Dimerization and Nuclear Localization of Ku Proteins*

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Ku, a heterodimer of Ku70 and Ku80, plays a key role in multiple nuclear processes, e.g. DNA repair, chromosome maintenance, and transcription regulation. Heterodimerization is essential for Ku-dependent DNA repair in vivo, although its role is poorly understood. Some lines of evidence suggest that heterodimerization is required for the stabilization of Ku70 and Ku80. Here we show that the heterodimerization of these Ku subunits is important for their nuclear entry. When transfected into Ku-deficient xrs-6 cells, exogenous Ku70 and Ku80 tagged with green fluorescent protein accumulated into the nucleus, whereas each nuclear localization signal (NLS)-dysfunctional mutant was undetectable in the nucleus. Our results support the idea that each Ku can translocate to the nucleus through its own NLS. On the other hand, the nuclear accumulation of each NLS-dysfunctional mutant was markedly enhanced by the presence of an exogenous wild-type counterpart. In Ku-expressing HeLa cells, each NLS-dysfunctional mutant, as well as wild-type Ku70 and Ku80, was still detectable in the nucleus, whereas the double mutant of each Ku subunit with decreased functions of both nuclear targeting and dimerization was undetectable in the nucleus. Our results indicate that each Ku subunit can translocate to the nucleus not only through its own NLS but also through heterodimerization with each other.

Ku is a complex composed of two protein subunits of 70 and 80 kDa, hereafter designated as Ku70 and Ku80, respectively (1). It was shown that Ku is the DNA-binding component of a DNA-dependent protein kinase (DNA-PK)1 that phosphorylates several nuclear proteins in vitro, e.g. p53, RNA polymerase II, or Ku itself and is involved in DNA double-strand break (DSB) repair and V(D)J recombination (2, 3). Besides this main function, the Ku protein has other functions, some of which may be independent of DNA-PK activity. Both Ku70- and Ku80-knockout mice exhibited not only deficiencies in DNA DSB repair but also growth retardation (4, 5). In addition, Ku70-knockout mice have small populations of mature T lymphocytes and a significant incidence of thymic lymphoma, but Ku80-knockout mice do not. Ku70 has been reported to show Ku80-dependent and -independent DNA binding, whereas Ku80 requires association with Ku70 for DNA binding (12). In addition, Ku70 may have unique functions that are independent of Ku80.

Ku was originally reported to be a nuclear protein, consistent with its functions as a subunit of DNA-PK. On the other hand, although Ku is thought to be localized and to function only in the nucleus, several studies have revealed the cytoplasmic or cell surface localization of Ku proteins in various cell types (18–21). The subcellular localization of Ku70 and Ku80 changes during the cell cycle (22), and the nuclear translocation of Ku70 precedes that of Ku80 in late telophase/early G1 cells (23). Furthermore, changes in the subcellular localization of Ku could be controlled by various external growth-regulating stimuli (24). CD40L treatment of the myeloma cells induces a translocation of Ku from the cytoplasm to the cell surface, and that cell surface Ku can mediate both homotypic and heterotypic adhesion (25). Morio et al. (26) have reported that DNA-PK activity of human B cells is, at least in part, regulated by the nuclear translocation of Ku. These data suggest that the control mechanism for subcellular localization of Ku70 and Ku80 may be complex, at least in part, a key role in regulating the physiological function of Ku in vivo, although the mechanism is poorly understood.

We have recently identified nuclear localization signals (NLSs) of Ku70 and Ku80 (23, 27). The structures of the NLSs of the two Ku protein subunits are different. NLSs of Ku80 and Ku70 belong to the single-basic type and the variant bipartite-basic type, respectively (23, 27). We have also shown that both Ku70 and Ku80 can translocate to the nucleus without forming a heterodimer using their own NLS (23, 28). Moreover, the subcellular localization of Ku70 is affected by somatostatin embryo fibroblasts due to the rapid loss of proliferating cells and have shown signs of senescence (4, 5). However, this appears not to be the case for cs DNA-PK-knockout mice (6). These findings suggest that Ku plays some role in growth regulation and/or senescence independent of the function of DNA-PK.

Ku has been generally believed to always exist and function as a heterodimer. The heterodimerization is essential for DNA DSB repair in vivo and is also important in activating DNA-PK, which is one of the main functions of Ku (7). The interacting regions of Ku70 and Ku80 have been identified by many research groups (8–12), but the role of this interaction in the Ku functions remains unknown. Loss of one of the Ku subunits results in a significant decrease in the steady-state level of the other (5, 13, 14), suggesting that the heterodimerization is, in part, required for the stabilization of each Ku subunit. On the other hand, there are some differences in the phenotype between Ku70- and Ku80-knockout mice (4, 5, 15, 16). For example, Ku70-knockout mice have small populations of mature T lymphocytes and a significant incidence of thymic lymphoma, but Ku80-knockout mice do not. Ku70 has been reported to show Ku80-dependent and -independent DNA binding, whereas Ku80 requires association with Ku70 for DNA binding (12). In addition, Ku70 may have unique functions that are independent of Ku80.

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‡ The abbreviations used are: DNA-PK, DNA-dependent protein kinase; DSB, DNA double-strand break; NLS, nuclear localization signal; GFP, green fluorescent protein; PI, propidium iodide; EYFP, yellow variant GFP; ECFP, cyan variant GFP; EGFP, enhanced GFP; GST, glutathione S-transferase.
treatment in CV-1 cells, but that of Ku80 is not (24). These results suggest that the nuclear translocation of Ku70 and Ku80 may be independently regulated.

In the present study, we examined the subcellular localization of chimeric constructs of green fluorescent protein (GFP) color variants, and Ku proteins to which mutations were introduced by the site-directed mutagenesis technique, and found that Ku70 and Ku80 translocate to the nucleus not only through their own NLS but also through the heterodimerization, suggesting that the heterodimerization of Ku is important for their nuclear entry and functional regulation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cultures—**Cells of established hamster cell lines of CHO-K1 and xrs-6 (derived from the CHO-K1 cell line on the basis of their sensitivity to ionizing radiation) were cultured in Ham’s F-12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. The cell line of human tumor, HeLa-S3, has been described in previous studies (11, 27). The cells were maintained in a humidified incubator at 37 °C under 5% CO2. The xrs-6 cells defective in Ku80 were kindly provided by Dr. P. Jeggo (14, 29).

**Transfection of DNAs into HeLa and xrs-6 Cells—**Cells were plated on a six-well dish (Falcon, Lincoln Park, NJ) at a density of 2 × 10^5 cells/well the night before transfection. Transient transfections were performed in these cells using Effectene (Qiagen Inc., Chatsworth, CA) according to the manufacturer’s protocol. DNA was stained with 50 μg/mL propidium iodide (PI) (Sigma) containing 200 μM/ml RNase (Sigma). After incubation, the cells were fixed in 0.2 M phosphate buffer (pH 7.4) with 4% paraformaldehyde and then examined under an Olympus IX 70 fluorescence microscope (Olympus, Tokyo, Japan) to determine nuclear localization, as described previously (27). Images were acquired with a Hamamatsu chilled 3-chip color charge-coupled-device camera (C5810-01) driven by the IP Lab imaging software (Signal Analitics Corp., Vienna, VA).

**Plasmid Construction—**cDNA for human Ku70 and Ku80 was derived from pEGFP-Ku70(1–609) or pEYFP-Ku80(1–732) (23, 27). Full-length Ku70 or Ku80 was cloned in pECFP-C1 or pEYFP-C1 (CLONTECH, Palo Alto, CA) using a previously described method (pECFP-Ku70(1–609) and pEYFP-Ku80(1–732), respectively) (23, 27). The junctions of both constructs were verified by sequencing. Ku70- or Ku80-site-specific mutants were formed by incorporating mutant oligonucleotides by strand extension reactions. The QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer’s recommendations (23). Following the application of the mutagenesis strategy, each mutant was identified by DNA sequencing as described previously (23).

**Immunoblotting and Immunoprecipitation—**The immunoblotting analysis was performed as described previously (24, 27). In brief, total lysates from cells were boiled and cleared by centrifugation, and the supernatants were electrophoresed on 5–15% SDS-polyacrylamide gels. The fractionated products were electrotransferred onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking of nonspecific binding sites with 1% bovine serum albumin, the membranes were incubated with a goat anti-Ku70 polyclonal antibody (C-19), goat anti-Ku80 polyclonal antibody (M-20), and anti sera of a Japanese patient OM (which contained both anti-Ku70 and -Ku80 antibodies). The corresponding proteins were visualized using a ProteoBlot Western blot AP system (Promega, Madison, WI) according to the manufacturer’s instructions (30). Immunoprecipitation was performed as described previously (23). The Ku products were immunoprecipitated with an anti-Ku70 monoclonal antibody (NSH10), anti-Ku80 polyclonal antibody (AH51), or anti-Ku70/Ku80 monoclonal antibody (162) in combination with protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). They were subjected to 5–15% SDS-polyacrylamide gel electrophoresis, and the fractionated products were transferred to membranes and immunoblotted as described above.

**RESULTS**

**The Sole NLS of Each Ku Is Functional—**There are some lines of evidence that the control mechanism for subcellular localization of the two Ku subunits may play a key role in regulating the physiological function of Ku (23–26), although the mechanism is poorly understood. Previously, we identified each NLS of human Ku70 (amino acids 539–556) and human Ku80 (amino acids 561–569), suggesting that each Ku translocates to the nucleus through its own NLS (23, 27). To further investigate the nuclear translocation mechanism of Ku subunits, we first evaluated whether the EGFP fusion Ku proteins were produced and able to associate with the other Ku subunits in the CHO-K1 mutant xrs-6 cells, which have no Ku80 protein. Whole-cell extracts prepared from CHO-K1, xrs-6, and three xrs-6 transfectants contain pEGFP-C2, pEYFP-Ku70(1–609), or pEYFP-Ku80(1–732), respectively. In the lysate of xrs-6 cells transformed with pEGFP-Ku70(1–609), a signal of EGFP-Ku70 with the expected molecular weight was detected by immunoblotting using an anti-Ku70 polyclonal antibody (C-19), but not in the other lysates (Fig. 1A). Expectedly, EGFP-Ku80 was detected by immunoblotting with an anti-Ku80 polyclonal antibody (M-20) in the only lysate of xrs-6 cells transformed with pEYFP-Ku80(1–732) (Fig. 1B, lane 5). These results indicated that the expression of EGFP-Ku70 or -Ku80 fusion proteins is successful in xrs-6 cells (Fig. 1). As observed previously, each Ku subunit is required to stabilize each other, and the absence of the Ku80 protein in the xrs-6 cells has been shown to result in loss of the Ku70 protein (14, 31). In addition, reintroduction of the Ku80 gene restores the expression of the Ku70 protein in xrs-6 cells (14, 31). We confirmed that hamster Ku70 was detected in extracts prepared from the pEGFP-Ku80(1–732) transfectants and CHO-K1 cells (Fig. 1A, lanes 1 and 5), suggesting that the exogenous human Ku80 tagged with EGFP, as well as hamster Ku80, also stabilizes hamster Ku70. Expectedly, hamster Ku80 was not detected in the xrs-6 and their transfectants, whereas it was detected in the CHO-K1 cells (Fig. 1B).

The Ku70 NLS (amino acids 539–556) contains a cluster of basic amino acids (Fig. 2, A and C) (27). When the fusion products of the Ku70 NLS fragment-substituted single amino acid (K553A), GST and GFP (GST-Ku70[539–556], K553A-GFP), were microinjected into the cytoplasm of HeLa cells, the purified recombinant proteins of this mutant completely lost their nuclear localization activity (27). On the other hand, the same mutation in the full-length Ku70 (EGFP-Ku70[1–609], K553A) affected its nuclear localization activity in a transient expression assay, confirming that Ku70 NLS is important in the nuclear translocation of Ku70. However, this construct showed significant residual nuclear localization (28). On the basis of these findings, we considered the possibility that the mutation in the full-length Ku70 may not completely abolish the NLS function and/or that Ku80 may contribute to the localization. To address this possibility, we examined the subcellular localization of chimeric constructs of EGFP and human Ku proteins to which mutations were introduced by the site-
directed mutagenesis technique in the xrs-6 cells, which have no Ku80 protein and markedly depressed levels of Ku70. We first confirmed that the wild-type Ku70 fusion proteins (EGFP-Ku70(1–609)) accumulated within the nucleus in the xrs-6 cells, which have undetectable Ku80 (Fig. 2E, panel a), as shown in our previous report (28). Then, the expression vectors of the NLS-less deletion mutants, pEGFP-Ku70(1–609, Y534*) or pEGFP-Ku70(1–609, Y530*), were separately transfected into xrs-6 cells. Both mutant proteins have a severely decreased nuclear localization activity, but not completely lost (Fig. 2E, panels b and c). The expression vectors of the two Ku70 NLS mutants, pEGFP-Ku70(1–609, K542A/K553A), and pEGFP-Ku70(1–609, K542A/R543A/K553A), were separately transfected into xrs-6 cells. The EGFP-Ku70(1–609, K542A/R543A/K553A) lost its nuclear localization activity, whereas EGFP-Ku70(1–609, K553A/K556A) has a severely decreased nuclear localization activity but not completely lost (Fig. 2E, panels f and g). These results support the idea that a mutation in the full-length Ku70 (K553A) did not completely abolish the NLS function. Moreover, these results indicated that Ku70 has a functional NLS, pEGFP-Ku70(1–609, L388R) and pEGFP-Ku70(1–609, L413R) were separately transfected into xrs-6 cells. As the normal fusion protein EGFP-Ku70(1–609) did (Fig. 2E, panel a), the two mutant fusion proteins, EGFP-Ku70(1–609, L388R) and EGFP-Ku70(1–609, L413R), accumulated within the nuclei (Fig. 2E, panels d and e). In contrast, when pEGFP-Ku70(1–609, L388R/K542A/R543A/K553A) was introduced, the double mutant lost its nuclear localization activity (Fig. 2E, panel h) as the NLS mutant EGFP-Ku70(1–609, K542A/R543A/K553A) did (Fig. 2E, panel g). These results indicate that a single L388R or L413R mutation does not affect the nuclear localization activity in xrs-6 cells.

Using the same methods, we examined whether mutations within the NLS site (amino acids 561–569) of Ku80 impair nuclear translocation of Ku80 (Fig. 2, B and D) (23). The expression vectors of the four Ku80 NLS mutants, pEGFP-Ku80(1–732, P562A), pEGFP-Ku80(1–732, K568A), pEGFP-Ku80(1–732, P562A/K565A/K566A), and pEGFP-Ku80(1–732, K565A/K566A/K568A), were separately transfected into xrs-6 cells. Two mutant proteins (EGFP-Ku80(1–732, P562A/K565A/K566A) and EGFP-Ku80(1–732, K565A/K566A/K568A)), which have triple mutations, lost their nuclear localization activity (Fig. 2F, panels d and e), indicating that the NLS was functional in Ku80. In contrast, EGFP-Ku80(1–732, K568A) was detected in both the nucleus and the cytoplasm (Fig. 2F, panel c), suggesting that the single mutation did not completely abolish the NLS function of Ku80. Moreover, EGFP-Ku80(1–732, K568A), as well as the wild-type Ku80 fusion protein (EGFP-Ku80(1–732)), was detected in the nucleus (Figs. 2F, panels a and b), suggesting that the single mutation did not affect the NLS function of Ku80. These results suggest that the nuclear transport of exogenous human Ku80 is not affected by dimerization with endogenous hamster Ku70, although the exogenous human Ku80 is also involved in stabilizing hamster Ku70 (Fig. 1). Next, pEGFP-Ku80(1–732, A453H/V454H) was transfected into xrs-6 cells. As the normal fusion protein EGFP-Ku80(1–732) did (Fig. 2F, panel a), the mutant proteins accumulated within the nuclei (Figs. 2F, panel f). In contrast, when pEGFP-Ku80(1–732, A453H/V454H/K565A/K566A) was introduced, the double mutant lost its nuclear localization activity (Fig. 2F, panel g) as the NLS mutant EGFP-Ku80(1–732, K565A/K566A/K568A) did (Fig. 2F, panel e). These results indicate that the A453H and V454H mutations do not affect the nuclear localization activity of Ku80 in xrs-6 cells. When pEGFP-Ku80(1–732, P410L) or pEGFP-Ku80(1–732, P410L/H411Y) was separately transfected into xrs-6 cells, both mutant proteins accumulated within the nuclei, suggesting that the P410L and H411Y mutations do not also affect the nuclear localization activity of Ku80 in xrs-6 cells (Fig. 2F, panels h and i). On the other hand, when the empty vector (pEGFP) was transfected into xrs-6 cells, EGFPs were localized throughout the cell (Fig. 2E, panel i), because they have a small molecular mass, which enables them to enter the nucleus by passive diffusion.

**Nuclear Entry of the Ku Mutants Lacking the Functional NLS in HeLa Cells**—As described above, each Ku has a functional NLS. We examined whether mutations specifically within their NLS sites impair nuclear translocation in human cells, which express Ku70 and Ku80 proteins. We first confirmed that the wild-type Ku70 fusion proteins (EGFP-Ku70(1–609)) accumulated within the nucleus (Fig. 3A, panel a) as shown in our previous report (27). Next, the expression vectors of the two Ku70 NLS mutants, pEGFP-Ku70(1–609, K553A/K556A) and pEGFP-Ku70(1–609, K542A/R543A/K553A), were
FIG. 3. Subcellular localization of EGFP-tagged wild-type Ku and their NLS mutant proteins in HeLa cells. Site-specific mutants of Ku70 or Ku80 were constructed in pEGFP-C2 as described under “Experimental Procedures.” Each mutant was transfected and expressed in HeLa cells. For the same cells, EGFP images (green) and PI-stained DNA images (red) are shown alone or merged as indicated. A: panel a, pEGFP-Ku70(1–609); panel b, pEGFP-Ku70(1–609, Y534*); panel c, pEGFP-Ku70(1–609, Y530*); panel d, pEGFP-Ku70(1–609, K553A/K556A); panel e, pEGFP-Ku70(1–609, K542A/R543A/K553A). Arrowheads indicate mainly nuclear localization (panels d and e); arrows indicate mainly cytoplasmic localization (panels b and c). B: panel a, pEGFP-Ku80(1–732); panel b, pEGFP-Ku80(1–732, P562A); panel c, pEGFP-Ku80(1–732, K568A); panel d, pEGFP-Ku80(1–732, P562A/K565A/K566A); panel e, pEGFP-Ku80(1–732, K565A/K566A/K568A). Amino acids changed are designated by listing the wild-type residue, the amino acid positions, and then the introduced mutant amino acid. Asterisks denote the introduction of a stop codon.

FIG. 4. Interaction of wild-type Ku and EGFP-tagged Ku mutant proteins in HeLa cells. Immunoblotting anti-Ku antiserum (Japanese patient OM) from immunoprecipitation with antibodies against Ku70 (N3H10) (lanes 1, 3, 6, 8, 10, 12, 14, and 16), Ku80 (AHP317) (lanes 4, 7, 9, 11, 13, and 17), and Ku70/Ku80 (lanes 2 and 5). Each Ku protein is indicated (Ku70, wild-type Ku70; Ku80, wild-type Ku80; Ku70*, wild or mutant Ku70-EGFP fusion protein; Ku80*, mutant Ku80-EGFP fusion protein). Lanes 1 and 2, pEGFP-Ku70(1–609)-transfected cells; lanes 3–5, pEGFP-Ku70(1–609, K542A/R543A/K553A)-transfected cells; lanes 6 and 7, pEGFP-Ku70(1–609, L385R/K542A/R543A/K553A)-transfected cells; lanes 8 and 9, pEGFP-Ku80(1–732, K565A/K566A/K568A)-transfected cells; lanes 10 and 11, pEGFP-Ku80(1–732, A453H/V454H/K565A/K566A/K568A)-transfected cells; lanes 12 and 13, pEGFP-Ku80(1–732, P410L)-transfected cells; lanes 14 and 15, pEGFP-Ku80(1–732, P410L)-transfected cells; lanes 16 and 17, pEGFP (Control)-transfected cells.
antibody (Fig. 4, lane 10). As shown in Fig. 4 (lanes 9 and 11), when the extracts were immunoprecipitated with the anti-Ku80 antibody, wild-type Ku70, Ku80, and EGFP-Ku80(1–732, K565A/K566A/K568A) or EGFP-Ku80(1–732, A453H/V454H/K565A/K566A/K568A) fusion proteins coprecipitated. These results indicate that mutations of Ku80 at amino acids 453, 454, 556, and 568, but not at amino acids 565, 566, and 568, significantly impaired the interaction of Ku80 with Ku70. Recently, Singleton et al. (29) reported that a mutation in hamster Ku80 corresponding to amino acid 410 abolished its ability to interact with Ku70 in the reticulocyte lysate in vitro translation system. We examined whether the same and other mutations in the EGFP-tagged human Ku80 abrogated its interaction with human Ku70 in human cells. When extracts of pEGFP-Ku80(1–732, P410L) or pEGFP-Ku80(1–732, P410L/H411Y) transformants were immunoprecipitated, wild-type Ku70, Ku80, and EGFP-Ku80(1–732, P410L) or EGFP-Ku80(1–732, P410L/H411Y) fusion proteins immunoprecipitated with the anti-Ku80 antibody (Fig. 4, lanes 13 and 15). In contrast, the two fusion proteins were hardly detected by immunoprecipitation with the anti-Ku70 antibody (Fig. 4, lanes 12 and 14). These results indicate that the mutations of human Ku80 significantly impaired its interaction with Ku70. On the other hand, when the empty vector (pEGFP) was transfected, only the wild-type Ku proteins immunoprecipitated as expected (Fig. 4, lanes 16 and 17).

We examined whether the Ku70 double mutant lacking both the functional NLS and the ability of heterodimerization is localized in the nucleus. When transfected into HeLa cells, the double mutant EGFP-Ku70(1–609, L385R/K542A/R543A/K553A) was detected in the cytoplasm (Fig. 5b). In contrast, the EGFP-Ku70(1–609, L385R) accumulated within the nucleus (Fig. 5a) as shown in our previous report (28). In addition, EGFP-Ku70(1–609, K542A/R543A/K553A) accumulated mainly in the nucleus in a large number of cells (Fig. 3a, panel e) (data not shown). These results suggest that the EGFP-Ku70(1–609, K542A/R543A/K553A), at least a part of it, can translocate to the nucleus through the heterodimerization with Ku80 in HeLa cells.

We next examined whether the Ku70 double mutant lacking both the functional NLS and the ability of heterodimerization is localized in the nucleus. EGFP-Ku80(1–732, A453H/V454H/K565A/K566A/K568A), when transfected into HeLa cells, was detected in the cytoplasm (Fig. 5d), whereas the EGFP-Ku80(1–732, K565A/K566A/K568A) and EGFP-Ku80(1–732, A453H/V454H) accumulated within the nucleus (Figs. 3b, panel e, and 5c) (23), suggesting that Ku80 can translocate to the nucleus through the heterodimerization with Ku70 independent of its NLS. On the other hand, when pEGFP was transfected into HeLa cells, EGFPs were distributed throughout the cell (Fig. 5g) as described previously (27).

Previously, we reported that a Ku80 mutant lacking the ability of heterodimerization is localized in the nucleus, suggesting that Ku80 can translocate to the nucleus without binding with Ku70 (23). We confirmed whether other Ku80 mutants lacking the ability of heterodimerization are localized in the nucleus (Fig. 4, lanes 12–15, and Fig. 5). When transfected into HeLa cells, the two mutants, EGFP-Ku80(1–732, P410L) and EGFP-Ku80(1–732, P410L/H411Y), were detected in the nucleus (Fig. 5, e and f), supporting the idea that Ku80 can translocate to the nucleus without binding with Ku70.

The Role of Heterodimerization in Nuclear Entry—As mentioned above, our findings suggest the possibility that Ku70 and Ku80 can translocate to the nucleus not only through their own NLS but also through heterodimerization. To further confirm this, the nuclear distribution of Ku70 and Ku80 was compared by coexpression of the two proteins. A yellow variant GFP (EYFP)-Ku80 and a cyan variant GFP (ECFP)-Ku70 were transiently expressed in xrs-6 cells. Fluorescence images of the GFP color variants were captured separately using appropriate filter sets (Fig. 6). First, the ECFP-tagged wild-type Ku70 (ECFP-Ku70(1–609)), the EYFP-tagged wild-type Ku80 (EYFP-Ku80(1–732)), or the EYFP-tagged Ku80 mutant (EYFP-Ku80(1–732, A453H/V454H)) translocated alone in the xrs-6 cells. As each ECFP-tagged fusion protein did in the xrs-6 cells (Fig. 2: E, panel a; F, panel a; and Fig. 5c), each variant GFP fusion protein accumulated within the nucleus (data not shown). Next, the NLS-dysfunctional mutant (ECFP-Ku70(1–609, K542A/R543A/K553A) or EYFP-Ku80(1–732, K565A/K566A/K568A)) or double mutant lacking both nuclear targeting and dimerization functions (ECFP-Ku70(1–609, L385R/K542A/R543A/K553A) or EYFP-Ku80(1–732, A453H/V454H/K565A/K566A/K568A)) transiently expressed in the xrs-6 cells. As each EGFP-tagged fusion protein did (Fig. 2: E, panel g; F, panel h; F, panels e and g), each variant GFP fusion protein lost its nuclear localization activity (data not shown). These results indicate that their distribution was not due to the tag. When EYFP-Ku80(1–732) and ECFP-Ku70(1–609) were coexpressed, the ECFP-Ku70(1–609) accumulated within the nucleus (Fig. 6, a and a’). On the other hand, when EYFP-Ku80(1–732) and the NLS-dysfunctional Ku70 mutant were coexpressed, the ECFP-Ku70(1–609, K542A/R543A/K553A) accumulated within the nucleus (Fig. 6, b and b’), supporting the idea that Ku70 can translocate to the nucleus independent of its own NLS. In addition, when the
Ku subunits. Ku80 plays an important role in the nuclear translocation of these proteins; results suggest that the heterodimerization between Ku70 and Ku80 is dependent on its interaction with the other subunit. We have also found that the heterodimerization between Ku70 and Ku80 can translocate from the cytoplasm to the nucleus without forming a heterodimer using their own NLS. Irradiation resulted in an up-regulation of the cellular level of Ku70, but not that of Ku80, and Ku70 accumulated within the nucleus (33). Moreover, the subcellular localization of Ku70 was affected by somatostatin treatment in CV-1 cells, but that of Ku80 was not (24). In addition, the nuclear translocation of Ku70 preceded that of Ku80 at the late telephase/early G1 phase during the cell cycle (23). Each Ku subunit may have a functional NLS to perform unique functions, which are independent of each other, although further studies will be necessary to confirm this. As described above, nuclear translocation of Ku70 and Ku80 can be independently regulated in vivo. The structures of NLSs of both Ku protein subunits are quite different, and NLSs of Ku80 and Ku70 are of the single-basic type and the variant bipartite-basic type, respectively (23, 27). Thus, we speculate that the nuclear translocation of Ku proteins is controlled at least in part at the NLS recognition step and that this is regulated by NLS receptors with various specificities in vivo.

We have also found that the heterodimerization between Ku70 and Ku80 plays an important role in their nuclear translocation. When two NLS-disfunctional Ku80 mutants were coexpressed, the EGFP-Ku80(1–732, K565A/K566A/K568A) and EGFP-Ku80(1–732, A453H/V454H/K565A/K566A/K568A) did not accumulate within the nucleus (Fig. 6, panels e and f), suggesting that the nuclear translocation of Ku80 through the heterodimerization with Ku70 is dependent on Ku70 NLS. On the other hand, when the Ku70 and Ku80 double mutants were coexpressed, both proteins localized in the cytoplasm as expected (Fig. 6, f and f’). In control experiments, when EGFP and EYFP were cotransfected into xrs-6 cells, both GFP-variant proteins localized throughout the cell (Fig. 6, g, g’, and g”). Taken together, these results suggest that the heterodimerization between Ku70 and Ku80 plays an important role in the nuclear translocation of these Ku subunits.

**DISCUSSION**

In general, it is known that nuclear proteins containing an intrinsic NLS enter the nucleus associated with NLS receptors (e.g., importin α/β) through their own NLSs (32). We had previously reported that Ku70 and Ku80 have an intrinsic NLS and that the NLS receptor can recognize the NLSs of Ku70 and Ku80 (23, 27). In this study, we have further examined the molecular basis of the regulation of Ku70 and Ku80 subcellular localization using site-directed mutagenesis. In agreement with our previous observations, we have found that each Ku subunit can translocate to the nucleus through its own NLS (23, 28). Our data have shown that each Ku subunit can translocate to the nucleus independent of its own NLS and that this translocation is dependent on its interaction with the other subunit.

Ku has been generally considered to always form and function as a heterodimer. In studies using knockout mice, however, inactivation of Ku70 resulted in some phenotype distinct from that of Ku80-knockout mice (4, 5, 15, 16), suggesting the possibility that Ku70 and Ku80 have unique functions that are independent of each other. Recently, we identified the NLSs of human Ku70 (amino acids 539–556) and Ku80 (amino acids 561–569) (23, 27). In this study, the EGFP-Ku80 fusion protein accumulated within the nuclei of xrs-6 cells (Fig. 2F, panel a). Furthermore, the NLS-disfunctional mutant (EGFP-Ku80(1–732, K565A/K566A/K568A) or EGFP-Ku80(1–732, P562A/K565A/K566A)) lost its nuclear localization activity in the xrs-6 cells (Fig. 2F, panels d and e), indicating that Ku80 contains a classical NLS and that this NLS is functional. In this and previous reports, we also showed that the EGFP-Ku70 fusion protein accumulated within the nuclei of xrs-6 cells (Fig. 2E, panel a) (28). On the other hand, the NLS-disfunctional mutant, EGFP-Ku70(1–609, K542A/R543A/K553A), lost its nuclear localization activity in the xrs-6 cells (Fig. 2E, panel g). These findings support the idea that both Ku70 and Ku80 can translocate from the cytoplasm to the nucleus without forming a heterodimer using their own NLS. Irradiation resulted in an up-regulation of the cellular level of Ku70, but not that of Ku80, and Ku70 accumulated within the nucleus (33). Moreover, the subcellular localization of Ku70 was affected by somatostatin treatment in CV-1 cells, but that of Ku80 was not (24). In addition, the nuclear translocation of Ku70 preceded that of Ku80 at the late telephase/early G1 phase during the cell cycle (23). Each Ku subunit may have a functional NLS to perform unique functions, which are independent of each other, although further studies will be necessary to confirm this. As described above, nuclear translocation of Ku70 and Ku80 can be independently regulated in vivo. The structures of NLSs of both Ku protein subunits are quite different, and NLSs of Ku80 and Ku70 are of the single-basic type and the variant bipartite-basic type, respectively (23, 27). Thus, we speculate that the nuclear translocation of Ku proteins is controlled at least in part at the NLS recognition step and that this is regulated by NLS receptors with various specificities in vivo.

We have also found that the heterodimerization between Ku70 and Ku80 plays an important role in their nuclear translocation. When two NLS-disfunctional Ku80 mutants were coexpressed into HeLa cells, both EGFP-Ku80(P562A/K565A/K566A) and EGFP-Ku80(K565A/K566A/K568A) translocated to the nucleus (Fig. 3B, panels d and e). However, EGFP-Ku80(A453H/V454H/K565A/K566A/K568A), which was a double mutant lacking both nuclear targeting and dimerization functions, did not translocate to the nucleus (Fig. 5d). These results suggest that endogenous Ku70 transports the NLS-disfunctional Ku80 mutants into the nucleus in HeLa cells. In cotransfection analysis using xrs-6 cells, the Ku80 mutant lack-
ing only the nuclear targeting function was localized in the nucleus by the exogenous wild-type Ku70 (Fig. 6, e and e'). In addition, when the same Ku80 mutant and NLS-dysfunctional Ku70 mutant (ECFP-Ku70/1–609, K542A/R543A/K553A) were coexpressed, EYFP-Ku80/1–732, K565A/K566A/K568A) did not accumulate within the nucleus (Fig. 6, d and d'). These results indicate that Ku70 transport Ku80 into the nucleus via its own NLS in xrs-6 cells. In this manner, one role of Ku70 may be to regulate the nuclear translocation of Ku80. On the other hand, the NLS-dysfunctional Ku70 mutant, but not the double mutant lacking both nuclear targeting and dimerization functions, was localized into the nucleus due to the presence of exogenous wild-type Ku80 (Fig. 6, b, b', c, and c'). When the same Ku70 mutant and NLS-dysfunctional Ku80 mutant (EYFP-Ku80/1–732, K565A/K566A/K568A) were coexpressed, ECFP-Ku70/1–609, K542A/R543A/K553A) did not accumulate within the nucleus (Fig. 6, d and d'). These results indicate that Ku80 can transport Ku70 into the nucleus via its own NLS in xrs-6 cells. However, the NLS-dysfunctional Ku80 mutant, which can still interact with Ku80, did not completely localize to the nucleus despite the presence of endogenous Ku80 in HeLa cells (Fig. 3a, panels d and e). The half-life of the Ku80 monomeric form is less than 2 h (34), whereas that of the EGFP-tagged protein was more than 24 h (17). Although the discrepancy between the results in HeLa and in xrs-6 cells remains unclear, this may be due to the differences in half-life between endogenous Ku80 in HeLa cells and exogenous EGFP-tagged Ku80 in xrs-6 cells. Alternatively, in HeLa cells, the Ku70 localization may be dependent not only on the nuclear import mechanism but also on the nuclear export mechanism. Further studies will be necessary to confirm that Ku80 transport Ku70 into the nucleus in vivo.

Ku70 and Ku80 play an important role in DNA DSB repair and V(D)J recombination in vivo (4, 5, 15, 16). Heterodimerization between Ku70 and Ku80 is essential for Ku-dependent DNA DSB repair in vivo (7), but the role of this interaction in Ku functions remains unknown. It is also reported that the heterodimerization is required for the stabilization of each Ku subunit (5, 13, 14). On the other hand, Morio et al. (26) reported that the DNA-PK activity of human B cells is, at least in part, regulated by the nuclear translocation of Ku. Recently, we have generated cell lines stably expressing the wild-type Ku80 (EGFP-Ku80) or NLS-dysfunctional Ku80 mutant tagged with EGFP (EGFP-Ku80(K565A/K566A/K568A)). We have found that the tagged wild-type Ku80 protein can complement a deficiency of the DNA DSB repair of xrs-6 cells (data not shown). In contrast, the tagged NLS-dysfunctional Ku80 mutant protein cannot complement a deficiency of the DNA DSB repair of xrs-6 cells, although the Ku80 mutant protein is stabilized by tagged with EGFP (data not shown).

In conclusion, we have shown a novel role of the heterodimerization of Ku70 and Ku80. Ku70 and Ku80 appear to have multiple functions as a monomeric form and a heterodimeric form. We speculate that the Ku subunits may use the NLS-dependent nuclear translocation pathway to perform some function(s) independent of each other, and Ku subunits may use the nuclear translocation pathway through heterodimerization to perform the same functions dependent on each other. The control mechanism for nuclear localization of Ku70 and Ku80 appear to play, at least in part, a key role in regulating the physiological function of Ku in vivo. Further studies to elucidate the molecular mechanisms of nuclear transport of the Ku subunits will lead to a better understanding of the regulation mechanism of nuclear proteins.

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