). The same immunoprecipitates as in the top panel were blotted for the expression of CD44, DNA-PKcs, Ku heterodimer and MMP-9 to assess the quality of the immunoprecipitations (lower panel). *The size of this immunoprecipitated protein corresponds to Ku70. (B) The expressions of CD44, Ku70, Ku80 and MMP-9 were analyzed by indirect immunofluorescence flow cytometry in unpermeabilized living cells. In these experiments, the cells that exhibited alterations in membrane permeability (as assessed by positive propidium iodide staining) were excluded from the analysis. Solid histogram: control isotypes; open histogram: indicated antigens.

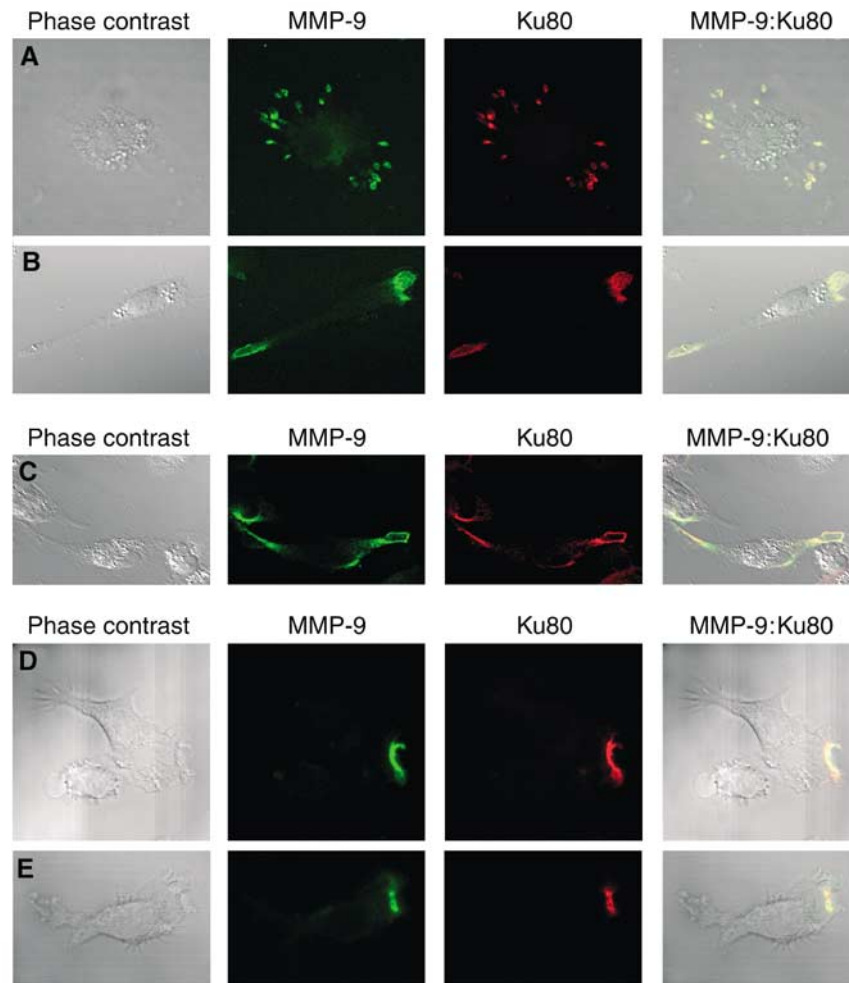


Figure 5 Colocalization of Ku80 and MMP-9 at the cell surface of TPA-treated HL-60 and THP-1 cells and migrating normal macrophages. (A–C) HL-60 or THP-1 cells were treated with TPA (50 nM, 72 h), and fixed cells were double stained with anti-Ku antibody (mAbS10B1) and anti-MMP-9 antibody (Ab805) (A) or Ab19092 (B, C) before detection with FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG labelling. The red and green images were merged and subjected to colocalization analysis. Digital images were taken at $\times 40$ magnification (zoom 4). Note that in these experimental conditions (fixation without cell permeabilization), the nuclear Ku is not stained. Nuclear Ku staining and cytoplasmic signal were observed when cells were permeabilized with 0.2% Triton X-100 (data not shown). (D, E) Similar experiments were performed in monocyte-derived macrophages obtained from healthy donors. Cells were costained with anti-Ku80 (mAbS10B1) and anti-MMP-9 antibody (Ab19092) as described above. Digital images were taken at $\times 100$ magnification (zoom 3). Note the typical fan-like shape of migrating macrophages.

Cellular invasion of collagen IV matrices is blocked by antibodies directed against Ku70 or Ku80 subunits

To assess the functional consequences of Ku/MMP-9 interaction on the proteolytic potential of the cells, the ability of cells to invade a three-dimensional collagen IV matrix in the presence of antibodies directed against Ku was evaluated. As previously reported (Boghaert *et al*, 1994), invasion of type IV collagen could be blocked by GM6001, a broad-spectrum inhibitor of MMP, and EDTA, whereas aprotinin used as control had no effect (Figure 6A). The notion that MMP-9 is required for collagen invasion is supported by the inhibitory effect of a functional blocking MMP-9 antibody (Figure 6B). An MMP-2 functional blocking antibody used as a control had no inhibitory effect. These results were expected since it has been previously demonstrated that MMP-9 is the major, if not unique, collagenase involved in TPA-treated monocytic cells invasion on collagen IV matrices (Watanabe *et al*, 1993). In accordance, we observed that

the gelatinolytic activity of MMP-2 was almost undetectable in the supernatant and in the cell-associated fractions of TPA-treated HL-60 or THP-1 cells (data not shown). Preincubation of TPA-treated THP-1 cells, with anti-Ku70 (mAbN3H10) or anti-Ku80 (mAbS10B1, epitope aa 8–221) antibodies had an inhibitory effect on collagen IV invasion as compared to control IgG, whereas preincubation with boiled antibody (S10B1) had no effect. In these experiments, we noted that preincubation with mAb111, which recognized the C-terminal part of Ku80 (aa 610–705), had no inhibitory effect (data not shown). To demonstrate formally that Ku was involved in invasion of TPA-treated cells, we tested whether the inhibition of Ku expression by antisense treatment could affect cell invasion on collagen IV matrices. On exposure to specific antisense oligonucleotides (ASOs, 200 nM) targeted to Ku80 messenger (Belenkov *et al*, 2002), the expression of Ku80 at the cell membrane, as evaluated by flow cytometry, was significantly reduced (Figure 6C). A significant decrease of

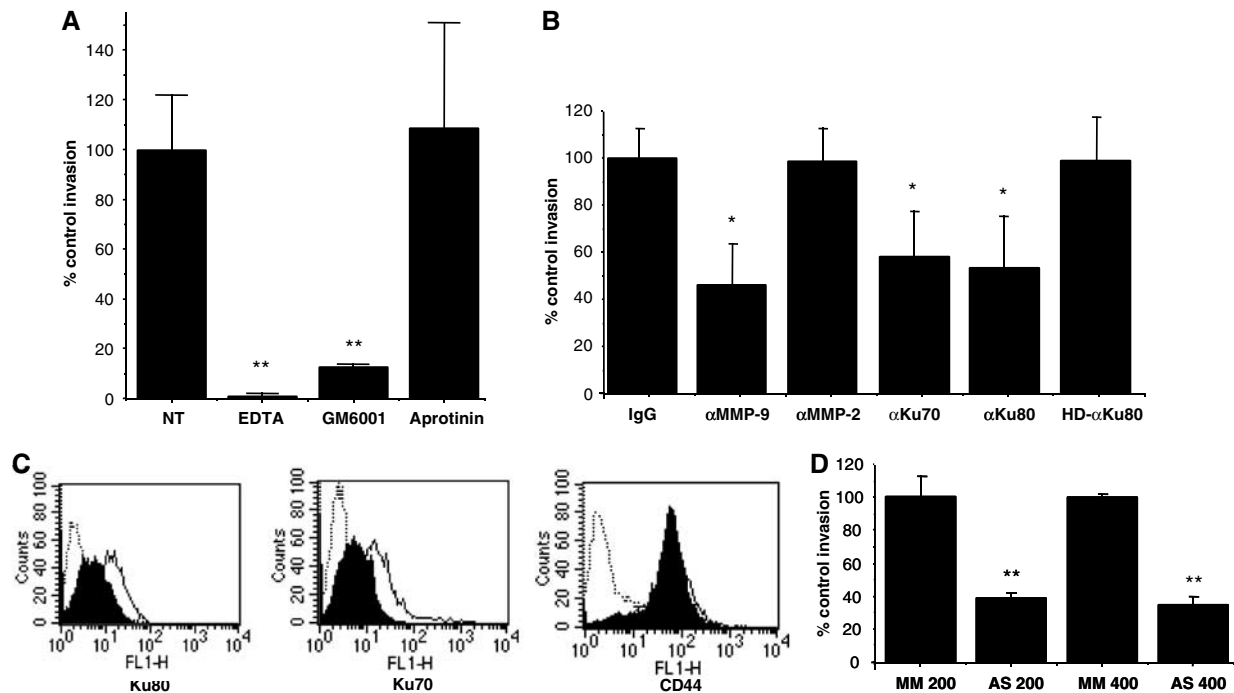


Figure 6 Invasion of TPA-treated THP-1 cells through a type IV collagen gel is inhibited by antibodies directed against Ku subunits. In all, 2×10^5 THP-1 cells treated with TPA (50 nM, 72 h) were added to collagen-coated Transwell chambers in serum-free medium and allowed to migrate for 72 h. Following invasion, noninvading cells were removed from the top chamber, membranes were fixed and stained, and invading cells were enumerated using an optical microscope. **(A)** Before adding cells to the Transwells, cells were incubated as indicated with EDTA (1 mM), aprotinin (20 μ g/ml) or the MMP inhibitor GM6001 (10 μ M) for 1 h prior to and during the invasion assay. Data are expressed as % of control invasion (cells without inhibitors), normalized to 100% (*statistically significant by Student's *t*-test, $P < 0.05$ relative to control). **(B)** Before adding cells to the Transwells, cells were incubated as indicated with MMP-9 (α MMP-9), MMP-2 (α MMP-2), Ku antibodies (α Ku70, α Ku80), heat-denatured α Ku80 (HD- α Ku80) or control IgG (20 μ g/ml) for 1 h prior to and during the invasion assay. Data are expressed as % of control invasion (cells with IgG), normalized to 100% (*statistically significant by Student's *t*-test, $P < 0.05$ relative to control). Cell preincubation with control IgG did not have any inhibitory effect on type IV collagen invasion as compared to untreated cells (data not shown). **(C)** Membrane expression of Ku70, Ku80 and CD-44 in TPA-treated THP-1 cells exposed to mismatch (open histogram) or antisense (solid histogram) oligonucleotide (200 nM) and analyzed by indirect immunofluorescence flow cytometry. The results obtained for secondary antibody alone are indicated (dashed lines) and were identical between mismatch and antisense oligonucleotide treated cells. **(D)** Invasion of TPA-treated THP-1 cells exposed to the indicated concentrations of mismatch or antisense oligonucleotides. Data are expressed as % of control invasion (cells exposed to mismatch oligonucleotides), normalized to 100% (*statistically significant by Student's *t*-test, $P < 0.05$ relative to control, **statistically significant by Student's *t*-test, $P < 0.01$ relative to control).

Ku70 cell surface expression was also observed suggesting that, as described previously (Sato *et al*, 1995; Li *et al*, 2002), each subunit of Ku protein is required to stabilize the other (Figure 6C). On the contrary, expression of CD44 exposed to antisense treatment was totally unaffected (Figure 6C). Similar results were obtained when 400 nM of oligonucleotides was used (data not shown). A consistent inhibition of cell invasion was observed, reaching 65% reduction compared with control, in these cells with reduced levels of Ku expression. A similar inhibitory effect on cell invasion was obtained for the two tested oligonucleotide concentrations (Figure 6D).

Discussion

We present here independent observations that document a specific interaction between a membrane form of the Ku heterodimer and a protein involved in ECM remodelling, MMP-9. First, Ku80 interacts with MMP-9 in yeast two-hybrid analyses. Second, the two proteins co-precipitate from membrane extracts of macrophage-differentiated cells with either Ku- or MMP-9-specific antibodies. Third, the two proteins colocalize *in vivo* at similar structures of the cell membrane

in differentiated leukemic cells or in monocyte-derived macrophages obtained from healthy donors. These biochemical and morphological observations are strong evidence of a complex between Ku and MMP-9 that is found at cell membrane of highly invasive cells of tumorous and normal origin. The presence of a Ku/MMP-9 complex at the cell membrane suggests that Ku serves as an MMP-9 'docking' molecule. Recent studies demonstrate that MMP-9 localization to the cell surface of mammary carcinoma cells or keratinocytes is mediated by binding to the main receptor for hyaluronan, CD44 (Bourguignon *et al*, 1998; Yu and Stamenkovic, 1999, 2000). The association between MMP-9 and CD44 is required for MMP-9 to promote tumor invasion and angiogenesis (Yu and Stamenkovic, 1999, 2000). The observations that cell surface collagen IV (Olson *et al*, 1998), integrins $\alpha_M\beta_2$ or $\alpha_L\beta_2$ (Stefanidakis *et al*, 2003) may also localize MMP-9 to the cell membrane emphasizes that MMP-9 is likely to be associated to the cell surface by more than one mechanism. In addition, the association between CD44 and MMP-9 is selective and is not observed in all cells that express both molecules (Stamenkovic, 2000). Interestingly, in membrane extracts obtained from either TPA-treated HL-60 or THP-1 cells, MMP-9 was not detected in the anti-CD44 immunoprecipi-

tates, suggesting that CD44 is not a docking molecule for MMP-9 in this model system of macrophage-differentiated cells (data not shown). We have identified the hemopexin domain of MMP-9 as responsible for Ku binding. The domain of MMP-9 responsible for the interaction with the previously described docking molecules has not been characterized. It is noteworthy that it has been previously described that the other gelatinase, MMP-2, binds through its hemopexin domain to $\alpha v \beta 3$ integrin on melanoma cells and angiogenic blood vessels, thereby enhancing tumor growth (Brooks *et al*, 1996). In MMP-2 and MMP-9, the hemopexin domain provides a docking site for binding for the carboxyl-terminal domain of a TIMP molecule, forming a zymogen/inhibitor complex (Ellerbroek and Stack, 1999). Thus, interaction of MMP with docking molecules via the hemopexin domain might represent a way of localizing TIMP-free MMP to cell surfaces, thereby facilitating their activation and proteolytic activities.

Our results suggest that Ku by its ability to interact with MMP-9 at the cell surface is involved in the localized degradation of basement membrane. First, the colocalization of Ku and MMP-9 at the leading edge of both leukemic cells and migrating macrophages strongly suggests a role in ECM degradation. Second, the ability of TPA-treated THP-1 cells to invade collagen IV is inhibited by antibodies that recognized either the Ku70 or Ku80 subunit. The inhibitory effect of Ku antibodies in living cells selectively demonstrates the implication of Ku membrane form in the invasion process. Based on co-immunoprecipitation experiments of proteins expressed in yeast, we have demonstrated that a core region of Ku80 (aa 222–372) is involved in MMP-9 binding. Interestingly, collagen IV invasion was inhibited by an antibody (mAbS10B1, aa 8–221) that recognizes a short upstream region of the characterized domain involved in Ku80/MMP-9 interaction, whereas cell invasion was unaffected by an antibody that recognizes the C-terminal part of the Ku80 molecule (mAb111, aa 610–705). In addition, collagen IV invasion was also inhibited by an antibody that recognizes the Ku70 protein (mAbN3H10). This result, in addition to the fact that MMP-9 also interacts with the Ku70 subunit in yeast two-hybrid experiments, strongly suggests that MMP-9 interacts with more than one domain of the Ku heterodimer. In agreement with the results obtained with Ku antibodies, inhibition of Ku expression resulted in a significant inhibition of cell invasion on collagen IV matrices. The membrane-associated Ku appears to play unexpected roles in cellular invasion. Indeed, Ku has been shown to be involved in the regulation of cell–cell interaction (Teoh *et al*, 1998; Lynch *et al*, 2001) and in tumor cells when exposed to hypoxia to participate to a more migratory phenotype (Ginis and Fallor, 2000). We have recently demonstrated that the Ku protein is involved in cellular adhesion to fibronectin (Monferran *et al*, 2004). These results, in addition to our present data, suggest that the membrane-associated form of Ku might have a coordinated function in cell adhesive and proteolytic processes, as previously described for integrins such as $\alpha v \beta 3$ (Hood and Cheresch, 2002), and underline the important and unexpected role of Ku in the interaction between cells and the microenvironment. At present, it remains unknown why all primate cells that express Ku in the nucleus do not express it on the cell surface also. Ku has been detected on the cell surface in a variety of tumor cells, including leukemia and

solid tumor cell lines, and in normal cells Ku cell surface expression has been detected to date only on endothelial cells (Ginis *et al*, 1995). Our present study demonstrates that Ku is also expressed on the cell surface of primary human macrophages (see the results of immunofluorescence experiments; Figure 5D and E). In addition, when Ku expression was investigated in blood mononuclear cells obtained from healthy donors, we were able to show that monocytes, but not T and B lymphocytes, were positive for Ku cell surface expression (see results in Supplementary Figure 4), these results being in accordance with the data obtained with transformed cell lines (our present study; Ginis *et al*, 1995 and data not shown). None of the Ku subunits contain a transmembrane domain, which may explain their anchoring to the cell membrane. In agreement with a previous study (Lucero *et al*, 2003), Ku at the cell surface of THP-1 cells behaves like a peripheral, rather than integral, membrane protein (see Supplementary Figure 5). According to these results, our current hypothesis is that Ku is secreted and associated to integral membrane proteins, identification of Ku interacting protein at the cell surface being under investigation in our laboratory. Finally, within its different subcellular localization, Ku appears to be involved in both prosurvival and proinvasive pathways. Indeed, it is now clearly established that Ku, as a key component of the nonhomologous end joining pathway, sustains cell survival and the maintenance of genomic stability after exposure to exogenous DNA-damaging agents, or during endogenous oxidative metabolism, which is a significant source of DSB (Jackson, 2002). In addition, it has been recently demonstrated that Ku70 has a cytoprotective function in the cytosol via the suppression of the apoptotic translocation of Bax to mitochondria (Sawada *et al*, 2003). The dual role of Ku in prosurvival and proinvasive pathways might be of critical importance in tissue destructive processes.

Materials and methods

Antibodies

Anti-Ku70 (clone N3H10), anti-Ku80 (clone 111 or S10B1), anti-Ku70/80 (clone 162), anti-DNA-PKcs (clone 18-2) and anti-CD44 (clone 156-3C11) monoclonal antibodies were from Neomarkers (Fremont, CA). The monoclonal anti-MMP-9 (mAb13415) and anti-MMP-2 (mAb13405) antibodies were from Chemicon (Temecula, CA). Polyclonal rabbit antibodies anti-MMP-9 (Ab19047, Ab805 and Ab19092) and anti-MMP2 (Ab809) were from Chemicon (Temecula, CA). Anti-epitope tag HA (clone HA-7) and anti-epitope tag c-myc (clone 9E10) were from Sigma (Saint Louis, MO) and Clontech (Palo Alto, CA), respectively.

Two-hybrid screen and immunoprecipitation of epitope-tagged protein in yeast extracts

The Matchmaker GAL4 Two-Hybrid system 3 from Clontech (Palo Alto, CA) was used. *Saccharomyces cerevisiae* strain AH109 was transformed with bait plasmid—DBDKu80—by a modified lithium acetate method (Gietz *et al*, 1992). The human bone marrow cDNA library introduced into *S. cerevisiae* strain Y187 was obtained from Clontech. The Y187 strains were mated with AH109 strain containing DBDKu80 and the resulting diploids (approximately 10^7 independent diploid cells) were screened for His⁺, Ade⁺, β -gal⁺ phenotypes. A total of 19 yeast positive clones were obtained and one positive clone (clone 5F) corresponded to the aa residues 440–707 of the human MMP-9 sequence. To delineate the regions responsible for Ku/MMP-9 interaction, truncation mutants of DBDKu80 and 5F were constructed (see Supplementary material for cDNA construction strategies). For immunoprecipitations, AH109 cells expressing cmyc-Ku80, cmyc-Ku1, cmyc-Ku2, cmyc-

Ku3 or cmyc-Ku70 were mated with Y187 cells expressing HA-MMP-9, HA-5-F, HA-HX or HA-Linker. Isolated diploid colonies were grown to early log phase and extracts, prepared as previously described (Belenguer *et al*, 1997), were immunoprecipitated with indicated antibodies as previously described (Muller *et al*, 2001). In these experimental conditions, no interaction between MMP-9 and lamin C was detected (data not shown).

Cell culture and induction of differentiation

HL-60, U-937 and THP-1 cells (both obtained from ATCC) were grown in RPMI 1640 medium. For induction of differentiation, exponentially growing cells were incubated in the presence or not of TPA (Sigma, Saint Louis, MO) at 50 or 150 nM for 24 or 48 h. To determine expression and secretion of MMP-9, the supernatants were collected and the differentiated attached cells were removed from the culture dishes by scraping and extracted as described below. The isolation and culture of macrophages were performed as described previously (Astarie-Dequeker *et al*, 2002).

Whole-cell and membrane extraction

WCEs were prepared as previously described (Muller *et al*, 2001). The preparations of nuclear, cytosolic and membrane extracts were performed as described previously (Yu and Stamenkovic, 1999). The quality of the subcellular fractionation was assessed by immunoblot analysis of the different fractions using antibodies directed against the integral membrane protein CD44 and the nuclear PARP-1 protein (both obtained from Neomarkers).

Immunoprecipitations, immunoblot analysis, gelatin zymography and cell surface biotinylation

Immunoprecipitations and Western blots were performed as described previously (Muller *et al*, 2001). Gelatin zymography of protein extracts or aliquots of conditioned culture medium was performed as described previously (Yu and Stamenkovic, 1999). Biotinylation of cell surface proteins was performed as described previously (Akimov *et al*, 2000).

Flow cytometry

For flow cytometry, TPA-treated HL-60 cells (50 nM, 48 h) detached by Accutase (Uptima-Interchim, Montluçon, France) were incubated 1 h at 4°C with 20 µg/ml of the indicated antibodies or matched control isotypes at similar concentrations. After washing with PBS containing 0.5% BSA and 2% FCS, cells were incubated for 30 min at 4°C with secondary fluorescein-labelled IgG. After washing with PBS, cells were incubated with propidium iodide (10 µg/ml final concentration) at 4°C for 5 min. The cells were analyzed in FACScan flow cytometer (Becton Dickinson, Franklin Lanes, NJ). In these experiments, the cells that exhibited alteration in membrane permeability (Zuliani *et al*, 2003) (as assessed by propidium iodide-positive labelling) were excluded from the analysis (less than 10% in the three independent experiments performed, data not shown).

Immunofluorescence and confocal microscopy

HL-60 or THP-1 cells treated with TPA (50 nM, 72 h) or monocyte-derived macrophages grown on coverslips were fixed in 3.7% paraformaldehyde for 20 min at room temperature (RT). After blocking with 10% FCS and 2% BSA in PBS for 30 min, cells were incubated with a mixture (8 µg/ml) of anti-Ku80 antibody (clone S10B1) and anti-MMP-9 antibody (Ab805 (A) or Ab19092 (B-E)) at

4°C overnight. After washing, antibody binding was detected with FITC-conjugated goat anti-rabbit IgG (Sigma, St Louis, MO) and with rhodamine-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands) for 30 min at RT. As controls, cells were incubated either with primary or secondary antibodies alone. Confocal images were obtained by means of a confocal laser microscopy system (TCS, NT) (Leica, St-Gallen, Switzerland). Images were collected by scanning stained cells sequentially under × 40 objective lens. Images were processed for publication using the Adobe PhotoShop 5.0 software program.

Treatment of THP-1 cells with oligonucleotides

The phosphorotriate ASO targeted to human Ku80 messenger 101209 and its respective mismatch control ASO 111449 have been described previously (Belenkov *et al*, 2002). Exponentially growing THP-1 cells were exposed to the indicated concentrations of antisense and mismatch oligonucleotides combined with Oligofectamine (Invitrogen, CA). Since we observed that in leukemic cells, the apparent half-life of Ku80 protein was greater than 3 days (data not shown) in accordance to previous reports (Ajmani *et al*, 1995), an additional transfection was performed 72 h after the initiation of the first transfection, to assess the effect of Ku80 antisense treatment on Ku80 protein suppression and related phenotypic changes. Cells were then treated with TPA for 48 h and processed as described below for the invasion assays. In these experimental conditions, the total content of both Ku70 and Ku80 proteins was also decreased by about two-fold in THP-1 exposed to antisense, but not mismatch control, oligonucleotide (data not shown).

Cellular invasion

Cellular invasion was performed with type IV collagen (Sigma, St Louis, MO) and Transwell chambers (12 µm, Costar, Cole Palmer) as previously described (Ellerbroek *et al*, 2001). In certain conditions, cells were preincubated for 1 h at RT with EDTA (1 mM), GM6001 (10 µM), aprotinin (20 µg/ml), monoclonal antibodies directed against Ku70 (N3H10), Ku80 (S10B1, 111), MMP-9 (13415), MMP-2 (13405), control IgG at 20 µg/ml or heat denatured antibody against Ku80 (S10B1). Cells were allowed to migrate for 72 h, noninvading cells were removed from inner wells using a cotton swab and invading cells adherent to the bottom of the filter were fixed with 100% methanol and stained with toluidine blue. A total of 35–40 fields per filter were counted under a × 20 objective using an optical microscope. Three independent experiments were performed. Data were normalized to appropriate control and compared by Student's *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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