

Dissociation of Akt1 from its negative regulator JIP1 is mediated through the ASK1–MEK–JNK signal transduction pathway during metabolic oxidative stress: a negative feedback loop

Jae J. Song and Yong J. Lee

Department of Surgery and Pharmacology, University of Pittsburgh, Pittsburgh, PA 15213

We have previously observed that metabolic oxidative stress-induced death domain-associated protein (Daxx) trafficking is mediated by the ASK1–SEK1–JNK1–HIPK1 signal transduction pathway. The relocalized Daxx from the nucleus to the cytoplasm during glucose deprivation participates in a positive regulatory feedback loop by binding to apoptosis signal-regulating kinase (ASK) 1. In this study, we report that Akt1 is involved in a negative regulatory feedback loop during glucose deprivation. Akt1 interacts with c-Jun NH₂-terminal kinase (JNK)–interacting protein (JIP) 1, and Akt1 catalytic activity is inhibited. The JNK2-mediated phosphorylation of JIP1 results in the dissociation of Akt1

from JIP1 and subsequently restores Akt1 enzyme activity. Concomitantly, Akt1 interacts with stress-activated protein kinase/extracellular signal-regulated kinase (SEK) 1 (also known as MKK4) and inhibits SEK1 activity. Knockdown of SEK1 leads to the inhibition of JNK activation, JIP1–JNK2 binding, and the dissociation of Akt1 from JIP1 during glucose deprivation. Knockdown of JIP1 also leads to the inhibition of JNK activation, whereas the knockdown of Akt1 promotes JNK activation during glucose deprivation. Altogether, our data demonstrate that Akt1 participates in a negative regulatory feedback loop by interacting with the JIP1 scaffold protein.

Introduction

We have previously observed that glucose deprivation increases the intracellular levels of hydroperoxide and glutathione disulfide (Lee et al., 1998). The increased steady-state levels of hydroperoxide and glutathione disulfide are sensed through thioredoxin and glutaredoxin and subsequently activate the apoptosis signal-regulating kinase (ASK) 1–MEK–MAPK–HIPK1 signal transduction pathway (Song et al., 2002; Song and Lee, 2003a,b,c). The activated HIPK1 phosphorylates the death domain-associated protein (Daxx), leading to the relocalization of Daxx from the nucleus to the cytoplasm (Song and Lee, 2004). The translocated Daxx binds to ASK1 and subsequently leads to ASK1 oligomerization (Song and Lee, 2003a). The association of Daxx with ASK1 may function as a positive feedback regulator to maintain/promote

ASK1–MEK–MAPK signal transduction through ASK1 oligomerization (Song and Lee, 2004). Unlike Daxx, CDC25A phosphatase and mouse GST Mu 1-1 physically associate with ASK1 and inhibit its oligomerization as well as its activity (Cho et al., 2001; Zou et al., 2001).

Biological regulatory systems usually have switchlike properties. Positive and negative feedback loops may produce bistable systems under stress conditions. Bistability can result from the combined effects of positive and negative regulators. Thus, we hypothesized that glucose deprivation elicits both positive and negative regulatory signaling pathways. As mentioned previously, Daxx-mediated ASK1 oligomerization may act as a positive feedback loop for ASK1–MEK–MAPK signal transduction by maintaining/promoting ASK1 activity. However, a fundamental question that remains unanswered is how glucose deprivation-induced ASK1–MEK–MAPK signal transduction can be negatively controlled. In this study, we postulated that Akt1 acts as a negative regulator. A previous study has shown that Akt interacts with ASK1 and negatively regulates ASK1 by phosphorylating ASK1 on Ser-83 residue

Correspondence to Yong J. Lee: leeyj@msx.upmc.edu

Abbreviations used in this paper: ASK1, apoptosis signal-regulating kinase 1; Daxx, death domain-associated protein; DLK, dual zipper-bearing kinase; JIP1, JNK-interacting protein 1; JNK, c-Jun NH₂-terminal kinase; MLK, mixed lineage protein kinase; MOI, multiplicity of infection; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase 1; si, small interference.

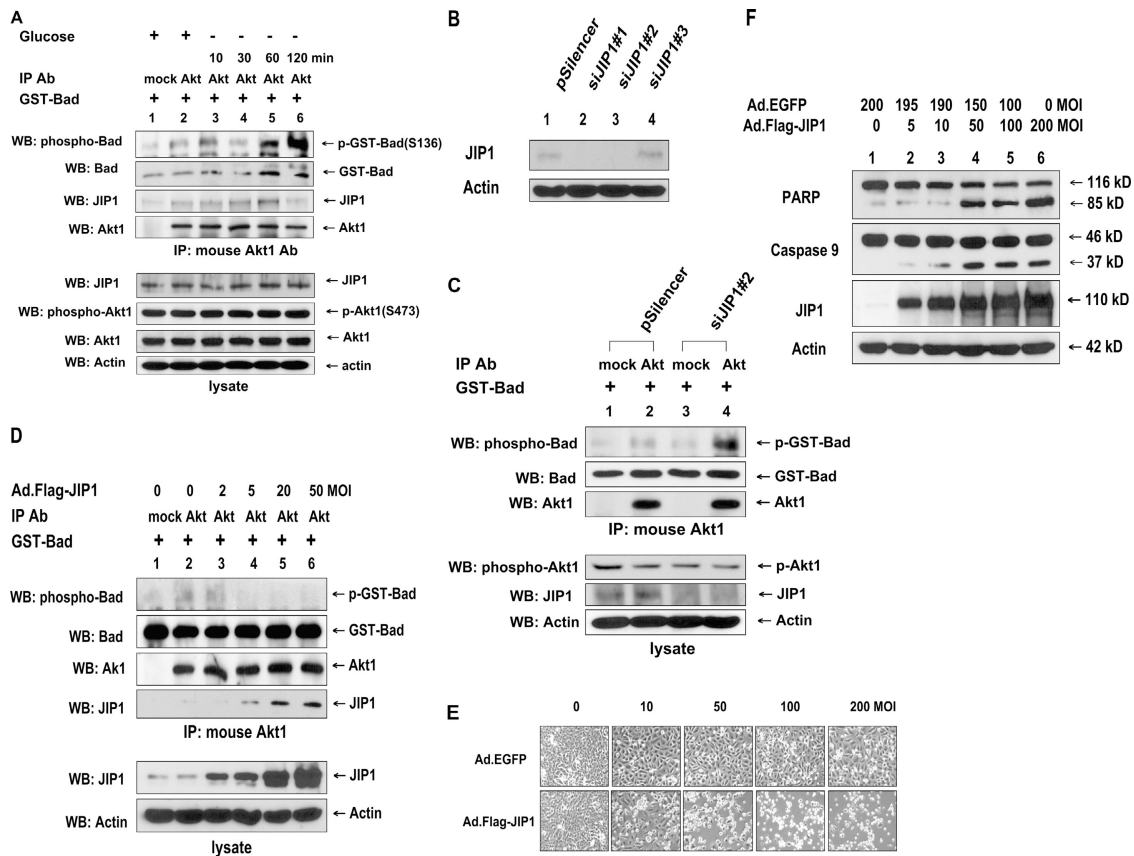


Figure 1. Role of JIP1 in Akt activity in DU-145 cells. (A) Cells were exposed to glucose-free medium for various times (10–120 min). Cells were lysed, and lysates were immunoprecipitated (IP) with anti-mouse Akt1 antibody. Immunoprecipitates were analyzed for the interaction of JIP1 with Akt1 (with anti-JIP1 antibody) and Akt1 catalytic activity in vitro using GST-Bad protein as a substrate (top). GST-Bad, phosphorylated GST-Bad, or Akt1 was detected with anti-Bad, anti-phospho-Ser-136-Bad, or anti-rabbit Akt1 antibody, respectively. Cell lysates (bottom) were immunoblotted with anti-JIP1, anti-phospho-Ser-473-Akt1, anti-Akt1, or antiactin antibody. (B) Immunoblot of JIP1 expression in control vector transfected (pSilencer) or pSilencer-siJIP1 stably transfected (siJIP1 #1–3) single cell clones from DU-145 cells. Lysates containing equal amounts of protein (20 μ g) were separated by SDS-PAGE and were immunoblotted with anti-JIP1 antibody. (C) Control plasmid or pSilencer-siJIP1 stably transfected siJIP1 #2 cells were lysed, and lysates were immunoprecipitated with anti-mouse Akt1 antibody. Akt1 catalytic activity in vitro was determined by using GST-Bad protein as a substrate (top). GST-Bad, phosphorylated GST-Bad, or Akt1 was detected with anti-Bad, anti-phospho-Ser-136-Bad, or anti-rabbit Akt1 antibody, respectively. Cell lysates (bottom) were immunoblotted with anti-phospho-Ser-473-Akt1, anti-JIP1, or antiactin antibody. (D) Cells were infected with adenoviral vector containing Flag-tagged JIP1 cDNA (Ad.Flag-JIP1) at various multiplicity of infections (MOIs; 2–50). After 48 h of infection, cells were lysed, and lysates were immunoprecipitated with anti-mouse Akt1 antibody. Immunoprecipitates were analyzed for Akt catalytic activity and JIP1 binding using immunoprecipitated Akt as described in Fig. 1 A (top). The presence of JIP1 or actin in the lysates was verified by immunoblotting (bottom). (E and F) Cells were infected with Ad.EGFP and/or Ad.Flag-JIP1 at various MOIs (10–200). After 48 h of infection, morphology was evaluated with a phase-contrast microscope (E), or cell lysates were immunoblotted with anti-PARP, anti-caspase-9, anti-JIP1, or antiactin antibody (F).

(Kim et al., 2001). Park et al. (2002) also observed that Akt phosphorylates stress-activated protein kinase/extracellular signal-regulated kinase (SEK)1 on Ser-78 residue, resulting in the inhibition of SEK1 enzyme activity. It is well known that the Akt family of Ser/Thr-directed protein kinases (Akt1–3) are important mediators of cell survival in response to growth factors, including insulin and insulin-like growth factor I (Bellacosa et al., 1998; Datta et al., 1999; Lawlor and Alessi, 2001; Leininger et al., 2004). Akt is activated by phosphoinositide-dependent kinases 1 and 2 through phosphorylation at Thr-308 and Ser-473 residues (Alessi et al., 1997; Toker and Newton, 2000). A number of proapoptotic proteins have been identified as direct Akt substrates, including Forkhead transcription factors, caspase-9, glycogen synthase kinase 3, and Bad (Cross et al., 1995; Datta et al., 1997; del Peso et al., 1997; Cardone et al., 1998; Pap and Cooper, 1998; Brunet et al., 1999; Kops et al., 1999; Hetman et al., 2000). The proapoptotic function of

these molecules is suppressed upon phosphorylation by Akt. Recently, Kim et al. (2002) reported an interaction between Akt1 and c-Jun NH₂-terminal kinase (JNK)-interacting protein (JIP) 1. The Akt1–JIP1 interaction is decreased concomitantly with an increase in an association between JIP1 and JNK (Kim et al., 2002). Based on that previous study, we hypothesized that JIP1 negatively regulates Akt1 by means of protein–protein interactions. The glucose deprivation-induced dissociation of Akt1 from JIP1 leads to the restoration of Akt1 enzyme activity.

JIP1 is a scaffold protein that integrates both positive and negative regulators of JNK. JIP1 assembles JNK, MKK7, and mixed lineage protein kinase (MLK) proteins on different regions of JIP1 and facilitates the JNK signaling pathway (Whitmarsh et al., 1998, 2001). A JNK negative regulator, MAPK phosphatase-7, also binds to JIP1 and inhibits JNK activation by dephosphorylating JNK (Willoughby et al., 2003). A recent

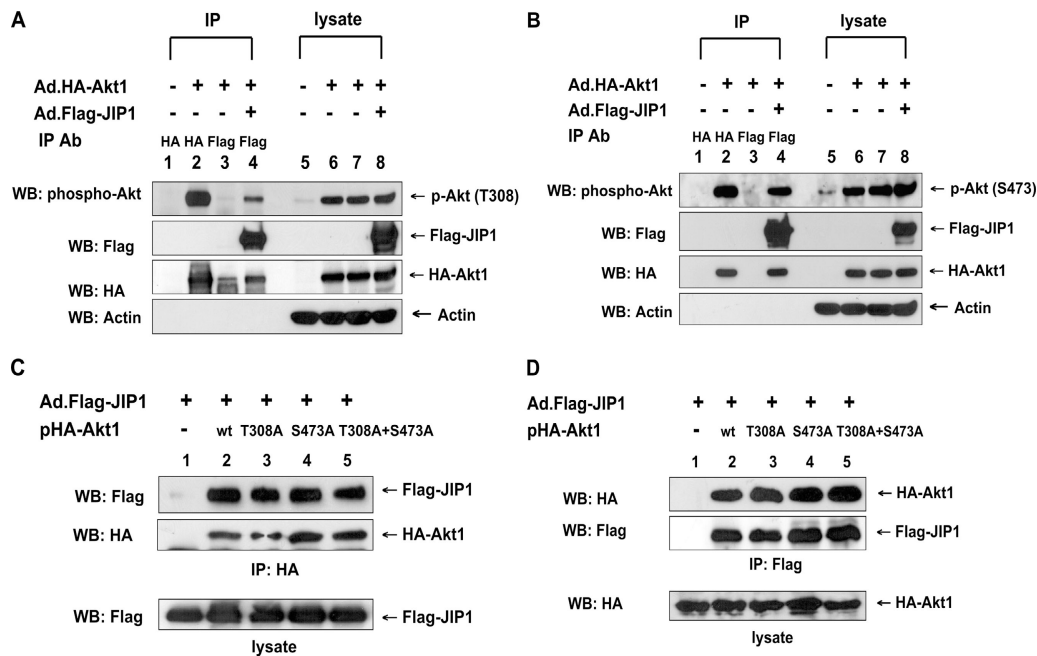


Figure 2. **Phosphorylation of Akt on Thr-308 and Ser-473 and its role in the association of Akt1 with JIP1.** (A and B) DU-145 cells were infected with adenoviral vector containing HA-tagged Akt1 (Ad.HA-Akt1) and/or Ad.Flag-JIP1 at an MOI of 10. After 48 h of infection, cells were lysed. Lysates were immunoprecipitated with anti-HA antibody or anti-Flag antibody. Immunoprecipitated proteins and lysates were separated by SDS-PAGE and were immunoblotted with anti-phospho-Thr-308-Akt, anti-phospho-Ser-473-Akt, anti-Flag, anti-HA, or antiactin antibody. (C and D) DU-145 cells were transfected with pHA-Akt1 (wild type), pHA-Akt1 (Thr-308A), pHA-Akt1 (Ser-473A), or pHA-Akt1 (Thr-308A + Ser-473A) plasmids and were infected with Ad.Flag-JIP1 at an MOI of 10. After 48 h of incubation, cells were lysed. Cell lysates were immunoprecipitated with anti-HA antibody (C) or anti-Flag antibody (D) and were immunoblotted with anti-Flag or anti-HA antibody (top). The presence of Flag-JIP1 or HA-Akt1 in the lysates was verified by immunoblotting with anti-Flag antibody or anti-HA antibody, respectively (bottom).

study revealed that the recruitment of JNK to JIP1 and the phosphorylation of JIP1 by JNK are prerequisites for activation of the JNK module (Nihalani et al., 2003). On the other hand, JNK activity can be antagonized by Akt kinase activity in numerous cellular systems (Levrresse et al., 2000; Kim et al., 2002; Barthwal et al., 2003; Aikin et al., 2004), and this cross talk may underlie many of the prosurvival effects of Akt.

In this study, we have demonstrated that JIP1–Akt1 plays an important role in the negative feedback loop during glucose deprivation. Glucose deprivation–induced JNK2 activation results in the phosphorylation of JIP1, which leads to the restoration of Akt1 activity by dissociating Akt1 from JIP1. Subsequently, Akt1 inhibits the glucose deprivation–induced ASK1–SEK1–JNK2 signal transduction pathway.

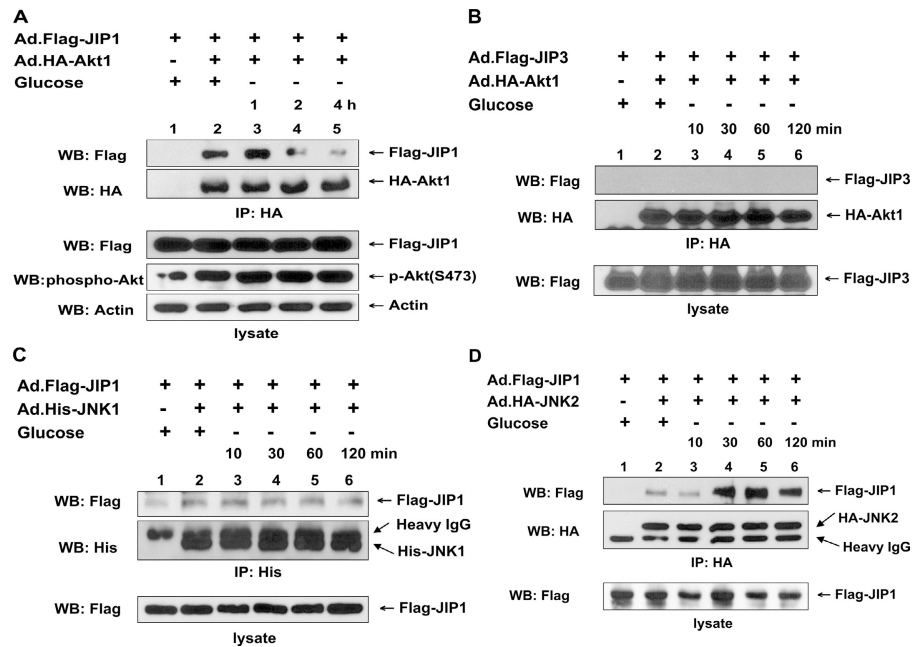
Results

Interaction between Akt1 and JIP1

A previous study has shown that Akt1 binds to JIP1, which is a JNK pathway scaffold protein (Kim et al., 2002). We hypothesized that JIP1 acts as a negative regulator of Akt1 and that JIP1–Akt1 interaction results in the inhibition of Akt1 catalytic activity. Metabolic oxidative stress may dissociate Akt1 from JIP1, thereby restoring Akt1 enzyme activity. To test the hypothesis, which was the first step in this study, we examined whether endogenous JIP1 associates with endogenous Akt1 and inhibits the enzymatic activity of Akt1 and whether metabolic oxidative stress dissociates Akt1 from JIP1, thereby restoring

Akt1 enzyme activity. DU-145 cells were exposed to glucose-free medium for various times (10–120 min). Cells were lysed and immunoprecipitated with anti-Akt1 antibody followed by immunoblotting with anti-JIP1 antibody, or Akt1 enzyme activity was measured by using an immune complex kinase assay. Fig. 1 A shows that endogenous Akt1 interacted with endogenous JIP1 (lane 2). However, Akt1 dissociated from JIP1 within 2 h during glucose deprivation (Fig. 1 A, lane 6). Fig. 1 A also shows that Akt1 phosphorylated an Akt-specific substrate, Bad (lane 6). These results suggest that Akt1 interacts with JIP1 and that Akt1 catalytic activity is inhibited. The dissociation of Akt1 from JIP1 restores Akt1 enzyme activity, and the role of JIP1 in Akt1 catalytic activity was further examined by the knockdown of JIP1 expression. Unlike pSilencer control plasmid–transfected cells, pSilencer–short interference (si)JIP1 stably transfected siJIP1#2 cells contained a low level of JIP1 (Fig. 1 B) and promoted Akt1 enzyme activity (Fig. 1 C). In contrast, when JIP1 was overexpressed, more JIP1 associated with Akt1, and the phosphorylation of Bad on Ser-136 was inhibited (Fig. 1 D, lanes 4–6). The inhibition of Bad phosphorylation was dependent on the level of JIP1 expression (Fig. 1 D). These results suggest that the interaction between Akt1 and JIP1 leads to the inhibition of Akt1 activity. Most interesting, the overexpression of JIP1 led to apoptosis, as shown by cell surface blebbing and the formation of apoptotic bodies (Fig. 1 E). These observations were consistent with poly (ADP-ribose) polymerase cleavage (Fig. 1 F), which is the hallmark feature of apoptosis, and with TUNEL assay (not depicted). Fig. 1 F also shows that pro-

Figure 3. Effect of glucose deprivation on Akt1–JIP1/3 interaction or JIP1–JNK1/2 interaction. DU-145 cells were coinfecting with Ad.HA-Akt1 and Ad.Flag-JIP1 (A) or Flag-tagged JIP3 (Ad.Flag-JIP3; B) at an MOI of 10. DU-145 cells were coinfecting with Ad.Flag-JIP1 and Ad.His-JNK1 (C) or Ad.HA-JNK2 (D) at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for various times. (A and B) Cell lysates were immunoprecipitated with anti-HA antibody and were immunoblotted with anti-Flag or anti-HA antibody (top). The presence of Flag-JIP1, Flag-JIP3, phospho-Akt, or actin in the lysates was verified by immunoblotting (bottom). (C and D) Lysates were immunoprecipitated with anti-His/anti-HA antibody and were immunoblotted with anti-Flag or anti-His/anti-HA antibody (top). The presence of Flag-JIP1 in the lysates was verified by immunoblotting (bottom).



caspase-9, the precursor form of caspase-9, was cleaved and activated in JIP1-overexpressing cells. Apoptosis was dependent on the level of JIP1 expression.

It is well known that Akt1 is activated by phosphoinositide-dependent kinase 1 through phosphorylation at the Thr-308 and Ser-473 residues (Alessi et al., 1997). Fig. 2 (A and B) shows that Akt1 phosphorylated on Thr-308 and that Ser-473 binds to JIP1. To determine whether both residues play an important role in the interaction between Akt1 and JIP1, site-directed mutagenesis was used to create point mutations in one or both residues, converting them to Ala residues (Thr-308A, Ser-473A, and Thr-308A + Ser-473A). Fig. 2 (C and D) clearly demonstrates that there is no difference between wild-type Akt1 and mutant-type Akt1s in terms of binding to JIP1. These results indicate that the phosphorylation of these residues is not essential for the binding of Akt1 to JIP1.

Effect of glucose deprivation on JIP1–Akt1 or JIP1–JNK1/2 interaction

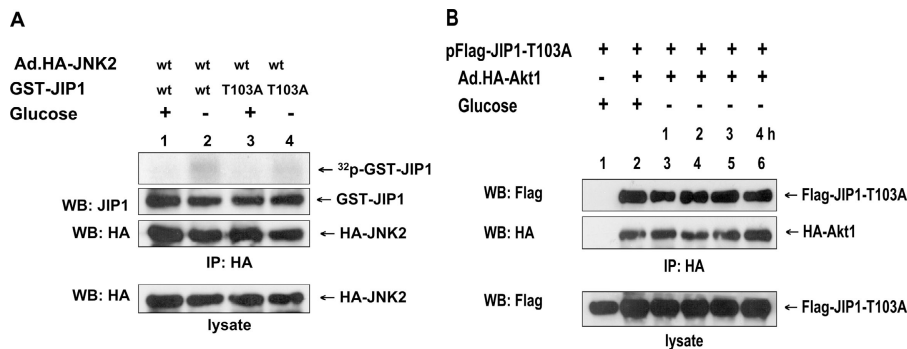
To investigate whether metabolic oxidative stress dissociates Akt1 from JIP1, DU-145 cells were infected with Ad.Flag-JIP1 and Ad.HA-Akt1. Cells were then exposed to glucose-free medium for various times, and the interaction between the two molecules was examined by immunoprecipitation. Fig. 3 A shows that Akt1 dissociated from JIP1 within 2 h during glucose deprivation. We further examined whether our findings could be generalized to other JIPs. Unlike JIP1, there was no interaction between Akt1 and JIP3 (Fig. 3 B) or JIP2 (not depicted), regardless of the glucose concentration of the medium. Next, we examined the mechanism by which Akt1 dissociates from JIP1 during glucose deprivation. We hypothesized that JNK is involved in this process. As a first step, we investigated the interaction between JIP1 and JNK1/2 during glucose deprivation. Fig. 3 (C and D) shows that the binding affinity of JNK2 to JIP1, but not that of JNK1 to JIP1, was enhanced during glucose deprivation.

Phosphorylation of JIP1 on Thr-103 is responsible for the dissociation of Akt1 from JIP1 during glucose deprivation

Previously, Nihalani et al. (2003) reported that JNK is involved in the Thr-103 phosphorylation of JIP1, leading to the dissociation of dual zipper-bearing kinase (DLK) from JIP1. JNK recruitment to JIP1 is related to the decreased affinity of JIP1 and DLK (Nihalani et al., 2001). We postulated that metabolic oxidative stress-induced JNK2 activation promotes the interaction between JNK2 and JIP1 and leads to the phosphorylation of JIP1 on Thr-103, thereby causing the dissociation of Akt1 from JIP1. To examine this possibility, we used site-directed mutagenesis to create a point mutation at residue Thr-103 (Thr → Ala) of JIP1 and measured the effect on the dissociation of Akt1 from JIP1 during glucose deprivation. Data from immune complex kinase assays show that the glucose deprivation–induced phosphorylation of JIP1 by JNK2 was diminished in Thr-103A mutant-type JIP1 (Fig. 4 A). Unlike wild-type JIP1, the interaction between Akt1 and Thr-103A mutant-type JIP1 was maintained during glucose deprivation (Figs. 3 A and 4 B). These data suggest that JNK2 binds to JIP1 and phosphorylates JIP1 on the Thr-103 residue during glucose deprivation. The phosphorylation of JIP1 on Thr-103 leads to the dissociation of Akt1 from JIP1.

JNK2–JIP1 interaction and JNK2-mediated phosphorylation of JIP1

Fleming et al. (2000) reported that phosphorylation of Thr-183 and Tyr-185 residues is required for the full activation of JNK2. We hypothesized that phosphorylation of both residues during glucose deprivation is essential for JNK2–JIP1 interaction and the phosphorylation of JIP1. To test this possibility, Thr-183 and Tyr-185 were replaced with Ala (Thr-183A) and phenylalanine (Y185F), respectively. Fig. 5 A shows that glucose deprivation increased the interaction between JIP1 and Thr-183A mutant-

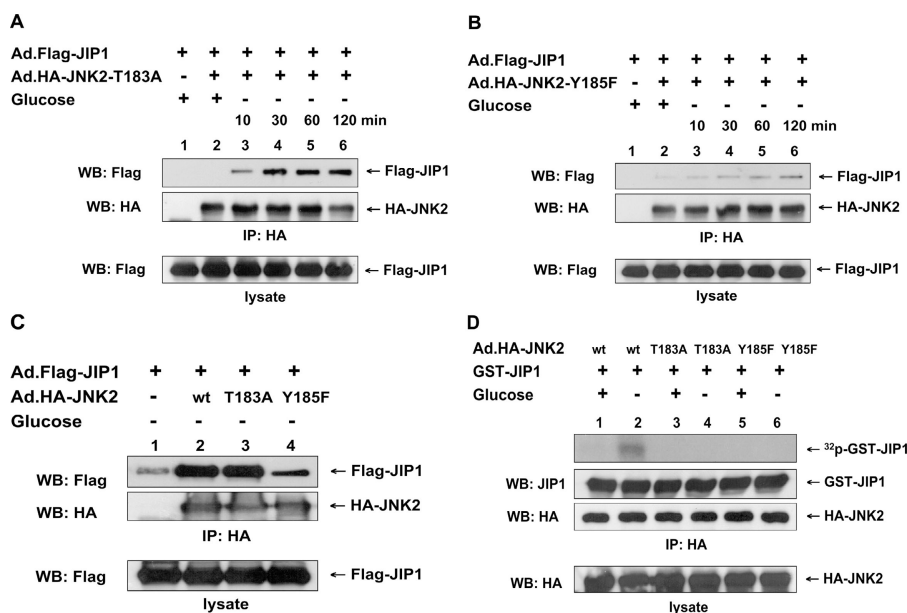


autoradiography. The presence of GST-JIP1 and HA-JNK2 in the kinase buffer was verified by immunoblotting with anti-JIP1 antibody and anti-HA antibody, respectively (top). The presence of HA-JNK2 in the lysates was verified by immunoblotting with anti-HA antibody (bottom). (B) DU-145 cells were transfected with pFlag-JIP1-Thr-103A and infected with Ad.HA-Akt1. After 48 h of incubation, cells were exposed to glucose-free medium for various times (1–4 h). Lysates were immunoprecipitated with anti-HA antibody and were immunoblotted with anti-Flag or anti-HA antibody (top). The presence of Flag-JIP1-Thr-103A in the lysates was verified by immunoblotting with anti-Flag antibody (bottom).

type JNK2. Fig. 5 B also shows that Y185F mutant increased its binding to JIP1 during glucose deprivation. However, the total amount of JIP1 bound to the Y185F mutant-type JNK2 is much less (Fig. 5 C). Data from densitometer tracings revealed that the ratio of the relative intensity of JIP1/JNK2 of wild type is similar to that of Thr-183A. However, the ratio of the relative intensity of JIP1/JNK2 of Y185F is only 35% of the wild-type value. These data suggest that the phosphorylation of Tyr-185 residue, but not that of Thr-183 residue, facilitates the binding of JNK2 to JIP1. We further investigated whether the phosphorylation of both JNK2 residues is required for the JNK2-mediated phosphorylation of JIP1. Data from an immune complex kinase assay shows that JIP1 was phosphorylated by only wild-type JNK2 during glucose deprivation, but not by either Thr-183A or Y185F mutant-type JNK2 (Fig. 5 D). These results suggest that phosphorylation of both the Thr-183 and Tyr-185 residues is necessary for full activation of the JNK2 enzyme.

Role of SEK1 in JNK2 activation, JIP1-JNK2 interaction, and Akt1-JIP1 binding

We previously observed that glucose deprivation activates the ASK1-SEK1-JNK pathway (Song et al., 2002). To examine whether glucose deprivation-activated SEK1 plays a role in the interaction between JIP1 and JNK and in the dissociation of Akt1 from JIP1, we attempted to silence SEK1 expression by using short hairpin RNAs. DU-145 cells were stably transfected with either pSilencer control plasmid or pSilencer-siSEK1 vector. We selected several stable transfectants, as described in Materials and methods. We chose three transfectant clones (pSilencer, siSEK1#1, and siSEK1#2) for further studies (Fig. 6). Fig. 6 A shows that the expression of SEK1 was effectively reduced in the siSEK1#2 transfectant. To examine the role of SEK1 in the activation of JNK, pSilencer and siSEK1#2 transfectants were exposed to glucose-free medium for various times (Fig. 6 B) or for 60 min (Fig. 6 C). Fig. 6 (B and C) shows that



JNK2 in the kinase buffer was verified by immunoblotting with anti-JIP1 antibody and anti-HA antibody, respectively (top). The presence of HA-JNK2 in the lysates was verified by immunoblotting with anti-HA antibody (bottom).

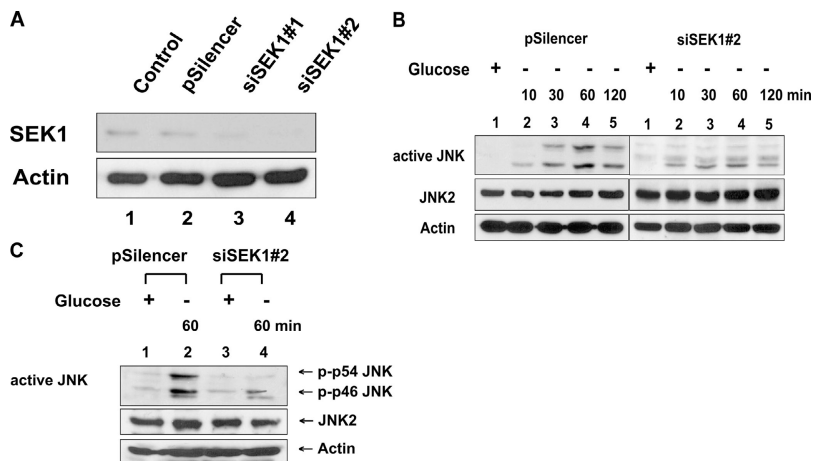
Figure 4. Phosphorylation of JIP1 on Thr-103 and its role in the dissociation of JIP1 from Akt1 during glucose deprivation. (A) DU-145 cells were infected with Ad.HA-JNK2 at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for 1 h and lysed. Cell lysates were immunoprecipitated with anti-HA antibody. To examine which amino acid residue of JIP1 can be phosphorylated by JNK2, 0.5 μ g GST-JIP1 (wild type) or GST-JIP1-Thr-103A (mutant type) was incubated with immunoprecipitated HA-JNK2 in kinase buffer containing 100 μ Ci/ml γ -[32 P]ATP at 30°C for 1 h. Phosphorylated proteins were resolved by SDS-PAGE and were analyzed by

autoradiography. The presence of GST-JIP1 and HA-JNK2 in the kinase buffer was verified by immunoblotting with anti-JIP1 antibody and anti-HA antibody, respectively (top). The presence of HA-JNK2 in the lysates was verified by immunoblotting with anti-HA antibody (bottom). (B) DU-145 cells were transfected with pFlag-JIP1-Thr-103A and infected with Ad.HA-Akt1. After 48 h of incubation, cells were exposed to glucose-free medium for various times (1–4 h). Lysates were immunoprecipitated with anti-HA antibody and were immunoblotted with anti-Flag or anti-HA antibody (top). The presence of Flag-JIP1-Thr-103A in the lysates was verified by immunoblotting with anti-Flag antibody (bottom).

Role of Thr-183 and Tyr-185 residues of JNK2 in glucose deprivation-induced JNK2-JIP1 interaction or JIP1 phosphorylation.

DU-145 cells were coinfecting with Ad.Flag-JIP1 and adenoviral vector containing HA-tagged wild-type JNK2 (Ad.HA-JNK2-wt), Thr-183A mutant-type JNK2 (Ad.HA-JNK2-Thr-183A), or Y185F mutant-type JNK2 (Ad.HA-JNK2-Y185F) at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for various times (A and B) or for 60 min (C and D) and were lysed. (A–C) Lysates were immunoprecipitated with anti-HA antibody and were immunoblotted with anti-Flag or anti-HA antibody (top). The presence of Flag-JIP1 in the lysates was verified by immunoblotting with anti-Flag antibody (bottom). (D) Cell lysates were immunoprecipitated with anti-HA antibody. To examine which types of JNK2 can phosphorylate JIP1, 0.5 μ g GST-JIP1 was incubated with immunoprecipitated HA-JNK2 in kinase buffer containing 100 μ Ci/ml γ -[32 P]ATP at 30°C for 1 h. Phosphorylated proteins were resolved by SDS-PAGE and were analyzed by autoradiography. The presence of GST-JIP1 and HA-JNK2 in the kinase buffer was verified by immunoblotting with anti-JIP1 antibody and anti-HA antibody, respectively (top). The presence of HA-JNK2 in the lysates was verified by immunoblotting with anti-HA antibody (bottom).

Figure 6. Glucose deprivation-induced JNK activation in control plasmid (pSilencer) or pSilencer-siSEK1 stably transfected DU-145 cells. (A) Immunoblot of SEK1 expression in control vector-transfected (pSilencer) or pSilencer-siSEK1 stably transfected (siSEK1#1 and siSEK1#2) single cell clones from DU-145 cells. Lysates containing equal amounts of protein (20 μ g) were separated by SDS-PAGE and were immunoblotted with anti-SEK1 antibody. Control, untransfected cells. (B and C) Control plasmid or pSilencer-siSEK1 stably transfected cells were exposed to glucose-free medium for various times (10–120 min; B) or for 60 min (C). After incubating, cells were harvested, and Western blot analysis was performed with anti-ACTIVE JNK, anti-JNK2, or antiactin antibody as the loading control.



the glucose deprivation-induced activation of JNK and the phosphorylation of p46 and p54 was suppressed in the siSEK1#2 transfectant. Relatively less suppression was observed in the siSEK1#1 transfectant (unpublished data). We further examined whether the knockdown of SEK1 mRNA and protein suppresses the interaction between JIP1 and JNK2 during glucose deprivation. Fig. 7 A shows that the binding of JNK2 to JIP1 increased during glucose deprivation in the pSilencer transfectant. However, this binding was markedly inhibited in the siSEK1#2 transfectant (Fig. 7, B and C). We also observed that glucose deprivation-induced JNK activation, the dissociation of Akt1

from JIP1, and the restoration of Akt1 activity were delayed in siSEK1#2 cells in comparison with pSilencer control vector-transfected cells (Fig. 7, E and D). These data suggest that SEK1 plays an important role in JNK2 activation, the binding of JNK2 to JIP1, and the subsequent dissociation of Akt1 from JIP1.

Glucose deprivation-induced interaction between Akt1 and SEK1

A previous study has shown that Akt binds to SEK1 and subsequently inhibits SEK1 activity by phosphorylating mouse SEK1 on Ser-78 residue (Park et al., 2002). We postulated that the glu-

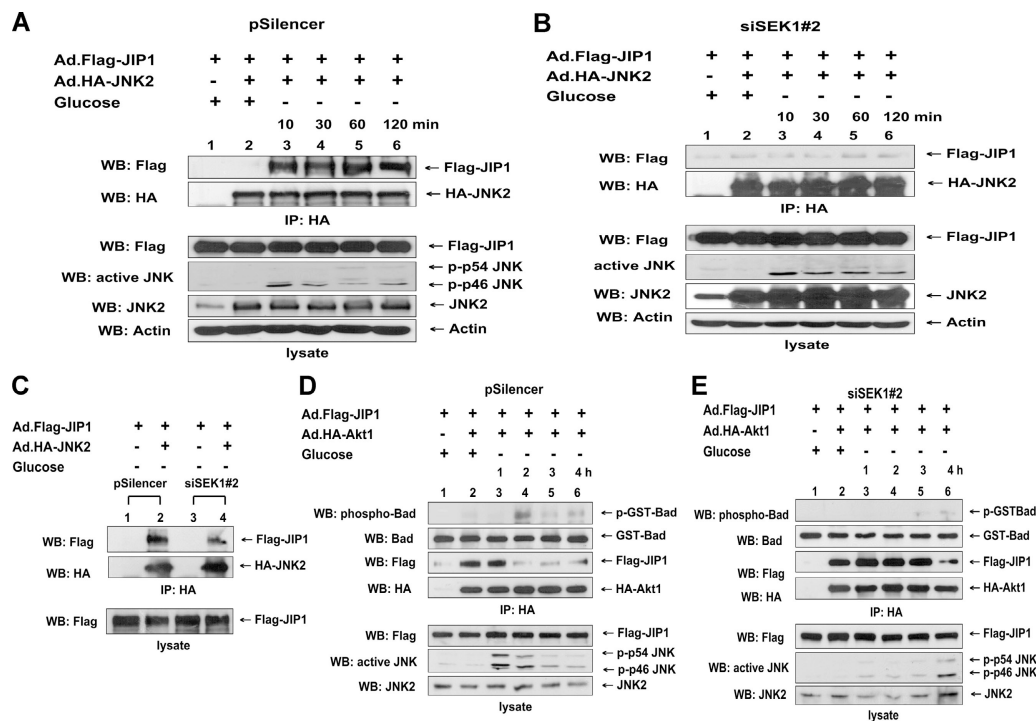


Figure 7. Role of SEK1 in the interaction between JIP1 and JNK2/Akt1 during glucose deprivation. Control plasmid (pSilencer) or pSilencer-siSEK1 stably transfected (siSEK1#2) cells were coinfecting with Ad.Flag-JIP1 and adenoviral vector containing HA-tagged JNK2 (Ad.HA-JNK2) or Ad.HA-Akt1 at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for various times (A, B, D, and E) or for 60 min (C) and were lysed. Lysates were immunoprecipitated with anti-HA antibody and were immunoblotted with anti-Flag or anti-HA antibody (A–E). Akt1 catalytic activity in vitro was determined by using GST-Bad protein as a substrate (D and E). GST-Bad or phosphorylated GST-Bad was detected with anti-Bad or anti-phospho-Ser-136-Bad antibody, respectively (top). Flag-JIP1, phosphorylated JNK, JNK2, or actin in the lysates was verified by immunoblotting with anti-Flag, anti-ACTIVE JNK, anti-JNK2, or antiactin antibody, respectively (bottom).

glucose deprivation-induced dissociation of Akt1 from JIP1 leads to the increased binding of Akt1 to SEK1. The interaction between Akt1 and SEK1 results in the inhibition of SEK1 enzyme activity. To test this hypothesis, we first examined Akt1–SEK1 binding during glucose deprivation. Fig. 8 (A and B) shows that binding of Akt1 to SEK1 gradually increased as a function of time during glucose deprivation without changes in the intracellular level of Akt1. We then investigated whether Akt1, which dissociates from JIP1 during glucose deprivation, is the Akt1 that interacts with SEK1. Fig. 8 (C and D) shows that endogenous Akt1 associated with endogenous SEK1 during glucose deprivation for 2 h and phosphorylated human SEK1 on Ser-80 residue. As shown previously in Fig. 4 B, Akt1 binds to JIP1 before glucose deprivation. However, Akt1 does not dissociate from the JIP1–Thr-103A mutant protein during glucose deprivation. This indicates that JIP1 is responsible for the dissociation of Akt1 from JIP1 during glucose deprivation. If it is true that the dissociation of Akt1 from JIP1 causes an increase in the interaction between Akt1 and SEK1, then that interaction should

likewise be diminished by the overexpression of JIP1–Thr-103A mutant protein. Fig. 8 E shows that glucose deprivation-induced Akt1–SEK1 interaction was suppressed by overexpressing mutant-type JIP1–Thr-103A but not wild-type JIP1. These results suggest that Akt1, which has been dissociated from JIP1, interacts with SEK1. To examine whether the interaction between Akt1 and SEK1 inhibits the catalytic activity of SEK1, cells were coinfecting with Ad.His-SEK1 and either Ad.HA-Akt1 wild type or Ad.HA-Akt1 mutant type (a dominant negative form carrying a point mutation that ablates the kinase activity by replacing Lys-179 residue with Met). Data from immune complex kinase assays show that GST-JNK1 was phosphorylated by His-SEK1 in cells that were deprived of glucose for 1 h (Fig. 8 F). This phosphorylation was reduced during 4 h of glucose deprivation in Ad.HA-Akt1 (wild type)-infected cells, but not in Ad.HA-Akt1-infected cells (Fig. 8 F). Altogether, these results suggest that the enzymatic activity of SEK1 is inhibited by its binding to Akt1 after several hours of glucose deprivation, resulting from activation of the negative feedback loop.

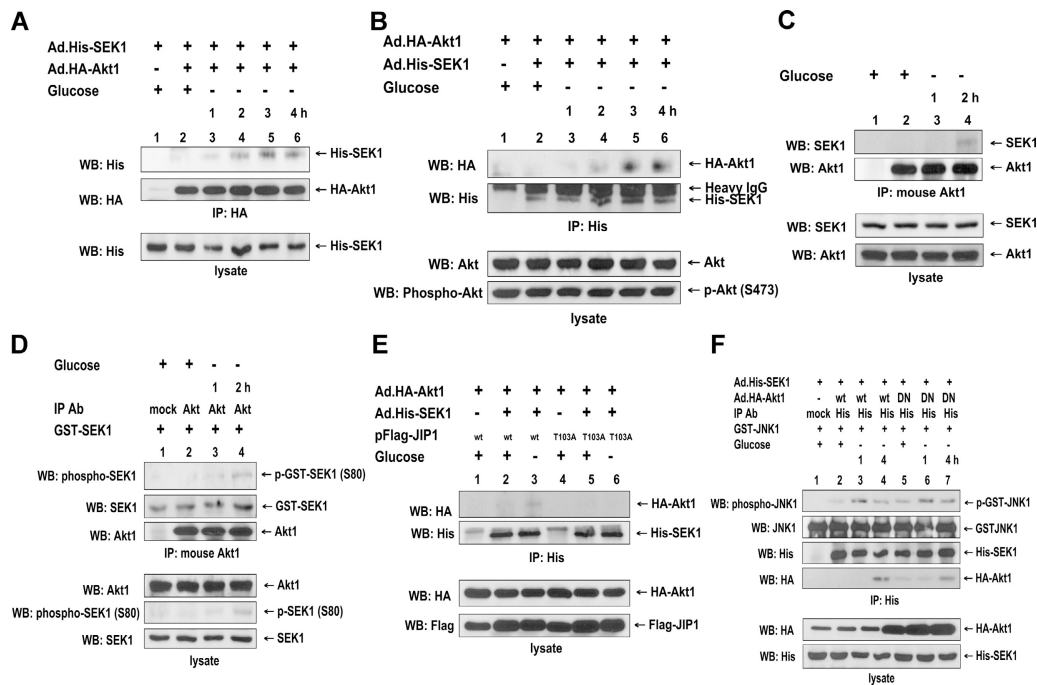


Figure 8. Interaction between SEK1 and Akt1 and its role in SEK1 enzyme activity during glucose deprivation. DU-145 cells were coinfecting with adenoviral vector containing His-tagged SEK1 (Ad.His-SEK1) and Ad.HA-Akt1 at an MOI of 10 (A, B, E, and F) and were transiently transfected with pFlag-JIP1-wt (wild type) or pFlag-JIP1–Thr-103A (mutant type; E). (A and B) After 48 h of incubation, cells were exposed to glucose-free medium for various times (1–4 h) and were lysed. Cell lysates were immunoprecipitated with anti-HA antibody (A) or anti-His antibody (B) and were immunoblotted with anti-His or anti-HA antibody (top). The presence of His-SEK1, Akt, or phospho-Akt in the lysates was verified by immunoblotting (bottom). (C and D) DU-145 cells were exposed to glucose-free medium for 1 or 2 h and were lysed. Lysates were immunoprecipitated with anti-mouse Akt antibody. (C) Immunoprecipitates were analyzed for SEK1 binding with anti-SEK1 or anti-rabbit Akt antibody (top). The presence of SEK1 and Akt in the lysates was verified by immunoblotting (bottom). (D) Immunoprecipitates were examined for the phosphorylation of SEK1 (Ser-80) by Akt. 0.5 μ g GST-SEK1 was incubated with immunoprecipitated Akt in kinase buffer containing ATP at 30°C for 1 h. Phosphorylated proteins were resolved by SDS-PAGE and were analyzed by immunoblotting with anti-phospho-SEK1 (Ser-80) antibody. The presence of GST-SEK1 or Akt in the immunoprecipitate was verified by immunoblotting with anti-SEK1 antibody or anti-rabbit Akt antibody, respectively (top). The presence of Akt, phospho-SEK1, or SEK1 in the lysates was verified by immunoblotting (bottom). (E) After 48 h of incubation, cells were exposed to glucose-free medium for 4 h and were lysed. Lysates were immunoprecipitated with anti-His antibody and were immunoblotted with anti-HA or anti-His antibody (top). The presence of HA-Akt1, Flag-JIP1 wild type, or Flag-JIP1–Thr-103A in the lysates was verified by immunoblotting (bottom). (F) DU-145 cells were coinfecting with Ad.His-SEK1 and Ad.HA-Akt1 wild type (wt) or Ad.HA-Akt1 dominant negative mutant type (DN) at an MOI of 10. After 48 h of incubation, cells were exposed to glucose-free medium for 1 or 4 h and were lysed. Lysates were immunoprecipitated with anti-His antibody. To examine the catalytic activity of SEK1, 0.5 μ g GST-JNK1 was incubated with immunoprecipitated His-SEK1 in kinase buffer containing ATP at 30°C for 1 h. Phosphorylated proteins were resolved by SDS-PAGE and were analyzed by immunoblotting with anti-ACTIVE JNK antibody. The presence of GST-JNK1, His-SEK1, or HA-Akt1 in the immunoprecipitates was verified by immunoblotting (top). The presence of HA-Akt1 (wt, DN) or His-SEK1 in the lysates was verified by immunoblotting (bottom).

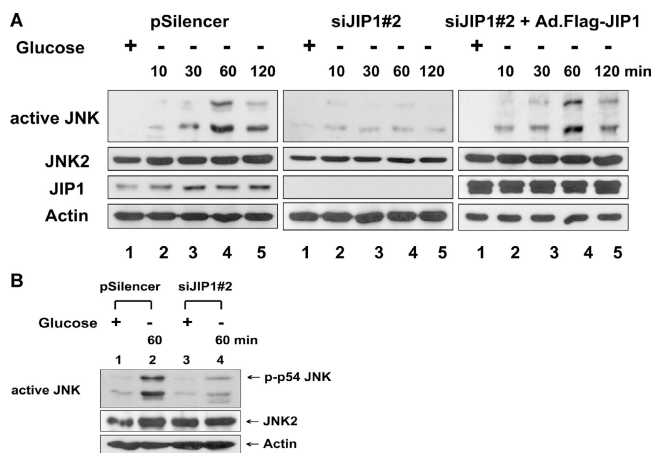


Figure 9. Glucose deprivation-induced JNK activation in control plasmid (pSilencer), pSilencer-siJIP1 stably transfected siJIP1#2, or Ad.Flag-JIP1-infected siJIP1#2 cells. Cells were exposed to glucose-free medium for various times (10–120 min; A) or for 60 min (B). Cell lysates were immunoblotted with anti-ACTIVE JNK, anti-JNK2, anti-JIP1, or antiactin antibody.

Role of JIP1 in glucose deprivation-induced JNK activation

To examine whether JIP1 plays an important role in JNK activation during glucose deprivation, pSilencer control plasmid-transfected cells or pSilencer-siJIP1 stably transfected siJIP1#2 cells were exposed to glucose-free medium for various times (Fig. 9 A) or for 60 min (Fig. 9 B). Fig. 9 shows that glucose deprivation-induced JNK activation was suppressed in siJIP1#2 cells. Because JIP1 was a knockdown, but not a knockout, in siJIP1#2 cells, basal levels of activated JNK was still observed. The activation of JNK was restored by overexpressing JIP1 in siJIP1#2 cells (Fig. 9 A, right).

Negative regulator role of Akt1 in glucose deprivation-induced JNK activation

Our studies have revealed that Akt1 acts as a negative regulator for the ASK1–SEK1–JNK pathway during glucose deprivation. To confirm our observations, cells were transfected with Akt1 or mock siRNA. Fig. 10 A shows that the expression of Akt1 was

effectively inhibited by siAkt1. Glucose deprivation-induced JNK activation (Fig. 10, B and C) and cytotoxicity (Fig. 10 D) were promoted in siAkt1-transfected cells. To exclude off-target effects of the siAkt construct, siAkt1-transfected cells were infected with Ad.HA-Akt1. Fig. 10 (C and D) shows that the overexpression of Akt1 in siAkt1-transfected cells suppressed glucose deprivation-induced JNK activation and cytotoxicity. These results suggest that Akt1 acts as a negative regulator for the ASK1–SEK1–JNK pathway during glucose deprivation.

Discussion

The cellular functions of scaffolding proteins are to maintain the specificity of signaling pathways and catalyze the activation of pathway components (Burack and Shaw, 2000). The JIP family of scaffolding proteins associate with MAPK, MAPK kinase, and MAPK kinase kinase and create a functional signaling module to control the specificity of signal transduction (Morrison and Davis, 2003). JIP1 scaffold facilitates JNK activation in an MLK-MKK7-dependent manner (Dickens et al., 1997; Whitmarsh et al., 1998; Yasuda et al., 1999; Kelkar et al., 2000). JIP3 mediates JNK signaling by interacting with ASK1, MEKK1, and SEK1 (Ito et al., 1999; Matsuura et al., 2002). JIP2 has been proposed to regulate p38 signaling modules (Schoorlemmer and Goldfarb, 2001; Matsuura et al., 2002). Most interesting, JIP1 and 2 interact and form homo- and heterooligomeric complexes with components of the JNK signaling pathway and facilitate signal transduction (Yasuda et al., 1999). However, our studies reveal that JIP1 also acts as a negative regulator (Fig. 1). Data from immune complex kinase assays show that JIP1 binds to Akt1 and inhibits Akt1 enzymatic activity (Fig. 1). Our observations somewhat contradicted a recent report (Kim et al., 2003) that JIP1 activates Akt1. We can only speculate that this discrepancy is a result of differences in analytical methods (immunoblotting assay vs immune complex kinase assay) because our data clearly demonstrate that immunoblotting with phosphospecific Akt antibodies does not truly reflect the catalytic activity of Akt. Data from immunoblot assays illustrate that phosphorylated (active form) Akt1 binds to JIP1; however, data from immune com-

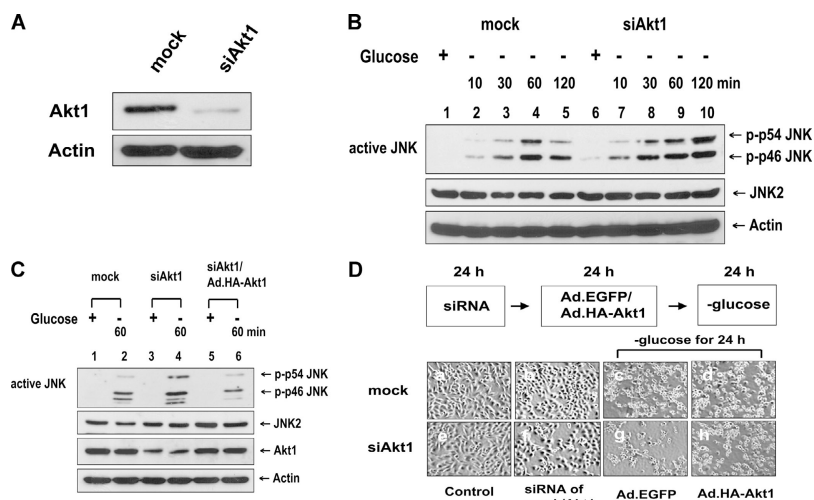


Figure 10. Role of Akt1 in glucose deprivation-induced JNK activation and morphological damage. (A and B) DU-145 cells were transfected with Akt1 siRNA or mock siRNA and were incubated for 36 h. (A) Akt1 protein expression was assessed by immunoblotting with anti-Akt1 antibody. (B) Cells were exposed to glucose-free medium for various times (10–120 min). Cell lysates were immunoblotted with anti-ACTIVE JNK, anti-JNK2, or antiactin antibody. (C and D) DU-145 cells were transfected with Akt1 or mock siRNA. After 24 h of incubation, cells were infected with Ad.EGFP or Ad.HA-Akt1 at an MOI of 10. After 24 h of infection, cells were exposed to glucose-free medium for 60 min (C) or for 24 h (D). (C) Cell lysates were immunoblotted with anti-ACTIVE JNK, anti-JNK2, anti-Akt1, or antiactin antibody. (D) Morphology was evaluated with a phase-contrast microscope.

plex kinase assays demonstrate that the enzymatic activity of Akt1 is suppressed by binding with JIP1 (Figs. 1 and 2).

The present studies reveal that JNK2-dependent JIP1 phosphorylation on Thr-103 regulates JIP1–Akt1 binding (Fig. 4). These results are consistent with a previous study that demonstrated that the JNK-mediated phosphorylation of JIP1 on Thr-103 is essential for regulating the binding of DLK to JIP1 (Nihalani et al., 2003). The dissociation of DLK from JIP1 results in DLK oligomerization, autophosphorylation, and, ultimately, in module activation (Nihalani et al., 2003). However, unlike DLK, phosphorylated Akt1 (active form) binds to JIP1 and is inactivated, whereas its dissociation from JIP1 results in the restoration of activity (Figs. 1–3). Akt1 then binds to SEK1 and negatively regulates SEK1 by phosphorylating Ser-80 residue (Park et al., 2002; Fig. 8). A previous study also revealed that Akt negatively regulates ASK1 by phosphorylating Ser-83 residue (Kim et al., 2001) and preventing oligomerization (unpublished data). Altogether, the Akt-mediated inhibition of SEK1 and/or ASK1 may act as a negative regulatory feedback loop for the ASK1–MEK–MAPK signal transduction pathway. Moreover, recent studies have demonstrated that during glucose deprivation, Akt phosphorylates and activates ARK5, which is a member of the AMP-activated protein kinase family (Suzuki et al., 2003a, 2004a). Activated ARK5 phosphorylates ataxia-telangiectasia mutated, a tumor suppressor, leading to the activation of p53 by phosphorylation (Suzuki et al., 2003a). The activation of ARK5, which is triggered by Akt during glucose deprivation, suppresses caspase activation and prevents cell death (Suzuki et al., 2003b, 2004b). It is possible that Akt-activated ARK5 is involved in the negative regulatory feedback loop for the ASK1–MEK–MAPK signal transduction pathway. This possibility needs to be investigated.

It was well known that HSP90 binds to Akt, and the inhibition of this Akt–HSP90 interaction by HSP90 inhibitors leads to the dephosphorylation and inactivation of Akt (Basso et al., 2002; Sato et al., 2000). Protein phosphatase 2A or protein phosphatase 1 may play an important role in the regulation of Akt dephosphorylation (Sato et al., 2000; Xu et al., 2003). In this study, we did not observe any significant reduction of the level of Akt1 phosphorylation during glucose deprivation (Fig. 3 A). Thus, HSP90 is not likely involved in the regulatory feedback loop for the ASK1–MEK–MAPK signal transduction pathway during glucose deprivation.

Previous studies have shown that JNK is activated by dual phosphorylation on the tripeptide motif Thr-X-Tyr, where X is any amino acid (Payne et al., 1991; Lawler et al., 1998). Two MAPK kinases, MKK4 and MKK7, synergistically activate JNK (Lawler et al., 1998; Ito et al., 1999; Matsuura et al., 2002; Kishimoto et al., 2003). MKK4 prefers Tyr-185 residue, and MKK7 prefers Thr-183 residue (Lawler et al., 1998; Fleming et al., 2000). Our data demonstrate that the full activation of JNK requires the phosphorylation of both residues (Fig. 5 D). However, the phosphorylation of JNK2 on Tyr-185 is a prerequisite for the recruitment of JNK2 to JIP1 (Fig. 5). Most interesting, the knockdown of SEK1 (MKK4) mRNA and protein by siRNA for SEK1 during glucose deprivation leads to the inhibition of JNK activation, JIP1–JNK2 binding, and the dissociation of

Akt1 from JIP1 (Figs. 6 and 7). These results suggest that SEK1-mediated JNK2 phosphorylation is necessary for the restoration of Akt1 enzyme activity. As mentioned in the Introduction, JIP1 specifically scaffolds JNK, MKK7, and members of the MLK family. In contrast, the ASK1–SEK1–JNK-signaling module preferentially interacts with JIP3 (Matsuura et al., 2002). Altogether, we postulate that two scaffolding proteins, JIP1 and 3, have a cross talk that leads to the regulation of the ASK1–SEK1–JNK signal during glucose deprivation. Glucose deprivation rapidly increases the interaction between ASK1 and JIP3, and the consequently activated ASK1 phosphorylates SEK1 on the Thr-261 residue. The activated SEK1 dissociates from JIP3 and phosphorylates JNK2 on the Tyr-185 residue. Phosphorylated JNK2 binds to JIP1, and the phosphorylation of the JNK2 Thr-183 residue occurs. Activated JNK2 phosphorylates JIP1 on the Thr-103 residue and leads to the dissociation of Akt1 from JIP1. Dissociated Akt1 binds to SEK1 and ASK1 and inhibits their enzyme activity by phosphorylating SEK1 on the Ser-80 residue and ASK1 on the Ser-83 residue. Although JIP3 and 1 are structurally distinct, we believe that cross talk takes place between these two scaffolding proteins. Obviously, further studies are necessary to understand the role of scaffolding proteins in the glucose deprivation-induced negative feedback loop. Our model will provide a framework for future studies.

Materials and methods

Cell culture

Human prostate adenocarcinoma DU-145 cells were cultured in DME with 10% FBS (HyClone) and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a humidified atmosphere containing 5% CO₂ and air at 37°C.

Reagents and antibodies

Polyclonal anti-SEK1, anti-phospho-Thr-308–Akt, anti-phospho-Ser-473–Akt, anti-Akt, anti-Bad, and anti-phospho-Ser-136–Bad were purchased from Cell Signaling, and anti-ACTIVE JNK was purchased from Promega. mAbs were purchased from the following companies: anti-JIP1 from Santa Cruz Biotechnology, Inc.; antiactin from ICN; anti-HA (clone 3F10) from Roche Diagnostics; anti-Flag (clone M2; mouse) from Sigma-Aldrich; and anti-His (penta-His; mouse) from QIAGEN.

Site-directed mutagenesis

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to make point mutations in Akt1, JNK2, or JIP1 protein. One Thr residue in Akt1 (Thr-308 Akt1) was replaced with Ala (Thr-308A Akt1). Sense primer (5'-GGTGCCACTATGAAGGCATTCTGCGGAACGC-3') and antisense oligonucleotides (5'-GCGTCCGCAGAAATGCCCTCATAGTGGCACC-3') were used for site-directed mutagenesis. One Thr residue in JNK2 (183 JNK2) phosphorylation site of Akt1, Ser-473 residue was changed to Ala. Sense (5'-TCCCCAGTTCGCCTACTCAGCCAGTG-3') and antisense primer oligonucleotides (5'-CACTGGCTGAGTAGGCGAACTGGGGGA-3') were used for site-directed mutagenesis. One Thr residue in JNK2 (183 JNK2) was replaced with Ala (Thr-183A). Sense (5'-CCAACITTTATGATGGCTCCTATGTGGTG-3') and antisense primer oligonucleotides (5'-CACCA-CATAGGGAGCCATCATAAAGTTGG-3') were used for site-directed mutagenesis. One Tyr residue in JNK2 (185 JNK2) was replaced with Phe (Y185F). Sense (5'-CTTTATGATGACTCCCTTTGTGGTGACACGGTAC-3') and antisense primer oligonucleotides (5'-GTACCGTGTACCCACAAA-GGGAGTATCATAAAG-3') were used for site-directed mutagenesis. One Thr residue in JIP1 (103 JIP1) was replaced with Ala (Thr-103A). Sense primer (5'-GGCAGGTGACGCTCCGGGCGCCG-3') and antisense oligonucleotides (5'-CGGCGCCCGGAGCGTCACTGCC-3') were used for site-directed mutagenesis. PCR reaction was prepared by adding 5 μl of 10× reaction buffer, 20 ng of double-stranded (ds) DNA template (pAdlox-HA-JNK2), 125 ng of each sense primer, 125 ng of each antisense primer, 1 μl deoxyribonucleotide triphosphate mix, double-distilled water

to a final volume of 50 μ l, and 1 μ l Pfu Turbo DNA polymerase (2.5 U/ μ l). PCR was performed with 14 cycles (95°C for 30 s; 58°C for 1 min; 68°C for 7 min) with initial incubation at 95°C for 30 s. After temperature cycling, the reaction was placed on ice for 2 min to cool the reaction. After PCR, 1 μ l DpnI restriction enzyme (10 U/ μ l) was added directly to each amplification reaction and incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. The DpnI-treated dsDNA was transformed into Epicurian Coli XL1-blue supercompetent cells. Colonies were selected, and each plasmid (pAdlox-HA-JNK2, pAdlox-HA-Akt1, and pAdlox-JIP1) was sequenced using primer (5'-GGATGCTAAGTTCAGG-3' for JNK2; 5'-CGAGAGCGCTGTCTCCGAG-3' for Akt1 308 and 473; and 5'-CATGACATCAGCCTGGAGGAG-3' for JIP1) to confirm mutation.

RNA interference by siRNA of SEK1 or Akt1

To stably express siRNA for long-term knockdown, pSilencer 2.1-U6 hygro vector (Ambion) was used for clonal cell lines. The inserts for hairpin siRNA into pSilencer were prepared by annealing two oligonucleotides. For human SEK1 siRNA, the top strand sequence was 5'-GATCCACGCAAGCACTGAAGTTGTTCAAGACAACCTCAGTCTTTCGCTTTTGGAAA-3', and the bottom strand sequence was 5'-AGCTTTTCAAAAAACGCAAGCACTGAAGTTGTTCTTGAACAACCTCAGTCTTTCGCTG-3'. The annealed insert was cloned into pSilencer 2.1-U6 hygro digested with BamH I and HindIII. The correct structure of pSilencer 2.1-U6 hygro-SEK1 was confirmed by nucleotide sequencing. The resultant plasmid, pSilencer-SEK1, was transfected into DU-145 cells, and 250 μ g/ml hygromycin B-resistant cell clones were isolated. The interference of SEK1 protein expression was confirmed by immunoblot using anti-SEK1 antibody.

To down-regulate Akt1, Akt1 siRNA (Santa Cruz Biotechnology, Inc.) was used. Cells were transfected with Akt1 siRNA and were incubated for 36 h. The interference of Akt1 protein expression was confirmed by immunoblotting using anti-Akt1 antibody (Upstate Biotechnology).

In vivo binding of Akt1 and JIP1 (or JIP3)

To examine the interaction between Akt1 and JIP1/3, DU-145 cells in 100-mm culture plates were coinfecting with the adenovirus of HA-tagged Akt1 (Ad.HA-Akt1) and Flag-tagged JIP1 (Ad.Flag-JIP1) or Flag-tagged JIP3 (Ad.Flag-JIP3). For immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1 mM PMSF, 80 μ M aprotinin, and 2 mM leupeptin, and the lysates were incubated with 0.5 μ g of rat anti-HA antibody for 2 h. After the addition of protein G agarose, the lysates were incubated for an additional 2 h. The beads were washed three times with the lysis buffer, separated by SDS-PAGE, and immunoblotted with mouse anti-Flag or rat anti-HA antibodies. The proteins were detected with the enhanced chemiluminescence reaction.

Shuttle vector construction

Adlox-HA-Akt1 was produced by inserting a HindIII-EcoRI fragment from pCMV6-HA-Akt1 into HindIII-EcoRI-cut pAdlox shuttle vector. pCMV5-Flag-JIP1 was provided by R. Davis (University of Massachusetts Medical School, Amherst, MA). pAdlox-Flag-JIP1 having Flag tagged at their NH₂-terminal and restriction enzyme recognition sites at the flanking sides (5', HindIII; 3', XbaI) was produced by PCR using pCMV5-Flag-JIP1 as a template. The sense primer was 5'-TAATAAGCTTGCAGGAGAGAGCGGCCTG-3', and the antisense primer was 5'-GCCGCTAGACTACTCAAGTAGATATCTTC-3'. PCR was performed for 30 cycles (94°C for 30 s, 55°C for 30 s, and 68°C for 2 min 30 s) with an initial incubation at 94°C for 1 min. For JIP1 (or JIP1-Thr103A) protein purification, a PCR product of mouse JIP1 having restriction enzyme sites at the flanking sides (5', EcoRI; 3', Sall) was produced using pcDNA3-T7-JIP1 (pAdlox-JIP1 Thr103A) as a template. The sense primer was 5'-TAATGAATTCGCGGAGCGAGAGAGCGGCCTG-3', and the antisense primer was 5'-GCCAGTCGACCTACTCCAAGTAGATATCTTCG-3'. pGEX-4T-1-JIP1 (or JIP1-Thr103A) was made by inserting an EcoRI-Sall fragment from JIP1 (or JIP1 Thr103A) PCR product into EcoRI-Sall-cut pGEX-4T-1 (Amersham Biosciences). pGEX-4T-1-JIP1 (or JIP1-Thr103A) was transformed into JM109, and JIP1 (JIP1-Thr103A) expression was confirmed by anti-JIP1 (Cell Signaling). GST-JIP1 (or JIP1-Thr103A) was purified by using glutathione Sepharose 4B column (Amersham Biosciences). pcDNA3-Flag-JIP3 was provided by K. Yoshioka (Kanazawa University, Kanazawa, Japan). pAdlox-Flag-JIP3 having Flag tagged at their NH₂-terminal and restriction enzyme recognition sites at the flanking sides (5', SphI; 3', AccI) was produced by PCR using pcDNA3-Flag-JIP3 as a template. The sense primer was 5'-CTGCGCATGCTGATGGACTACAAAGACGATGACGACAAGCT-3', and the antisense primer was 5'-CATCTAGTCGACTACT-

CAGGGGTGTAGGACCTGCC-3'. pcDNA3.1-His C-SEK1 was made by inserting the BamHI fragment from pEBG-SEK1. Adlox-His-SEK1 was made by inserting the SpeI-XbaI fragment from pcDNA3.1-His C-SEK1 into SpeI-XbaI-cut pAdlox shuttle vector. pLNCX-HA p54 JNK2 α were provided by L.E. Heasley (University of Colorado Health Sciences Center, Denver, CO). pLNCX-HA p54 JNK2 α were digested with HindIII-Clal, and their fragments were subcloned into HindIII-AccI-digested pAdlox.

Adenoviral vector construction

All recombinant adenoviruses were constructed by using the Cre-lox recombination system (Hardy et al., 1997). The selective cell line CRE8 has a β -actin-based expression cassette driving a Cre recombinase gene with an NH₂-terminal nuclear localization signal stably integrated into 293 cells. Transfections were done by using Lipofectamine Reagent (Invitrogen). 5 \times 10⁵ cells were split into a 6-well plate 1 d before transfection. For the production of recombinant adenovirus, 2 μ g SfiI-digested Adlox/HA-Akt1, Adlox/Flag-JIP1, Adlox/Flag-JIP3, His-SEK1, HA-JNK2, and HA-JNK2 (Thr183A and Y185F) and 2 μ g of ψ 5 viral genomic DNA were cotransfected into CRE8 cells. The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and ψ 5 viral DNA. A new virus has an intact packaging site and carries a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of each component to adenovirus was confirmed by Western blot analysis after the infection of corresponding recombinant adenovirus into DU-145 cells.

Immune complex kinase assay

For the immune complex kinase assay, DU-145 cells were infected with Ad.HA-Akt1/Ad.Flag-JIP1 at a multiplicity of infection (MOI) of 10. After 48 h of infection, cells were lysed in a buffer solution containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution (Sigma-Aldrich). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with rat anti-HA antibody (3F10; Roche Diagnostics) or mouse anti-Flag antibody (Sigma-Aldrich) and protein G agarose (Santa Cruz Biotechnology, Inc.). The beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution, were washed once with the kinase buffer solution, and were subjected to kinase assays. To examine whether the JIP1-bound Akt1 has a catalytic activity, GST-tagged fusion Bad protein (Santa Cruz Biotechnology, Inc.) was used as a substrate of Akt1. 1 μ g Bad was incubated with immunoprecipitated HA-Akt1 or Flag-JIP1-HA-Akt1 complex in kinase buffer containing 20 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 1 mM sodium orthovanadate, 2 mM DTT, and 20 μ M ATP at 30°C for 1 h. Finally, the reaction was stopped by adding 2 \times Laemmli buffer. Phosphorylated proteins were resolved by SDS-PAGE and were analyzed by immunoblotting by using anti-phospho-Bad (Ser-136) antibody (Cell Signaling). For in vitro kinase assay of JIP1 phosphorylation by JNK2, DU-145 cells were infected with various types of Ad.HA-JNK2 (wild type, Thr-183A, and Y185F) at an MOI of 10. After 48 h of infection, cells were incubated in glucose-free medium for 1 h and were lysed in a buffer solution containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution (Sigma-Aldrich). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with rat anti-HA antibody and protein G agarose. The beads were washed three times with the same solution as the beads above, were washed once with the kinase buffer solution, and were subjected to kinase assays. 0.5 μ g GST-JIP1 or GST-JIP1 (Thr103A) was incubated with various immunoprecipitated types of HA-JNK2 in kinase buffer containing 20 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 1 mM sodium orthovanadate, 2 mM DTT, 20 μ M ATP, and 100 μ Ci/ml γ -[³²P]ATP at 30°C for 1 h. Finally, the reaction was stopped by adding 2 \times Laemmli buffer. Phosphorylated proteins were resolved by SDS-PAGE and were analyzed by autoradiography.

Immunoblot analysis

Cell lysates were subjected to electrophoresis on 10% polyacrylamide gels containing SDS under reducing conditions, and the proteins in the gels were transferred onto a polyvinylidene difluoride membrane. The membranes were incubated with 7% (wt/vol) skim milk in PBST (PBS containing 0.1% [vol/vol] Tween 20) and were reacted with primary antibodies. After washing three times with PBST, the membranes were incubated with HRP-conjugated anti-IgG. The proteins were then detected with the ECL reagent.

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