Activation of Caspase-12, an Endoplasmic Reticulum (ER) Resident Caspase, through Tumor Necrosis Factor Receptor-associated Factor 2-dependent Mechanism in Response to the ER Stress*

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Takunari Yoneda‡¶, Kazunori Imaizumi§, Kayoko Oono‡¶, Daishi Yui‡¶, Fumi Gomi‡¶, Taiichi Katayama‡¶, and Masaya Tohyama‡¶

From the ‡Department of Anatomy and Neuroscience, Osaka University Medical School, 2–2 Yamadaoka, Suita, Osaka 565-0871, Japan, §Department of Cellular and Structural Biology, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama Iloga, Nara 630-0101, Japan, and the ¶Core Research for Evolutional Science and Technology, Japan Science and Technology, Kawaguchi, Saitama, 332-0012, Japan

When accumulation of a malformed protein in the endoplasmic reticulum (ER) is induced by various adverse conditions, such as hypoxia, glucose starvation, and perturbation of calcium homeostasis, cells respond to the stress by increasing transcription of genes encoding ER molecular chaperones, a process known as unfolded protein response. The signaling is initiated by IRE1s, ER stress sensors. Alternatively, excessive stress to the ER results in apoptosis. Caspase-12 is known to be essential for this ER stress-induced apoptosis. In this study, we analyzed the detailed regulatory mechanisms of IRE1s during ER stress. We identified c-Jun N-terminal inhibitory kinase (JIK) as a binding partner of IRE1α, and JIK was seen to modulate IRE1α-TRAF2 (tumor necrosis factor receptor-associated factor 2) complex formation and the resultant alteration to c-Jun N-terminal kinase signaling from IRE1s in response to ER stress. We also demonstrated that TRAF2 interacts with procaspase-12 and promotes the clustering of procaspase-12 and its activation by cleavage in response to ER stress. These results indicate that TRAF2 plays crucial roles not only in the signaling of the c-Jun N-terminal kinase pathway but also in activation of caspase-12 to transduce signals from IRE1s. Thus, we provide a missing link in the ER stress-induced apoptosis-signaling pathway, one which connects the stress sensor molecule IRE1 and the activation of caspase-12.

The endoplasmic reticulum (ER) is sensitive to alterations in homeostasis from a variety of different stimuli, such as glucose deprivation, perturbation of calcium homeostasis, and exposure to free radicals. Under such conditions, perturbation of protein folding and the accumulation of malformed proteins in the ER induce ER stress (1).

ER stress elicits two major cellular-protecting responses. One is the attenuation of protein synthesis, and the other is the up-regulation of genes encoding chaperones that facilitate the protein folding process in the ER known as the unfolded protein response (UPR). Both responses reduce the accumulation and aggregation of malformed proteins in the compartments of the cells (1).

IRE1α (2) and IRE1β (3) are believed to be ER stress sensor proteins and play important roles in transducing the stress signals initiated by the accumulation of malformed proteins from the ER to the cytoplasm and nucleus. IRE1s are known to participate in the UPR and control the expression of ER molecular chaperones. When cells are exposed to excess levels of stimuli causing ER stress, apoptotic signals are transduced from the ER and promote apoptotic cell death. It is reported that c-Jun N-terminal kinases (JNKs) are activated by the accumulation of malformed proteins in the ER (4). JNKs constitute a family of signal transducers that are activated by a variety of exogenous stimuli, such as growth factor deprivation, Fas or Tumor necrosis factor α (TNF α) treatments (5, 6), anticancer drug treatments, and UV light irradiation. JNKs regulate gene expression through the phosphorylation and activation of transcription factors such as cJUN or the activator protein-1 family (7). The activation of JNK requires TNF receptor-associated factor 2 (TRAF2), a member of the TRAF family of proteins, which transduce signals from IREs. In addition, dominant-negative TRAF2, which is truncated in the N-terminal RING effector domain of TRAF2, inhibits the activation of JNK by signals from IRE1α (4).

During apoptosis induced by ER stress, caspase-12 is localized to the ER and is activated (8). The activation of caspase-12 is not mediated by other stimuli. Furthermore, it has also been reported that caspase-12-deficient mice are resistant to ER stress-induced apoptosis, but their cells are led to apoptosis in response to other stimuli (8). Although it has been shown that caspase-12 is activated during ER stress-induced apoptosis, the mechanisms of its activation by ER apoptotic signals are still unknown, and even less is known about how TRAF2 can transduce ER stress signals from IRE1s to its downstream signaling events. To address these issues in this study, we describe the identification and the characterization of interactions among the ER stress-associated molecules, and we present a picture of how they are coupled to the activation of this apoptotic signal cascade.

EXPERIMENTAL PROCEDURES

Cell Lines, Reagents, and Antibodies—Human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 mg/ml streptomycin at 37 °C in a 5%
**RESULTS**

**Interactions among IRE1α, TRAF2, and JIK**—It is believed that IRE1αs sense ER stress through their luminal domains (10, 13936). The mechanism of caspase-12 activation in response to ER stress is shown to be mediated by interactions involving IRE1α, TRAF2, and JIK.

**Metabolic-labeling Experiments**—Metabolic labeling with $^{32}$P was performed as described in our previous paper (9). HEK293T cells in 6-well plates were incubated at 37 °C for 3 h with or without ER stress after the addition of $^{32}$P. Radiolabeled lysates from each sample were immunoprecipitated with anti-FLAG antibody or anti-TRAF2 antibody. The immunoprecipitates were separated by 12% SDS-polyacrylamide gel electrophoresis and subsequently electrotransferred onto polyvinylidene difluoride filters (Millipore). The filters were then exposed to x-ray film for the detection of $^{32}$P. To control for loading in this procedure, the filters were stained with the specific antibody after autoradiography.

**FIG. 1.** Association of JIK with IRE1α and TRAF2. A, interactions between IRE1α and JIK. Coimmunoprecipitation analyses were performed as described under “Experimental Procedures.” Specific bindings between JIK and IRE1α are shown in the lanes from coexpressed cell lysates. Molecular mass markers are indicated on the left. B, interaction between JIK and TRAF2. Coimmunoprecipitation experiments revealed interactions between JIK and TRAF2.

**FIG. 2.** Modulations of JNK activation in HEK293T cells in response to ER stress. $2 \times 10^6$ HEK293T cells transfected with 0.5 μg expression vectors were treated with or without 2.5 μg/ml tunicamycin (Tm) for 3 h. Phosphorylation of c-Jun at Ser-63 was measured by Western blotting using the phosphospecific anti-c-Jun antibody. The cell lysates were also immunoprecipitated with anti-c-Jun antibody (a). Activations of JNK were also detected by immunoblotting analysis using the phosphospecific anti-JNK antibody. To control for loading, Western blotting analyses of lysates were performed with anti-JNK1 antibody (b). JIK promoted the activation of both JNK and c-Jun in response to ER stress, and the overexpression of mutant JIK suppressed these activations as much as did dominant-negative TRAF2. The relative intensities of protein bands were determined using the NIH Image software.
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11). The cytosolic parts of IRE1s, the kinase domains and RNaseL domains, are reported to transduce ER stress signals to the downstream events to promote the transcription of ER molecular chaperones. These transduced signals are known to mediate the increase of gene expressions, such as ER molecular chaperones, prolyl peptidyl isomerases, and disulfide exchange proteins (12, 13). In contrast, excess levels of stress in the ER result in apoptosis (14, 15). We hypothesized that the cytosolic portion of ER stress sensor molecules can activate not only the UPR pathway but also the apoptotic-signaling pathway. To examine this hypothesis, the yeast two-hybrid systems were employed to search for genes that interact with the cytosolic kinase domain of IRE1α. As a result of screening the human fetus brain cDNA library, JIK was identified as a possible binding partner of IRE1α (data not shown). It has been reported that JIK is a human STE20-related serine/threonine kinase and that JIK activity is decreased upon epidermal growth factor receptor activation, but it is not modulated by other exogenous stimuli, such as UV irradiation, TNF-α, NaCl, H₂O₂, and anisomycin treatments (16). However, there is no information about JIK in the ER stress signaling. Because JIK is implicated in the JNK signaling, we suspected that JIK also interacts with TRAF2 and forms complexes with both IRE1s and TRAF2.

To demonstrate the interactions of these molecules in mammalian cells, we performed coimmunoprecipitation experiments in HEK293T cells cotransfected with various combinations of expression plasmids for IRE1α–FLAG, JIK-HA, TRAF2, and mock control. Immunoprecipitations of full-length JIK-HA with the anti-HA antibody and Western blot analyses showed that JIK could be coimmunoprecipitated with both IRE1α–FLAG and TRAF2 (Fig. 1, A and B). The results suggested that IRE1α, TRAF2, and JIK form complexes and might influence the functions of one another. The same results were obtained when the order of precipitation was reversed (Fig. 1, A and B). During ER stress condition, it was reported that activated IRE1s could recruit TRAF2 to the ER (4). We also characterized the complex both in normal and the UPR-induced states. Treatments of 1.0 μg/ml and 2.5 μg/ml tunicamycin for 3 h resulted in no significant alteration of the bindings between JIK and IRE1α compared with that of no treated control. On the other hand, the complex formation between JIK and TRAF2 was facilitated by the same stimulations as described above (data not shown). These observations mean that TRAF2 is recruited to the JIK-IRE1α complex in response to the ER stress.

JIK Functions as a Regulator of the JNK-signaling Pathway during ER Stress—It is well known that ER stress activates JNKs (4). We speculated that JIK might play regulatory roles in JNK activation under ER stress conditions. Therefore, we measured the relative levels of JNK activity in cells that were treated with various manipulations by using an immune complex kinase activity assay. After treatments with 2.5 μg/ml tunicamycin for 3 h, JNK was activated in 293T cells transfected with mock vectors. Overexpression of JIK resulted in the acceleration of JNK activation induced by treatment with tunicamycin. Alternatively, transfection of catalytically inactive mutant JIK (A181F183) (16) inhibited the activation of JNK by the same stress (Fig. 2a). The cell lysates were also immunoprecipitated with phosphospecific anti-JNK antibody. Immunoprecipitated proteins were detected by immunoblotting analy-
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**Interactions between TRAF2 and Procaspase-12**—It is still unknown whether the activation of JNK signaling by TRAF2 during ER stress is directly implicated in apoptosis. On the other hand, another group reported that ER resident caspase-12, which is one of the cystein-protease family, plays an essential role in ER stress-induced cell death (8). Procaspase-12 contains the caspase recruitment domain in its N-terminal region, which is known as a predicted prodomain (8). Because caspase recruitment domain is known as the domain that interacts with apoptosis-associated proteins, such as TRAF2, Apaf1, and other caspases (17), we tried to examine whether procaspase-12 is able to interact with TRAF2.

Total cell lysates from HEK293T cells transfected with procaspase-12 and/or TRAF2 expression vectors were immunoprecipitated using anti-TRAF2 antibody and were immunoblotted with anti-caspase-12 antibody. As shown in Fig. 4, procaspase-12 immunoreactive 60-kDa protein was detected only in TRAF2 and procaspase-12-cotransfected cells (Fig. 4, lane 3). No bands were detected in the cells transfected with either vector alone (Fig. 4, lanes 1 and 2). These results indicate that TRAF2 directly associates with procaspase-12 under normal conditions. On the other hand, treatments of TRAF2 and procaspase-12-cotransfected cells with 2.5 μg/ml tunicamycin for 3 h inhibited the interactions between these molecules (Fig. 4, lanes 4 and 5). The same findings, such as the reduction of the coprecipitated procaspase-12 by TRAF2 during ER stress, were observed when IRE1β was overexpressed (Fig. 4, lanes 6 and 7). Reverse experiments also showed similar consistent results. We ascertained that the results documented above were not attributed to the reduction of protein expression by using direct Western blotting (Fig. 4, each lower panel).

It is known that procaspase-12 is cleaved in response to ER stress (8). We examined whether TRAF2 overexpression affected the cleavage of procaspase-12. Our results showed that the coexpression of TRAF2 with procaspase-12, in fact, emphasized the cleavage of procaspase-12 (Fig. 4(a)). This acceleration of the cleavage seems to be based on the activations of procaspase-12 as initiated by TRAF2.

**Caspase-12 Homodimerization—Overexpression of procaspase-12**—In cells transfected with procaspase-12 (data not shown). We hypothesized that after procaspase-12 was recruited to the ER stress signal transducer in response to ER stress, this protease was activated through homodimerization and cleavage by some protein such as calpain (18). Initially, we tried to demonstrate procaspase-12 dimerization through coimmunoprecipitation experiments. Procaspase-12-tagged HA epitope at its C-terminal was coexpressed in 293T cells with procaspase-12-tagged FLAG at its C-terminal. After immunoprecipitation of procaspase-12 with a FLAG antibody, coprecipitating HA-tagged procaspase-12 was detected by Western blotting with an anti-HA antibody. As we expected, procaspase-12-tagged HA was coprecipitated only from the lysates expressing both the HA-tagged procaspase-12 and the FLAG-tagged procaspase-12 (Fig. 5, lane 3). The same results
The TRAFN, which forms a tetramer, inhibited the activation of JNK in response to ER stress, and the catalytically overexpressed inactive mutant increased JNK phosphorylation and JNK activity during ER stress. This hypothesis is supported by the findings that overexpression of JIK increased JNK phosphorylation and JNK activity during ER stress, and that the catalytically overexpressed inactive mutant JIK inhibited the activation of JNK in response to ER stress as much as the dominant-negative form of TRAF2. As phosphorylated TRAF2 was up-regulated in sync with the activation of JNK when JIK was overexpressed, this JIK action on JNK signaling was possibly because of increased phosphorylated forms of TRAF2. However, it is not known whether TRAF2 is phosphorylated directly or indirectly by JIK.

Previously, JIK was reported to inhibit the JNK activation induced by epidermal growth factor receptor (16). This finding is not consistent with our present data that JIK promoted the activation of the JNK-signaling pathway in response to ER stress. According to Tassi et al. (16), any stimuli except treatment with epidermal growth factor did not affect the JNK pathway, indicating that JIK may play diverse roles in modulating the signaling upstream of JNK pathway in response to various stimuli. However, at present the reasons for the discrepancy in the function of JIK are unclear. To clarify the mechanisms of the activation of JNK through JIK under ER stress conditions, further analyses are needed including identification of specific substrate(s) for JIK.

Caspase-12 is known to be essential for cell death induced by...
ER stress. Indeed, procaspase-12 is cleaved, and the activated forms are accumulated under ER stress conditions (8). However, to date, the mechanisms of the activation of procaspase-12 in response to ER stress have not been demonstrated. This study showed that TRAF2 plays an essential role in the activation of caspase-12. In unstressed cells, TRAF2 formed a stable complex with procaspase-12. The stimuli that induce ER stress led to the dissociation of procaspase-12 from TRAF2, and simultaneously dimerization (or oligomerization) of procaspase-12 was promoted. These findings raise the possible mechanisms that the dissociation of TRAF2 from caspase-12 is a trigger for the activation of caspase-12 during ER stress and that the resultant-procaspase-12 is clustered to the ER.

Several recent studies demonstrate that procaspases, such as caspase-2, -8, and -10, can be activated through dimerization/oligomerization mediated through their prodomains. Specific adapter molecules are reported to be able to interact with these procaspases. For example, the prodomains of caspase-8 and -10 interact with the adapter molecule Fas-associated death domain protein (23–25). In a similar manner, caspase-2 and -10 interact with the adapter molecule RAIDD (24). It is suggested that the primary role of these adapter molecules may be to bring procaspase molecules into close proximity with each other to enable dimerization. The mechanism regarding the activation of procaspase-12 might be similar to these recent reports. Although we could not show the recruitment of procaspase-12 to IRE1s during ER stress in the present study, we cannot negate the possibility that procaspase-12 is recruited to IRE1s before oligomerization and that TRAF2 plays a role as an adapter molecule that recruits procaspase-12. Further studies are needed to elucidate the more detailed mechanisms responsible for the activation of caspase-12 focusing on the recruitment of procaspase-12 to IRE1s under ER stress conditions.

In conclusion, in this study we provide a missing link in the ER stress-induced apoptosis-signaling pathway, which connects between the stress sensor molecule IRE1 and the caspase-12. We demonstrate that TRAF2 is a key mediator that transduces the signals from the ER to cytosol during ER stress. Therefore, TRAF2 might become a target molecule with which we can try to control ER stress-induced apoptosis.

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