

Glutamine-dependent Antiapoptotic Interaction of Human Glutaminyl-tRNA Synthetase with Apoptosis Signal-regulating Kinase 1*

Received for publication, July 13, 2000, and in revised form, October 26, 2000
Published, JBC Papers in Press, November 28, 2000, DOI 10.1074/jbc.M006189200

Young-Gyu Ko‡, Eun-Kyoung Kim‡, Taeho Kim‡, Heonyong Park‡, Hee-Sae Park§, Eui-Ju Choi§, and Sunghoon Kim‡¶

From the ‡National Creative Research Initiatives Center for ARS Network, Sung Kyun Kwan University, Suwon, Kyunggi-do 440-746, Korea and the §National Creative Research Initiatives, Center for Cell Death, Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Glutamine has been known to be an apoptosis suppressor, since it blocks apoptosis induced by heat shock, irradiation, and c-Myc overexpression. Here, we demonstrated that HeLa cells were susceptible to Fas-mediated apoptosis under the condition of glutamine deprivation. Fas ligation activated apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase (SAPK)) in Gln-deprived cells but not in normal cells, suggesting that Gln might be involved in the activity control of ASK1 and JNK/SAPK. As one of the possible mechanisms for the suppressive effect of Gln on ASK1, we investigated the molecular interaction between human glutaminyl-tRNA synthetase (QRS) and ASK1 and found the Gln-dependent association of the two molecules. While their association was enhanced by the elevation of Gln concentration, they were dissociated by Fas ligation within 5 min. The association involved the catalytic domains of the two enzymes. The ASK1 activity was inhibited by the interaction with QRS as determined by *in vitro* kinase and transcription assays. Finally, we have shown that QRS inhibited the cell death induced by ASK1, and this antiapoptotic function of QRS was weakened by the deprivation of Gln. Thus, the antiapoptotic interaction of QRS with ASK1 is controlled positively by the cellular concentration of Gln and negatively by Fas ligation. The results of this work provide one possible explanation for the working mechanism of the antiapoptotic activity of Gln and suggest a novel function of mammalian ARSs.

Apoptosis is a genetically regulated process that is essential for correct morphogenesis during embryogenesis and the maintenance of tissue homeostasis (1, 2). Since dysregulation of apoptosis has been implicated in autoimmune disease and atherosclerosis as well as neurodegenerative disorders and cancer (3–5), tumor suppressor and proapoptotic genes should tightly regulate apoptosis (6). Apoptosis is induced by cytokines such

as Fas ligand and tumor necrosis factor (TNF),¹ growth factor withdrawal, ischemia, and amino acid deprivation (5, 7, 8).

Glutamine is a nonessential amino acid, but it is heavily utilized as a major metabolic fuel as well as a precursor for nucleotide synthesis in fibroblasts, lymphocytes, and macrophages (9). Since Gln deprivation induces apoptosis in intestinal epithelial cells, whereas methionine deprivation does not (8), Gln might play an important role in protecting cells from apoptosis induced by different stimuli. For example, Gln supplementation reduces apoptosis induced by heat shock, irradiation, and c-Myc overexpression (10–12). Furthermore, Gln stimulates intestinal cell proliferation and activates mitogen-activated protein kinases such as p42/p44 MAPK and JNK/SAPK (13, 14), suggesting that Gln could regulate signal transduction pathways for cellular proliferation and apoptosis. However, the molecular mechanism of Gln in suppressing apoptosis and stimulating cellular proliferation remains to be explained.

Aminoacyl-tRNA synthetases (ARSs) catalyze aminoacylation of their cognate tRNAs and thus play an essential role in protein synthesis. ARSs have been found in cytoskeleton- or endoplasmic reticulum-associated structures or in cytoplasm, but they are also found in the nucleus and even in the nucleolus (15–17), suggesting that ARSs have various noncanonical functions in addition to tRNA aminoacylation from eukaryotes. In particular, methionyl-tRNA synthetase is involved in rRNA biogenesis at its localization site in the nucleolus (17).

Upon cellular exposure to apoptosis condition, mammalian tyrosyl-tRNA synthetase is secreted and split into two fragments with distinct cytokine activities by leukocyte elastase and extracellular protease (18, 19). The split cytokines contain an endothelial monocyte-activating polypeptide II-like domain and an interleukin-8-like domain. Thus, tyrosyl-tRNA synthetase has a potent apoptosis-inducing activity after proteolytic cleavage, since their split polypeptides stimulate the production of TNF and tissue factor from target cells and have leukocyte chemotaxis activity to scavenge apoptotic corpses. The precursor of endothelial monocyte-activating polypeptide II is associated with the N-terminal noncatalytic extension of arginyl-tRNA synthetase and enhances aminoacylation activity (20). Like human tyrosyl-tRNA synthetase, the cytokine domain of this precursor is released upon apoptosis and exerts its proapoptotic function (21, 22).

* This work was supported by a grant from the National Creative Research Initiatives of the Ministry of Science and Technology of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence may be addressed: National Creative Research Initiatives Center for ARS Network, Sung Kyun Kwan University, 300 Chunchundong, Jangangu Suwon, Kyunggi-do 440-746, Korea. Tel.: 82-31-290-5680; Fax: 82-31-290-5682; E-mail: shkim@yurim.skku.ac.kr.

¹ The abbreviations used are: TNF, tumor necrosis factor; ARS, aminoacyl-tRNA synthetase; QRS, glutaminyl-tRNA synthetase; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPK, mitogen-activating protein kinase; HA, hemagglutinin; MBP, myelin basic protein.

Here, we studied a novel regulatory role of human glutamyl-tRNA synthetase (QRS) in inhibiting apoptosis signal-regulating kinase 1 (ASK1). We first searched for the effect of Gln in suppressing Fas-mediated apoptosis and reducing the activation of ASK1 as well as JNK/SAPK. We also observed that QRS inhibits ASK1 kinase activity and apoptosis by binding to ASK1. The molecular interaction of QRS and ASK1 was dependent on glutamine concentration, suggesting that glutamine could repress Fas-mediated apoptosis and ASK1 activation via QRS.

EXPERIMENTAL PROCEDURES

Materials—QRS (native N-terminal 236 amino acids) was overexpressed as a His-tagged protein using *Escherichia coli* and then purified using nickel affinity chromatography following the manufacturer's protocol (Invitrogen). Rabbit polyclonal antibody was then raised against these proteins as described previously (20). The IgG from antiserum was purified by protein A affinity chromatography according to the manufacturer's protocol (Bio-Rad). Anti-hemagglutinin (HA), -ASK1, and -Myc antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-JNK/SAPK, p38, phospho-p38, p42/p44 MAPK, and phospho-p42/p44 MAPK antibodies were from New England Biolabs. Anti-Fas antibody for apoptosis induction was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The caspase-3 colorimetric assay kit was obtained from Promega.

The cDNAs encoding the full-length, N-terminal 236 amino acids (QRS-N) and C-terminal 539 amino acids (QRS-C) of human QRS were generated using polymerase chain reaction from pM191 (Dr. Shiba, Cancer Institute, Tokyo, Japan) and ligated into pcDNA3 (Invitrogen) containing Myc and FLAG tags using the *EcoRI* and *NotI* sites. The cDNAs encoding the full-length, N-terminal 649 amino acids (ASK1-N) and C-terminal 725 amino acids (ASK1-C) of human ASK1 were cloned into pcDNA3 with an HA epitope tag at the 3'-end (kind gift of Dr. Hidenori Ichijo, Tokyo Medical and Dental University).

Cell Cultures, DNA Transfection, and Immunoprecipitation—Human embryonic kidney 293 and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 50 μ g/ml penicillin and streptomycin in a 5% CO₂ incubator. 100-mm dishes of 293 cells were transfected with pcDNA3-HA-ASK1 and pcDNA3-Myc-QRS using Geneporter (Gene Therapy Systems) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were lysed with 20 mM Tris-HCl (pH 7.5) buffer containing 12 mM β -glycerolphosphate, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 3 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 μ g/ml aprotinin. ASK1 in the cell lysate was reacted with anti-HA antibody (5 μ g) at 4 °C for 1 h. After the addition of 50 μ l of protein A-agarose, the mixture was incubated at 4 °C for an additional 4 h. The beads were washed four times with 20 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl, 5 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. The precipitated proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. The immunoprecipitates were analyzed by immunoblotting with anti-QRS and -HA antibodies.

ASK1 Kinase Assay and Reporter Gene Assay—The kinase activity of ASK1 was determined using the immunoprecipitated ASK1 prepared as described above. The precipitated ASK1 was washed three times with 20 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl, 5 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 and once with a reaction buffer of 20 mM Tris-HCl (pH 7.5) and 20 mM MgCl₂. The reaction was carried out in the reaction buffer in the presence of 0.5 μ Ci of [γ -³²P]ATP for 10 min at 30 °C using myelin basic protein (MBP) (40 μ g/ml) (Sigma) as an exogenous substrate (23). The samples were resolved by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography, and the phosphorylated MBP was quantified by a phosphor image analyzer (Fuji; FLA-3000).

For the serum response element-Luc reporter gene assay, 293 cells grown in 24-well plates were cotransfected with the indicated plasmids with 50 ng of serum response element-Luc reporter construct. After an incubation of 24 h, cells were lysed in 150 μ l of reporter lysis buffer (Promega), and 20 μ l of the lysate was assayed in a Luminometer (MicroLumatPlus; EG&G) with a luciferase assay system (Promega).

DNA Fragmentation Assay—To determine the degradation of chromosomal DNA into nucleosome-sized fragments, a 500- μ l aliquot of the lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to the cell pellet (2 $\times$ 10⁵

cells) and incubated at 37 °C overnight. DNA was obtained by ethanol precipitation, separated in a 1.8% agarose gel, and visualized under UV light.

Measurement of Caspase-3 Activity—Caspase-3 activity was measured according to the manufacturer's protocol. HeLa cells in 100-mm dishes were lysed with 300 μ l of chilled cell lysis buffer. After microcentrifugation (14,000 $\times$ g, 20 min, 4 °C), 60 μ g of total protein from the clear supernatant was mixed with 32 μ l of caspase assay buffer, 2 μ l of Me₂SO, 10 μ l of 100 mM dithiothreitol, and 2 μ l of 10 mM Asp-Glu-Val-Asp-p-nitroanilide. After incubation at 37 °C for 4 h, samples were read at 405 nm.

Apoptosis Assay—293 cells were transiently transfected with 0.5 μ g of pcDNA3-HA-ASK1-C and 1 μ g each of pcDNA3-Myc-QRS-F, -N, and -C along with 0.5 μ g of pcDNA3-EGFP. Total amounts of the transfected DNA were adjusted to be the same with pcDNA3. The cells were washed with PBS twice 20 h after transfection, and then normal or Gln-free medium was added. The transfected cells were fixed with 3% paraformaldehyde with 4,6-diamidino-2-phenylindole 48 h after transfection, and then the cell death was determined by counting the apoptotic nuclei using fluorescence microscopy.

RESULTS

Glutamine Deprivation Sensitizes HeLa Cells to Fas-mediated Apoptosis—Since c-Myc-dependent apoptosis is reduced by Gln supplementation (12) and is dependent on Fas signaling (24, 25), it is tempting to conjecture that Gln itself suppresses Fas-mediated apoptosis. To address this issue, we incubated HeLa cells in Gln-free medium overnight and administered anti-Fas antibody for inducing apoptosis after supplementing Gln (0 and 4 mM, respectively). Following the treatment with anti-Fas for 8 h, cell morphology was observed under an inverted microscope. Blebbing morphology appeared in cells in the absence of Gln but not in the presence of Gln (Fig. 1A). Since the blebbing morphology was not observed in cells untreated with anti-Fas antibody, Gln deprivation makes cells susceptible to apoptosis by Fas ligation. The same results were obtained with respect to intranucleosomal DNA laddering. Following treatment with anti-Fas antibody for 24 h, DNA fragmentation appeared in cells incubated in Gln-free medium (Fig. 1B). However, there was very little DNA fragmentation in cells untreated with anti-Fas or supplemented with Gln.

To make these data more convincing, the extent of apoptosis activation after anti-Fas treatment was determined by monitoring caspase-3. Cells incubated in Gln-free medium showed higher caspase-3 activity than those supplemented with Gln, and only the cells in Gln-free medium were sensitive to Fas-induced apoptosis (Fig. 1C). Apoptosis of HeLa cells in Gln-free medium was increased by the anti-Fas antibody treatment in a dose-dependent manner, whereas the cells in the normal medium were not sensitive to Fas ligation (Fig. 1D). Based on these data, we conclude that Gln suppresses apoptosis induced by Fas ligation.

Glutamine Deprivation Sensitizes HeLa Cells to SAPK and ASK1 Activation by Fas Ligation—To explore the molecular mechanism for suppressing Fas-mediated apoptosis by Gln, we investigated the activation/phosphorylation of p42/p44 MAPK, JNK/SAPK, p38 MAPK, and ASK1 after Fas ligation in cells incubated with or without Gln. To measure the activation status of endogenous JNK/SAPK and ASK1, we performed an *in vitro* kinase assay using polyclonal antibodies that specifically recognize the phosphorylated active forms of these enzymes. The phosphorylation of p42/p44 MAPK was shown in 10 min after anti-Fas antibody treatment both in cells grown with and without Gln (Fig. 2, top row). However, p38 MAPK was not phosphorylated after Fas ligation in either treatment of the cells (data not shown). Next, we analyzed JNK/SAPK activation by an immunocomplex kinase assay. The results showed that stimulation of the Gln-starved HeLa cells with anti-Fas antibody increased the JNK/SAPK activity starting 10 min after stimulation of Fas up to 60 min (Fig. 2, middle row). In

FIG. 1. Gln deprivation sensitizes HeLa cells to Fas-mediated apoptosis. *A*, HeLa cells were incubated in Gln-free medium for 12 h, supplemented with 0 or 4 mM Gln 1 h before the treatment with anti-Fas antibody (100 ng/ml). Eight hours after Fas ligation, cellular morphology was observed under an inverted microscope. *B*, HeLa cells were incubated in Gln-free medium for 12 h and supplemented with 0, 0.1, 0.2, 0.5, 1, and 2 mM Gln 1 h before the treatment with anti-Fas antibody (100 ng/ml). DNA ladder was determined according to "Experimental Procedures" 24 h after anti-Fas antibody treatment (100 ng/ml). *C*, HeLa cells were incubated in Gln-free medium for 12 h and supplemented with 0 or 4 mM Gln. Caspase activity was measured 12 h after anti-Fas treatment according to "Experimental Procedures." *D*, HeLa cells in the normal or Gln-free medium were treated with the indicated amounts of anti-Fas antibody, and the apoptotic cells were counted.

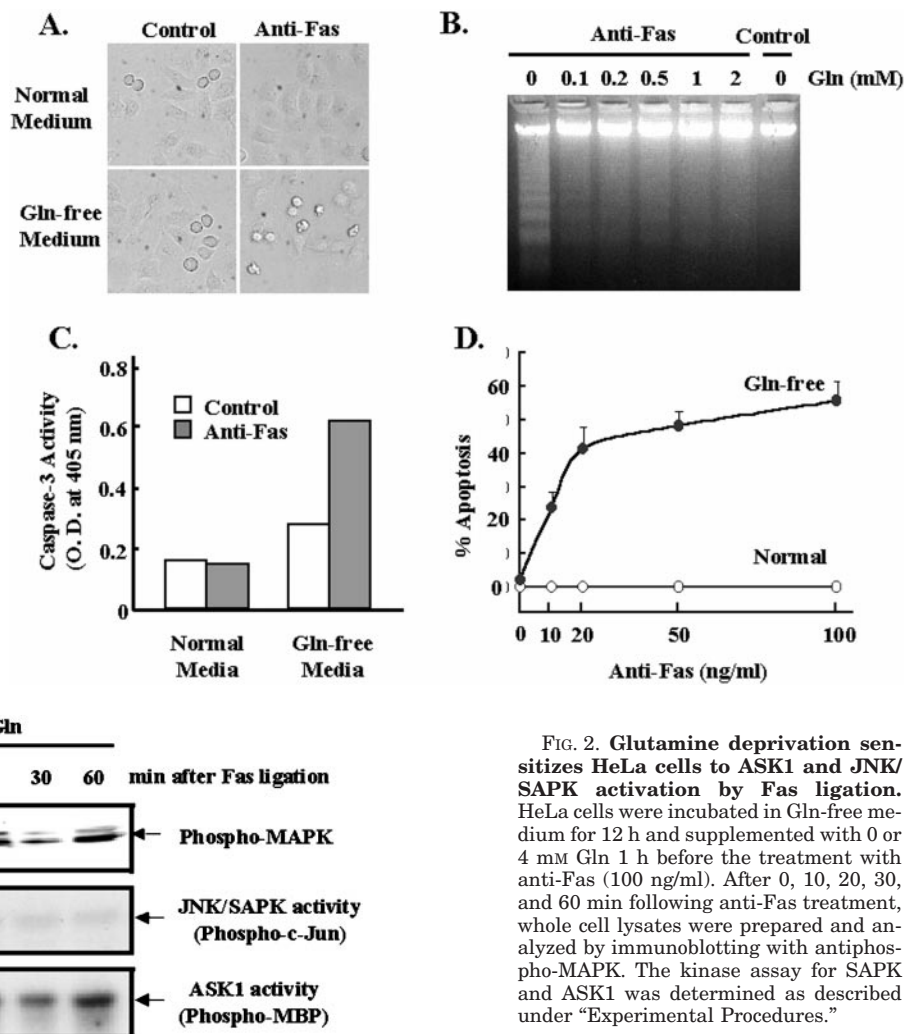


FIG. 2. Glutamine deprivation sensitizes HeLa cells to ASK1 and JNK/SAPK activation by Fas ligation. HeLa cells were incubated in Gln-free medium for 12 h and supplemented with 0 or 4 mM Gln 1 h before the treatment with anti-Fas (100 ng/ml). After 0, 10, 20, 30, and 60 min following anti-Fas treatment, whole cell lysates were prepared and analyzed by immunoblotting with antiphospho-MAPK. The kinase assay for SAPK and ASK1 was determined as described under "Experimental Procedures."

contrast, there was no JNK/SAPK activation by Fas ligation in cells incubated in Gln-containing medium.

Since JNK/SAPK is activated by ASK1 after Fas ligation, we next investigated the activation of ASK1 after Fas ligation. ASK1 kinase assay was determined by immunoprecipitation with rabbit polyclonal anti-ASK1 antibody and reaction with MBP and [γ - 32 P]ATP. As shown in Fig. 2, ASK1 was significantly activated 10 min after Fas ligation in cells incubated in Gln-free medium. However, there was, if any, little activation of ASK1 in cells incubated in 4 mM Gln-containing medium. Taken together, we can conclude that Gln suppresses ASK1 and JNK/SAPK activation by Fas ligation.

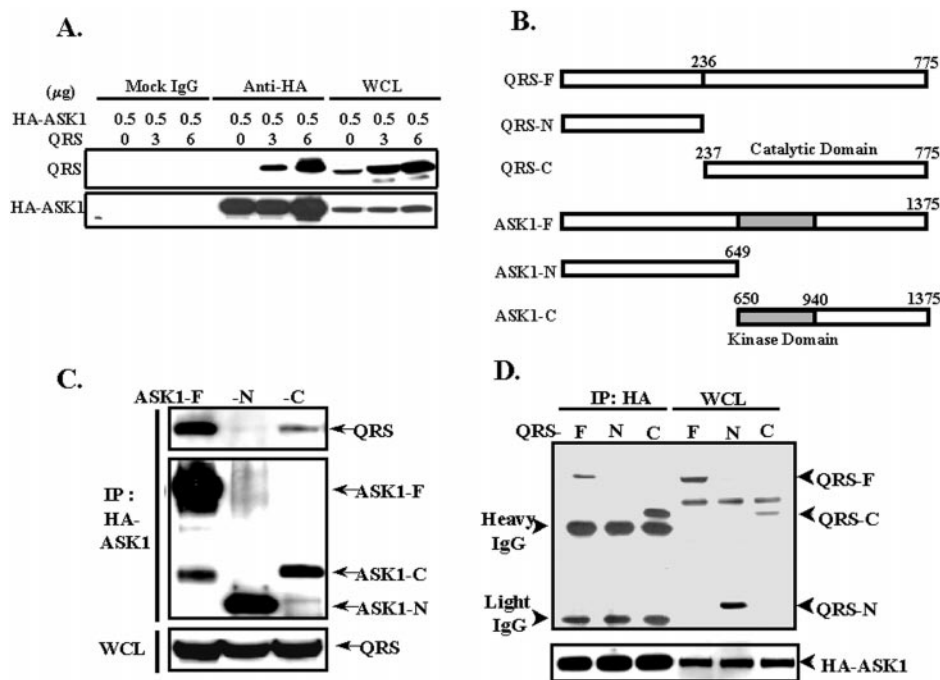
QRS Is Associated with ASK1—Gln might suppress Fas-mediated apoptosis by inhibiting ASK1 or JNK/SAPK that is involved in the apoptosis pathway. Although various metabolic enzymes could control the steady state level of cellular free Gln, we were interested in QRS that ligates Gln to its cognate tRNA. Since human QRS is one of the enzymes utilizing free Gln, we thought that it could be a good candidate to explain Gln effect on ASK1 and JNK/SAPK inhibition. To address the issue, we determined the molecular interaction of QRS with ASK1 in 293 cells. HA epitope-tagged ASK1 was coexpressed with Myc-QRS in 293 cells and immunoprecipitated with anti-HA antibody. The immune complexes were subjected to immunoblotting with anti-QRS antibody. As shown in Fig. 3A, QRS was found to associated with ASK1 in a dose-dependent manner. Interaction of QRS with ASK1 was not detected by immunoprecipitation with mock IgG control antibody. However, we failed to detect

QRS coprecipitated with ASK1 at its endogenous level. Normally, most of QRS is associated with other ARSs to form a macromolecular protein complex (26), and thus only a small portion of QRS would be available for the interaction with ASK1. Also, the steady state level of ASK1 is low and associated with various cellular signaling molecules (23, 27–32). We suspect that these may be the reason for the difficulty to detect the association of endogenous QRS and ASK1 by coimmunoprecipitation.

To map the interaction regions of QRS and ASK1, various deletion mutants were generated (Fig. 3B). ASK-F, -N, or -C was precipitated with anti-HA antibody, and coimmunoprecipitated QRS was detected by immunoblotting with anti-QRS antibody. QRS was precipitated with ASK1-F or -C but not with ASK1-N, indicating that ASK1-C is responsible for the interaction (Fig. 3C). Then Myc-tagged QRS derivatives (Fig. 3B) were transiently expressed with HA-ASK1 and immunoprecipitated with anti-HA antibody. The precipitated complex was immunoblotted with anti-Myc antibody. QRS-F and QRS-C were precipitated with ASK1, whereas QRS-N was not (Fig. 3D). Thus, the catalytic domains of QRS and ASK1 are involved in their association.

QRS Represses ASK1 Activity—Since QRS interacted with ASK1 as shown above, we then tested the effect of QRS on ASK1 activity. HA-ASK1 was co-overexpressed with different Myc-QRS derivatives and immunoprecipitated with anti-HA antibody. The precipitated complexes were subjected to a kinase assay using myelin basic protein as a substrate of ASK1. ASK1 was strongly repressed by QRS-F and QRS-C but not by

FIG. 3. QRS interacts with ASK1. *A*, plasmids pcDNA-Myc-QRS (0, 3, or 6 μ g) and pcDNA-HA-ASK1 (0.5 μ g) were co-transfected into 293 cells grown in 100-mm dishes. Twenty-four hours after transfection, the samples were immunoprecipitated with anti-HA antibody and immunoblotted with anti-QRS. *B*, schematic presentation of Myc-QRS and HA-ASK1 deletion mutants used for coimmunoprecipitation assays. *C*, interaction of HA-ASK1-F, -N, and -C with Myc-QRS-F. Each of the ASK1 deletion mutants was immunoprecipitated (IP) with anti-HA antibody, and the coprecipitation of QRS was determined with anti-Myc antibody. Whole cell lysate (WCL) of each transfectant contains a similar amount of QRS. *D*, interaction of Myc-QRS-F, -N, and -C with HA-ASK1-F. HA-ASK1 was immunoprecipitated with anti-HA antibody, and coprecipitation of QRS mutants was determined with anti-Myc antibody. Each of the cells contains a similar amount of HA-ASK1.



QRS-N (Fig. 4A), consistent with the interaction result in Fig. 3D.

We confirmed the QRS-mediated repression of ASK1 by determining the ability of ASK1 to stimulate the serum response factor (SRF). JNK/SAPK phosphorylated by ASK1 activates the SRF, which then induces transcription from the promoter containing the serum response element (33). Using this principle, the effect of QRS binding to ASK1 was tested by the induction of luciferase, the expression of which is under the control of SRF (34). Expression of ASK1 alone in 293 cells increased the expression of luciferase about 5-fold. However, coexpression of ASK1 with QRS-F or QRS-C abolished the induction of SRF-luciferase (Fig. 4B).

Molecular Interaction between QRS and ASK1 Is Affected by Fas Ligation and Glutamine—To address the physiological significance and control of the interaction between QRS and ASK1, we investigated whether the interaction of QRS and ASK1 is affected by an apoptotic signal. Since ASK1 is activated after cellular exposure to H_2O_2 , TNF, or agonistic anti-Fas antibody, we added these reagents to 293 cells, and the interaction between QRS and ASK1 was tested by coimmunoprecipitation. The two molecules were dissociated as early as 5 min after the treatment with anti-Fas antibody but were reassociated in 30 min (Fig. 5A). However, the interaction was not affected by H_2O_2 or TNF in this experimental condition (Fig. 5B).

Since Gln suppresses ASK1 and JNK/SAPK activation by Fas ligation, Gln itself might affect the molecular interaction of QRS to ASK1. To explore the possibility, 293 cells were coexpressed with Myc-QRS and HA-ASK1 in the presence of 0, 2, 4, or 20 mM Gln. Twenty-four hours after transfection, the molecular association of QRS to ASK1 was determined by immunoprecipitation. To further confirm the effect of Gln on the interaction of the two proteins, we also checked whether the addition of Gln to the immunoprecipitation mixture affects the association of the two molecules. The expression level of QRS and ASK1 was not affected by Gln concentration in medium (Fig. 6A, lower panel), whereas the molecular interaction between QRS and ASK1 was significantly increased when the cells were cultivated in the presence of Gln (Fig. 6A, upper panel). Moreover, the addition of 20 mM Gln to the immuno-

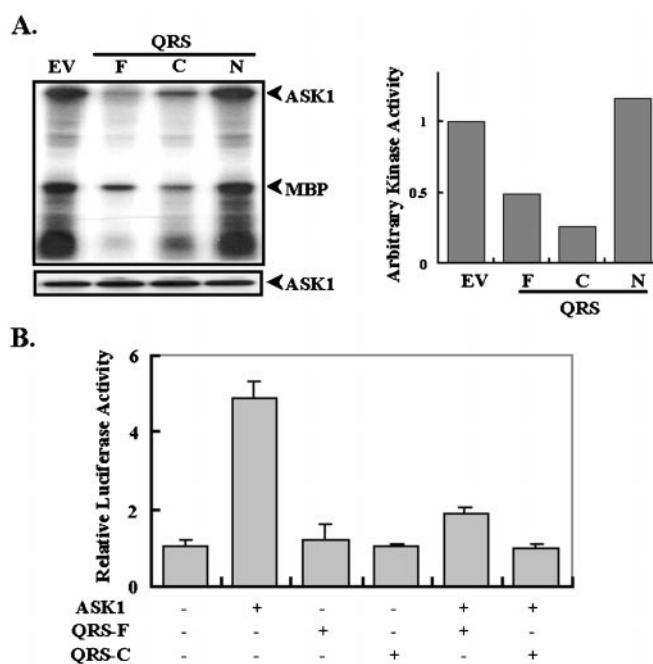


FIG. 4. QRS represses ASK1 activity. *A* (left), HA-ASK1 was coexpressed in 293 cells with Myc-QRS-F, -N, or -C. HA-ASK1 was precipitated with anti-HA antibody, and the kinase activity of the immunoprecipitated ASK1 was determined using MBP as an exogenous substrate in the presence of [γ - 32 P]ATP. The phosphorylated MBP and autophosphorylated ASK1 were visualized by autoradiography. *EV*, empty vector. *Right*, the amount of phosphorylation was quantified using a phosphor image analyzer. *B*, the ASK1 activity was determined by JNK/SAPK-dependent activation of the reporter gene, luciferase. The reporter plasmid in which the expression of luciferase is under the control of SRF was transfected into 293 cells with the combination of HA-ASK1 and Myc-QRS. The expression of luciferase was monitored using luminometer, and three independent experiments were carried out.

precipitation buffer fortified the interaction of QRS and ASK1 even when the cells were cultivated without Gln. Moreover, the ASK1 activity was greatly reduced in the presence of 20 mM Gln (Fig. 6B), consistent with the effect of Gln on the interaction of QRS and ASK1. Thus, it is clear that Gln positively

FIG. 5. QRS is dissociated from ASK1 by Fas ligation. 293 cells transfected with pcDNA3-Myc-QRS (1 μ g) and pcDNA3-HA-ASK1 (0.3 μ g) were treated with agonistic anti-Fas antibody (2 μ g/ml) (A), TNF (200 ng/ml) (B, left), and H_2O_2 (1 mM) (B, right). The cells were harvested at the indicated times. The proteins were extracted from the harvested cells, and HA-ASK1 was immunoprecipitated with anti-HA antibody. The amount of coimmunoprecipitated QRS was determined by anti-QRS antibody.

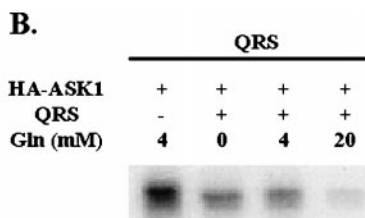
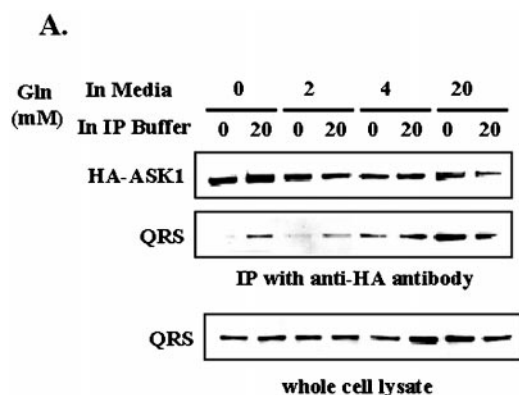
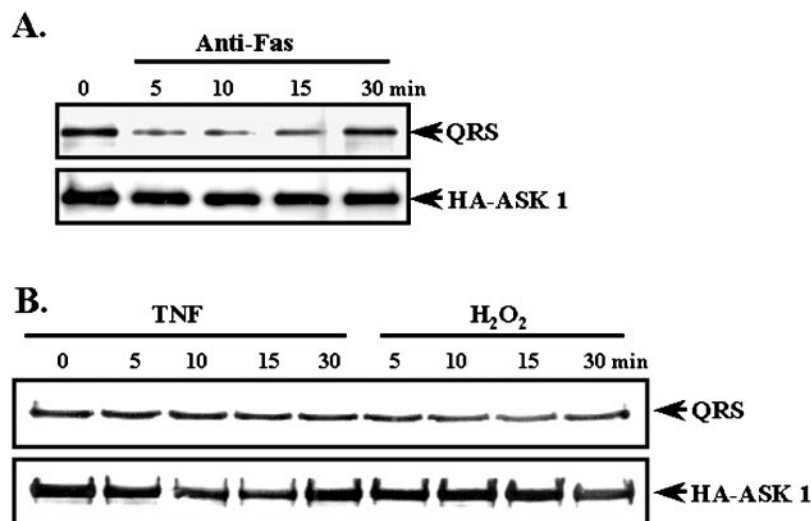


FIG. 6. Gln fortifies the molecular interaction of QRS to ASK1. A, 293 cells transfected with Myc-QRS (2 μ g) and HA-ASK1 (0.5 μ g) in the presence of 0, 2, 4, and 20 mM Gln were subjected to immunoprecipitation with anti-HA antibody. The precipitated complexes were analyzed by immunoblotting with anti-Myc antibody. Gln (0 or 20 mM) was added to the immunoprecipitation (IP) buffer in order to observe the effect of Gln on the interaction between QRS and ASK1 more clearly. B, HA-ASK1 was cotransfected with Myc-QRS to 293 cells in the presence of 0, 2, 4, and 20 mM Gln. Immunoprecipitates by anti-HA antibody were subjected to *in vitro* kinase assay for ASK1 activity according to "Experimental Procedures."

controls the suppressive association of QRS with ASK1.

QRS Blocks ASK1-induced Apoptosis—We finally investigated whether QRS can inhibit the cell death mediated by ASK1. Since the N-terminal domain of ASK1 is inhibitory to its own proapoptotic kinase activity (35), the truncation of this domain generates the constitutively active mutant, ASK1-C (also called ASK1 Δ N). Apoptosis of 293 cells was induced by the transient transfection of ASK1-C to about 25% in normal medium, and it was further enhanced to 35% when the cells were cultivated in Gln-free medium (Fig. 7). The ASK1-C-induced cell death was decreased by the coexpression of QRS-F or -C but not with QRS-N in the normal medium (Fig. 7, left). However, the antiapoptotic activity of QRS-F or -C was less apparent in Gln-free medium (Fig. 7, right). This result is consistent with the molecular association of QRS with ASK and its *in vitro* effect on the activity of ASK1 shown above.

DISCUSSION

Aminoacyl-tRNA synthetases are a family of enzymes essential for protein synthesis. However, it has been discovered that these enzymes are actively involved in a broad repertoire of other critical cellular activities as well as protein synthesis (36). It has been previously demonstrated that ARSs have idiosyncratic distribution in cytoplasm, nucleus, and nucleolus (17, 37). For instance, methionyl-tRNA synthetase is localized in the nucleolus of rapidly proliferating mammalian cells and is responsible for ribosomal RNA biosynthesis (17). Seshiah and Andrew (38) also showed that each ARS is uniquely expressed in different tissues and developmental stages of *Drosophila*. Thus, the differential expression and cellular localization of each ARS imply that each ARS has its noncanonical function in addition to the catalytic activity for tRNA aminoacylation. In-

deed, different ARSs play roles in tRNA maturation (proofreading and nuclear export), cytokine-like activity, mitochondrial RNA splicing, and transcriptional and translational regulation (36).

Here, we found that human QRS is not only the enzyme for cell proliferation but also the protein that plays a regulatory role in cell death through an antagonistic interaction with ASK1, a protein kinase that plays a critical role in apoptosis. QRS has been classified as a component of the multi-tRNA synthetase complex (39, 40). However, QRS not associated with the multi-tRNA synthetase complex has been found (41) and shown to be catalytically active (26). Thus, QRS bound to ASK1 may be in dynamic equilibrium with that in the multi-tRNA synthetase complex.

ASK1 is a MAPK kinase kinase that activates SEK1/MKK4 and MKK3/MKK6, which in turn activate JNK/SAPK and p38, respectively (42). The kinase activity of ASK1 is stimulated by a variety of apoptosis stimuli such as Fas ligation, TNF, reactive oxygen species, and anti-cancer drugs cisplatin and paclitaxel (27, 30, 35, 42, 43). Since ASK1 overexpression induces apoptosis, and a kinase-inactive mutant of ASK1 reduces TNF, Fas and Daxx-induced apoptosis and JNK activation (35, 42), ASK1 is thought to be a pivotal kinase in the TNF and Fas signaling pathway leading to apoptosis. Indeed, ASK1 directly interacts with TRAFs and Daxx in the TNF- and Fas-mediated signal pathways (28, 35). In addition, it interacts with various negative and positive modulators such as thioredoxin, p21Cip1/WAF1, and 14-3-3 (31, 32). Binding of thioredoxin to ASK1 is controlled by its oxidation and reduction, thereby linking the cellular redox potential with the apoptotic process (31). We now add QRS as a novel negative modulator of ASK1 that mediates

FIG. 7. QRS blocks ASK1-induced apoptosis in Gln-dependent manner. 293 cells were cultivated in the normal or Gln-free medium. Apoptosis of 293 cells was induced by transient transfection of ASK1-C, which is a constitutive active mutant. The effect of QRS-F, -N, and -C on ASK1-C-induced apoptosis was monitored by counting apoptotic cells. The values are the average of two independent experiments.

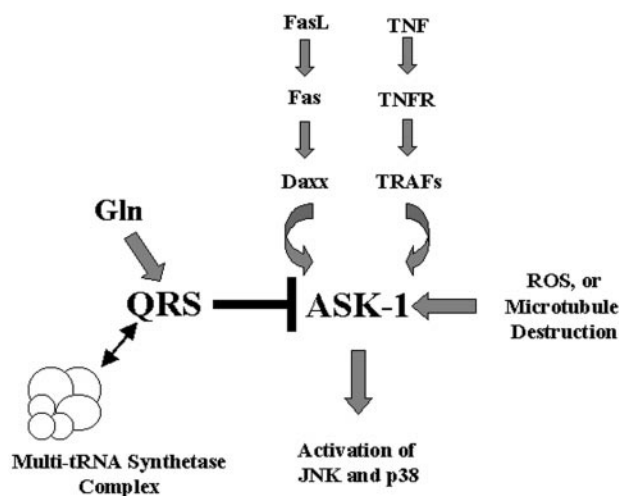
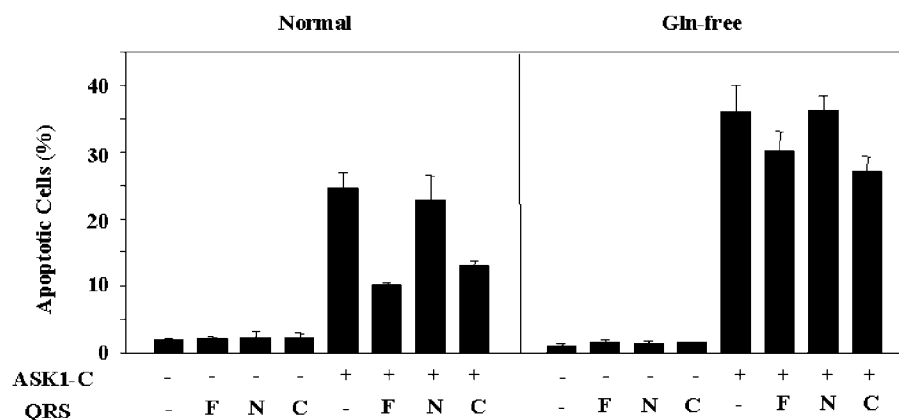


FIG. 8. Simplified working model explaining the role of Gln and QRS in ASK1 activity regulation. The ASK1 activity is repressed by the interactions with thioredoxin or QRS. (For simplicity, other ASK1 modulators and signaling pathways are not shown.) When thioredoxin is oxidized by reactive oxygen species (ROS) induced by TNF, it is dissociated from ASK1. The antagonistic interaction of QRS with ASK1 is broken by the competition with Daxx that is activated by Fas ligation. QRS associated with ASK1 may be in dynamic equilibrium with that associated with the multi-ARS complex.

the Fas-induced apoptosis in a manner controlled by Gln (Figs. 5–7).

Extensive investigations have revealed important effects of exogenous Gln in nutritional rehabilitation, stimulating intestinal villus regrowth after damage from chemotherapy and improving intestinal barrier function. In addition, Gln blocks apoptosis induced by heat shock, irradiation, and c-Myc-overexpression. In Figs. 1 and 2, we show that Gln prevents the activation of stress kinases such as ASK1 and JNK/ASK1 and then apoptosis initiated by Fas ligation, suggesting that Gln is an apoptosis suppressor. The cellular susceptibility to Fas-mediated signaling by Gln deprivation could not be explained by the protein synthesis inhibition, because ASK1 and JNK/SAPK were activated within 10 min after Fas ligation in Gln-free cells but not in normal cells. Therefore, Gln could suppress apoptosis by inhibiting ASK1 and then JNK/SAPK.

Although the similar amount of exogenous QRS was expressed in cells grown in complete and Gln-free medium, the molecular interaction between QRS and ASK1 was fortified and then the ASK1 activity was strongly inhibited in the presence of Gln (Fig. 6), indicating the positive effect of Gln on the interaction of the two molecules. It has been already demonstrated that amino acids themselves can be signaling molecules. For instance, glutamate acts as a messenger in glucose-induced insulin exocytosis because exogenous glutamate

directly stimulates insulin exocytosis, independently of mitochondrial function in permeabilized cells (44). Here, we suggest that Gln could be a signaling messenger for blocking ASK1 activation and suppressing apoptosis via binding to QRS.

Some types of apoptosis are suppressed by the activation of AKT/protein kinase B and MAPK. Activation of AKT/protein kinase B by extracellular survival factors prevents apoptosis by inactivating caspase-9, BAD, Forkhead, and IKK α that are involved in the apoptosis pathway (45–51). Since Fas-mediated apoptosis is prevented by activating MAPK through overexpression of K-Ras and basic fibroblast growth factor (52) and is accelerated by inhibiting MAPK with PD 98059 (53), MAPK could be another survival signal inhibiting apoptosis. In addition to AKT/protein kinase B and MAPK, we propose that Gln and QRS are intracellular survival factors for regulating apoptosis. Although further investigations are necessary to better understand the mechanism and control for the role of QRS in apoptosis, the results of this work provide the first evidence that one of the mammalian ARSs has a direct antagonistic interaction with a kinase that plays a key role in apoptosis.

Acknowledgments—We thank Drs. Guy A. Thompson, Jr. and Wongi Seol for critical discussions.

REFERENCES

- Vaux, D. L., and Korsmeyer, S. J. (1999) *Cell* **96**, 245–254
- Rich, T., Watson, C. J., and Wyllie, A. (1999) *Nat. Cell Biol.* **1**, E69–E71
- Chervonsky, A. V. (1999) *Curr. Opin. Immunol.* **11**, 684–688
- Bennett, M. R., and Boyle, J. J. (1998) *Atherosclerosis* **138**, 3–9
- Lee, J. M., Zipfel, G. J., and Choi, D. W. (1999) *Nature* **399**, A7–A14
- King, K. L., and Cidlowski, J. A. (1998) *Annu. Rev. Physiol.* **60**, 601–617
- Ashkenazi, A., and Dixit, V. M. (1998) *Science* **281**, 1305–1308
- Papacostas, H. T., Hwang, K. O., Rajaraman, S., Hellmich, M. R., Townsend, C. M., Jr., and Ko, T. C. (1998) *Surgery* **124**, 152–160
- Neu, J., Shenoy, V., and Chakrabarti, R. (1996) *FASEB. J.* **10**, 829–837
- Chow, A., and Zhang, R. (1998) *J. Nutr.* **128**, 1296–1301
- Santoso, J. T., Lucci, J. A., III, Coleman, R. L., Hatch, S., Wong, P., Miller, D., and Mathis, J. M. (1998) *Gynecol. Oncol.* **71**, 359–363
- Xu, Y., Nguyen, Q., Lo, D. C., and Czaja, M. J. (1997) *J. Cell. Physiol.* **170**, 192–199
- Rhoads, J. M., Argenzio, R. A., Chen, W., Rippe, R. A., Westwick, J. K., Cox, A. D., Berschneider, H. M., and Brenner, D. A. (1997) *Am. J. Physiol.* **272**, 943–953
- Rhoads, J. M., Argenzio, R. A., Chen, W., Graves, L. M., Licato, L. L., Blikslager, A. T., Smith, J., Gatzky, J., and Brenner, D. A. (2000) *Gastroenterology* **118**, 90–100
- Dang, C. V., Yang, D. C., and Pollard, T. D. (1983) *J. Cell Biol.* **96**, 1138–1147
- Schimmel, P., and Wang, C. C. (1999) *Trends. Biochem. Sci.* **24**, 127–128
- Ko, Y.-G., Kang, Y.-S., Kim, E.-K., Park, S. G., and Kim, S. (2000) *J. Cell Biol.* **149**, 567–574
- Wakasugi, K., and Schimmel, P. (1999) *Science* **284**, 147–151
- Wakasugi, K., and Schimmel, P. (1999) *J. Biol. Chem.* **274**, 23155–23159
- Park, S. G., Jung, K. H., Lee, J. S., Jo, Y. J., Motegi, H., Kim, S., and Shiba, K. (1999) *J. Biol. Chem.* **274**, 16673–16676
- Knies, U. E., Behrendorf, H. A., Mitchell, C. A., Deutsch, U., Risau, W., Drexler, H. C. A., and Cluass, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12322–12327
- Schwarz, M. A., Kandel, J., Brett, J., Li, J., Hayward, J., Schwarz, R. E., Chappey, O., Wautier, J.-L., Chabot, J., Gerfo, P. L., and Stern, D. (1999) *J. Exp. Med.* **190**, 341–353
- Saitoh, M., Nishitoh, H., Makino, F., Tekeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *EMBO J.* **17**, 2596–2606

24. Hueber, A.-O., Zornig, M., Lyon, D., Suda, T., Nagata, S., and Evan, G. I. (1997) *Science* **278**, 1305–1309
25. Brunner, T., Kasibhatla, S., Pinkoski, M. J., Fruttschi, C., Yoo, N. J., Echeverri, F., Mahboubi, A., and Green, D. R. (2000) *J. Biol. Chem.* **275**, 9767–9772
26. Kim, T., Park, S. G., Kim, J. E., Seol, W., Ko, Y.-G., and Kim, S. (2000) *J. Biol. Chem.* **275**, 21768–21772
27. Wang, T. H., Wang, H. S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T., and Wimalasena, J. (1998) *J. Biol. Chem.* **273**, 4928–4936
28. Nishitoh, H., Saitoh, M., Mochida, Y., Tateda, D., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) *Mol. Cell* **2**, 385–395
29. Berestetskaya, Y. V., Faure, M. P., Ichijo, H., and Voyno-Yasenetskaya, T. A. (1998) *J. Biol. Chem.* **273**, 27816–27823
30. Gotoh, Y., and Cooper, J. A. (1998) *J. Biol. Chem.* **273**, 17477–17482
31. Asada, M., Yamada, T., Ichijo, H., Delia, D., Miyazono, K., Fukumuro, K., and Mizutani, S. (1999) *EMBO J.* **18**, 1223–1234
32. Zhang, L., Chen, J., and Haian, F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8511–8515
33. Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell* **81**, 1159–1170
34. Yang, X., Khosravi-Far, R., Chang, H. Y., and Baltimore, D. (1997) *Cell* **89**, 1067–1076
35. Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998) *Science* **281**, 1860–1863
36. Martinis, S. A., Plateau, P., Cavarelli, J., and Florentz, C. (1999) *EMBO J.* **18**, 4591–4596
37. Popenko, V. I., Ivanova, J. L., Cherny, N. E., Filonenko, V. V., Beresten, S. F., Wolfson, A. D., and Kisselev, L. L. (1994) *Eur. J. Cell Biol.* **65**, 60–69
38. Seshiah, P., and Andrew, D. J. (1999) *Mol. Biol. Cell* **10**, 1595–1608
39. Kisselev, L. L., and Wolfson, A. D. (1994) *Prog. Nucleic Acids Res. Mol. Biol.* **48**, 83–142
40. Yang, D. C. H. (1996) *Curr. Top. Cell. Regul.* **34**, 101–136
41. Pahuski, E., Klekamp, M., Condon, T., and Hampel, A. E. (1983) *J. Cell. Physiol.* **114**, 82–87
42. Ichijo, H., Nishida, E., Irie, K., Dijde, P. T., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) *Science* **275**, 90–94
43. Wang, T. H., Popp, D. M., Wang, H. S., Sitoh, M., Mural, J. G., Henley, D. C., Ichijo, H., and Wimalasena, J. (1999) *J. Biol. Chem.* **274**, 8208–8216
44. Maechler, P., and Wollheim, C. B. (1999) *Nature* **402**, 685–689
45. Khwaja, A. (1999) *Nature* **401**, 33–34
46. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282**, 1318–1321
47. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91**, 231–241
48. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96**, 857–868
49. Kops, G. J., de Ruiter, N. D., De Vries-Smits, A. M., Powell, D. R., Bos, J. L., and Burgering, B. M. (1999) *Nature* **398**, 630–634
50. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* **401**, 82–85
51. Romashkova, J. A., and Makarov, S. S. (1999) *Nature* **401**, 86–90
52. Kazama, H., and Yonehara, S. (2000) *J. Cell Biol.* **148**, 557–566
53. Holmstrom, T. H., Tran, S. E., Johnson, V. L., Ahn, N. G., Chow, S. C., and Eriksson, J. E. (1999) *Mol. Cell Biol.* **19**, 5991–6002