Nuclear Akt interacts with B23/NPM and protects it from proteolytic cleavage, enhancing cell survival

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B23/NPM is a major nucleolar phosphoprotein that has a critical role in cell proliferation and cell death. Here, we show that it forms a complex with Akt on growth factor (GF) stimulation in both the cytoplasm and the nucleus, for which Akt activation is indispensable. The C terminus of B23 (239–294 residues) potently binds pleckstrin homology (PH) domain of Akt. Akt binding to B23 protects it from proteolytic degradation by caspase-3, leading to the up-regulation of cell survival. Interestingly, unsumoylated B23 K263R, but not wild-type B23, strongly interacts with Akt in the nucleoplasm in the absence of GFs. Furthermore, we show that Akt2, but not other isoforms, specifically regulates B23 sumoylation and protein stability. Also, nuclear Akt regulates the cell cycle progression activity of B23. Therefore, our findings support that nuclear Akt binds and stabilizes B23 in the nucleoplasm, and regulates its activities in cell survival and cell cycle.

Akt/PKB | caspase-3 | stability | sumoylation

The serine/threonine protein kinase, PKB/Akt, has a critical role in cell survival (1). Akt predominantly localizes in the cytoplasm, but it also occurs in the nucleus or translocates to the nucleus on stimulation (2, 3). For example, after 20–30 min of nerve (N)GF treatment, Akt translocates to the nucleus. Akt-associated pathways and substrate proteins leading to antiapoptotic effects and cellular survival are complex, but recent studies demonstrate that nuclear Akt impinges on the apoptotic machinery either by phosphorylation of nuclear substrates or by interacting with nuclear proteins (4–6). For example, nuclear Akt phosphorylates acinus, which induces chromatin condensation after caspase-3 dependent cleavage, and protects acinus cleavage, thereby inhibiting chromatin condensation (7).

Nucleophosmin (NPM)/B23 are a major nucleolar phosphoprotein involved in ribosome biogenesis (8). B23 also localizes in the cytoplasm and mediates centrosome duplication (9). B23 is a dynamic protein constantly shuttling between the nucleus and cytoplasm (10), and from the nucleolus to the nucleoplasm during S phase of the cell cycle, or under certain anticancer drug treatment (11, 12). In addition, unsumoylated B23 accumulates in the nucleoplasm (13), supporting the notion that B23 trafficking may be critical for its cellular functions. Overexpression of B23 induces a p53-dependent cell cycle arrest in normal fibroblasts, whereas it promotes S-phase entry in the absence of p53. Conversely, the loss of B23 expression attenuates cell proliferation and increases apoptosis (8, 14), suggesting that B23 may have both oncogenic potential and tumor suppressing functions.

In this article, we show that Akt interacts with B23 in response to NGF stimulation and prevents its proteolytic cleavage *in vitro* and *in vivo*, thus enhancing cell survival. In addition, B23 K263R, the major sumoylation site mutant that is essential for B23 nucleolar localization and resistance against apoptotic cleavage, more strongly interacts with Akt, implying that this interaction may occur in the nucleoplasm to compromise B23 destabilization. Also, we demonstrate that knocking down of Akt2 but not other isoforms dramatically decreases B23 protein level, resulting in its nucleoplasmic distribution. Therefore, Akt binds B23 and protects it from apoptotic cleavage, enhancing cell survival.

Results

Akt Interacts with B23. Akt translocates into the nucleus and prevents DNA fragmentation, and B23 resides mostly in the nucleus and protects against DNA cleavage during apoptosis on NGF stimulation (15, 16). To determine whether Akt and B23 physically interact, we conducted a binding assay with various B23 deletion mutants (Fig. 1*A Left*). GST pull-down assay revealed that HA-Akt strongly bound to the Δ14–107 and C-terminal fragment Δ1–239, but only a negligible interaction occurred with full length (FL) B23. Interestingly, the binding did not occur with fragment Δ240–294 or other truncations of B23, suggesting that amino acids 240–294 are essential for binding to Akt (Fig. 1*A Right*). Because FL-B23 barely interacts with Akt, we wondered whether the association between FL-B23 and Akt requires GF stimulation. On NGF stimulation for 45 min, Akt associated with GST-B23 clearly, but not with GST-alone (Fig. 1*B*), suggesting that this interaction can be regulated by GFs. To ascertain the interaction between Akt and B23, we performed coimmunoprecipitation assay with cotransfected mammalian expressing GST-Akt and various GFP-B23 constructs, followed by NGF treatment for 45 min. Immunoblotting revealed that the FL-B23 and C-terminal domain (152–294) of B23 bound to Akt, but not the N-terminal domain (1–152), supporting that the C-terminal domain is essential for B23 association with Akt (Fig. 1*C*). A mapping experiment with GST-tagged Akt fragments revealed that endogeneous B23 evidently bound to FL-Akt and the N-terminal PH domain of Akt, but not the catalytic domain or C-terminal tail domain of Akt, indicating that the N-terminal PH domain is necessary for Akt to interact with B23 (Fig. 1*D*).

Active Nuclear Akt Interacts with B23. We noticed that Akt interaction with B23 requires NGF stimulation, implying that Akt activation might regulate the complex formation. To examine whether NGF regulates Akt/B23 association in cells, we conducted coimmunoprecipitation assay with anti-Akt antibody in PC12 cells. The binding between endogenous Akt and B23

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The authors declare no conflict of interest.

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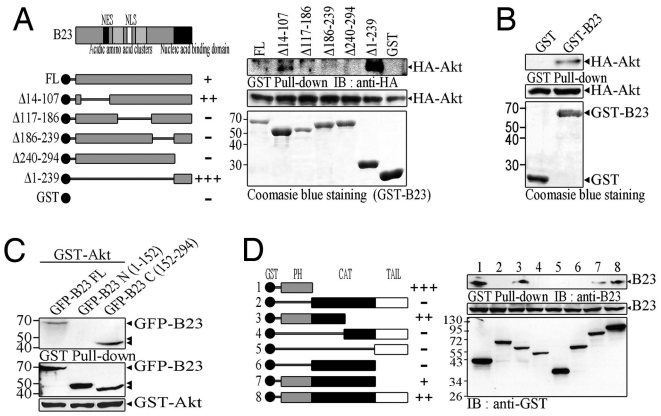

Fig. 1. Akt interact with B23. (*A*) Schematic representation of B23 FL, deletion mutants, and their respective affinity with Akt (*Left*). GST-B23 interacts with Akt *in vitro*. Purified GST-B23 FL and deletion proteins were incubated with lysates of PC12 cells transfected with HA-Akt. The C terminus of B23 strongly binds to Akt (*Top Right*). Transfection and GST-recombinant proteins were verified (*Middle Right* and *Bottom Right*). (*B*) Purified GST and GST-B23 proteins were incubated with HA-Akt transfected PC12 cell lysate after NGF treatment for 45 min, and the samples were probed with anti-HA (*Top*). Transfection and GST-recombinant proteins were verified (*Middle* and *Bottom*). (*C*) PC12 cells were cotransfected with GST-Akt FL and GFP-B23 FL, GFP-B23-N, or GFP-B23-C; 500 μg of cell lysate was applied for the GST-pull down assay and probed with anti-GFP (*Top*). The expressions of GFP-B23 and GST-Akt were verified (*Middle* to *Bottom*). (*D*) Schematic representation of Akt-FL, deletion mutants, and their respective affinity with B23 (*Left*). PC12 cells were transfected with GST-Akt-FL or truncated constructs. After GST pull down, the samples were probed with anti-B23 (*Right*).

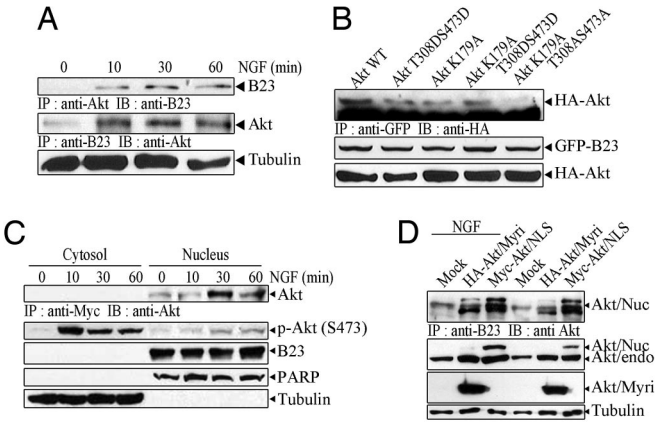

Fig. 2. Active nuclear Akt interact with B23. (*A*) NGF mediates endogenous Akt/B23 interaction. PC12 cells were serum-starved overnight followed by 100 ng/ml NGF; 500 μg of cell lysate was immunoprecipitated with anti-Akt or anti B23 antibodies, and probed with anti-B23 or anti-Akt antibodies, respectively (*Top* and *Middle*). Tubulin was used as a loading control (*Bottom*). (*B*) B23 selectively associates with active (phosphorylated) Akt. Various HA-Akt constructs (active Akt T308DS473D; kinase-dead K179A; active but kinase deficient K179AT308DS473D; and dominant negative K179AT308AS473A) were cotransfected with GFP-B23 into PC12 cells. Equal levels of GFP- and HA-constructs were expressed (*Middle* and *Bottom*). (*C*) B23 binds Akt in the nucleus. Myc-B23 cells were cultured in induced media for 24 h and fractionated to cytoplasm and nucleus after stimulation with NGF; 500 μg of each cytosolic or nuclear fraction were coimmunoprecipitated with anti-myc antibody. Phosphorylation and nuclear translocation of Akt on NGF stimulation were examined by p-Akt antibody (second lane). The induction of B23 was verified (third lane). PARP and Tubulin antibodies were used as markers for each fraction (fourth and fifth lanes). (*D*) Nuclear Akt binds to B23. HEK293 cells were infected with adenovirus expressing mock, HA-tagged myristoylated Akt, or NLS-Akt, and treated with or without NGF for 30 min. The expression of myc-NLS-Akt and HA-myristoylated Akt was verified (second and third lanes). The tubulin was used as a loading control.

occurred at 10 to 60 min on NGF stimulation (Fig. 2*A Top*). The reciprocal immunoprecipitation was conducted with anti-B23 antibody and revealed similar results (Fig. 2*A Middle*). To explore whether Akt phosphorylation status has any role in regulating the interaction between B23 and Akt, we cotransfected myc-B23 into HEK293 cells with wild-type and various mutant HA-Akt constructs, and treated with EGF for 20 min. The strongest binding occurred between B23 and constitutively active Akt (T308DS473D), a phosphorylation mimetic mutant. Nonetheless, WT, a kinase inactive mutant (K179A), and a kinase inactive but phosphorylation mimetic mutant (K179AT308DS473D) were able to bind B23. By contrast, the kinase inactive and nonphosphorylated mutant (K179AT308AS473A) failed to interact with B23. This finding underscores that Akt phosphorylation, but not kinase activity, is required for its association with B23 (Fig. 2*B*). Also, NGF triggered a demonstrable association between Akt and B23, and wortmannin, pharmacological inhibitor of PI 3-kinse pretreatment completely disrupted the interaction, whereas control or MEK1 inhibitor, PD98059 pretreatment failed to impede the interaction in myc-B23 cells [supporting information (SI) Fig. S1*A*] (15). Hence, these data support the notion that Akt activation might be indispensible for the interaction with B23. Fitting with our previous observation that phosphorylated Akt accumulated in the nucleus on NGF stimulation (3), subcellular fractionation demonstrated that endogenous B23 potently bound nuclear translocated Akt after 30-min treatment by NGF, but not cytoplasmic Akt, indicating that B23 is a potential binding partner of nuclear Akt (Fig. 2*C Top*), Akt phosphorylation was coupled to the interaction with B23 (Fig. 2*C* second lane). To explore whether the nuclear translocation of Akt is required for the interaction with B23, we infected PC12 cells with adenovirus expressing HA-tagged myristoylated-Akt or nuclear localized signal (NLS)-Akt (4). Nuclear Akt bound to B23 regardless of NGF, but plasma membrane Akt associated with cytoplasmic B23 in response to NGF treatment (Fig. 2*D Top*). However, a GFP-B23 and red fluorescent protein (RFP)-Akt cotransfection study showed that Akt colocalized with B23 in the nucleus on NGF induction (Fig. S1*B*). Thus, activation of Akt and its nuclear translocation are essential for the interaction with B23.

Akt Binding Prevents B23 Proteolytic Cleavage and Enhances Cell Survival. B23 is a substrate of caspase-3 (17). To explore whether Akt binding has any role in mediating the apoptotic cleavage of B23, we used stably transfected PC12 cells with an inducible form of active myc-NLS tagged Akt (CA) constructs or empty vector (EV) alone (16), and induced apoptotic cleavage of B23 by treatment of staurosporine. Apoptotic stimuli clearly demonstrated B23 protein cleavage in control EV cells. In contrast, B23 in CA cells was almost intact, underscoring that nuclear-targeted Akt prevents B23 apoptotic degradation (Fig. 3*A*). In cultured hippocampal neurons, depletion of Akt using shRNA adenovirus (7) significantly increased B23 cleavage on glutamate insult, whereas mock virus infection did not alter B23 protein level (Fig. 3*B*), suggesting that NGF-mediated Akt interaction protects B23 from apoptotic degradation. Similarly, our *in vitro* cleavage assay showed that in the absence of active Akt, B23 was potently cleaved in a time-dependent manner. In contrast, preincubation with active Akt substantially decreased caspase-3-mediated B23 cleavage (Fig. S2 *Left*), supporting that active Akt protects B23 from caspase-3 dependent cleavage.

To evaluate whether the Akt/B23 complex is critical for preventing apoptosis, we infected NLS-Akt or shRNA-Akt adenovirus into myc-B23 cells and induced apoptosis by stauro-

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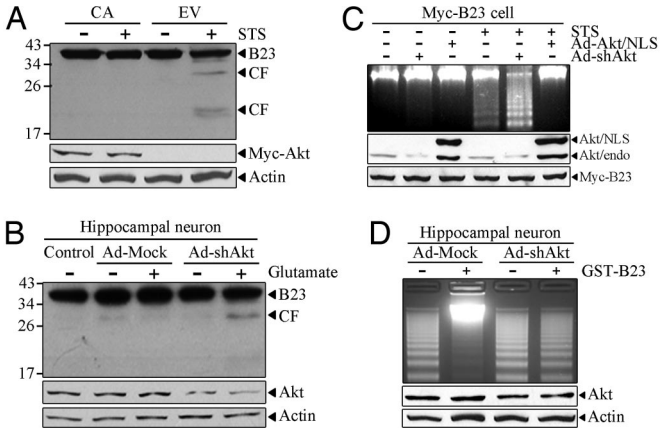

Fig. 3. Akt binding prevents B23 proteolytic cleavage and enhances cell survival. (*A*) CA and EV stably transfected PC12 cells were treated with or without 250 nM of STS for 18 h. Verification of myc-Akt induction and equal sample loading is shown by anti-myc and anti-β actin antibodies. (*B*) Knocking down of Akt destabilizes B23 in hippocampal neurons. Primary hippocampal neurons were cultured for 3 days and infected with control or adenovirus expressing shRNA-Akt for 24 h, followed by 1 mM of glutamate insult for 10 h. Immunoblot analysis was performed with anti-B23 antibody. The efficient Akt depletion and equal loading control are shown by anti-Akt and anti-β-actin antibodies. (*C*) NLS-Akt enhances the antiapoptotic effect of B23, but depletion of Akt reduces its antiapoptotic effect. Myc-B23 stably transfected PC12 cells were infected with Ad-mock, Ad-shAkt, or Ad-NLS-Akt virus. After 24 h, the cells were treated with or without 250 nM of STS for 18 h; 10 μg of genomic DNA was loaded on to a 2% agarose gel. Verification of viral infection and equal sample loading is shown by anti-Akt and anti-β actin antibodies (*Middle* and *Bottom*). (*D*) Ablation of Akt from hippocampal neurons enhances DNA fragmentation in the presence of B23. Hippocampal neurons were infected with Ad-Mock or Ad-shAkt virus for 24 h. Then, the cells were used for nuclei extraction and subsequent *in vitro* DNA fragmentation assay (*Top*). Similar protein quantities and viruses were verified (*Middle* and *Bottom*).

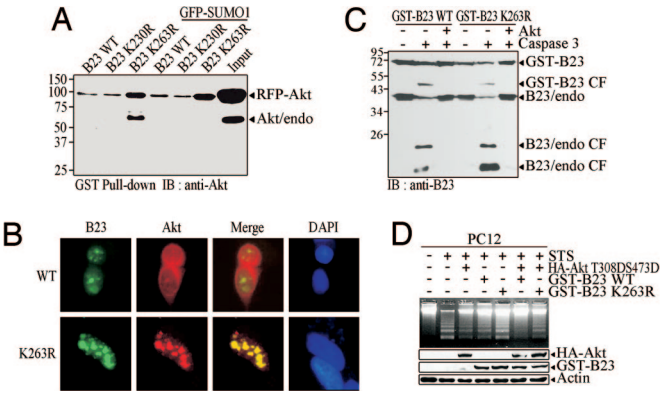

Fig. 4. B23 sumoylation has a role in mediating B23 binding to nuclear Akt. (*A*) The K263R mutant constitutively binds to Akt. Various GST-B23 constructs were cotransfected into HEK293 cells with GFP-Sumo1 and RFP-Akt. (*B*) B23 K263R and Akt colocalized in the nucleus. GFP-B23 WT and K263R were cotransfected into HEK293 cells with RFP-Akt. (*C*) Active Akt reduces the instability of B23 K263R. PC12 cells were transfected with GST-B23 WT and K263R. After 24 h, cell lysates were incubated with active caspase-3 with or without active Akt. Reaction mixtures were analyzed by immunoblotting. (*D*) Active Akt decreases DNA fragmentation in both B23 WT and B23 K263R mutant transfected cells. PC12 cells were transfected with HA-AKT T308DS473D or/and GST-B23 WT or K263R, followed by 250 nM of STS treatment for 18 h. Verification of transfected proteins and identical protein loading are shown by using anti-HA, anti-GST, and anti-β actin antibodies.

sporine. Depletion of Akt in myc-B23 cells does not evoke DNA fragmentation in control cells. However, remarkable DNA fragmentation occurred in Akt depleted cells on staurosporine treatment, which was blocked in NLS-Akt expressing cells (Fig. 3*C*). We also extended similar study into primary hippocampal neurons (Fig. 3*D*). ShRNA-Akt expressing cells revealed remarkable DNA fragmentation regardless of GST-B23 protein, whereas mock-virus infected cells showed demonstrable DNA fragmentation in the absence of GST-B23 protein. These data precisely correlate with the binding ability of B23 with Akt, implying that the B23/Akt interaction is important for antiapoptotic effect of B23.

B23 Sumoylation Has a Role in Mediating Its Binding to Nuclear Akt. A recent study (13) shows that B23 can be sumoylated on both Lys 230 and Lys 263 residues, whereas Lys 263 is the major sumoylation site. To explore whether B23 sumoylation has any role in the association between B23 and Akt, we cotransfected various GST-B23 constructs into HEK293 cells with GFP-Sumo1 and RFP-Akt. GST-pull down experiments demonstrated that sumoylation enhanced the interaction between B23 and Akt. Interestingly, the unsumoylated K263R mutant bound to both endogenous Akt and transfected RFP-Akt with much stronger affinity than that of wild-type and K230R (Fig. 4*A*). Expression level of transfected proteins are shown in Fig. S3. To further examine the effect of sumoylation on the interaction between B23 and Akt, we conducted immunofluorescent staining studies. Akt is mostly distributed in the cytoplasm, and did not colocalize with in wild-type transfected cells. Surprisingly, Akt translocated into the nucleus and colocalized with the B23 K263R mutant, which distributed in the nucleoplasm (Fig. 4*B*). Therefore, B23 sumoylation has an essential role in dictating B23 association with Akt. Sumoylation protects B23 from apoptotic degradation, and unsumoylated K263R is more sensitive to caspase-3 cleavage and apoptosis (13). To explore whether the nuclear targeting of Akt compromises the sensitivity of the unsumoylated B23 mutant for apoptotic degradation, we performed *in vitro* apoptotic cleavage assays with active caspase-3 and purified recombinant B23 in the absence or presence of active Akt. Compared with wild-type B23, the K263R mutant was more severely degraded. Nonetheless, Akt completely desensitized both wild-type and K263R B23 from apoptotic degradation (Fig. 4*C*). A DNA fragmentation assay revealed that the constitutively active form of Akt decreased DNA fragmentation either B23 wild-type or K263R mutant cotransfected cells (Fig. 4*D*). Collectively, these data suggest that Akt association with B23 has an essential role in protecting unsumoylated B23 from apoptotic degradation, thereby promoting cell survival.

Akt2 Specifically Regulates B23 Stability and Sumoylation. The three mammalian isoforms of Akt (Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ) share a high sequence homology and structural similarity. Each isoform of Akt has distinctive as well as common roles in cells (18–20). To determine whether B23 regulation by Akt is isoform specific, we used mouse embryonic fibroblast (MEF) from the following genotype combinations: WT; 1KO, Akt1/PKBα (−/−); 2KO, Akt2/PKBβ (−/−); DKO, Akt1/PKBα (−/−); and Akt2/PKBβ (−/−) (21). Interestingly, B23 relocalized from the nucleolus to the nucleoplasm in MEF 2KO and DKO cells (Fig. 5*A*). Immunofluorescent staining revealed that the elimination of Akt2 by siRNA Akt2 elicited endogenous B23 nucleoplasmic distribution in HEK 293 cells as shown in 2KO and DKO MEF cells, suggesting that Akt2 may regulate B23 nucleolar localization (Fig. 5*B*). Because B23 stability is tightly involved its nucleolar localization (22), if Akt2 is indeed critical for B23 nucleolar localization, ablation of Akt2 should affect on B23 stability. We tested this hypothesis by using MEF cell lines and half-life assay of B23 with isoform specific siRNA-Akt1 and Akt2. B23 protein levels were dramatically reduced in MEF 2KO and DKO cells, whereas the B23 protein level in MEF 1KO as

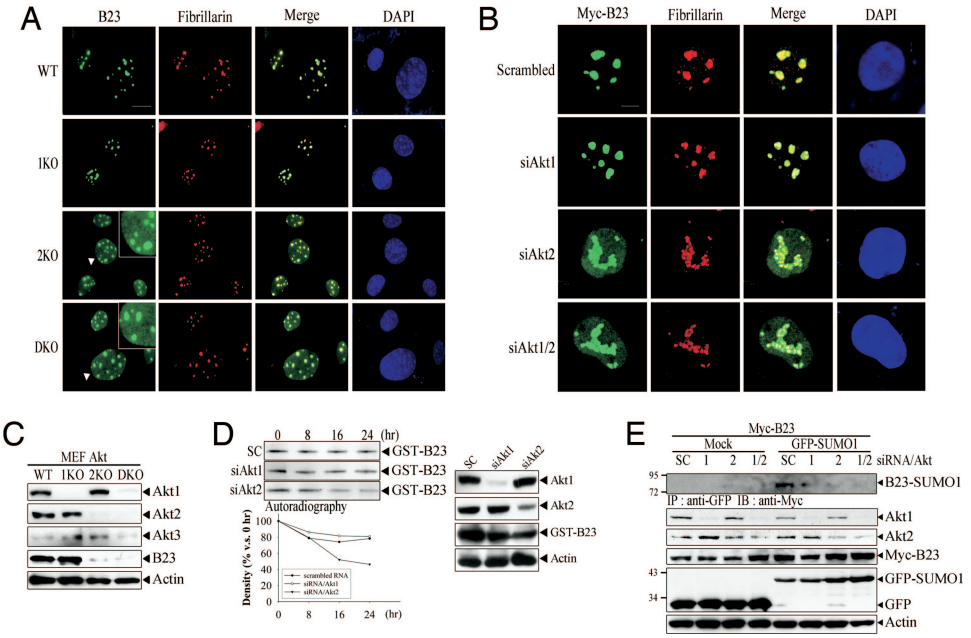

Fig. 5. Akt2 specifically regulates B23 stability and sumoylation. (*A*) Delocalization of B23 from nucleoli in MEF 2KO and DKO cells. MEF cells were immunostained with anti-B23 and antifibrillarin. Fibrillarin indicates nucleoli. Nuclei were counterstained with DAPI. An arrowhead indicates the magnified position in the white inset. Green, B23; red, fibrillarin; blue, DAPI. (Scale bar, 10 μm.) (*B*) Delocalization of B23 from nucleoli by depletion of Akt2. Transfected HEK293 cells with siRNA (Akt1, Akt2, Akt1 and 2, or scrambled siRNA) were immunostained following above. An arrowhead indicates the magnified position in the white inset. (Scale bar, 5 μm.) (*C*) Reduced B23 level in MEF 2KO and DKO cells. Immunoblots were probed by using antibodies against anti-Akt1, anti-Akt2, anti-Akt3, and anti-B23. Actin was used for a loading control. (*D*) B23 stability is affected by depletion of Akt2. PC12 cells were cotransfected with GST-B23 and siRNAs. Metabolic labeled GST-B23 with ^{32}S-methionine was applied for GST-pull down and subjected to SDS/PAGE (*Upper Left*). B23 stability was estimated by densitometry. Bar indicated SE (*Lower Left*). Control blots for protein expression and knock down effect were probed with anti-Akt1, Akt2, GST, and actin antibodies (*Right*). (*E*) Sumoylation of B23 is specifically regulated by Akt2. HEK293 cells were cotransfected with myc-B23, GFP-SUMO1 (or Mock), and siRNAs. Sumoylated B23 was immunoprecipitated by using anti-GFP antibody and immunoreacted with anti-myc antibody (*Top*).

well as MEF WT cells was not altered (Fig. 5*C*). A pulse–chase experiment showed that B23 in scramble siRNA transfected cells was a stable protein, revealing intact form of protein during 24 h. B23 in siRNA-Akt1 transfected cells was also a very stable protein with a half life as long as that of the B23 in scrambles siRNA expressing cells, whereas B23 in siRNA-Akt2 transfected cells was much shorter lived relative to control, showing increased instability (Fig. 5*D*). These data suggest that Akt 2 has a pivotal role in mediating B23 protein stability, probably through regulating its nucleolar localization, which agrees with our previous observation that abrogation of B23 nucleolar residency destabilizes the B23 protein. To better determine whether lower B23 protein level in Akt2 depleted cells regulates B23 sumoylation, we diminished the each isoforms of Akt protein level. Fig. 5*E* shows that a reduction of the Akt2 protein level in HeLa cells markedly altered B23 sumoylation compared with control or siRNA-Akt1 transfected cells. Notably, in the siRNA-Akt2 transfected cells, cotransfected myc-B23 was aggregated and distributed in both the nucleolus and the nucleoplasm, altering colocalization with Sumo1 (Fig. S4), suggesting Akt2 is responsible for dictating B23 nucleolar residency, thereby regulating its sumoylation.

Nuclear Akt Regulates Cell Cycle Progression Activity of B23. Because Akt is known to promote cell growth by regulating cell cycle progression and B23 has been proposed coordinately to regulate ribosome biogenesis and cell cycle progression (23), we attempted to investigate whether Akt regulates the effect of B23 in cell proliferation. Induction of B23 accelerates S-phase entry in myc-B23 cells (Fig. S5*A*). FACS analysis with myc-B23 cells infected with shRNA-Akt or NLS-Akt adenovirus showed that knocking down Akt reduced the S-phase population, whereas NLS-Akt expression increased the S-phase population, suggesting that Akt may regulate the role of B23 in cell cycle progression (Fig. 6*A*). However, we conducted FACS analysis with MEF WT, 1KO, and 2KO cells transfected with shRNA-B23 or GFT-B23 for 30 h (Fig. 6*B*). Overexpression of B23 in MEF WT cells enhanced the S-phase population (Fig. S5*B*). Remarkably, MEF

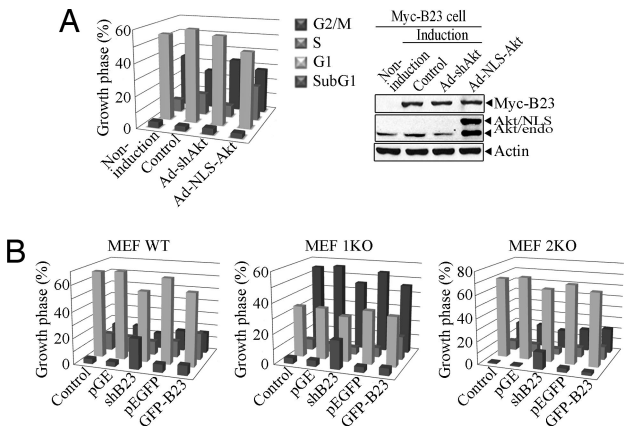

Fig. 6. Nuclear Akt regulates cell cycle progression activity of B23. (*A*) Akt is required for B23 mediating cell cycle progrssion. Myc-B23 cells were cultured in the induced or noninduced condition for 30 h. Then, the cells were infected with Ad-Mock control, Ad-siAkt, or Ad-NLS-Akt virus for 24 h before FACS analysis. Verification of B23 induction, effective virus, and equal protein loading is shown in the *Right*. (*B*) MEF WT, MEF 1KO, MEF 2KO, and MEF DKO cell lines were transfected with pGE-1(control vector), shRNA-B23, pEGFP-C2, or pEGFP-B23 constructs for 30 h. Then the cells were stained with PI before FACS analysis. The cell phase distribution is shown in the representation.

1KO cells revealed G_2/M phase arrest, whereas MEF 2KO cells accumulated in G_1 phase, implying differential regulation of cell cycle by Akt isoforms. Forced expression of GFP-B23 in 1KO alleviated cells from G_2 phase, raising S-phase cells. In 2KO cells, B23 slightly promoted S-phase entry, decreasing G_1 population. However, depletion of B23 in any of MEF cells substantially increased cells in the sub G_1 phase, but did not alter S phase, in agreement with its antiapoptotic role. The detailed cell cycle profile and protein expression are shown in Fig. S5*B*. Collectively, our data suggest that Akt isoforms distinctively regulate cell cycle progression and B23 may have some role in G_2/M transition, thus enhancing the S-phase population.

Discussion

In this article, we demonstrated that B23 acts as a novel nuclear Akt interacting protein. On GF stimulation, Akt directly interacts with B23 and protects it from apoptotic cleavage. Specifically, we found that Akt2 is a major isoform that regulates B23 biological functions including sumoylation, degradation, and cell cycle. B23 is a highly abundant nucleolar protein but also redistributes from the nucleolus to nucleoplasm. Here, we observed that the Akt/B23 interaction is exhibited in the nucleoplasm (Fig. S1*B*). This notion was supported by our finding that the K263R mutant, which was dispersed to the nucleoplasm from nucleolus, strongly binds to Akt even in the absence of GF (Fig. 4 *A* and *B*).

B23 acts as a physiological substrate of caspase-3 (17). Here, we show that Akt binding has a protective role in preventing B23 from apoptotic degradation. Our previous finding suggests that the B23/phosphatidylinositol 3,4,5-trisphosphate (PIP3) complex associates with caspase-activated DNase (CAD) and inhibits its nuclease activity (4, 15). In agreement with its susceptibility to caspase cleavage, our data demonstrate that both purified GST-B23 and endogenous B23 are degraded in the absence of either purified active Akt or stably transfected active Akt in cells by caspase-3 or staurosporine insult, respectively (Fig. 3). Consistent with these observations, depletion of Akt in myc-B23 cells or hippocampal neurons displayed demonstrable DNA fragmentation compared with that of the control. Surprisingly, K263R, which has been shown to have a higher susceptibility to caspase cleavage compared with wild-type (13), was completely blocked from caspase-induced degradation in the presence of active Akt (Fig. 4*C*), underscoring that B23/Akt association protects B23 from degradation. Correlatively, K263R transfected PC12 cells notably reduced DNA fragmentation in the presence of active Akt expression (Fig. 4*D*). Presumably, Akt preferentially binds to nucleoplasmic B23 to prevent its degradation, thereby enhancing cell survival. Interestingly, specific depletion of the Akt2 isoform in cells disperses B23 both inside and outside of the nucleolus and substantially decreases its protein level, affecting protein stability. Correlatively, the specific knocking down of Akt2 with siRNA-Akt2 disturbs the sumoylation of B23. Conceivably, Akt2 regulates B23 nucleolar localization, protein stability, and proper sumoylation.

In addition to regulating protein stability of B23, Akt also mediates the cell cycle progression activity of B23. B23 promotes S-phase entry (Fig. S5*A*) and depletion of B23 results in an abundant sub-G_1 population, confirming the cell survival effect of B23 (Fig. 6*B* and Fig. S5*B*). However, knocking down of Akt from myc-B23 cells abrogates S-phase entry and overexpression of nuclear Akt increases S-phase population (Fig. 6*A*), implying that Akt and B23 together might regulate cell cycle progression. Akt regulates the G_1 phase through activation of cyclin/Cdk kinase and inactivation of Cdk inhibitors. It also promotes cell cycle progression at the G_2/M transition through inactivation of WEE1Hu. Here, we show that Akt1 is involved in regulating G_2/M phase, and Akt2 promotes cell proliferation by regulating G_1 phase (Fig. 6*B*). Nevertheless, cell cycle profile is not affected in transient transfection of siRNA-Akt1, si-Akt2, and si-Akt1/2 in HeLa or 293 cells (Fig. S6), which might be due to the redundant functions of Akt isoforms. It is possible that Akt mediates the cell cycle regulatory proteins, including B23, in an isoform specific manner. It is unknown whether specific isoform of Akt binds to B23 and redundant function of Akt isoform is responsible for the specific regulation of the cell cycle regulatory proteins. Thus, our findings provide mechanistic insights into how nuclear Akt pathway mediates cell survival through interacting with nucleolar protein B23.

Materials and Methods

Cell Cultures and Reagents. PC12 cells were maintained in medium A (DMEM; Cellgro) with 10% FBS, 5% horse serum, and 100 units of penicillin-streptomycin. PC12 cells with stably inducible form of B23 or Akt constructs were cultured in medium B (medium A with 100 μg/ml G418, 100 μg/ml hygromycin B, and 2 μg/ml tetracycline) and induced in medium B without 2 μg/ml tetracycline for 24 h. MEF WT, MEF Akt Knockout cells, and 293A cells were maintained in DMEM containing 10% FBS in a humidified incubator at 37°C and 5% CO_2. For primary hippocampal neuron culture, the hippocampus was dissected from a new-born rat. After trypsinization, the cells were cultured in neurobasal-A/B27 media at 37°C, under a 5% $C0_2$ atmosphere in a humidified incubator. Akt (pan), Akt1, Akt2, Akt3, phosphoAkt (S473), GST, and PARP antibodies were obtained from Cell Signaling; α-Tubulin, β-Actin, Fibrillarin, and c-myc (9E10) antibodies were from Santa Cruz. Mouse anti-NPM polyclonal antibody was generated in our laboratory. Active Akt and active caspase-3 proteins were obtained from Upstate. All other chemicals were obtained from Sigma.

The siRNA Constructs. All siRNA oligonucleotides against Akt1 (sense, 5′-CCAUGAACGAGUUUGAGUACCUG AA-3′, antisense, 5′-UUCAGGUACUCAAACUCGUUCAUGGUC-3′), Akt2 (sense, 5′-UCC AUCAUCUCAGAUGUGGAAGAGUCA-3′, antisense, 5′-AGGUAGUAGAGUCUACACC UUCUCA-3′), and scrambled siRNA (Negative control, sense, 5′-CUUCCUCUCUUUCU CUCCCUUGUGA-3′, antisense, 5′-AGGAAGGAGAGAAAGAGAGGGAACACU-3′) were obtained from Integrated DNA Technologies.

DNA Fragmentation Assay. Oligonucleosomal fragmentation of genomic DNA was determined as described below. Briefly, 3×10^6 cells in 10 ml of medium were incubated with 250 nM of staurosporine (STS) for 18 h or 1 mM glutamate for 10 h. After incubation, the cells were lysed on ice for 60 min in 500 μl lysis buffer of 0.02% SDS/1% Nonidet P-40/0.2 mg/ml proteinase K in PBS. Genomic DNA was extracted by phenol/chloroform method. The pellet was dissolved in 50 μl of TE buffer (10 mg/ml RNase) for 2 h at 37°C. A total of 10 μg of DNA was loaded on a 2% agarose gel and visualized under UV light.

Immunostaining. Cells grown on coverslips in 24 well plates were fixed in PBS containing 4% paraformaldehyde for 15 min, permeabilized in PBS containing 0.25% Triton X-100 for 10 min, and blocked in 2% BSA for 30 min. Cells were immunostained by using antibodies against B23 and fibrillarin with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit. Nuclei were counterstained with DAPI. Cells were visualized under a fluorescent microscope or a Zeiss LSM confocal fluorescence microscope.

Half Life Assay (Metabolic Labeling). PC12 cells were transfected with the scrambled RNA, siRNA-Akt1 or siRNA-Akt2 prior for withdrawal of methionine for 1 h at 37°C in DMEM lacking methionine, cystein (Gibco). Cells were pulse-labeled with 50 μCi/ml of [^{35}S]Methionine (NEN Renaissance Products, Perkin–Elmer). After 2 h, the cells were washed in PBS and chased with complete culture medium. Later, cells were washed and performed GST-pull down assay. Harvested samples were subjected to SDS/PAGE followed by autoradiography. Protein half-life was estimated by using densitometry.

Flow Cytometry Assay. We fixed 10^6 cells with 70% ethanol at −20°C for 24 h and cells were washed with PBS, and resuspended in 100 μl phosphate/citrate buffer (0.2 M Na_2HPO_4, 0.1 M citrate, pH 7.5) for 30 min at room temperature. After washing with PBS, the samples were incubated with propidium iodide solution (20 μg/ml PI, 20 μg/ml RNase in PBS) for 30 min at RT and analyzed with a FACSCalibur system (Becton Dickinson).

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