Src family tyrosine kinases are widely expressed in many cell types and participate in a variety of signal transduction pathways. Despite the significance of Src in suppression of apoptosis, its mechanism remains poorly understood. Here we show that Src acts as an effector for Ku70-dependent suppression of apoptosis. Inhibition of endogenous Src activity promotes UV-induced apoptosis, which is impaired by Ku70 knockdown. Src phosphorylates Ku70 at Tyr-530, being close to the possible acetylation sites involved in promotion of apoptosis. Src-mediated phosphorylation of Ku70 at Tyr-530 decreases acetylation of Ku70, whereas Src inhibition augments acetylation of Ku70. Importantly, knockdown-rescue experiments with stable Ku70 knockdown cells show that the nonphosphorylatable Y530F mutant of Ku70 reduces the ability of Ku70 to suppress apoptosis accompanied by augmentation of Ku70 acetylation. Our results reveal that Src plays a protective role against hyperactive apoptotic cell death by reducing apoptotic susceptibility through phosphorylation of Ku70 at Tyr-530.

Protein-tyrosine phosphorylation is one of the key posttranslational modifications that controls a wide variety of cellular events, such as cell proliferation, differentiation, and cell adhesion (1, 2). The cross-talk between different types of posttranslational modifications is reported to include the interactions between phosphorylation events, between ubiquitination, and between phosphorylation and SUMOylation (3–5).

Src-family tyrosine kinases, which are non-receptor-type kinases, consist of proto-oncogene products and structurally related proteins and include at least 8 highly homologous proteins: c-Src, Lyn, Fyn, c-Yes, c-Fgr, Hck, Lck, and Blk (6, 7). c-Src, Lyn, Fyn, and c-Yes are widely expressed in various cell types, whereas c-Fgr, Hck, Lck, and Blk are found primarily in hematopoietic cells (7). Multiple combinations of Src members are expressed in many cell types and are involved in individual and overlapping signaling pathways (6, 7).

It is widely accepted that Src members are predominantly located at the cytoplasmic face of the plasma membrane through posttranslational myristoylation and palmitoylation, but in fact appreciable fractions are found in a variety of intracellular locations, such as late endosomes, lysosomes, the Golgi apparatus, secretory granules, and the nucleus (8–20). We have shown that Lyn, a Src member, is imported into and rapidly exported from the nucleus (21) and that nuclear tyrosine phosphorylation has a role in global changes of chromatin structure and histone modifications (13, 22–24). To explore in detail the roles of protein-tyrosine phosphorylation in cell functions, we recently performed phosphoproteomic analyses (5, 25) and revealed novel roles for tyrosine phosphorylation of nuclear proteins, such as the heterochromatin protein KAP1 (KRAB-associated protein 1)/TIF1β/TRIM28 (5), the transcription factors JunB (26) and FoxA1 (27), and the chromatin-associated protein A-KAP8 (A-kinase anchoring protein 8) (24).

Src has been reported to suppress apoptosis by down-regulating proapoptotic genes (28, 29) and up-regulating anti-apoptotic genes (30, 31). Although Src is well known as an activator of the major downstream pathways involved in growth and survival signaling, such as the Ras-mitogen-activated-protein-kinase (Ras-MAPK) pathway, the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) pathway, and the STAT3 pathway (32–35), it was reported that caspase-8 and the growth arrest and DNA damage protein 34 (GADD34) are associated with suppression of apoptosis through Src-mediated tyrosine phosphorylation (36, 37). Despite the significance of Src in suppression of apoptosis, the mechanism of Src-mediated suppression of apoptosis remains poorly understood.

In this study we identify Ku70 as a Src substrate involved in apoptosis signaling. Inhibition of the kinase activity of endogenous Src enhances the level of apoptosis upon apoptotic stimulation. Phosphorylation of Ku70 at Tyr-530 by

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* This work was supported in part by Grants-in-aid for Scientific Research 15K07922 (to Na. Y.) and 16K08227 (to No. Y.) and the LGS Program for Leading Graduate Schools (Chiba University Nurture of Creative Research Leaders in Immune System Regulation and Innovative Therapeutics), the Global COE Program (Global Center for Education and Research in Immune Regulation and Treatment) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Chiba Foundation for Health Promotion and Disease Prevention (to No. Y.), and the Japan Foundation for Applied Enzymology (to Na. Y.). The authors declare that they have no conflicts of interest with the contents of this article.

1 LGS research assistants supported by Grants-in-aid for the Japan Society for the Promotion of Science (JSPS) Research Fellow 16J04363 (to M. M.) and 16J04141 (to T. H.).

2 Global COE research assistants.

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Src inhibits acetylation of Ku70, resulting in suppression of apoptosis. Our results provide the first demonstration of a novel role of Ku70’s tyrosine phosphorylation in suppression of apoptosis.

Results

Tyrosine Phosphorylation of Ku70—We recently performed phosphoproteomic analyses of tyrosine-phosphorylated proteins in HeLa S3 cells overexpressing the Src-family kinase Lyn (5). Purified tyrosine-phosphorylated proteins using anti-phosphotyrosine (Tyr(P)) antibody were digested with trypsin. We found that the tyrosine-phosphorylated peptides detected by LC/MS/MS contained Ku70, a ubiquitously expressed protein, as a candidate substrate of Src-family kinases. To examine whether Ku70 was actually tyrosine-phosphorylated by Src-family kinases, cells were cotransfected with myc-tagged wild-type Ku70 (myc-Ku70-WT)\(^5\) plus c-Src or myc-Ku70-WT plus c-Src(kinase-dead (KD)) in the presence or absence of the Src inhibitor PP2, and cell lysates were subjected to immunoprecipitation and Western blotting analysis. In fact, myc-Ku70-WT was capable of being tyrosine-phosphorylated by c-Src but not c-Src(KD), and PP2 treatment inhibited tyrosine phosphorylation of myc-Ku70-WT (Fig. 1A).

\(^5\) The abbreviations used are: Ku70-WT, wild-type Ku70; Ku70-YF, Ku70-Y530F mutant; Ku70-YE, Ku70-Y530E mutant; KD, kinase dead; NHEJ, non-homologous end joining; ADR, adriamycin; TR, tetracycline repressor; Dox, doxycycline; ROS, reactive oxygen species; NAC, N-acetylcysteine; PKcs, protein kinase catalytic subunit; NLS, nuclear localization sequence.
Tyrosine Phosphorylation of Ku70

A

myc

DNA binding
Ku80 binding
Bax interaction
flexible linker
LVYPDPYNPEGKVTKRKHDEGSGSKRPKVE

myc-Ku70-WT
myc-Ku70-Y530F

B

COS-1

Input

IP: anti-myc

WB: anti-myc

anti-pTyr

anti-Src

myc-Ku70-WT
myc-Ku70-Y530F

myc-Ku70-WT or myc-Ku70-Y530F

myc-Ku70-WT or myc-Ku70-Y530F

Relative tyrosine phosphorylation

FIGURE 2. Tyrosine phosphorylation of Ku70 at Tyr-530. A, schematic representations of myc-Ku70-WT and myc-Ku70-Y530F. Gray bars indicate the sites of tyrosine residues on Ku70. myc, myc tag; DNA binding, DNA binding domain; Ku80 binding, Ku80 binding domain; Bax interaction, Bax-interaction domain; flexible linker, the C-terminal flexible linker region. B, COS-1 cells cotransfected with c-Src plus the indicated plasmid were cultured for 24 h. myc-Ku70-WT and myc-Ku70-Y530F were immunoprecipitated (IP) with anti-myc antibody. Western blotting (WB) was performed with the indicated antibodies. The level of tyrosine phosphorylation of myc-Ku70-Y530F is expressed as the value relative to that of myc-Ku70-WT after normalization with the protein levels of myc-Ku70-WT and myc-Ku70-Y530F.

Tyrosine Phosphorylation of Ku70 at Tyr-530—Our recent phosphoproteomic analysis (5) showed a tyrosine-phosphorylated peptide of Ku70 that contains phospho-Tyr-530 (518LGSLVDEFKELVpYPPDYNPEGK539; pY, phospho-Tyr). A recent study showed that NIH3T3 cells transformed by src and pp60c-Src have increased nuclear tyrosine phosphorylation of Ku70 compared with parental NIH3T3 cells (25). Furthermore, 1270F, a Src-overexpressing fibrosarcoma cell line, shows increased tyrosine phosphorylation of Ku70. These results suggest that Ku70 directly tyrosine-phosphorylates Ku70.

Tyrosine Phosphorylation of Ku70 at Tyr-530—Our recent phosphoproteomic analysis (5) showed a tyrosine-phosphorylated peptide of Ku70 that contains phospho-Tyr-530 (518LGSLVDEFKELVpYPPDYNPEGK539; pY, phospho-Tyr). A recent study showed that NIH3T3 cells transformed by src and pp60c-Src have increased nuclear tyrosine phosphorylation of Ku70 compared with parental NIH3T3 cells (25). Furthermore, 1270F, a Src-overexpressing fibrosarcoma cell line, shows increased tyrosine phosphorylation of Ku70. These results suggest that Ku70 directly tyrosine-phosphorylates Ku70.

Lack of Association of Ku70 Phosphorylation at Tyr-530 with Non-homologous End Joining (NHEJ) Repair—It is well known that Ku70 majorly forms a heterodimer with Ku80, which is required for repairing DNA double-strand breaks through NHEJ (38–40). To examine whether tyrosine phosphorylation of Ku70 could associate with NHEJ repair, we analyzed the formation of the Ku70/Ku80 heterodimer by coimmunoprecipitation of myc-Ku70 with FLAG-Ku80. In fact, expression of nucleo-localized Lyn (nuclear-localized sequence-tagged Lyn (NLS-Lyn)) did not affect heterodimerization of Ku70 with Ku80 (Fig. 3, A and B), although Ku70 and Ku80 were both tyrosine-phosphorylated upon NLS-Lyn expression (Fig. 3B). Because the Ku70/Ku80 heterodimer recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to DNA ends to form the active DNA-PKcs-Ku70/Ku80 complex (38–40), we treated myc-Ku70- and FLAG-Ku80-coexpressing cells with adriamycin (ADR) and examined whether tyrosine phosphorylation of Ku70 could affect the level of DNA-PKcs phosphorylation at Ser-2056, which is required for NHEJ repair (41, 42) (Fig. 3C). The phosphorylation levels of DNA-PKcs were not changed by NLS-Lyn expression upon ADR treatment (Fig. 3D). These results suggest that tyrosine phosphorylation of Ku70 at Tyr-530 does not appear to associate with NHEJ repair.

Involvement of Src and Ku70 in Suppression of Apoptosis—Previous studies reported that Ku70 plays a role in suppression of apoptosis (43–48). To understand the regulation of the ability of Ku70 to suppress apoptosis, we attempted to establish stable Ku70-knockdown cell lines. We first constructed two short-hairpin RNAs (shRNAs) targeting the sequences in the coding sequence (shKu70-CDS) and the 3′-untranslated region (shKu70–3′-UTR) of human Ku70 (see “Experimental Procedures”), and transiently transfected HeLa S3 cells with shKu70-CDS or shKu70–3′-UTR. At 48 h after transfection, Ku70 immunostaining showed that expression of shKu70-CDS greatly decreased the level of endogenous Ku70 compared with that of shKu70–3′-UTR. Nonetheless, the use of shKu70–3′-UTR, but not shKu70-CDS, enabled us to establish stable Ku70-knockdown cell lines (HeLa S3/shKu70) (Fig. 4, A and B), implying that a drastic decrease in endogenous Ku70 expression may affect cell proliferation. This observation is supported by previous studies that no Ku70-null human HCT116 clones were established (49). Then, using HeLa S3/shKu70 cells, we examined the effect of Ku70 knockdown on UV-induced apoptosis. We treated parental HeLa S3 cells and HeLa S3/shKu70 cells with UV light and measured the percentage of apoptotic cells by staining for cleaved caspase-3 and/or nuclear condensation/fragmentation (Fig. 4C). It was found that Ku70 knockdown potentiated UV-induced apoptosis prominently at low doses of UV (Fig. 4D), in agreement with previous studies (43).

A recent study showed that NIH3T3 cells transformed by expression of a temperature-sensitive v-Src mutant were strongly resistant to a variety of apoptosis inducers, including UV light (29). To examine the involvement of Src-family tyrosine kinases in UV-induced apoptosis, we treated parental HeLa S3 cells and HeLa S3/shKu70 cells with UV light in the presence or absence of PP2 or SU6656, another Src inhibitor, and found that Src inhibition increased the level of apoptosis in parental HeLa S3 cells prominently at a low dose of UV (Fig. 4E, open bars). These results suggest that the kinase activity of endogenous Src is involved in a reduction in susceptibility to apoptosis. Notably, Src inhibition did not affect the level of...
apoptosis in Ku70-knockdown cells at low and high doses of UV (Fig. 4E, solid bars). To substantiate that the kinase activity of Src was critical for suppression of apoptosis, parental HeLa S3 cells and HeLa S3/shKu70 cells were transfected with the Src-family kinase c-Src or Lyn and then subjected to UV irradiation. c-Src overexpression significantly suppressed UV-induced apoptosis compared with Lyn (Fig. 4F, open bars). However, c-Src overexpression did not appear to suppress apoptosis in Ku70-knockdown cells under these conditions (Fig. 4F, solid bars). Taken together, these results suggest that Src suppresses UV-induced apoptosis through Ku70. In addition, the level of ADR-induced apoptosis was decreased by inducible expression of c-Src in HeLa S3 cells (Fig. 4G), suggesting that high expression of Src leads to a reduction in susceptibility to apoptosis.

Reactive oxygen species (ROS) leads to Src activation (50, 51), and UV irradiation is thought to increase ROS-dependent...
Src activity. To examine whether Src-mediated suppression of UV-induced apoptosis through Ku70 was dependent on the production of ROS, we treated parental HeLa S3 cells and Ku70-knockdown cells with UV light in the presence or absence of N-acetylcysteine (NAC), a ROS scavenger. We also treated with ADR, which induces ROS-mediated apoptosis (52, 53), and observed that the level of ADR-induced apoptosis was markedly decreased by NAC treatment due to inhibition of ROS production. In contrast, the levels of UV-induced apoptosis were not affected but rather increased by NAC treatment of HeLa S3 cells or Ku70-knockdown cells (Fig. 4H). These results suggest that Ku70 suppresses UV-induced apoptosis in a ROS-independent manner.

Inability of Src to Be Activated upon UV Irradiation—To examine whether Src was activated by UV irradiation in our culture conditions, we assessed autophosphorylation levels of endogenous Src by Western blotting with anti-Src(Tyr(P)-416) antibody. EGF stimulation for 5 min strongly induced autophosphorylation of endogenous Src and increased the levels of tyrosine phosphorylation of cellular proteins, including ERK.

Tyrosine Phosphorylation of Ku70

**Figures and graphs:**

A. Cells: Parental HeLa S3, HeLa S3 / shKu70
   - Ku70
   - DNA

B. Cells: Parental HeLa S3, HeLa S3 / shKu70
   - WB: anti-Ku70, anti-actin

C. UV (J/m²)
   - None, 20, 100
   - Cleaved caspase-3, DNA

D. Parental HeLa S3, HeLa S3 / shKu70
   - Apoptotic cells (%)

E. Parental HeLa S3, HeLa S3 / shKu70
   - Treatment: DMSO, PP2, SU6656
   - UV: None, 20 J/m², 100 J/m²

F. Parental HeLa S3, HeLa S3 / shKu70
   - Transfection: Vector, c-Src, Lyn
   - UV: 100 J/m²

G. Inducible expression:
   - WB: anti-c-Src, anti-actin
   - Inducible expression:
     - None, c-Src-HA

H. Parental HeLa S3, HeLa S3 / shKu70
   - NAC: None, 20 J/m² UV, 100 J/m² UV, ADR
   - Apoptotic cells (%)
Tyrosine Phosphorylation of Ku70

Role for Tyrosine Phosphorylation of Ku70 at Tyr-530 in Suppression of Apoptosis—
To verify tyrosine phosphorylation of endogenous Ku70, we treated cells with or without UV light in the presence of pervanadate, an extremely potent protein-tyrosine phosphatase inhibitor, and immunoprecipitated endogenous Ku70 with anti-Ku70 antibody. Upon pervanadate treatment, we succeeded in detecting tyrosine phosphorylation of endogenous Ku70 irrespective of UV irradiation (Fig. 6A). Importantly, treatment with PP2 inhibited tyrosine phosphorylation of endogenous Ku70 (Fig. 6A). Furthermore, we examined the effect of knockdown of Src members on apoptosis in HeLa S3 cells. c-Src knockdown decreased the level of tyrosine phosphorylation of Ku70 (Fig. 6B), and significantly increased the levels of UV- and ADR-induced apoptosis (Fig. 6C). The levels of tyrosine phosphorylation of endogenous Ku70 were slightly decreased in Fyn-knockdown cells but, rather, increased in Lyn knockdown cells (Fig. 6D). In contrast to single knockdown of Fyn or Lyn, Fyn/Lyn double knockdown markedly decreased the levels of tyrosine phosphorylation of endogenous Ku70 irrespective of UV irradiation (Fig. 6D) and significantly increased the levels of UV- and ADR-induced apoptosis (Fig. 6E), consistent with the result showing that PP2 treatment increased the level of UV-induced apoptosis (Fig. 4E). Taken together, these results suggest that c-Src, Fyn, and Lyn, the major Src members in HeLa S3 cells, play a redundant role in tyrosine phosphorylation of Ku70 and suppression of apoptosis.

Contribution of Src Members to Suppression of Apoptosis—
To examine the role of glutamic acid residue (Tyr\(^\text{Y530E}\)) on Bax-induced apoptosis, HeLa S3 cells, which can express anti-Ku70 antibody. Upon pervanadate treatment, we succeeded in detecting tyrosine phosphorylation of endogenous Ku70 irrespective of UV irradiation (Fig. 6A). Importantly, treatment with PP2 inhibited tyrosine phosphorylation of endogenous Ku70 (Fig. 6A). Furthermore, we examined the effect of knockdown of Src members on apoptosis in HeLa S3 cells. c-Src knockdown decreased the level of tyrosine phosphorylation of Ku70 (Fig. 6B), and significantly increased the levels of UV- and ADR-induced apoptosis (Fig. 6C). The levels of tyrosine phosphorylation of endogenous Ku70 were slightly decreased in Fyn-knockdown cells but, rather, increased in Lyn knockdown cells (Fig. 6D). In contrast to single knockdown of Fyn or Lyn, Fyn/Lyn double knockdown markedly decreased the levels of tyrosine phosphorylation of endogenous Ku70 irrespective of UV irradiation (Fig. 6D) and significantly increased the levels of UV- and ADR-induced apoptosis (Fig. 6E), consistent with the result showing that PP2 treatment increased the level of UV-induced apoptosis (Fig. 4E). Taken together, these results suggest that c-Src, Fyn, and Lyn, the major Src members in HeLa S3 cells, play a redundant role in tyrosine phosphorylation of Ku70 and suppression of apoptosis.

Role for Tyrosine Phosphorylation of Ku70 at Tyr-530 in Suppression of Apoptosis—Replacement of a tyrosine residue with a glutamic acid residue (Tyr\(\rightarrow\)Glu) would exert a phosphomimetic effect due to its negative charge. To examine the role of tyrosine phosphorylation of Ku70 at Tyr-530 in suppression of apoptosis, we generated HeLa S3 cells, which can express myc-Ku70-WT, its nonphosphorylatable Y530F mutant, or its phosphomimetic Y530E mutant. Treatment with doxycycline (Dox), an analog of tetracycline, successfully induced expression of myc-Ku70-WT, myc-Ku70-Y530F, or myc-Ku70-Y530E (Fig. 7A). Expression of myc-Ku70-WT suppressed UV-induced apoptosis (Fig. 7B), in agreement with previous studies (43). Notably, expression of myc-Ku70-Y530E suppressed UV-induced apoptosis to a similar extent as that of myc-Ku70-WT, but expression of myc-Ku70-Y530F could not suppress UV-induced apoptosis so much (Fig. 7B). In the presence of PP2 there was no difference in the levels of UV-induced apoptosis between myc-Ku70-WT-expressing cells and myc-Ku70-Y530F-expressing cells (Fig. 7C). To further confirm these results, HeLa S3/shKu70 cells, in which endogenous Ku70 was knocked down, were transiently rescued with myc-Ku70-WT and its mutants (Fig. 7D). In our knockdown-rescue experiments, similar results were obtained with HeLa S3/shKu70 cells and parental HeLa S3 cells upon treatment with UV light or ADR in the presence or absence of PP2 (Fig. 7, E–G). However, expression of myc-Ku70-Y530F was not sufficient enough to suppress UV- or ADR-induced apoptosis to a similar extent as that of myc-Ku70-WT (Fig. 7, E–G). Considering that Src inhibition reduced the ability of Ku70 to suppress apoptosis (Fig. 4E) and that inhibition or knockdown of Src led to a decrease in the levels of tyrosine phosphorylation of Ku70 (Fig. 6, A, B, and D), it is very likely that Ku70 is consistently tyrosine-phosphorylated to some extent by endogenous Src and that tyrosine phosphorylation of Ku70 at Tyr-530 plays a role in suppression of apoptosis.

Next, apoptosis can be initiated by activation of the proapoptotic factor Bax (54, 55), and Ku70 suppresses Bax-mediated apoptosis by sequestering Bax away from mitochondria through the Ku70-Bax interaction (43–48). Cotransfection of Bax with control vector, c-Src, or v-Src, a highly activated variant of c-Src, showed that the levels of Bax-mediated apoptosis were decreased by expression of c-Src and v-Src (Fig. 7H). To examine the effect of tyrosine phosphorylation of Ku70 at Tyr-530 on Bax-induced apoptosis, HeLa S3 cells, which can express v-Src, were transfected with Bax plus myc-Ku70-WT or Bax plus myc-Ku70-Y530F (Fig. 7I). Expression of myc-Ku70-WT

**FIGURE 4. Involvement of Src and Ku70 in suppression of apoptosis.** A, parental HeLa S3 cells and HeLa S3/shKu70 cells in which endogenous Ku70 had been knocked down were double-stained with anti-Ku70 antibody and propidium iodide for DNA. Scale bars, 20 \(\mu\)m. B, whole cell lysates from parental HeLa S3 cells and HeLa S3/shKu70 cells were subjected to Western blotting (WB), using the indicated antibodies. C, parental HeLa S3 cells were irradiated with 20 or 100 J/m\(^2\) UV and cultured for 12 h. Cells were double-stained with anti-cleaved caspase-3 antibody and propidium iodide. D, parental HeLa S3 cells and HeLa S3/shKu70 cells were subjected to Western blotting (WB) using indicated antibodies. E, parental HeLa S3 cells and HeLa S3/shKu70 cells were irradiated with 20 or 100 J/m\(^2\) UV and cultured for 12 h in the presence of 20 \(\mu\)M PP2, 10 \(\mu\)M SU6656, or DMSO (dimethyl sulfoxide, solvent control). The percentage of apoptotic cells was quantitated. Results (%) represent the means ± S.D. from three independent experiments. Asterisks indicate significant differences (**, \(p < 0.01\); *, \(p < 0.05\); NS, not significant) calculated by Student’s t test. In each experiment, 136–249 cells were counted. F, parental HeLa S3 cells and HeLa S3/shKu70 cells cotransfected with GFP plus the indicated plasmid were cultured for 10–12 h. The percentage of apoptotic cells in GFP-positive cells was quantitated. Results (%) represent the means ± S.D. from four independent experiments. Asterisks indicate the significant difference (**, \(p < 0.01\); NS, not significant) calculated by Student’s t test. The results of no treatment (None) are the means from two independent experiments. In each experiment 136–419 cells were counted. F, parental HeLa S3 cells and HeLa S3/shKu70 cells cotransfected with GFP plus the indicated plasmid were cultured for 10–12 h, then irradiated with 100 J/m\(^2\) UV and further cultured for 10–12 h. The percentage of apoptotic cells in GFP-positive cells was quantitated. Results (%) represent the means ± S.D. from four independent experiments. Asterisks indicate the significant difference (**, \(p < 0.01\); NS, not significant) calculated by Student’s t test. In each experiment, 35–128 cells were counted. G, parental HeLa S3/TR cells and HeLa S3/TR cells, which can express c-Src-HA, were incubated with Dox for 5 h. Whole cell lysates were subjected to Western blotting (WB) (left panels). After a 5-h incubation with Dox, cells were treated with 1 \(\mu\)g/ml ADR and incubated for a further 13 h. Results (%) of a representative experiment (225–249 cells/each sample) are shown (right panel). H, parental HeLa S3 cells and HeLa S3/shKu70 cells pretreated with 40 mM N-acetylcycteine (NAC, (+)) or PBS (solvent control, (−)) for 1 h were treated with 1 \(\mu\)g/ml ADR for 13 h in the presence or absence of 40 mM NAC. Pretreated cells with or without 40 mM NAC were irradiated with 20 or 100 J/m\(^2\) UV and then cultured for 12 h in the presence or absence of 40 mM NAC. The percentage of apoptotic cells was quantitated. Results (%) represent the means ± S.D. from three or four independent experiments. Asterisks indicate significant differences (**, \(p < 0.01\); *, \(p < 0.05\); NS, not significant) calculated by Student’s t test. In each experiment, 39–249 cells were counted.
suppressed Bax-induced apoptosis (Fig. 7I), in agreement with previous studies (43). In contrast, expression of myc-Ku70-Y530F showed a significant reduction in suppression of Bax-induced apoptosis compared with that of myc-Ku70-WT (Fig. 7I). Taken together, these results suggest that Src-mediated phosphorylation of Ku70 at Tyr-530 is important for suppression of Bax-mediated apoptosis.

Role of Ku70 Phosphorylation at Tyr-530 in Acetylation of Ku70—Previous studies reported that the multiple lysine residues in Ku70 are targets for acetylation and that acetylation of Ku70 in the C-terminal flexible linker region adjacent to the Bax-interaction domain chiefly contributes to promote apoptosis by possible inactivation of the Ku70 Bax-interaction domain (43–45, 48). Our findings indicate that phosphorylation of Ku70 on Tyr-530 can modulate the acetylation status of Ku70, which may further influence its function in apoptosis regulation.

**FIGURE 5. Inability of Src to be activated upon UV irradiation.** A, HeLa S3 cells cultured in serum-free medium for 20 h were treated with 100 ng/ml EGF for the indicated times (lanes 2 and 3) or serum-free medium for 5 min (lane 1). HeLa S3 cells were cultured in medium containing 5% serum (lane 4). B, HeLa S3 cells were treated with 0, 20, or 100 J/m² UV light and then cultured for 5 min (left panels) and 30 min (right panels). C, HeLa S3 cells cultured with 20 μM PP2 or DMSO for 12 h were treated with 0 or 100 J/m² UV light and then cultured for 5 min in medium containing 20 μM PP2 or DMSO. D, HeLa S3 cells and HeLa S3/shKu70 cells were treated with 0, 20, or 100 J/m² UV light and then cultured for 5 min. A–D, whole cell lysates were subjected to Western blotting (WB) using the indicated antibodies. Levels of cellular tyrosine phosphorylation are expressed as values relative to that in each control sample after normalization with actin or α-tubulin levels.
Ku70 at Tyr-530 is important for suppression of apoptosis (Fig.
7, B, C, E, F, G, and I) and that the phosphorylation site of Ku70
is located close to the acetylation sites in the C-terminal flexible
linker region (Fig. 2), which may lead to the intriguing hypoth-
thesis that tyrosine phosphorylation of Ku70 interferes with
Ku70’s acetylation. We thus examined the levels of Ku70 acety-

FIGURE 6. Contribution of Src members to suppression of apoptosis. A, HeLa S3 cells were irradiated with 100 J/m² UV and then treated with pervanadate for 30 min. Endogenous Ku70 was immunoprecipitated with anti-Ku70 antibody or control antibody (Control Ab), and Western blotting (WB) was performed with the indicated antibodies. Cells were pretreated with or without 20 μM PP2 for 12 h. B and C, HeLa S3 cells were transfected with an episomal vector encoding sh(c-Src) (pEBmulti/neo/H1-sh(c-Src)), and then cells expressing sh(c-Src) were selected in 0.5–1 mg/ml G418. B, whole cell lysates were subjected to Western blotting. Amounts of endogenous c-Src are expressed as values relative to that in control after normalization with actin (left panels). Cells were treated with pervanadate for 30 min. Endogenous Ku70 was immunoprecipitated (IP) with anti-Ku70 antibody, and Western blotting was performed with the indicated antibodies (right panels). C, cells were irradiated with 20 or 100 J/m² UV and cultured for 12 h, or cells were treated with 1 μg/ml ADR for 12 h. The percentage of apoptotic cells was quantitated. Results (%) represent the means ± S.D. from three or four independent experiments. Asterisks indicate significant differences (**, p < 0.01; *, p < 0.05; NS, not significant) calculated by Student’s t test. The results of no treatment (None) are the means from two independent experiments. In each experiment, 137–374 cells were counted.

FEBRUARY 3, 2017 • VOLUME 292 • NUMBER 5 • JOURNAL OF BIOLOGICAL CHEMISTRY 1655

Tyrosine Phosphorylation of Ku70
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We treated cells expressing myc-Ku70-WT with UV light in the presence or absence of PP2 and performed immunoprecipitation assays using anti-myc antibody. myc-Ku70-WT immunoprecipitates were immunoblotted with mouse monoclonal anti-acetylated lysine antibody (anti-Ac-K (mAb)). In fact, Src inhibition augmented the level of acetylation of myc-Ku70-WT upon UV irradiation (Fig. 8, A and B). Conversely, acetylated myc-Ku70-WT was immunoprecipitated with rabbit polyclonal anti-acetylated lysine antibody (anti-Ac-K (polyAb)) (Fig. 8C), and acetylation of myc-Ku70-WT was also augmented by Src inhibition upon UV irradiation (Fig. 8, D and E). These results suggest that Src-mediated tyrosine phosphorylation of Ku70 inhibits acetylation of Ku70. Furthermore, by means of knockdown-rescue, we examined the levels of acetylation of myc-Ku70-WT and myc-Ku70-Y530F upon UV irradiation (Fig. 8F). Notably, expression of myc-Ku70-Y530F significantly augmented the level of its acetylation compared with that of myc-Ku70-WT (Fig. 8G). Taken together, these results suggest that the level of phosphorylation at Tyr-530 on Ku70 is inversely correlated with the level of Ku70 acetylation.

Discussion

Ku70 is known to suppress apoptosis by negatively regulating Bax activation (43–48). Upon apoptotic stimulation, acetylation of Ku70 in the C-terminal flexible linker region results in activating Bax, thereby promoting apoptosis (43–45, 48). In the present study we show that Src acts as an effector for Ku70-de-
Tyrosine Phosphorylation of Ku70

Ku70 majorly forms a Ku70/Ku80 heterodimer, which is required for NHEJ repair (38–40). However, Ku70 has also been shown to regulate Bax-mediated apoptosis (43–48). Ku70 suppresses not only Bax-mediated apoptosis independently of Ku80 but also staurosporine-induced apoptosis without DNA damage (43, 45). Considering that tyrosine phosphorylation of Ku70 does not affect the Ku70/Ku80 heterodimer formation and the levels of DNA-PKcs phosphorylation (Fig. 3) and that tyrosine phosphorylation of Ku70 enhances the ability of Ku70 to suppress Bax-mediated apoptosis (Fig. 7J), our observations support the notion that Src suppresses apoptosis through Ku70 independently of NHEJ repair. Although many studies have shown the functional importance of Src in suppression of apoptosis (29–32, 35), there are a limited number of reports regarding Src substrates, which describe that Src-mediated tyrosine phosphorylation of caspase-8 and GADD34 negatively regulates apoptosis (36, 37). The mechanism of Src-mediated suppression of apoptosis through Src substrates still remains poorly understood, possibly because protein-tyrosine phosphorylation is extremely unstable within cells. We, therefore, assume that phosphorylation levels of substrates are very rapidly regulated by a balance of the activities between tyrosine kinases and tyrosine phosphatases, like ON/OFF switching in a microprocessor (5, 22–27, 57). Nonetheless, carefully using a high dose of the potent tyrosine phosphatase inhibitor NaVO₃, we are able to detect tyrosine phosphorylation of endogenous proteins and have shown dependent suppression of apoptosis. Our conclusion is supported by the following evidence: (i) inhibition of the activity of endogenous Src enhances susceptibility to apoptotic stimuli, (ii) Ku70 knockdown impairs c-Src-mediated suppression of apoptosis, (iii) Src phosphorylates Ku70 at Tyr-530, (iv) similar to UV-induced apoptosis, phosphorylation of Ku70 at Tyr-530 suppresses ADR- or Bax-induced apoptosis, (v) inhibition of the activity of endogenous Src increases the level of Ku70 acetylation, and (vi) Ku70 mutation at Tyr-530 → Phe augments acetylation of Ku70. Therefore, we propose a model for reduced apoptotic susceptibility by Src (Fig. 8H). Src phosphorylates Ku70 at Tyr-530, which is located close to the possible acetylation sites in the C-terminal flexible linker region (Fig. 2A, note the five lysine residues in the region ranging from Lys-539 to Lys-556; Ref. 43). Phosphorylation of Ku70 at Tyr-530 decreases acetylation of Ku70, resulting in a reduction in susceptibility to apoptotic stimuli. Inhibition of the activity of Src decreases phosphorylation of Ku70 at Tyr-530 and concomitantly augments the level of acetylation of Ku70, thereby promoting Bax-mediated apoptosis. Our results suggest the intriguing possibility that endogenous Src activity raises the cellular threshold to apoptotic stimuli through tyrosine phosphorylation of Ku70 in every cell. Taken together with ubiquitous expression of Src and Ku70, we hypothesize that Src is a safety device for Ku70-mediated apoptosis to protect our bodies from hyperactive apoptotic cell death.

Multiple Src members are co-expressed in various combinations in a cell type-dependent manner (6, 7). One possibility is to assume that cellular localizations among Src members are involved in their functional specificity and redundancy. Our previous studies indicate the importance of palmitoylation for distinct trafficking pathways between nonpalmitoylated c-Src and palmitoylated Fyn and Lyn (15, 18). c-Src is rapidly exchanged between the plasma membrane and intracellular organelles possibly through its cytosolic release (15). We show that, unlike Fyn and Lyn, c-Src overexpression strongly induced phosphorylation of Ku70 (Fig. 1B) and significantly suppressed apoptosis (Fig. 4F). Single knockdown of c-Src significantly increased UV- and ADR-induced apoptosis (Fig. 6C) compared with Lyn and Fyn single-knockdown (Fig. 6E). However, Fyn/Lyn double-knockdown increased UV- and ADR-induced apoptosis (Fig. 6E) accompanied by a strong decrease in the levels of tyrosine phosphorylation of Ku70 (Fig. 6D). Taken together, these results suggest that Src members can compensate for each other in reducing apoptotic susceptibility through tyrosine phosphorylation of Ku70.

**FIGURE 7. Role of Ku70 phosphorylation at Tyr-530 in suppression of apoptosis.** A–C, parental HeLa S3/TR cells and HeLa S3/TR cells, which can express myc-Ku70-WT, myc-Ku70-Y530F, or myc-Ku70-Y530E, were incubated with Dox for 24 h. A, cells were double-stained with anti-myc antibody and propidium iodide. Scale bars, 40 μm. B, cells were irradiated with 100 J/m² UV and cultured for 12 h. The percentage of apoptotic cells was quantitated. Results (%) represent the means ± S.D. from three independent experiments. In each experiment 61–269 cells were counted. Asterisks indicate the significant difference (**p < 0.01; NS, not significant) calculated by Student’s t test. C, HeLa S3/TR cells, which can express myc-Ku70-WT or myc-Ku70-Y530F, were irradiated with 100 J/m² UV and then cultured for 12 h in the presence or absence of 20 μM PP2. The percentage of apoptotic cells was quantitated. Results (%) represent the means ± S.D. from three independent experiments. In each experiment 51–94 cells were counted. Asterisks indicate the significant difference (**p < 0.01; NS, not significant) calculated by Student’s t test. D–F, HeLa S3/shKu70 cells transfected with myc-Ku70-WT, myc-Ku70-Y530F, or myc-Ku70-Y530E were cultured for 24 h. D, whole cell lysates were subjected to Western blotting (WB) using the indicated antibodies. E, cells were irradiated with 20 J/m² UV and cultured for 12 h. The percentage of cells exhibiting cleaved caspase-3 was quantitated. Results (%) represent the means ± S.D. from three independent experiments. In each sample, 405 cells were counted. Asterisks indicate the significant difference (**p < 0.01; NS, not significant) calculated by Student’s t test. F, cells were cultured for 24 h and treated with 1 μg/ml ADR for 12 h or 18 h. The percentage of apoptotic cells was quantitated. Results (%) represent the means ± S.D. from three independent experiments. In each experiment, 113–405 cells were counted. Asterisks indicate the significant difference (**p < 0.01) calculated by Student’s t test. G, HeLa S3/shKu70 cells were transfected with an episomal vector encoding myc-Ku70-WT or myc-Ku70-Y530F, and then cells expressing myc-Ku70-WT or myc-Ku70-Y530F were selected in 1.5 μg/ml puromycin. Cells were treated with 1 μg/ml ADR for 12 h. The percentage of apoptotic cells was quantitated. Results (%) represent the means ± S.D. from three independent experiments. In each experiment 94–460 cells were counted. An asterisk indicates the significant difference (**p < 0.01; NS, not significant) calculated by Student’s t test. H, HeLa S3 cells cotransfected with FLAG-Bax plus the indicated plasmid were cultured for 10 h. The percentage of apoptotic cells in Bax-positive cells was quantitated. Results (%) represent the means ± S.D. from three independent experiments. In each experiment 107–266 cells were counted. An asterisk indicates the significant difference (**p < 0.01; NS, not significant) calculated by Student’s t test. I, HeLa S3/TR-v Src cells cotransfected with the indicated plasmid were cultured for 12 h. Whole cell lysates were subjected to Western blotting using the indicated antibodies. An arrowhead indicates nonspecific bands. The percentage of cells exhibiting cleaved caspase-3 was quantitated. Results (%) represent the means ± S.D. from three independent experiments. Asterisks indicate the significant difference (**p < 0.01) calculated by Student’s t test. The result of vector alone is the means from two independent experiments. In each experiment, 49–80 cells were counted.

Tyrosine Phosphorylation of Ku70

Tyrosine phosphorylation of Ku70 majorly forms a Ku70/Ku80 heterodimer, which is required for NHEJ repair (38–40). However, Ku70 has also been shown to regulate Bax-mediated apoptosis (43–48). Ku70 suppresses not only Bax-mediated apoptosis independently of Ku80 but also staurosporine-induced apoptosis without DNA damage (43, 45). Considering that tyrosine phosphorylation of Ku70 does not affect the Ku70/Ku80 heterodimer formation and the levels of DNA-PKcs phosphorylation (Fig. 3) and that tyrosine phosphorylation of Ku70 enhances the ability of Ku70 to suppress Bax-mediated apoptosis (Fig. 7J), our observations support the notion that Src suppresses apoptosis through Ku70 independently of NHEJ repair. Although many studies have shown the functional importance of Src in suppression of apoptosis (29–32, 35), there are a limited number of reports regarding Src substrates, which describe that Src-mediated tyrosine phosphorylation of caspase-8 and GADD34 negatively regulates apoptosis (36, 37). The mechanism of Src-mediated suppression of apoptosis through Src substrates still remains poorly understood, possibly because protein-tyrosine phosphorylation is extremely unstable within cells. We, therefore, assume that phosphorylation levels of substrates are very rapidly regulated by a balance of the activities between tyrosine kinases and tyrosine phosphatases, like ON/OFF switching in a microprocessor (5, 22–27, 57). Nonetheless, carefully using a high dose of the potent tyrosine phosphatase inhibitor NaVO₃, we are able to detect tyrosine phosphorylation of endogenous proteins and have shown...
the significance of tyrosine-phosphorylated substrates (5, 24, 26, 27). Because we have learned from our own experiences that the inhibitory potency of Na$_3$VO$_4$, albeit an inorganic compound, against the activity of protein-tyrosine phosphatases is drastically lost (i) at neutral pH and (ii) by freeze-thawing, we dissolve Na$_3$VO$_4$ in H$_2$O just before use or store a Na$_3$VO$_4$ stock solution in single-use aliquots at $-20^\circ$C. To prevent dephosphorylation of proteins, Na$_3$VO$_4$ is added at a final concentration of 10 mM to both immunoprecipitation buffer and SDS sample buffer, and the samples heated at 95°C for 5 min in SDS sample buffer are immediately kept at 4°C and subjected to SDS-PAGE without freeze-thawing (see also “Experimental Procedures”). Thus, by minimizing inactivation of Na$_3$VO$_4$, we succeeded in finding quite a few tyrosine-phosphorylated proteins by our phosphoproteomic analysis (5, 25). Furthermore, we were successfully able to detect Src-mediated tyrosine phosphorylation of endogenous Ku70 in the presence of pervanadate, a highly potent protein-tyrosine phosphatase inhibitor.
Tyrosine Phosphorylation of Ku70

A previous proteomics-based study showed the possibility that Ku70 could be tyrosine-phosphorylated due to the presence of Ku70 in the anti-phosphotyrosine immunoprecipitates from human breast cancer MCF-7 cells stimulated with nerve growth factor (58), but one cannot exclude the possibility that Ku70 might be a non-phosphorylated protein that is merely associated with the tyrosine-phosphorylated immunoprecipitates. It remains to be determined whether Ku70 would be actually tyrosine-phosphorylated by nerve growth factor and what role for tyrosine phosphorylation of Ku70 would play upon stimulation with nerve growth factor. In the present study we demonstrate that Ku70 is indeed tyrosine-phosphorylated by Src (Fig. 1, A and B). With in vitro kinase assays, we verify direct phosphorylation of Ku70 by Src (Fig. 1, D and E). In our phosphoproteomic analysis and the following site-directed mutagenesis, we identified Tyr-530 as a major phosphorylation site among the total 21 tyrosine residues found on Ku70 (Fig. 2). Taken together with our findings that tyrosine phosphorylation of Ku70 is involved in suppression of apoptosis (Figs. 7 and 8), we showed that Src-mediated phosphorylation of Ku70 at Tyr-530 is critical for suppression of apoptosis.

We demonstrate that the levels of Ku70 acetylation were augmented by Src inhibition (Fig. 8, A, B, D, and E) and by the mutation of Ku70 at Tyr-530 → Phe (Fig. 8, F and G). Previous studies showed that Ku70 is acetylated at multiple sites, including the sites located in the C-terminal flexible linker region and the DNA binding domain (43, 56), and that acetylation of Ku70 in the C-terminal flexible linker region negatively regulates Ku70-mediated suppression of apoptosis (43–45, 48). Ku70 is acetylated by the acetyltransferases CBP (cAMP-response element-binding protein (CREB)-binding protein) and p300/CBP-associated factor (PCAF) (43, 48) and deacetylated by the deacetylases SIRT1, SIRT3, and HDAC6 (44, 49, 50). Although an increase in the levels of Ku70 acetylation was seen in the immunoprecipitates using anti-Ac-K polyAb (Fig. 8C), a decrease in the levels of Ku70 acetylation was detected when myc-Ku70-WT was immunoprecipitated using anti-myc antibody and immunoblotted with anti-Ac-K mAb at 6 h after UV irradiation (Fig. 8, A and F). This discrepancy might be explained by the following possibilities such as (i) activation of deacetylation under UV irradiation, (ii) overlapping and distinct acetylation sites on Ku70 recognized by anti-Ac-K mAb and anti-Ac-K polyAb, and (iii) a small proportion of acetylated Ku70 in total amounts of Ku70. It should be emphasized that Src-mediated phosphorylation of Ku70 at Tyr-530 decreased the levels of Ku70 acetylation, leading to suppression of apoptosis (Figs. 7 and 8). There is no report about the interaction between acetylation and phosphorylation of Ku70 in apoptosis thus far. The cross-talk between acetylation and phosphorylation has been documented among histone and non-histone proteins (61–63). Considering that the phosphorylation site of Ku70 at Tyr-530 is located close to the C-terminal flexible linker region (Fig. 2), we hypothesize that phosphorylation at Tyr-530 may interfere with the association of Ku70 with acetyltransferases or may enhance the association of Ku70 with deacetylases. More extensive experiments are needed to fully elucidate the relationship between tyrosine phosphorylation and acetylation on Ku70.

Src is overexpressed and activated in numerous types of human cancers and plays important roles in cancer development, progression, and resistance to anticancer therapy (64–67). Our results suggest that, upon UV or ADR treatment, high expression of Src markedly reduces susceptibility to apoptosis and leads to promotion of cell survival (Fig. 4, F and G). We further show that inhibition of Tyr-530 phosphorylation enhances susceptibility to apoptosis (Fig. 7), raising the promising possibility that development of inhibitors for tyrosine phosphorylation of Ku70 might potentiate the sensitivity of cancer cells to anticancer drugs.

In conclusion, we identify Ku70 as a substrate of Src. This is a first report of the significance of Src-mediated phosphorylation of Ku70 at Tyr-530 in suppression of apoptosis. Further studies will help us to understand the relationship between tyrosine phosphorylation of Ku70 and cancer cell survival or therapeutic resistance.

Experimental Procedures

Plasmids—Knockdown of Ku70 was performed with shRNA for silencing Ku70 (shKu70) (5′-GAAGAGTCTACCCGA-CATAAG-3′ in the 3′-untranslated region (shKu70–3′-UTR) (Sigma MISSION shRNA library) and 5′-GATGAGTCATAACA-GATGAGTCATAACA-

FIGURE 8. Role of Ku70 phosphorylation at Tyr-530 in acetylation of Ku70. A–E, HeLa S3/TR cells, which can express myc-Ku70-WT (HeLa S3/TR-myc-Ku70-WT), incubated with DMSO for 2 h followed by UV treatment. A, myc-Ku70-WT was immunoprecipitated (IP) with anti-myc antibody. Western blotting (WB) was performed with anti-Ac-K antibody and mouse monoclonal anti-acetylated lysine antibody (anti-Ac-K MAb). Amounts of acetylated myc-Ku70-WT at 2 h and 6 h after UV irradiation are expressed as relative values. B, relative amounts of acetylated myc-Ku70-WT at 6 h after UV irradiation were quantitated. Results represent the means ± S.D. from 4 independent experiments. An asterisk indicates the significant difference (*, p < 0.05) calculated by Student’s t test. C, cell lysates were immunoprecipitated with rabbit polyclonal anti-acetylated lysine antibody (anti-Ac-K polyAb) or control antibody (Control Ab). Western blotting was performed with anti-myc and anti-actin antibodies. D, acetylated myc-Ku70-WT was immunoprecipitated with anti-Ac-K (polyAb). Western blotting was performed with anti-myc and anti-actin antibodies. E, relative amounts of acetylated myc-Ku70-WT at 6 h after UV irradiation are expressed as relative values. F, relative amounts of acetylated myc-Ku70-WT at 6 h after UV irradiation were quantitated. Results represent the means ± S.D. from 4 independent experiments. An asterisk indicates the significant difference (*, p < 0.05) calculated by Student’s t test. G, HeLa S3/myc-Ku70 cells were transfected with an episomal vector encoding myc-Ku70-WT or myc-Ku70-Y530F, and then cells expressing myc-Ku70-WT or myc-Ku70-Y530F were selected in 1.5 μg/ml puromycin. Cells were irradiated with 20 J/m² UV and cultured for 6 h. myc-Ku70-WT or myc-Ku70-Y530F was immunoprecipitated with anti-myc antibody. F, Western blotting was performed with anti-myc antibody and anti-Ac-K MAb. Amounts of acetylated myc-Ku70-WT and myc-Ku70-YF upon UV irradiation are expressed as relative values. G, relative amounts of acetylated myc-Ku70-WT or acetylated myc-Ku70-Y530F were quantitated. Results represent the means ± S.D. from 4 independent experiments. An asterisk indicates the significant difference (*, p < 0.05; NS, not significant) calculated by Student’s t test. H, model for Src-mediated reduction in susceptibility to apoptotic stimuli. Upon apoptotic stimulation, acetylation of Ku70 in the C-terminal flexible linker region results in promoting apoptosis. Src-mediated phosphorylation of Ku70 at Tyr-530 decreases the level of acetylation of Ku70, thereby reducing susceptibility to apoptotic stimuli. When the activity of Src was inhibited, a decrease in phosphorylation of Ku70 at Tyr-530 and a concomitant increase in acetylation of Ku70 take place in order to enhance susceptibility to apoptotic stimuli and promote apoptosis. P, phosphorylation; Ac, acetylation; Y, tyrosine residue; K, lysine residue.
Tyrosine Phosphorylation of Ku70

GAGGATCAT-3’ in the coding sequence (shKu70-CDS)). Knockdown of c-Src was performed with shRNA for silencing c-Src (sh(c-Src); 5’-GCTCCAGAATTGCTCAACAC-3’) (68). Knockdown of Fyn was performed with shRNA for silencing Fyn (sh(Fyn); 5’-GGAAGAGCTCTGAAATTA-3’) (69). Knockdown of Lyn was performed with shRNA for silencing Lyn (sh(Lyn); 5’-GGACAGAGTGTCAACTCGA-3’) (69). The nucleotides for shRNA were annealed and subcloned into the BglII-XhoI site of the pENTR4/H1 vector (provided by H. Miyoshi) (19, 70–72). The EBNA1-based episomal pEBmulti/neomycin vector (Wako Pure Chemical Industries, Osaka, Japan) by replacing the CAG promoter with the H1 promoter as described (24, 71). The Tyr 5 residue of Ku70 was created by PCR using Ku70-WT as a template with 5’-CTCAATCTGAGTATGGAAGGTTA-3’ (antisense) and the SpeI fragment of the PCR product was subcloned into the SpeI site of the pEBmulti/neomycin vector (24, 71). To construct myc-tagged wild-type Ku70 (myc-Ku70-WT), the BamHI and XhoI sites were created at both ends of human wild-type Ku70 (Ku70-WT; pcDNA3/myc-Ku70-WT) (57). The Tyr 5 residue of Ku70 was created by PCR using Ku70-WT as a template with 5’-CTCAATCTGAGTATGGAAGGTTA-3’ (antisense) and the SpeI fragment of the PCR product was subcloned into the SpeI site of the pEBmulti/neomycin vector (24, 71). To construct myc-tagged wild-type Ku70, the BamHI and XhoI sites were created at both ends of human wild-type Ku70 (Ku70-WT; pcDNA3/myc-Ku70-WT) (57). To construct myc-tagged wild-type Ku70 (myc-Ku70-WT), the BamHI and XhoI sites were created at both ends of human wild-type Ku70 (Ku70-WT; pcDNA3/myc-Ku70-WT) (57). The Tyr 5 residue of Ku70 was created by PCR using Ku70-WT as a template with 5’-CTCAATCTGAGTATGGAAGGTTA-3’ (antisense) and the SpeI fragment of the PCR product was subcloned into the SpeI site of the pEBmulti/neomycin vector (24, 71).

Cells and Transfection—COS-1 and HeLa S3 cells (Japanese Collection of Research Bioresources, Osaka, Japan) were cultured in Iscove’s modified Dulbecco’s medium (IMDM) containing 5% bovine serum. HeLa S3/TR cells, which can express v-Src (HeLa S3/TR/v-Src) or c-Src-HA, were prepared recently (79, 80). HeLa S3/sh(Fyn) cells, HeLa S3/sh(Lyn) cells, and HeLa S3/sh(Fyn)+sh(Lyn) cells were generated using pENTR4/H1/sh(Fyn) and/or pENTR4/H1/sh(Lyn) with a plasmid containing the hygromycin resistant gene as described previously (69). Cells seeded in a 35-mm (60-mm) culture dish were transiently transfected with 1 μg (3 μg) of plasmid DNA using 5 μg (15 μg) of linear polyethyleneimine (25 kDa) (Poly-science, Inc.) (78). To establish a stable Ku70-knockdown cell line (HeLa S3/shKu70), HeLa S3 cells were cotransfected with pENTR4/H1/shKu70 and a plasmid containing the hygromycin resistant gene and were selected in 250 μg/ml hygromycin. Although we could not establish any Ku70-knockdown cell lines by using shKu70-CDS, stable Ku70-knockdown cell lines were successfully established by using shKu70-3’-UTR. To generate stable cell lines for tetracycline-inducible myc-Ku70-WT, myc-Ku70-Y530F, or myc-Ku70-Y530E expression, HeLa S3/TR cells (23) transfected with pCDNA4/TO/puro/myc-Ku70-WT, pCDNA4/TO/puro/myc-Ku70-Y530F, or pCDNA4/TO/puro/myc-Ku70-Y530E were selected in 350 ng/ml puromycin. Expression of myc-Ku70-WT, myc-Ku70-Y530F, or myc-Ku70-Y530E was induced by the addition of 1 μg/ml Dox,
a tetracycline derivative. To stably express myc-Ku70-WT or myc-Ku70-Y530F in HeLa S3/shKu70 cells, cells transfected with pEBmulti/puro/mvc-Ku70-WT and pEBmulti/puro/myc-Ku70-Y530F were selected in 1.5 μg/ml puromycin. To generate c-Src-knockdown cells, HeLa S3 cells transfected with pEBmulti/neO/H1 or pEBmulti/neO/H1-sh(c-Src) were selected in 0.5–1 mg/ml G418. To inhibit Src-mediated tyrosine phosphorylation, cells were treated with 20 μM PP2 (Sigma) or 10 μM SU6656 (Sigma). To inhibit deacetylation of Ku70, cells were treated with 65 nm or 100 nm trichostatin A (TSA) (Wako Pure Chemical Industries or Sigma) (23). For growth factor stimulation, cells were serum-starved for 20 h before treatment with 100 ng/ml EGF (16).

Antibodies—The following mouse monoclonal antibodies were used: myc (9E10), phosphotyrosine (Tyr(P)) (4G10; Upstate Biotechnology, Inc; provided by T. Tamura and T. Yoshimoto) (81), Lyn (H6; catalog #sc-7274; Santa Cruz Biotechnology, and Lyn9; catalog #016-14251; Wako Pure Chemical Industries), Src (GD11; catalog #05-184; Millipore), actin (C4; catalog #MAB1501; Chemicon International), Ku70 (N3H10; catalog #sc-56129; Santa Cruz Biotechnology), acetylated-lysine (Ac-K mAb) (Ac-K-103; catalog #9681; Cell Signaling Technology), FLAG (M2; catalog #F3165; Sigma), phospho-p44/42 MAPK Thr-202/Tyr-204 (pERK1/2) (E10; catalog #9106; Cell Signaling Technology), and a control antibody (MOPC21; M9269; Sigma). The following rabbit polyclonal antibodies were used: Ku70 (catalog #ABE558; Millipore), cleaved caspase-3 (Asp-175) (catalog #9661; Cell Signaling Technology), acetylated-lysine (Ac-K polyAb) (catalog #9441; Cell Signaling Technology), FLAG (catalog #F7425; Sigma), Src(Tyr(P)-416) (phospho-Src family (Tyr416); catalog #2101; Cell Signaling Technology), FLAG (catalog #F7425; Sigma), phospho-p44/42 MAPK Thr-202/Tyr-204 (pERK1/2) (E10; catalog #9106; Cell Signaling Technology), and a control antibody (MOPC21; M9269; Sigma). The following rabbit polyclonal antibodies were used: Ku70 (catalog #ABE558; Millipore), cleaved caspase-3 (Asp-175) (catalog #9661; Cell Signaling Technology), FLAG (catalog #F7425; Sigma), Src(Tyr(P)-416) (phospho-Src family (Tyr416); catalog #2101; Cell Signaling Technology), and a control antibody (MOPC21; M9269; Sigma). The following rabbit polyclonal antibodies were used: Ku70 (catalog #ABE558; Millipore), cleaved caspase-3 (Asp-175) (catalog #9661; Cell Signaling Technology), acetylated-lysine (Ac-K polyAb) (catalog #9441; Cell Signaling Technology), FLAG (catalog #F7425; Sigma), Src(Tyr(P)-416) (phospho-Src family (Tyr416); catalog #2101; Cell Signaling Technology), ERK2 (C14; catalog #sc-154; Santa Cruz Biotechnology), phospho-DNA-PKcs Ser-2056 (phospho-DNA-PKcs) (catalog #ab18192; Abcam), Lyn (Lyn44; catalog #sc-2027; Santa Cruz Biotechnology), myc (A14; catalog #sc-789; Santa Cruz Biotechnology), HA (Y11; catalog #sc-805; Santa Cruz Biotechnology), Fyn (Fyn3; catalog #sc-16; Santa Cruz Biotechnology), Src (N16; catalog #sc-19; Santa Cruz Biotechnology), myc (A14; catalog #sc-789; Santa Cruz Biotechnology), and flag (catalog #ab9109; Abcam), and a control antibody (catalog #sc-2027; Santa Cruz Biotechnology). The following rabbit monoclonal antibodies were used: β-actin (13E5; catalog #4970; Cell Signaling Technology), phospho-p44/42 MAPK Thr-202/Tyr-204 (pERK1/2) (D13.14.4E, XP®; catalog #4370; Cell Signaling Technology), and Src (EPR5496; catalog #ab109381; Abcam). The following rat monoclonal antibody was used: α-tubulin (catalog #MCA78G; Serotec). The following goat polyclonal antibodies were used: myc (A14-G; catalog #sc-789-G; Santa Cruz Biotechnology), HA (Y11-G; catalog #sc-805-G; Santa Cruz Biotechnology), and FLAG (catalog #NB600-344; Novus Biologicals). Horseradish peroxidase (HRP-) F(ab’)2 of anti-mouse IgG (catalog #NA9310), anti-rabbit IgG (catalog #NA9340), and anti-rat IgG (catalog #NA9350) antibodies were purchased from Amersham Biosciences. HRP-F(ab’)2 of anti-goat IgG (catalog #705-036-147), anti-mouse IgG light chain (catalog #115-035-174), and anti-rabbit IgG light chain (catalog #211-032-171) antibodies were purchased from Jackson ImmunoResearch Laboratories. Alexa Fluor-labeled IgG secondary antibodies purchased from Invitrogen were used: Alexa Fluor 488-labeled donkey anti-mouse IgG (catalog #A21202), anti-rabbit IgG (catalog #A21206) and anti-goat IgG (catalog #A11055) antibodies, Alexa Fluor 546-labeled donkey anti-mouse IgG (catalog #A10036) and anti-goat IgG (catalog #A11056) antibodies, and Alexa Fluor 647-labeled donkey anti-rabbit IgG (catalog #A31573) antibody.

Western Blotting and Immunoprecipitation—Western blotting was performed with enhanced chemiluminescence (Millipore) as described previously (14, 24, 25). Whole cell lysates prepared in Laemmli SDS sample buffer were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes. Protein bands were detected with appropriate antibodies and analyzed with a ChemiDoc XRSPPlus image analyzer (Bio-Rad). Sequential reprobing of membranes with a variety of antibodies was performed after the complete removal of primary and secondary antibodies from membranes in stripping buffer, 0.2 M glycine-HCl buffer, pH 2.5, or inactivation of HRP by 0.1% NaN3, as described previously (82). Immunoprecipitation was performed using an appropriate primary antibody together with protein-G beads, as described previously (19, 21, 83, 84). To detect tyrosine phosphorylation of Ku70, cell lysates were prepared as described recently (57). In brief, cells were suspended in SDS lysis buffer (0.1% SDS, 50 mM Tris-HCl, pH 7.5, 10 mM Na3VO4, 10 mM unbuffered HEPES, 4 μg/ml aprotinin, 1.6 μg/ml pepstatin A, 4 μg/ml leupeptin, 1 mM EDTA, and 1 mM PMSF). Then Triton X-100 was added to the cell lysates at a final concentration of 0.5%. After centrifugation, the cell lysates were subjected to immunoprecipitation. It is extremely important to note that the inhibitory potency of Na3VO4 against the activity of protein-tyrosine phosphorylases is rapidly lost (i) at neutral pH and (ii) by freeze-thawing. Accordingly, Na3VO4 was dissolved in H2O just before use or stored as a 100 mM or 300 mM stock solution in single-use aliquots at −20 °C. For detection of tyrosine phosphorylation of immunoprecipitates, the immunoprecipitates obtained within a 2-h primary antibody incubation were lysed in Laemmli SDS sample buffer to which 10 mM unbuffered HEPES and 10 mM Na3VO4 were added just before use, and the samples heated at 95 °C for 5 min were subjected to SDS-PAGE without freeze-thawing. Carefully using Na3VO4 for analysis of tyrosine phosphorylation signaling, we were eventually able to detect a variety of tyrosine-phosphorylated substrates of Src and c-Abl (5, 24–27). To detect acetylation levels of Ku70, cells were lysed in Dulbecco’s phosphate-buffered saline (PBS) containing 1% Triton X-100, 4 μg/ml aprotinin, 1.6 μg/ml pepstatin A, 4 μg/ml leupeptin, 1 mM EDTA, 1 mM PMSF, and 1 mM trichostatin A. In brief, acetylated-myc-Ku70 was immunoprecipitated using anti-Ac-K polyAb and immunoblotted with anti-myc antibody. Conversely, myc-Ku70 was immunoprecipitated using anti-myc antibody and immunoblotted with anti-Ac-K mAb but not anti-Ac-K polyAb, because acetylation of Ku70 was not detected by Western blotting using anti-Ac-K polyAb. To analyze the formation of the Ku70/Ku80 heterodimer by coimmunoprecipitation of myc-Ku70 with FLAG-Ku80, cells were lysed in 0.5% Triton lysis buffer (0.5% Triton X-100, 30 mM HEPES, pH 7.8, 100 mM NaCl, 10 mM Na3VO4, 10 mM unbuffered
Tyrosine Phosphorylation of Ku70

HEPES, 10% glycerol, 4 μg/ml aprotinin, 1.6 μg/ml pepstatin A, 4 μg/ml leupeptin, 1 mM EDTA, and 1 mM PMSF. The intensity of chemiluminescence was measured using Quantity One software (Bio-Rad). Composite figures were prepared using the GNU Image Manipulation Program version 2.6.2 software (GIMP) and Illustrator 16.0 software (Adobe).

Phosphoproteomic Analysis—We recently performed phosphoproteomic analysis of tyrosine-phosphorylated proteins upon Src overexpression and identified various proteins as candidate substrates of Src, including Ku70 (5). In brief, parental HeLa S3 or HeLa S3/NLS-Lyn cells incubated with 0.5 mM Na3VO4 for 1.5 h were lysed with SDS lysis buffer (100 mM Tris-HCl, pH 6.8, 3% SDS, 20% glycerol, and 10 mM Na3VO4). Cell lysates were heated at 95 °C for 5 min and sonicated. Before immunoprecipitation, wash buffer (30 mM HEPES, pH 7.4, 300 mM NaCl, and 1.0% Triton X-100) was added to dilute SDS to 0.1%. Tyrosine-phosphorylated proteins collected on anti-Tyr(P) antibody-coupled beads from cell lysates were digested with trypsin. Molecular mass analysis of trypsin fragments was performed by LC/MS/MS (5).

Pervanadate Treatment—Pervanadate (10 mM) was prepared in PBS for 5 min at 4 °C from a 100 mM Na3VO4 stock solution and 30% H2O2 at final concentrations of 10 mM and 0.1%, respectively. Excess H2O2 was removed by adding catalase (Wako Pure Chemical Industries) for 5 min at room temperature (85). The resulting pervanadate solution was immediately added to culture medium, and cells were incubated with 0.1 or 0.5 mM pervanadate for 30 min.

Immunofluorescence—Confocal images were obtained using a Fluoview Fv500 confocal laser scanning microscope with a 20 × 1.00 NA or 40 × 1.00 NA objective (Olympus, Tokyo), as described (23, 80). One planar (xy) section slice images (0.6- or 2.0-μm thickness) were shown. Cells fixed in PBS containing 4% paraformaldehyde and then treated with 200 μg/ml pepstatin A, 20 μg/ml propidium iodide for 0.5–1 h. After staining, cells were mounted in PBS containing 0.1% saponin and 3% bovine serum albumin at room temperature (86). Cells were subsequently reacted with appropriate primary antibodies for 1 h, washed with PBS containing 0.1% saponin, and stained with Alexa Fluor 488-, Alexa Fluor 546-, or Alexa Fluor 647-conjugated secondary antibodies for 1 h. For DNA staining, cells were fixed in PBS containing 4% paraformaldehyde and then treated with 200 μg/ml RNase A and 20 μg/ml propidium iodide for 0.5–1 h. After staining, cells were mounted in PBS containing 50% glycerol and 1 mg/ml p-phenylenediamine. To detect Ku70, cells fixed in PBS containing 4% paraformaldehyde for 20 min were extracted in PBS containing 0.05% Triton X-100 for 5 min and blocked in PBS containing 0.1% saponin and 3% bovine serum albumin at room temperature. Composite figures were prepared using GIMP 2.6.2 and Illustrator 16.0. To detect phospho-DNA-PKcs, cells fixed in PBS containing 4% paraformaldehyde for 20 min were extracted in 0.5% Triton lysis buffer for 3 min on ice and blocked in PBS containing 0.1% saponin and 3% bovine serum albumin at room temperature. For quantitation of the number of phospho-DNA-PKcs foci per cells, fluorescence intensities of immunostaining were measured using ImageJ software (National Institutes of Health).

Apoptosis Assay—To induce apoptotic responses, cells were treated with UV (UV-C) light using a CL-1000 UV cross-linker (UVP LLC) or with Adriamycin (Wako Pure Chemical Industries). For UV irradiation, cells were treated with or without UV light after removal of medium and cultured in fresh medium for 10–12 h. For ADR treatment, cells were incubated with culture medium containing 1 μg/ml ADR for the indicated times. Cells were stained with propidium iodide for detection of nuclear condensation/fragmentation (Figs. 4 and 7, B, C, and F–H) or with anti-cleaved caspase-3 antibody for detection of caspase-3 activation (Fig. 7, E and I). The percentages of apoptotic cells were quantitated as the ratio of the number of apoptotic cells to the total number of cells from more than three randomly selected fields. The number of apoptotic cells in each field was counted from myc-positive cells among myc-Ku70-WT- and its mutant-transfected cells and from vector-transfected or untransfected cells.

In Vitro Kinase Assay—Cell lysates were prepared in Triton X-100 lysis buffer (30 mM HEPES, pH 7.4, 0.5% Triton X-100, 100 mM NaCl, 4 mM EDTA, 10 mM NaF, 50 μg/ml aprotinin, 100 μM leupeptin, 25 μM pepstatin A, and 2 mM PMSF) as described (5). Immunoprecipitation was performed using anti-myc or anti-HA antibody-precoated protein-G beads. In vitro kinase assays were performed as described (13, 14, 84, 87). In brief, c-Src was immunoprecipitated with anti-HA antibody from Triton X-100 lysates of COS-1 cells transfected with c-Src (c-Src-HA) or c-Src(KD) (c-Src[KD]-HA). myc-Ku70-WT was immunoprecipitated with anti-myc antibody from Triton X-100 lysates of COS-1 cells transfected with myc-Ku70-WT. After washing, myc-Ku70-WT was eluted with 0.2 M glycine-HCl buffer, pH 2.5, and immediately neutralized with 1 M Tris-HCl, pH 8.8. Equal amounts of c-Src immunoprecipitates bound to the beads were reacted with eluted myc-Ku70-WT in kinase buffer (40 mM HEPES, pH 7.4, 0.1% Triton X-100, 5 mM MnCl2, 5 mM MgCl2, and 1 mM Na3VO4) containing 100 μM unlabeled ATP at 30°C for the indicated periods. Phosphorylated bands were immunodetected with anti-Tyr(P) antibody, and the intensity of chemiluminescence was measured using Quantity One software (Bio-Rad). Composite figures were prepared using GIMP 2.6.2 and Illustrator 16.0.

Author Contributions—M. M. and Na. Y. conceived of the study, designed the experiments, and wrote the manuscript. M. M. carried out the majority of experiments. S. K., T. T., and T. K. performed phosphoproteomic analysis. S. K. performed some of the Western blotting experiments. M. M., S. K., T. H., R. Y., T. M., No. Y., and Na. Y. analyzed and discussed the data. All authors reviewed and approved the manuscript.

Acknowledgments—We are grateful to Dr. Donald J. Fujita, Dr. Stéphane A. Laporte, Dr. Tadashi Yamamoto, Dr. Toshiki Tamura, Dr. Takayuki Yoshimoto, and Dr. Hiroyuki Miyoshi for invaluable plasmids and antibodies.

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Tyrosine Phosphorylation of Ku70

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