Serum- and glucocorticoid-inducible kinase 1 (SGK1) controls Notch1 signaling by downregulation of protein stability through Fbw7 ubiquitin ligase

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Summary

Notch is a transmembrane protein that acts as a transcriptional factor in the Notch signaling pathway for cell survival, cell death and cell differentiation. Notch1 and Fbw7 mutations both lead the activation of the Notch1 pathway and are found in the majority of patients with the leukemia T-ALL. However, little is known about the mechanisms and regulators that are responsible for attenuating the Notch signaling pathway through Fbw7. Here, we report that the serum- and glucocorticoid-inducible protein kinase SGK1 remarkably reduced the protein stability of the active form of Notch1 through Fbw7. The protein level and transcriptional activity of the Notch1 intracellular domain (Notch1-IC) were higher in SGK1-deficient cells than in SGK1 wild-type cells. Notch1-IC was able to form a trimeric complex with Fbw7 and SGK1, thereby SGK1 enhanced the protein degradation of Notch1-IC via a Fbw7-dependent proteasomal pathway. Furthermore, activated SGK1 phosphorylated Fbw7 at serine 227, an effect inducing Notch1-IC protein degradation and ubiquitylation. Moreover, accumulated dexamethasone-induced SGK1 facilitated the degradation of Notch1-IC through phosphorylation of Fbw7. Together our results suggest that SGK1 inhibits the Notch1 signaling pathway via phosphorylation of Fbw7.

Key words: Notch1-IC, Fbw7, SGK1, Dexamethasone, Protein degradation

Introduction

The Notch1 signaling pathway is associated with cell fate determination, cell polarity establishment, differentiation, cell proliferation, cell survival and cell death (Artavanis-Tsakonas et al., 1995; Egan et al., 1998; Lai, 2004; Weinmaster, 1998). The Notch1 receptor is a large single-pass type I transmembrane receptor harboring a large extracellular domain involved in ligand binding and a cytoplasmic domain involved in signal transduction (Kopan and Cagan, 1997; Mumm and Kopan, 2000; Weinmaster, 1997). The Notch1 receptor is produced by furine cleavage in the trans-Golgi network, resulting in the release of an active Notch fragment (Logeat et al., 1998). It is targeted to the cell membrane as a heterodimer. Activation of the Notch1 pathway occurs by direct contact with the ligands Delta 1–Delta 4, and Serrate/Jagged 1 and 2 (Lindsell et al., 1996; Lindsell et al., 1995). This binding triggers a cascade of proteolytic cleavages by ADAM metalloproteases, and subsequently cleaves the Notch1 receptor by a γ-secretase protein complex that is composed of four subunits: presenilin (PS1), nicastrin (NCT), anterior pharynx defective (APH-1) and presenilin enhancer-2 (PEN-2), after which the Notch1 intracellular domain (Notch1-IC) is finally released from the membrane (Brou et al., 2000; De Strooper, 2003; De Strooper et al., 1999; Francis et al., 2002; Goutte et al., 2002; Kimberly et al., 2003; Kopan and Goate, 2002; Lee et al., 2002; Luo et al., 2003; Struhl and Greenwald, 1999; Wolfe and Haass, 2001; Ye et al., 1999; Yu et al., 2000). Then, Notch1-IC is translocated into the nucleus and binds to the cofactors CSL (CBF1/suppressor of hairless/Lag-1), MAML-1 (mastermind-like-1), and p300/CBP, leading to transcriptional activation of downstream target genes such as those encoding Hes1, Hes5, Hes7, Hey1, Hey2, HeyL, and Cyclin D (Abu-Issa and Cavicchi, 1996; Bessho et al., 2001; de Celis et al., 1996; Fischer et al., 2004; Jennings et al., 1994; Jouve et al., 2000; Leimeister et al., 2000; Ligoxygakis et al., 1998; Maier and Gessler, 2000; Ohtsuka et al., 1999; Ronchini and Capobianco, 2001). The regulation of Notch signaling occurs at multiple levels, including patterns of ligand and receptor expression, Notch–ligand interactions, receptor and ligand trafficking, and a variety of covalent modifications, including glycosylation, phosphorylation, acetylation, nitration and ubiquitylation (Baron et al., 2002; Bash et al., 1999; Espinosa et al., 2003; Foltz et al., 2002; Goto et al., 2001; Haines and Irvine, 2003; Kim, M. Y. et al., 2007; Kim et al., 2008; Kimble and Simpson, 1997; Mitsiadis et al., 1999; Mo et al., 2007; Nijjar et al., 2002; Oberg et al., 2001; Pfister et al., 2003; Qiu et al., 2000; Shaye and Greenwald, 2005; Taniguchi et al., 2001; Weber et al., 2006; Wu et al., 2001).

Recent studies have emphasized the role of ubiquitylation in regulating Notch signaling (Lai, 2002). Five known E3 ligases regulate Notch itself, Notch ligands, or known Notch antagonists. LNX is a positive regulator of Notch signaling responsible for degradation of Numb, a membrane-associated protein that inhibits the function of the Notch receptor (Nie et al., 2002). Neuralized (*neur*) and Mind bomb (*mib*) promote the monoubiquitylation and endocytosis of Delta (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Pavlopoulos et al., 2001). Itch associates with the

intracellular domain of Notch via its WW domains and promotes ubiquitylation of Notch1-IC through its HECT domain (Qiu et al., 2000). Finally, Notch1-IC is also degraded in the nucleus by the ubiquitin–proteasome system with Fbw7, an E3 ligase for the ubiquitylation of Notch1-IC (Lai, 2002; Minella and Clurman, 2005; Mo et al., 2007; Oberg et al., 2001; Wu et al., 2001). To date, little is known about the regulation of Notch1-IC protein stability via Fbw7.

Several studies have reported mutations of the *Fbw7* gene in various tumors, including endometrial cancersa and T cell acute lymphoblastic leukemia (T-ALL), and inactivation of Fbw7 has been shown to lead to an increased genetic instability (Malyukova et al., 2007; Rajagopalan et al., 2004; Spruck et al., 2002). Loss-of-function mutations of the *Fbw7* gene also lead to activation of the Notch1 signaling pathway by inhibition of ubiquitin-mediated degradation of the activated form of Notch1 (Nakayama and Nakayama, 2005; Oberg et al., 2001). Human *NOTCH1* was first discovered as a gene activated at the breakpoint of the t(7;9), a very rare chromosomal translocation. These truncated Notch1 isoforms (TAN1) are temporally and spatially expressed in the manner of the T-cell receptor β-locus in lymphoblast of T-ALL patients (Ellisen et al., 1991). Definitive evidence for a central role of Notch1 in human T-ALL came from a recent study in which 50% of human T-ALL cell lines and primary patient samples were shown to harbor somatic activating mutations in Notch1 that result in aberrant Notch1 signaling (Weng et al., 2004). Small molecule inhibitors of the γ-secretase complex (GSIs) are now available that effectively inhibit Notch1 signaling in vitro. Inhibition of Notch1 signaling with GSIs in T-ALL results in rapid clearance of Notch1-IC and transcriptional downregulation of Notch1 target genes (Palomero et al., 2006a; Palomero et al., 2006b; Weng et al., 2004). Hence, modulation of the Notch signaling cascade is important for developing novel and specific therapeutic strategies for T-ALL as well as for solid tumors.

The serum- and glucocorticoid-inducible protein kinase SGK1 was previously cloned as a serum- and glucocorticoid-inducible transcript expressed in rat mammary tumor cells (Webster et al., 1993). The expression of SGK1, but not of SGK2 or SGK3, is acutely regulated by glucocorticoids and serum (Kobayashi et al., 1999). SGK1 is an important regulator of diverse cellular processes including metabolism, proliferation, channel conductance, cell volume, cell survival and differentiation (BelAiba et al., 2006; Lang and Cohen, 2001; Mikosz et al., 2001). SGK shares close sequence identity with the catalytic domain of Akt and with the phosphorylated serine and threonine residues that lie in the RxRxx[S/T] motif (Kobayashi et al., 1999). SGK1 is controlled in a phosphoinositide 3-kinase-dependent manner in response to insulin or growth factor signaling (Brunet et al., 2001). Activated SGK1 can phosphorylate glycogen synthase kinase-3β (GSK-3β), b-Raf, IKKβ, FKHRL1, Nedd4-2, p27, Fe65 and SEK1 (Brunet et al., 2001; Flores et al., 2005; Hong et al., 2008; Kim, M. J. et al., 2007; Lee et al., 2008; Tai et al., 2009; Zhang et al., 2001). SGK1 is an inhibitor of γ-secretase, and SGK1-mediated phosphorylation and degradation of Nicastrin might be relevant to the mechanism by which SGK1 suppresses γ-secretase activity (our unpublished results). In fatty livers, glucocorticoids suppress *Hes1* gene expression through the glucocorticoid receptor (Lemke et al., 2008). SGK1 has been identified as a probable negative regulator of *Hes5* gene expression and regulates contextual fear memory formation (Lee et al., 2007). Despite these observations, the precise mechanisms underlying the connection between Notch1 and SGK1 signaling pathways remain to be accurately delineated.

Therefore, in this study, we evaluated the signal crosstalk occurring between Notch1 and SGK1 signaling. We demonstrated that SGK1 modulates the Notch1 signaling pathway through the downregulation of protein stability via Fbw7. The phosphorylation of Fbw7 by SGK1 induces Notch1-IC protein degradation and ubiquitylation. Collectively, our findings indicate that SGK1 plays an inhibitory role in the Notch1 signaling pathway via the phosphorylation of Fbw7.

Results

SGK1 suppresses the transcriptional activity of Notch1

To determine whether SGK1 is involved in regulating the transcriptional activation of Notch1 target genes, a reporter assay was performed in HEK293 cells using luciferase reporter genes. We investigated the effects of SGK1 on Notch1-IC transcriptional activity. Notch1-IC-induced 4×CSL luciferase reporter activity was inhibited by the co-transfection of SGK1 using 4×CSL-Luc reporter genes (Fig. 1A). We also found similar results using Hes1-Luc and Hes5-Luc reporter systems (data not shown). To investigate whether the kinase activity of SGK1 is necessary for the downregulation of the transcriptional activation of Notch1 target genes, we used a dominant-negative, kinase-deficient SGK1 mutant (SGK1-KD) to block the kinase activity of SGK1. In the luciferase reporter gene assay of the HEK293 cells, SGK1-KD was transfected instead of SGK1, and the effects of this transfection on the transcriptional activation of Notch1-IC target genes were then assessed using 4×CSL-Luc. The transcriptional activity of Notch1-IC was inhibited by SGK1, but was not inhibited by the co-transfection of Notch1-IC and SGK1-KD (Fig. 1B). To determine the role of endogenous SGK1 in Notch1 signaling, we performed transcription reporter assays using SGK1 wild-type ($SGK1^{+/+}$) and SGK1-deficient ($SGK1^{-/-}$) fibroblast cells. The transcriptional activity of Notch1-IC in SGK1-deficient fibroblasts was threefold higher than in SGK1 wild-type cells (Fig. 1C). We also found that Notch1-IC transcriptional activity was suppressed by expression of SGK1 in SGK1-deficient cells (Fig. 1D). These results indicated that SGK1 suppresses the transcriptional activity of Notch1 in intact cells.

SGK1 disrupts the binding of Notch1-IC to RBP-Jk

To determine whether SGK1 is involved in regulating the interactions between Notch1-IC and the transcriptional regulator RBP-Jk, coimmunoprecipitation was performed in SGK1 wild-type and SGK1-deficient fibroblast cells. Endogenous binding between Notch1-IC and RBP-Jk in SGK1-deficient fibroblasts was much higher than in SGK1 wild-type cells (Fig. 2A). The physical interaction of endogenous Notch1-IC and RBP-Jk in SGK1-deficient fibroblast was suppressed by ectopic expression of SGK1 in SGK1-deficient cells (Fig. 2B).

To observe the effects of SGK1 on the molecular interactions between Notch1-IC and RBP-Jk, coimmunoprecipitation was performed in HEK293 cells by co-transfection of Myc-tagged Notch1-IC, FLAG-tagged RBP-Jk, and HA-tagged SGK1. Notch1-IC and RBP-Jk were coimmunoprecipitated, but when co-transfected with SGK1, the band of Notch1-IC that interacted with RBP-Jk disappeared (Fig. 2C). The cell lysates were oppositely immunoprecipitated by anti-Myc antibody and immunoblotted with anti-FLAG M2 antibody. Results reconfirmed the disruption of the Notch1-IC and RBP-Jk complex in the presence of SGK1 (data not

shown). Surprisingly, from the immunoblot of the cell lysates, the protein level of Notch1-IC was downregulated upon co-transfection of SGK1 (Fig. 2C, lane 6), which shows that SGK1 might regulate the protein level of Notch1-IC.

Next, we determined whether SGK1 plays a role in the regulation of the Notch1-IC protein level by using western blot analysis of HEK293 cells. The cells were co-transfected with Myc-tagged Notch1-IC and HA-tagged SGK1. We found downregulation of the Notch1-IC protein level with coexpression of SGK1 (Fig. 2D). The Notch1-IC protein level was reduced upon co-transfection of SGK1, but was not reduced upon co-transfection of SGK1-KD (Fig. 2D). This result showed that the kinase activity of SGK1 is essential for regulation of the Notch1-IC protein level.

To test the involvement of GSK-3β in the protein downregulation of Notch1-IC by SGK1, we used a GSK-3β inhibitor (LiCl) and a GSK-3β dominant-negative mutant [GSK-3β(S9A), in which serine 9 was replaced by alanine]. The results showed that the protein downregulation of Notch1-IC by SGK1 was independent of GSK-3β (Fig. 2E,F). These results indicated that the Notch1-IC protein level is downregulated by SGK1, which is dependent on an intact SGK1 kinase, but independent of the downstream kinase GSK-3β.

SGK1 negatively regulates Notch1 signaling via the E3 ligase Fbw7

The half-life of endogenous Notch1-IC was determined using cycloheximide, an inhibitor of protein translation. SGK1 wild-type and SGK1-deficient fibroblast cells were exposed to cycloheximide, and the amount of Notch1-IC was analyzed using immunoblotting. The steady-state level and the half-life of endogenous Notch1-IC were higher in SGK1-deficient fibroblasts than in wild-type cells (Fig. 3A). Next, we evaluated the involvement of SGK1 in the Notch1-IC proteasome-dependent degradation pathway by performing luciferase reporter gene assays. The transcriptional activity of Notch1-IC was inhibited by SGK1, but recovered in the presence of the protease inhibitor MG132 (Fig. 3B). Moreover, the endogenous Notch1-IC protein level was higher in SGK1-deficient cells than in SGK1 wild-type cells (Fig. 3C, lanes 1 and 3), and the treatment of MG132 enhanced the endogenous Notch1-IC protein level by inhibiting proteasomal degradation (Fig. 3C, lanes 2 and 4). These results reveal that the stability of the endogenous Notch1-IC protein is downregulated by SGK1 through the proteasome-dependent pathway.

To determine whether SGK1 promotes Itch-dependent Notch1-IC degradation, we measured protein levels of Notch1-IC in the presence of the Itch wild-type or the catalytically inactive dominant-negative mutant of Itch. We found that co-transfection of Itch wild-type with SGK1 greatly decreased Notch1-IC expression. Also, Notch1 protein level was decreased when the Itch inactive mutant was coexpressed with SGK1 (Fig. 3D). These results indicate that protein downregulation of Notch1-IC by SGK1 occurred via the Itch-independent pathway. We tested the involvement of Fbw7 in the degradation of Notch1-IC by SGK1. When Fbw7 was co-transfected with Notch1-IC and SGK1, the steady state level of Notch1 proteins was slightly decreased. However, the Notch1-IC protein level was found to be decreased by Fbw7 in a dose-dependent manner (Fig. 3E). We tested the involvement of Fbw7 by using the F-box deleted, and hence dominant-negative, mutant form of Fbw7 (Fbw7ΔF). Notch1-IC transcriptional activity was decreased in the presence of SGK1, but was significantly restored by co-transfection with Fbw7ΔF (Fig. 3F). Western blot analysis showed the Notch1-IC protein level to be decreased by Fbw7 and SGK1, and was remarkably restored by the co-transfection of Fbw7ΔF (Fig. 3G). These results indicate that Fbw7ΔF can recover and enhance the transcriptional activity and protein level of Notch1-IC in the presence of SGK1. Accordingly, we suggest that SGK1 negatively regulates Notch1-IC through Fbw7.

Next, we evaluated the involvement of SGK1 in the physical association between Fbw7 and Notch1-IC by

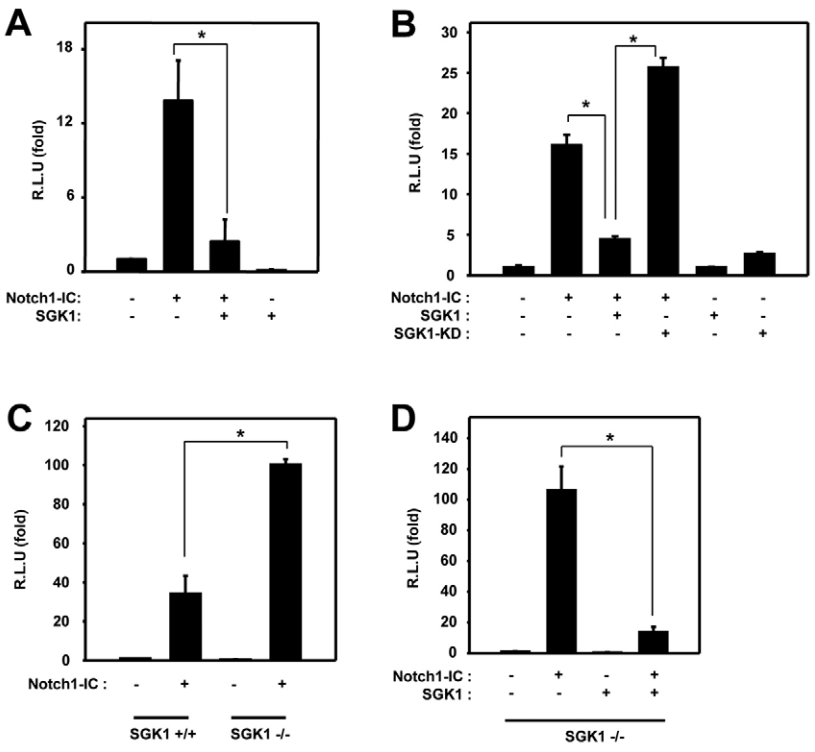

Fig. 1. SGK1 suppresses the transcriptional activity of Notch1. (**A**) HEK293 cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase, along with Notch1-IC and SGK1, as indicated. (**B**) HEK293 cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase, along with Notch1-IC, SGK-KD and SGK1, as indicated. (**C**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase, along with Notch1-IC. (**D**) *SGK1*$^{-/-}$ MEF cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase, along with Notch1-IC and SGK1. After 48 hours of transfection, the cells were lysed and the luciferase activity determined. The data were normalized against β-galactosidase activity. The results represent the means + s.e.m. of three independent experiments. RLU, relative luciferase units. The data were evaluated for significant difference using the Student's *t*-test; ANOVA, **P*<0.001.

coimmunoprecipitation. Immunoblot analysis of Myc immunoprecipitates from the transfected cells using anti-GFP antibody revealed that SGK1 facilitated the physical association between Fbw7 and Notch1-IC in the cells (Fig. 3H). These results indicate that the downregulation of Notch1-IC protein by SGK1 occurs via an Fbw7-dependent pathway.

Notch1 can form a trimeric complex with SGK1 and Fbw7 in intact cells

Because our results suggested that Notch1 is a target of SGK1, we next investigated whether these two proteins interact physically in intact cells. In vitro binding studies showed that purified glutathione *S*-transferase (GST) and GST–Notch1-IC proteins were immobilized onto glutathione (GSH)–agarose. FLAG–SGK1 expressing the cell lysates was incubated either with GST or GST–Notch1-IC immobilized onto GSH–agarose. The interaction between GST–Notch1-IC and SGK1 was detected on bead complexes (Fig. 4A). We then evaluated the physical interactions occurring between Notch1-IC and SGK1. HEK293 cells were co-transfected with vectors encoding Myc-tagged Notch1-IC and FLAG-tagged SGK1, and were then subjected to co-immunoprecipitation analysis (Fig. 4B). Immunoblot analysis using

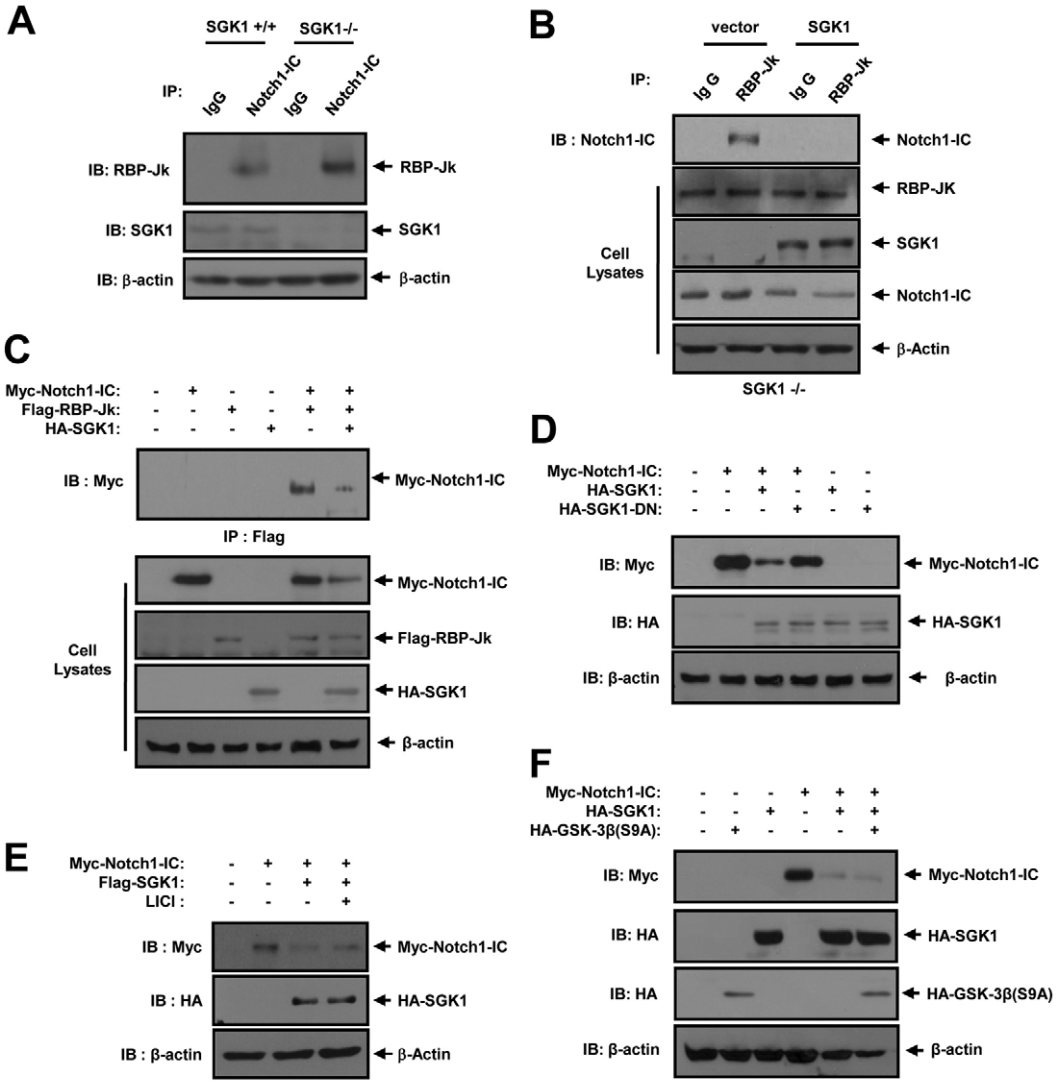

Fig. 2. SGK1 disrupts the binding of Notch1-IC to RBP-Jk. (**A**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells were lysed and subjected to immunoprecipitation with IgG and anti-Notch1-IC antibodies as indicated. The immunoprecipitates were immunoblotted with anti-RBP-Jk antibody. Cell lysates were immunoblotted with anti-SGK1. (**B**) *SGK1*$^{-/-}$ MEF cells expressing either control vector or HA–SGK1 were lysed and subjected to immunoprecipitation with IgG and anti-RBP-Jk antibodies as indicated. Immunoprecipitates were immunoblotted with an anti-Notch1-IC. Cell lysates were immunoblotted with anti-SGK1, anti-Notch1-IC and RBP-Jk. (**C**) HEK293 cells were transfected with expression vectors for Myc–Notch1-IC, FLAG–RBP-Jk and HA–SGK1. The cell lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates immunoblotted with anti-Myc antibody. The cell lysates were subjected to immunoblot analysis with anti-Myc and anti-FLAG antibodies. (**D**) HEK293 cells were transfected with expression vectors for Myc–Notch1-IC, HA–SGK1-DN and HA–SGK1. Cell lysates were subjected to immunoblot analysis with anti-Myc or anti-HA antibodies. (**E**) HEK293 cells were transfected with expression vectors for Myc–Notch1-IC and HA–SGK1. After 42 hours of transfection, the cells were treated with LiCl (2 mM) for 6 hours and the cell lysates were subjected to immunoblot analysis with anti-Myc or anti-HA antibodies. (**F**) HEK293 cells were transfected with expression vectors for Myc–Notch1-IC, HA–SGK1 and HA–GSK-3β(S9A). The cell lysates were subjected to immunoblot analysis with anti-Myc or anti-HA antibodies. Antibody to β-actin was used as a loading control.

anti-FLAG antibody of the Myc immunoprecipitates from the transfected cells revealed that SGK1 physically associated with Notch1-IC in the cells. By contrast, immunoblot analysis using anti-Myc antibody of the FLAG immunoprecipitates also showed interaction between the two proteins (Fig. 4B). Notch1-IC harbors a CDC 10/Ankyrin domain that includes a RAM domain and seven Ankyrin repeats, an OPA domain and a PEST domain within its structure. We attempted to determine which, if any, of these domains might be involved in the interaction between Notch1-IC and SGK1. We utilized a variety of FLAG–Notch1-IC deletion mutants: Notch1-IC-N (CDC 10 domain), Notch1-IC-ΔNΔC (OPA domain), and Notch1-IC-C (PEST domain). We conducted coimmunoprecipitation assays using the three Notch1-IC deletion mutants and HA–SGK1. Our results showed that SGK1 bound to

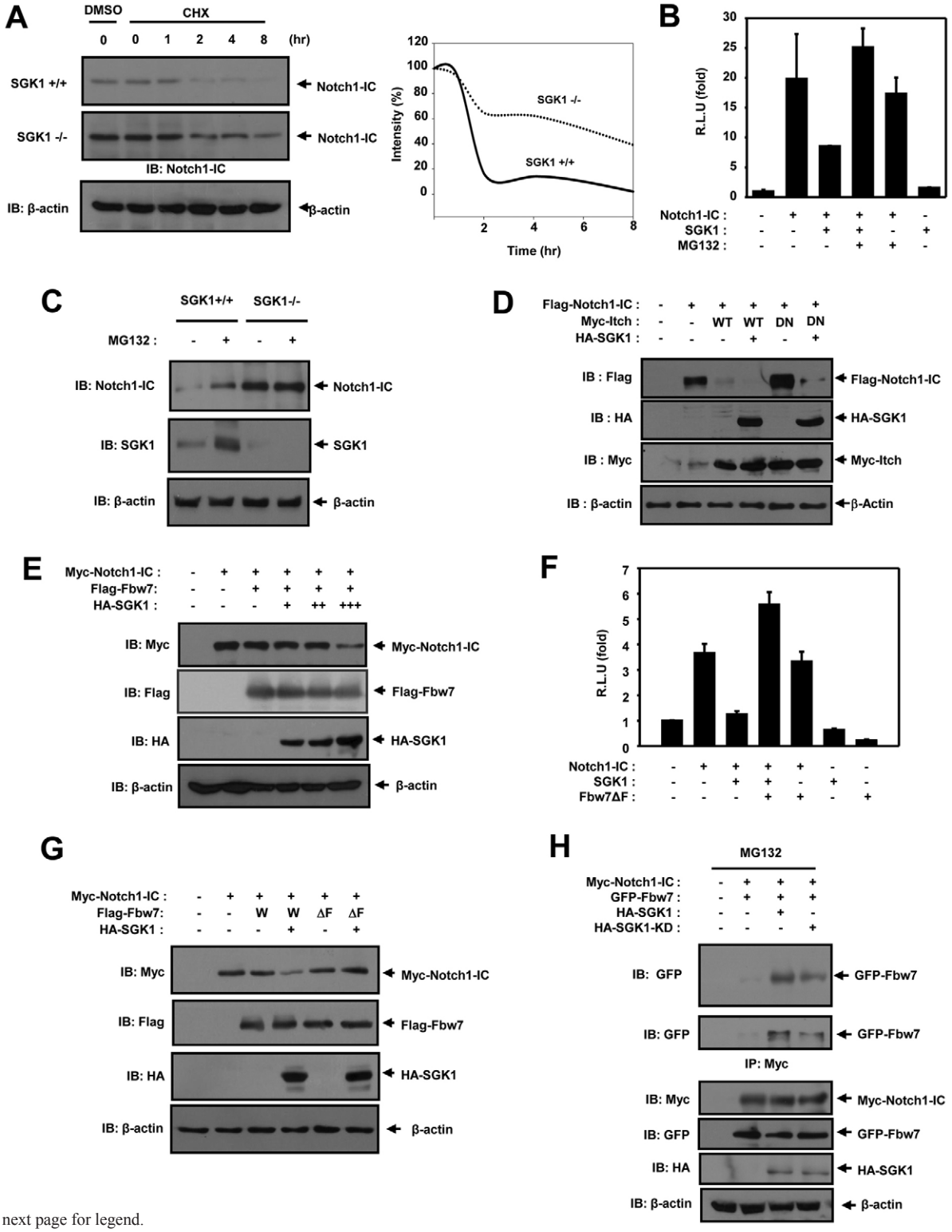

Fig. 3. See next page for legend.

Notch1-IC-N but not to Notch1-IC-ΔNΔC or Notch1-IC-C (Fig. 4C). To confirm the physical binding between Notch1-IC and Fbw7, we performed coimmunoprecipitation assays using the three Notch1-IC deletion mutants and Myc–Fbw7. Our data indicated that Fbw7 bound to Notch1-IC-N but not to Notch1-IC-ΔNΔC or Notch1-IC-C (Fig. 4D).

At this point, we evaluated the formation of a trimeric complex between Notch1-IC and SGK1 or Fbw7, in order to define more precisely the role of SGK1 in the negative regulation of Notch1 signaling. We found binding not only between Notch1-IC and SGK1, but also between Notch1-IC and Fbw7 (Fig. 4E). From these results, we postulate that Notch1-IC forms a trimeric complex with SGK1 and Fbw7. To confirm this, HEK293 cells were co-transfected with vectors encoding Myc–Notch1-IC, FLAG–SGK1 and FLAG–Fbw7, and were then subjected to co-immunoprecipitation analysis. Immunoblot analysis revealed that Notch1-IC formed a trimeric complex with SGK1 and Fbw7 (Fig. 4E). We found a gradually decreased expression level of Notch1-IC in the presence of Fbw7 or SGK1 and Fbw7 coexpression, although no change in the binding affinity between Notch1-IC and Fbw7. Therefore, the results indicated that Notch1-IC interacts with Fbw7 and that this association is significantly stronger in the presence of SGK1 (Fig. 4E). We also attempted to confirm the effect of SGK1 on the physical association occurring between Notch1-IC and Fbw7 using SGK1 wild-type and SGK1-null MEF cells. Coimmunoprecipitation assays with endogenous Notch1-IC and Fbw7 revealed binding in the SGK1 wild-type cells and these effects were prevented in the SGK1-null cells (Fig. 4F). Furthermore, we attempted to confirm the effect of SGK1 on the physical association occurring between Notch1-IC and Fbw7 using rat fibroblast Rat2 cells expressing control siRNA or rat SGK-specific siRNA. Coimmunoprecipitation assays with endogenous Notch1-IC and Fbw7 revealed binding in the rat fibroblast Rat2 cells and these effects were prevented in the SGK1 knockdown in Rat2 cells. Thus, our data indicate that Notch1 might be the principal target protein of SGK1 via Fbw7.

Fig. 3. The E3 ligase, Fbw7, mediates the negative regulation of Notch1 by SGK1. (**A**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells were treated with 100 μM DMSO or 100 μM cycloheximide (CHX) for the indicated periods of time, and the cell lysates immunoblotted with anti-Notch1-IC antibody (left). We quantified the intensity of each band using a densitometer and plotted relative intensities (right). (**B**) HEK293 cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase, along with Notch1-IC and SGK1, as indicated. After 42 hours of transfection, the cells were treated with MG132 (10 μM) for 6 hours and then the cells were lysed and the luciferase activity determined. The data were normalized against β-galactosidase activity. The results represent the means + s.e.m. of three independent experiments. RLU, relative luciferase units. (**C**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells were treated with MG132 (3 μM) for 6 hours, and the cell lysates immunoblotted with anti-Notch1-IC and anti-SGK1 antibodies. (**D**) HEK293 cells were co-transfected with expression vectors encoding FLAG–Notch1-IC, Myc–Itch (WT), Myc–Itch-DN (DN) and HA–SGK1 in the indicated combinations. After 48 hours of transfection, the cell lysates were immunoblotted with anti-Myc, anti-FLAG and anti-HA antibodies. (**E**) HEK293 cells were transfected with expression vectors encoding Myc–Notch1-IC, FLAG–Fbw7 and HA–SGK1 in the indicated combinations. After 48 hours of transfection, the cell lysates were immunoblotted with anti-Myc, anti-FLAG and anti-HA antibodies. (**F**) HEK293 cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase, along with Notch1-IC, SGK1 and Fbw7ΔF as indicated. After 48 hours of transfection, the cells were lysed and the luciferase activity determined. The data were normalized against β-galactosidase activity. The results represent the means + s.e.m. of three independent experiments. RLU, relative luciferase units. (**G**) HEK293 cells were transfected with expression vectors encoding Myc–Notch1-IC, FLAG–Fbw7 (W), FLAG–Fbw7ΔF (ΔF) and HA–SGK1 in the indicated combinations. After 48 hours of transfection, the cell lysates were immunoblotted with anti-Myc, anti-FLAG and anti-HA antibodies. (**H**) HEK293 cells were transfected with expression vectors for Myc–Notch1-IC, HA–SGK1, HA–SGK1-KD and GFP-Fbw7 as indicated. After 42 hours of transfection, the cells were treated with MG132 (10 μM) for 6 hours and the cell lysates immunoprecipitated with anti-Myc antibody and the immunoprecipitates immunoblotted with anti-GFP antibody. Cell lysates were subjected to immunoblot analysis with anti-Myc, anti-HA or anti-GFP antibodies.

SGK1 phosphorylate Fbw7 on serine 227, which facilitates degradation of Notch1-IC

Given that our results suggest that Notch1 is a target of SGK1 via Fbw7, we next investigated whether SGK1 and Fbw7 interact physically in intact cells. HEK293 cells were co-transfected with a vector encoding His-tagged Fbw7 and FLAG-tagged SGK1 and then subjected to coimmunoprecipitation analysis (Fig. 5A). Immunoblot analysis of the His immunoprecipitates from the transfected cells with an anti-FLAG antibody revealed that Fbw7 physically associated with SGK1 in the cells (Fig. 5A). Next, we attempted to ascertain whether SGK1 was able to physically associate with Fbw7 in intact cells. Using MDA-MB-231 and HeLa cells, immunoblot analysis of the SGK1 immunoprecipitates with an anti-Fbw7 antibody indicated the physical association of endogenous SGK1 and Fbw7 (Fig. 5B,C).

We next conducted an in vitro kinase assay with constitutive active (SGK1-CA) and dominant-negative SGK1 (SGK1-DN), and purified GST–Notch1-IC or GST–Fbw7. The SGK1 immunocomplexes prepared from the HEK293 cells catalyzed the phosphorylation of purified recombinant GST–Fbw7, but not GST–Notch1-IC (Fig. 5D). Dominant-negative SGK1 did not phosphorylate these two proteins (Fig. 5D). We also attempted to confirm the effect of SGK1 on the phosphorylation of Fbw7 using SGK1 wild-type and SGK1-null MEF cells. Immunoblot assays with phospho-serine/threonine antibody revealed phosphorylation of Fbw7 by dexamethasone induced endogenous SGK1 in SGK1 wild-type cells, but not in SGK1-null cells (Fig. 5E). We also found that the phosphorylation of Fbw7 was substantially increased by overexpressed SGK1 in SGK1-deficient cells (Fig. 5E). We attempted to ascertain whether or not endogenous SGK1 could phosphorylates Fbw7 and whether phosphorylated Fbw7 could interact with Notch1-IC in intact cells, using MEF cells from SGK1 wild-type and SGK1-deficient mice. The total cell lysates were first immunoprecipitated with anti-Notch1-IC antibody, and then immunoprecipitates were secondly immunoprecipitated with anti-Fbw7 antibody. Immunoblot analysis of the second immunoprecipitates from MEF cells with an anti-phospho-serine antibody revealed that Notch-IC was physically associated with phosphorylated Fbw7 in SGK1 wild-type cells, but not in SGK1-null cells (Fig. 5F).

SGK preferentially phosphorylates substrate protein serine and threonine residues that lie in an Arg-Xaa-Arg-Xaa-Xaa-[Ser/Thr] motif (Kobayashi et al., 1999). In silico studies have shown that the Fbw7 N-terminus contains a possible conserved serine residue in vertebrates; this serine residue is accessible and is located in front of the F-box domain. This motif is present at amino acids 222–227 of human Fbw7 (or amino acids 144–149 of mouse Fbw7). Furthermore, via site-directed mutagenesis, we determined that the replacement of serine 227 of Fbw7 with alanine caused a reduction

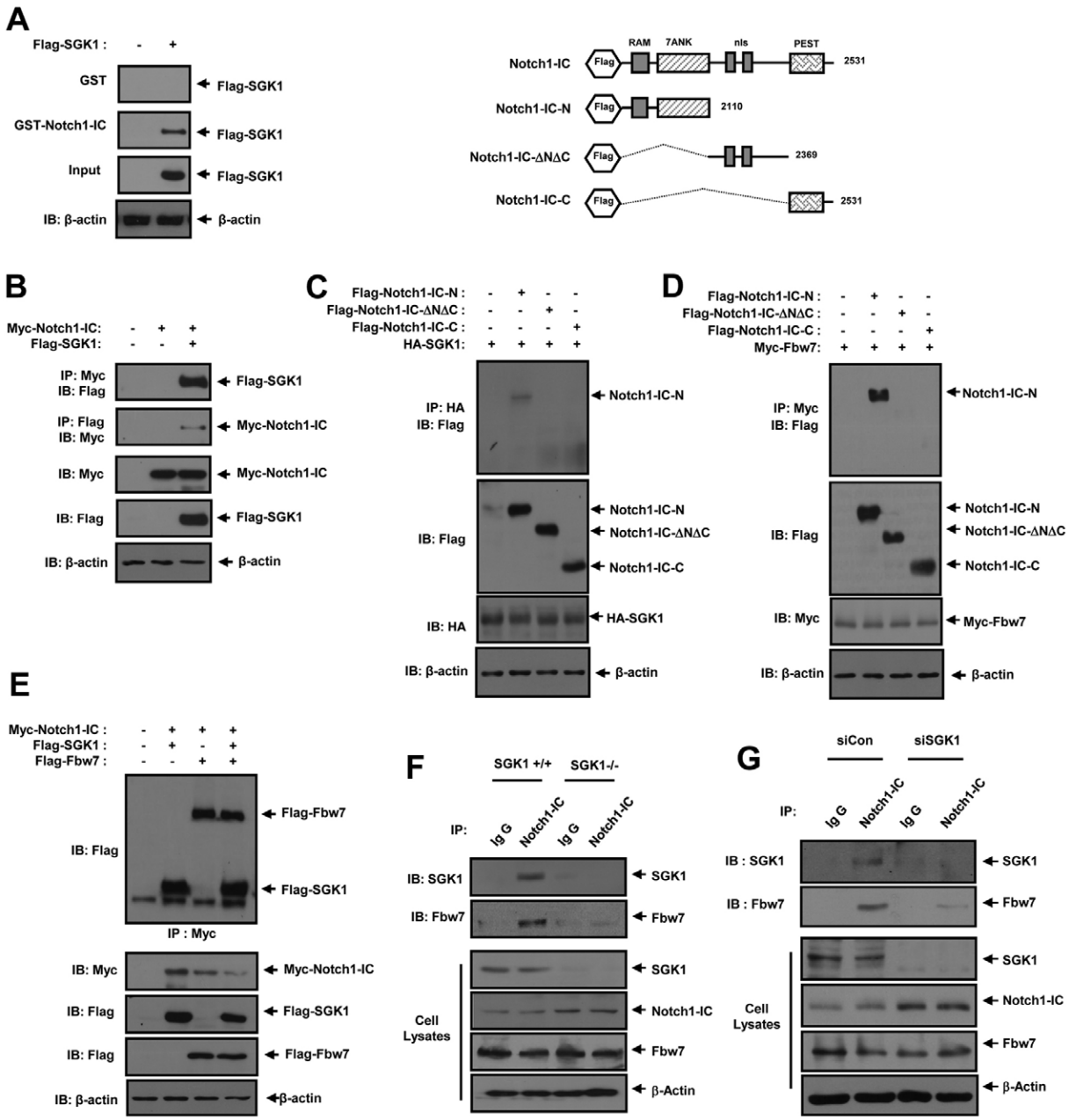

Fig. 4. SGK1 facilitates physical association between Notch1-IC and Fbw7 in intact cells. (**A**) Left: Recombinant GST or GST–Notch1-IC proteins were immobilized onto GSH–agarose. HEK293 cells were transfected for 48 hours with the expression vector encoding FLAG–SGK1 or empty vector. After transfection, the cell lysates were subjected to GST pull-down experiments with immobilized GST or GST–Notch1-IC. Proteins bound to GST or GST–Notch1-IC were analyzed via immunoblotting with anti-FLAG antibody. Right: FLAG–Notch1-IC deletion mutants used in the experiments shown in C and D. (**B**) HEK293 cells were transfected for 48 hours with expression vectors encoding Myc–Notch1-IC and FLAG–SGK1, as indicated. After transfection, the cell lysates were subjected to immunoprecipitation with anti-Myc or anti-FLAG antibodies. The immunoprecipitates and cell lysates were then immunoblotted with anti-FLAG or anti-Myc antibodies. (**C**) HEK293 cells were transfected for 48 hours with expression vectors encoding FLAG–Notch1-IC-N, Notch1-IC-ΔNΔC, FLAG–Notch1-IC-C and HA–SGK1. After transfection, the cell lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were then immunoblotted with anti-FLAG antibody. The cell lysates were also immunoblotted with anti-FLAG and anti-HA antibodies. (**D**) HEK293 cells were transfected for 48 hours with expression vectors encoding three FLAG–Notch1-IC deletion mutants and Myc–Fbw7. After transfection, the cell lysates were subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were then immunoblotted with anti-FLAG antibody. The cell lysates were also immunoblotted with anti-FLAG and anti-HA antibodies. (**E**) HEK293 cells were transfected for 48 hours with expression vectors encoding Myc–Notch1-IC, FLAG–SGK1, and FLAG–Fbw7. After transfection, the cell lysates were subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were then immunoblotted with anti-FLAG antibody. The cell lysates were also immunoblotted with anti-Myc and anti-FLAG antibodies. (**F**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells were lysed and subjected to immunoprecipitation with IgG and anti-Notch1-IC antibodies as indicated. The immunoprecipitates were immunoblotted with anti-SGK1 or anti-Fbw7 antibody. The cell lysates were immunoblotted with anti-Notch1-IC, anti-SGK1 and anti-Fbw7. (**G**) Rat fibroblast Rat2 cells expressing either control siRNA (siCon) or rat SGK1-specific siRNA (siSGK1) were lysed and subjected to immunoprecipitation with IgG and anti-Notch1-IC antibodies as indicated. The immunoprecipitates were immunoblotted with anti-SGK1 or anti-Fbw7 antibodies. The cell lysates were immunoblotted with anti-Notch1-IC, anti-SGK1 and anti-Fbw7. Antibody to β-actin was used as a loading control.

in the in vitro phosphorylation of the recombinant protein by the SGK1 immunoprecipitates (Fig. 5G). Moreover, we ascertained that Fbw7(S227A) is resistant to SGK1-induced degradation of Notch1-IC, which implies that the SGK1-induced phosphorylation of Fbw7 is crucial for the degradation of the Notch1-IC protein (Fig. 5H). We then attempted to characterize the involvement of

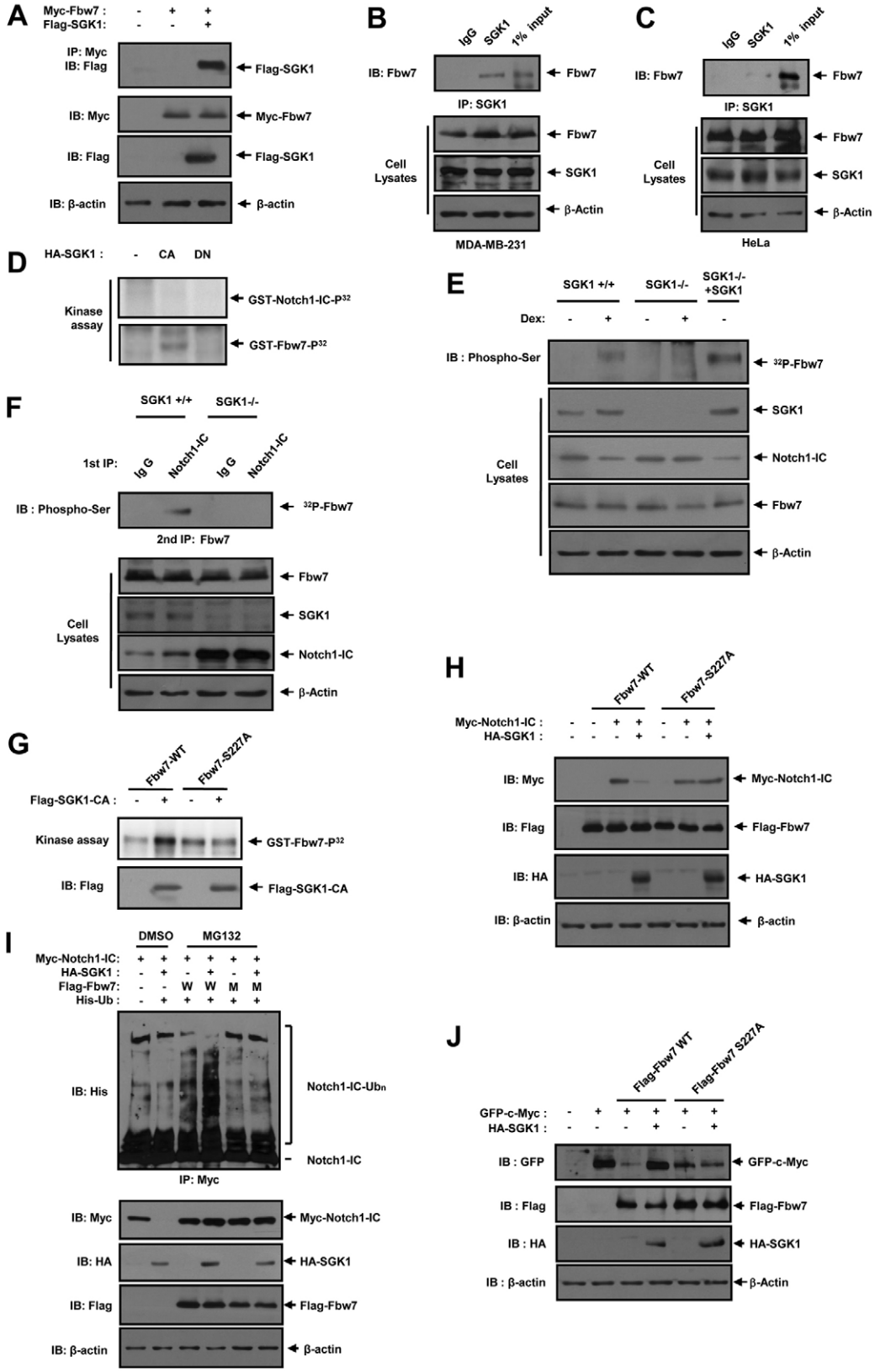

Fig. 5. See next page for legend.

phosphorylation in the poly-ubiquitylation of Notch1-IC by Fbw7. HEK293 cells were co-transfected with vectors coding for Myc-tagged Notch1-IC, HA-tagged SGK1, FLAG-tagged Fbw7 wild-type and S227A mutants, and His-tagged ubiquitin, and were then subjected to ubiquitylation analysis. Immunoblot analysis of the His immunoprecipitates from the transfected cells with an anti-Myc antibody showed that SGK1 and the wild type Fbw7 facilitated the ubiquitylation of Notch1-IC and that mutant Fbw7 prevented the ubiquitylation of Notch1-IC (Fig. 5I).

Previous reports showed that Fbw7 regulates phosphorylation dependent c-Myc degradation (Welcker et al., 2004; Yada et al., 2004). Therefore, we investigated the role of Fbw7 phosphorylation by SGK1 in the degradation of c-Myc protein. Whereas Fbw7 facilitate the degradation of c-Myc protein, SGK1 was found to robustly restore the level of c-Myc protein (Fig. 5J). However, SGK1 could not restore the degradation of c-Myc protein by Fbw7(S227A) (Fig. 5J). Thus, our data show that SGK1 prevents Fbw7-mediated degradation of c-Myc protein through Fbw7 phosphorylation. These results showed that the phosphorylation of Fbw7 by SGK1 is crucial to its ability to degrade Notch1-IC or c-Myc via different mechanism.

Fig. 5. SGK1-mediated phosphorylation of Fbw7 on serine 227 facilitates the degradation of Notch1-IC. (**A**) HEK293 cells were transfected for 48 hours with expression vectors encoding Myc–Fbw7 and FLAG–SGK1, as indicated. After transfection, the cell lysates were subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were then immunoblotted with anti-FLAG antibody. The cell lysates were also immunoblotted with anti-Myc and anti-FLAG antibodies. (**B**) MDA-MB-231 and (**C**) HeLa cells were lysed and subjected to immunoprecipitation with anti-SGK1 antibody or rabbit control IgG as indicated. Immunoprecipitates were immunoblotted with anti-Fbw7 antibody. Cell lysates were immunoblotted with anti-Fbw7, and anti-SGK1. (**D**) HEK293 cells were transfected with expression vectors encoding FLAG–SGK1-CA and FLAG–SGK1-DN as indicated. After 48 hours of transfection, the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting precipitates were examined for SGK1 kinase activity by an immune complex kinase assay using GST–Notch1-IC and GST–Fbw7. (**E**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells treated with 5 mM dexamethasone (Dex) for 24 hours. The cell lysates were subjected to immunoprecipitation with an anti-Fbw7 antibody, and the immunoprecipitates were immunoblotted with an anti-phospho-serine antibody. Cell lysates were immunoblotted with anti-Notch1-IC, anti-SGK1 and anti-Fbw7. (**F**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells were lysed and subjected to immunoprecipitation with IgG and anti-Notch1-IC antibodies as indicated. First immunoprecipitates (1st IP) were denatured by heating in the presence of SDS and DTT, diluted, and immunoprecipitated again (2nd IP) by using the anti-Fbw7 antibody. Washed immunoprecipitates were immunoblotted with an anti-phospho-serine antibody. Cell lysates were immunoblotted with anti-Notch1-IC, anti-SGK1 and anti-Fbw7. (**G**) HEK293 cells were transfected with expression vectors encoding FLAG–SGK1-CA, as indicated. After 48 hours of transfection, the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting precipitates were examined for SGK1 kinase activity by an immune complex kinase assay using GST–Fbw7 and GST–Fbw7(S227A). The cell lysates were also immunoblotted with anti-FLAG antibody. (**H**) HEK293 cells were transfected with expression vectors encoding Myc–Notch1-IC, FLAG–Fbw7 and FLAG–Fbw7(S227A), and HA–SGK1 in the indicated combinations. After 48 hours of transfection, the cell lysates were immunoblotted with anti-Myc, anti-FLAG and anti-HA antibodies. (**I**) HEK293 cells were transfected with expression vectors for Myc–Notch1-IC, HA–SGK1, FLAG–Fbw7 (W), FLAG–Fbw7(S227A) (M) and His-ubiquitin as indicated. After 42 hours of transfection, the cells were treated with DMSO (100 μM) and MG132 (10 μM) for 6 hours as indicated, and the cell lysates immunoprecipitated with anti-His antibody. Immunoprecipitates were immunoblotted with anti-Myc antibody. The cell lysates were subjected to immunoblot analysis with anti-Myc, anti-HA and anti-FLAG antibodies. (**J**) HEK293 cells were co-transfected with expression vectors encoding GFP–c-Myc, FLAG–Fbw7 and FLAG–Fbw7(S227A), and HA–SGK1 in the indicated combinations. After 48 hours of transfection, the cell lysates were immunoblotted with anti-GFP, anti-FLAG and anti-HA antibodies. Antibody to β-actin was used as a loading control.

Dexamethasone inhibits Notch1 signaling through phosphorylation of Fbw7

Because SGK1 expression is induced by glucocorticoid receptor activation in many cell types, we hypothesized that glucocorticoids can contribute to the regulation of Notch1 signaling through the induction of SGK1. To investigate whether dexamethasone can modulate Notch1 signaling, we first examined the effects of dexamethasone on Notch1 signaling using the luciferase reporter system. The expression of Notch1-IC was found to induce a significant activation of the 4×CSL-Luc reporter system. When the cells were treated with dexamethasone, the transcriptional activity of Notch1-IC was suppressed, and this effect occurred in a dose-dependent manner (Fig. 6A). Western blot analysis, showed that the Notch1-IC protein level was decreased by dexamethasone. Consistent with previous reports, we observed that dexamethasone mediated the induction of SGK1 (Fig. 6B). To investigate the endogenous effects of SGK1-mediated Notch1 inhibition by the suppressive effects of dexamethasone on the Notch1 signaling pathway, we studied SGK1 wild-type and SGK1-null MEF cells. dexamethasone inhibited endogenous Notch1 transcriptional activity in the SGK1 wild-type MEF cells, but not in the SGK1-null MEF cells (Fig. 6C). Thus, SGK1 deficiency abolished the dexamethasone-dependent inhibition of Notch1 activity. Furthermore, via site-directed mutagenesis, we determined that the replacement of serine 227 of Fbw7 with alanine caused a reduction in phosphorylation by SGK1 (Fig. 6D). Moreover, we ascertained that Fbw7(S227A) is resistant to SGK1-induced degradation of Notch1-IC, which implies that the SGK1-induced phosphorylation of Fbw7 is crucial for the degradation of Notch1 protein (Fig. 6E). These results showed that the phosphorylation of Fbw7 by SGK1 plays a crucial role in the regulation of Notch1 protein stability.

Discussion

Several downstream targets of Notch signaling have been identified, including *Enhancer of split* [*E(spl)*] complex genes and the mammalian homologs of the *Hairy* and *E(spl)* genes, *Hes1* and *Hes5* (Abu-Issa and Cavicchi, 1996; de Celis et al., 1996; Jennings et al., 1994; Jouve et al., 2000; Ligoxygakis et al., 1998; Ohtsuka et al., 1999). A recent study has suggested that glucocorticoids suppress *Hes1* gene expression through the glucocorticoid receptor (Lemke et al., 2008). One such glucocorticoid receptor-inducible gene, SGK1, has been identified as a probable negative regulator of *Hes5* gene expression (Lee et al., 2007). However, the direct relationship between SGK1 and Notch1 signaling is not well known. In this study, we found that SGK1 inhibited the transcriptional activity of Notch1-IC, and that the kinase activity of SGK1 was essential for this function. SGK1 attenuated Notch-1 IC protein stability by stimulating the proteasomal degradation of Notch1-IC in a E3 ligase Fbw7-dependent manner.

Several research groups have shown that Fbw7 binds to phosphorylated Notch-IC via its WD40 domains, and mediates its ubiquitylation and subsequent rapid degradation (Gupta-Rossi et al., 2001; Hubbard et al., 1997; Oberg et al., 2001; Wu et al., 2001). Our results show that the inhibitory mechanism functions

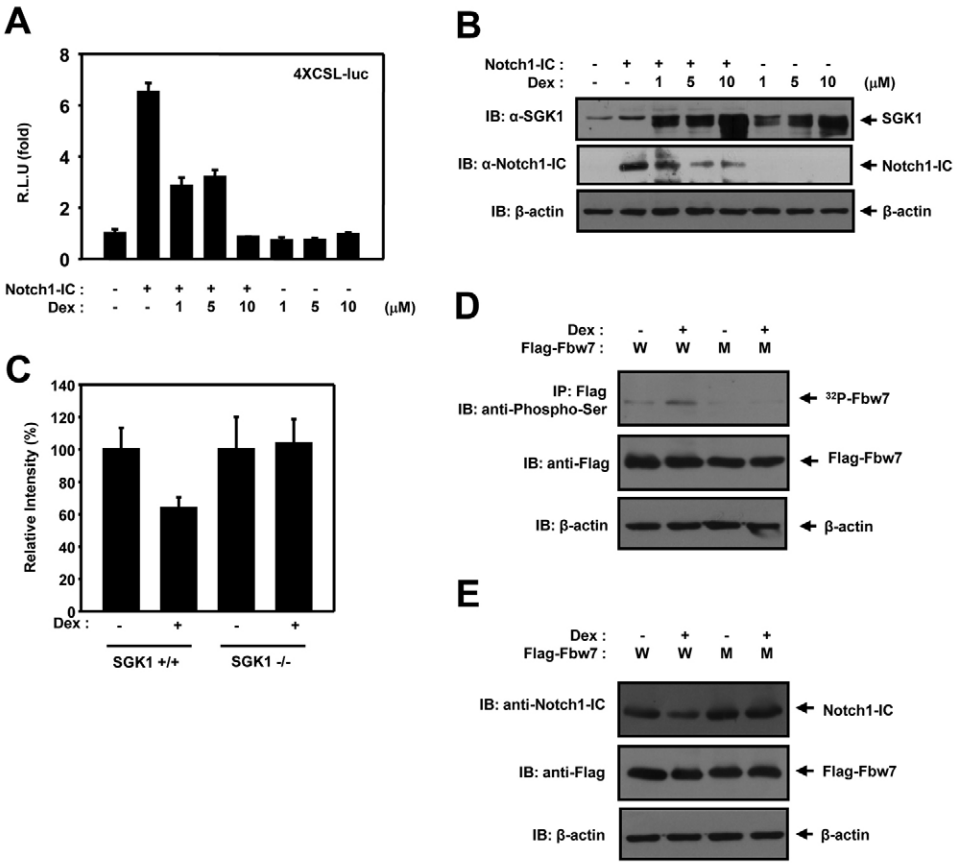

Fig. 6. Dexamethasone inhibits Notch1 signaling through the phosphorylation of Fbw7. (**A**) HEK293 cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase, along with Notch1-IC. After 24 hours of transfection, the cells were treated with dexamethasone (Dex) for 24 hours in the indicated amount. The cells were lysed, and the luciferase activity was determined. The data were normalized against β-galactosidase activity. The results represent the means + s.e.m. of three independent experiments. RLU, relative luciferase units. (**B**) HEK293 cells were transfected with expression vectors for Notch1-IC. After 24 hours of transfection, the cells were treated with dexamethasone for 24 hours and the cell lysates were subjected to immunoblot analysis with anti-Notch1-IC or anti-SGK1 antibodies. (**C**) *SGK1*$^{+/+}$ and *SGK1*$^{-/-}$ MEF cells were transfected with expression vectors for 4×CSL-Luc and β-galactosidase. After 24 hours of transfection, the cells were treated with 5 μM dexamethasone for 24 hours. The cells were lysed, and the luciferase activity was determined. The data were normalized against β-galactosidase activity. The results represent the means + s.e.m. of three independent experiments. (**D**) HEK293 cells were transfected with expression vectors for FLAG–Fbw7 (W) or FLAG–Fbw7(S227A) (M). After 24 hours of transfection, the cells were treated with dexamethasone for 24 hours, the cell lysates immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates immunoblotted with anti-phospho-serine antibody. The cell lysates were subjected to immunoblot analysis with anti-FLAG antibody. (**E**) HEK293 cells were transfected with expression vectors for FLAG–Fbw7 or FLAG–Fbw7(S227A). After 24 hours of transfection, the cells were treated with dexamethasone for 24 hours and the cell lysates subjected to immunoblot analysis with anti-Notch1-IC or anti-FLAG antibodies.

through the suppression of the interaction of Notch1-IC and RBP-Jk due to the downregulation of Notch1-IC protein stability; this is also dependent on the kinase activity of SGK1 and independent of GSK-3β. In this study, we found that SGK1 stimulated the proteasomal degradation of ectopically expressed Notch1-IC. The reduction of endogenous Notch1-IC by SGK1 was also observed as a proteasome-dependent regulation in SGK1-null cells. Collectively, our findings reveal that SGK1 kinase activity plays a crucial role in the proteasomal degradation of Notch1-IC. SGK1 preferentially phosphorylates serine and threonine residues that lie in RxRxx[S/T] motifs (Kobayashi et al., 1999). The phosphorylation of Notch1-IC by ILK, GSK-3β, CDK8, and possibly other kinases, regulates its half-life in a positive or negative fashion (Fryer et al., 2004; Mo et al., 2007). However, Notch1-IC does not contain consensus sites for SGK1 phosphorylation and is not phosphorylated by SGK1.

Interestingly, Fbw7 contains one conserved consensus site for phosphorylation by SGK1 kinase and is phosphorylated by SGK1 in vitro and in vivo. The negative regulation of Notch1-IC by SGK1 is further supported by our observation that endogenous SGK1, when activated, physically interacts with endogenous Notch1-IC and Fbw7, thereby forming a trimeric complex in intact cells. Furthermore, in this study we demonstrate that SGK1-mediated Fbw7 phosphorylation on serine 227 results in a decrease of the Notch1-IC protein level and an increase in the ubiquitylation of Notch1-IC. This study suggests a novel role of SGK1 in reducing Notch signaling. Because SGK1 activation increases Fbw7 phosphorylation, it is expected to enhance Notch1-IC ubiquitylation and degradation. Thus, enhancement of the interaction between Notch1-IC and Fbw7 might be a possible mechanism for SGK1-mediated phosphorylation and proteasomal degradation of Notch1-IC.

SGK1 was originally identified as a glucocorticoid-regulated mRNA in mammary epithelial cells and was termed SGK to reflect its regulation at the transcriptional level by serum and glucocorticoids (Webster et al., 1993). Therefore, the cellular expression of SGK1 is increased in response to dexamethasone, resulting in the cellular accumulation and activation of SGK1. Consistent with the results of a previous report, we determined that the exposure of HEK293 cells to dexamethasone induced the accumulation of SGK1 in living cells, thereby suppressing Notch1 transcriptional activity. However, the downregulation of endogenous SGK1 expression, using SGK1-null cells, resulted in an increase in Notch1 transcriptional activity. In this study, we demonstrate that dexamethasone-induced SGK1 facilitates Fbw7 phosphorylation and results in a decrease in the Notch1-IC protein level. From our results, we can postulate that Fbw7 is a direct substrate of SGK1, and that the phosphorylation of Fbw7 is one of the key factors in the regulation of the Notch1 signaling pathway (Fig. 7).

Recently, several reports concentrating on the functions of the Notch signaling cascade have shown that it influences normal development by regulating differentiation, proliferation and apoptosis (Hirashima, 2009; Sainson and Harris, 2006; Sjolund et al., 2005; Watt et al., 2008). Notch1 and Fbw7 mutations both lead the activation of the Notch1 pathway and are found in the majority of T-ALL patients. Activation of the Notch signaling pathway is firmly established in T-ALL cells and is probably involved in the genesis of many other tumor cell types (Grabher et al., 2006; Gridley, 2004; Palomero et al., 2007; Real et al., 2009; Vilimas et al., 2007). One of the most promising approaches has been to inhibit Notch signaling using GSIs as a novel therapeutic strategy for T-ALL as well as solid tumors. In addition, glucocorticoids such as dexamethasone have been administered to suppress T-ALL (Kiefer et al., 1995; Nakao et al., 1981). However, the molecular mechanism by which SGK1 mediates the antileukemic effect of glucocorticoids has remained unclear. Our results now indicate that Fbw7 phosphorylation by SGK1 contributes to the blocking of Notch signaling, and that activation of SGK1-mediated Fbw7 phosphorylation might represent a potential therapeutic strategy for improving the effectiveness of antileukemic drugs in T-ALL cells. Further studies on the relationships between SGK1 and Fbw7 should improve the understanding of the pathogenesis of T-ALL- and Notch1-related cancers.

Materials and Methods

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humidified incubator with an atmosphere of 95% O_2 and 5% CO_2. SGK wild-type and SGK-null mouse embryonic fibroblasts were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For plasmid DNA transfection, the cells were plated at a density of 50–60% confluence, grown overnight, and transfected with appropriate expression vectors in the presence of indicated combinations of plasmid DNAs by using the calcium phosphate method or Lipofectamine-PLUS reagent (Kim M. J. et al., 2007; Mo et al., 2007).

Site-directed mutagenesis

Site-directed mutagenesis of cDNA encoding Fbw7 was performed with a QuikChange kit (Stratagene), and the mutagenic primer was S227A (5′-CAACGACGCCGAATTACAgCTGTCCAGCCACCTACA-3′) (mismatches with the Notch1-IC cDNA template are indicated by lowercase letters). The mutations were verified by automatic DNA sequencing.

Cloning and preparation of recombinant protein

A human *FBW7* gene was constructed via standard PCR and inserted into the bacterial expression vector pGEX4T-3 (Amersham Pharmacia). Expression of the recombinant GST–Notch1-IC proteins within the transformed bacteria was induced using 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma). GST–Fbw7 and its mutant proteins were purified with GSH–agarose (Sigma) in accordance with the manufacturer's instructions.

Immunoblot analysis

After 48 hours of transfection, the cultured HEK293 cells were harvested and lysed in RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 2 μg/ml each of leupeptin and aprotinin] for 30 minutes at 4°C. The cell lysates were subjected to 20 minutes of centrifugation at 12,000 ***g*** and 4°C. The resultant soluble fraction was boiled in Laemmli buffer and subjected to SDS-PAGE. After gel electrophoresis, the separated proteins were transferred via electroblotting onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were then blocked with Tris-buffered saline solution (pH 7.4) containing 0.1% Tween 20 and 5% nonfat milk. The blotted proteins were then probed with anti-Myc antibody (9E10), anti-HA (12CA5) antibody, or anti-FLAG M2 antibody (Sigma), followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). The blots were developed using enhanced chemiluminescence.

Protein stability assay

For the protein stability assay, the cells were seeded at a density of 50–60% confluence and incubated overnight. The cells were treated with 0.1 mM cycloheximide for 0, 1, 2, 4 or 8 hours by its addition to the medium to block the synthesis of new proteins; the cells were collected at each time point and the total lysates were then lysed in RIPA or Laemmli buffer. Protein levels of endogenous Notch1 were determined by immunoblotting with anti- Notch1 at dilutions of 1:3000.

Immunocomplex kinase assay

The cultured cells were harvested and lysed in buffer A, containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 25 mM glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% NP-40, 0.5% deoxycholate and 0.1% SDS for 30 minutes at 4°C. The cell lysates were then subjected to centrifugation at 12,000 ***g*** for 20 minutes at 4°C. The soluble fraction was incubated for 3 hours with appropriate antibodies against the indicated protein kinase at 4°C. The immunocomplexes were then coupled to Protein G–agarose during an additional hour of incubation at 4°C, after which they were pelleted via centrifugation. The immunopellets were rinsed three times with lysis buffer and then twice with 20 mM HEPES (pH 7.4). The immunocomplex kinase assays were conducted via the incubation of the immunopellets for 30 minutes at 30°C with 2 μg of substrate protein in 20 μl of reaction buffer that contained 0.2 mM sodium orthovanadate, 10 mM $MgCl_2$, 2 μCi [^{32}P]ATP and 20 mM HEPES (pH 7.4). The phosphorylated substrates were separated by SDS-PAGE and quantified using a Fuji BAS 2500 PhosphoImager. The GST fusion proteins to be used as substrates were expressed in *Escherichia coli* using pGEX-4T (Pharmacia) and purified using GSH–Sepharose, as described previously. The protein concentrations were determined by the Bradford method (Shimadzu).

Reporter assay

The cells were lysed in chemiluminescence lysis buffer (18.3% 1 M K_2HPO_4, 1.7% 1 M KH_2PO_4, 1 mM PMSF and 1 mM DTT) and assayed for luciferase activity with a luciferase assay kit (Promega). The activity of the luciferase reporter protein in the transfected cells was normalized by reference to the β-galactosidase activity in the same cells.

In vitro binding assay

The recombinant GST-Notch1-IC protein was expressed in the *E. coli* BL21 strain, using the pGEX system as indicated. The GST fusion protein was then purified with GSH–agarose beads (Sigma) in accordance with the manufacturer's instructions. An equal amount of GST or GST–Notch1-IC fusion protein was incubated with the lysates of the HEK293 cells, which had been transfected for 3 hours with combinations of expression vectors at 4°C, with rotation. After incubation, the beads were washed three times with ice-cold phosphate-buffered saline (PBS) and boiled with 20 μl of Laemmli sample buffer. The precipitates were separated via SDS-PAGE, and the pull-down proteins were detected via immunoblotting with specific antibodies.

Coimmunoprecipitation assays

The cells were lysed in 1 ml of RIPA buffer for 30 minutes at 4°C. After centrifugation at 12,000 ***g*** for 20 minutes, the supernatants were subjected to immunoprecipitation with appropriate antibodies coupled to Protein A–agarose beads (Peptron). The resulting immunoprecipitates were washed three times with PBS (pH 7.4). Laemmli sample buffer was then added to the immunoprecipitated pellets; the pellets were heated at 95°C for 5 minutes and then analyzed by SDS-PAGE. The western blot was performed with the indicated antibodies.

Double immunoprecipitations

Cells were washed twice with cold PBS and lysed in EBC buffer (50 mM Tris-HC1 pH 8.0, 120 mM NaC1, 0.5% Nonidet P-40) containing 10 pg/ml aprotinin, 10 pg/ml leupeptin, and 1 mM PMSF. After 20 minutes on ice, the cell lysates were centrifuged at 16,000 ***g*** for 20 minutes, and the supernatant subjected to immunoprecipitation with appropriate antibodies at 4°C for 3 hours with rotation. After incubation, the complex containing the immunoprecipitate was then incubated with 30 µl Protein A–agarose beads (Peptron) at 4°C for 1 hour. The immunoprecipitates were washed four times with EBC buffer and then solubilized by heating at 95°C for 3 minutes in 50 µl of SDS lysis buffer (20 mM Tris-HC1 pH 7.5, 50 mM NaC1, 1% SDS) containing 1 mM DTT. When a second immunoprecipitation of the dissociated immunocomplex was required, the supernatant was diluted with 500 µl of high salt buffer (50 mM Tris-HC1 pH 8.0, 300 mM NaCl, 1% Triton X-100) and subjected to the second immunoprecipitation using appropriate antibodies at 4°C for 3 hours with rotation. The resulting immunoprecipitates were washed three times with PBS (pH 7.4). Laemmli sample buffer was then added to the immunoprecipitated pellets; the pellets were heated at 95°C for 5 minutes and then analyzed by SDS-PAGE. The western blot was performed with the indicated antibodies.

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