Activation Signal of Nuclear Factor-κB in Response to Endoplasmic Reticulum Stress is Transduced *via* IRE1 and Tumor Necrosis Factor Receptor-Associated Factor 2

Masayuki KANEKO, Yoshifumi NIINUMA, and Yasuyuki NOMURA*

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University; Sapporo 060–0812, Japan. Received March 29, 2003; accepted April 11, 2003; published online April 14, 2003

Conditions that perturb the function of the endoplasmic reticulum (ER) lead to an accumulation of proteins and subsequent induction of several responses, such as an increased expression of ER-resident chaperones involved in protein folding and activation of c-jun N-terminal kinase (JNK). These responses are mediated by a transmembrane kinase/ribonuclease, IRE1, which transduces the signal from the ER lumen to the cytosol. Although nuclear transcription factor-κB (NF-κB) is also activated by ER stress, whether this response depends on IRE1 is unknown. In this study, we show that IRE1 is involved in the activation of NF-κB induced by ER stress. NF-κB was activated by ER stress-inducing agents, thapsigargin and tunicamycin. The activation was inhibited by a dominant-negative IRE1. In addition, a dominant-negative TRAF2 also suppressed the activation of NF-κB by ER stress. These results suggest that ER stress-induced NF-κB activation is also mediated by the IRE1-TRAF2 pathway, as well as JNK activation.

Key words endoplasmic reticulum (ER) stress; nuclear transcription factor-κB (NF-κB); IRE1; TRAF2

Secretory and membrane proteins are correctly folded and glycosylated in the endoplasmic reticulum (ER) by ER-resident molecular chaperones. The ER stores intracellular calcium and is a major signal-transducing organelle releasing calcium in the cytoplasm. Exposure of cells to various pharmacological agents that disturb ER functions, such as calcium depletion from the ER lumen, inhibition of glycosylation or reduction of disulfide bonds, and in physiological conditions, expression of mutant proteins or overexpression of some wild-type proteins by viral infection, leads to an accumulation of proteins in the ER lumen. Under these conditions called ER stress, two distinct signal transduction pathways, termed the unfolded protein response (UPR) and the ER-overload response (EOR), are activated to transduce the signal from the ER to the nucleus.[1,2]

The UPR leads to an increase in the expression of ER stress-response genes, including ER chaperones and CHOP/GADD153.[3,4] This response is mediated by an ER-transmembrane molecule, IRE1 (α and β), whose cytosolic domain contains a Ser/Thr protein kinase and an endoribonuclease domain with its C-terminus.[5,6] Accumulation of unfolded proteins in the ER lumen initiates oligomerization and *trans*-autophosphorylation of IRE1, resulting in activation of the kinase/RNase activities. Activated IRE1 then cleaves off the intron of XBP-1, a transcription factor that binds to the ER stress-response element (ERSE), to increase the transcription of target genes.[7—10]

The EOR triggers the activation of nuclear factor-κB (NF-κB) through accumulation of proteins in the ER.[11] NF-κB is activated by a wide variety of stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and viral products. The activation of NF-κB induced by ER stress has been suggested to require the release of calcium from the ER through overloading of the ER with accumulated proteins and subsequent production of reactive oxygen species (ROS).[12] On the other hand, production of GRP78/Bip, an ER chaperone, as the UPR was independent of the activation of NF-κB, since overexpression of NF-κB subunits fails to induce GRP78/Bip.[11] Therefore, the EOR appears to be distinguishable from the UPR.

c-Jun N-terminal kinase (JNK) is also activated by ER stress.[13,14] This signaling pathway is mediated by the association of IRE1s with tumor necrosis factor-α (TNF-α) receptor-associated factor 2 (TRAF2).[15] However, it has been unclear whether ER stress-induced NF-κB activation depends on the IRE1-TRAF2-mediated response. A signal initiated by TNF-α diverges from TRAF2 to two pathways leading to NF-κB and JNK signaling.[16,17] Thus, IRE1 seems to activate NF-κB through TRAF2, as well as JNK. We here show that ER stress-induced NF-κB activation is mediated by IRE1 and TRAF2, similar to the JNK activation.

MATERIALS AND METHODS

Expression Vector Construction Human full-length IRE1α (the region corresponding to amino acids; 1—977) and ΔRN-IRE1α (a truncated form of the C-terminal RNase domain; 1—786) with a hemagglutinin (HA) epitope at the C-terminus were amplified by PCR. K599A-IRE1α mutant was constructed by PCR using the overlapping method. All were ligated into the vector pCR3.1 (Invitrogen). Human full-length TRAF2 (1—508) and ΔN-TRAF2 (a N-terminus-truncated form; 87—508) were amplified by PCR, and ligated into the vector pcDNA6/Myc-His (Invitrogen), expressing Myc (c-Myc) and polyhistidine (6×His) epitopes at the C-terminus of the inserted sequence.

Cell Culture and Stable Cell Lines Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2/95% air atmosphere. 293 cell lines stably expressing K599A-IRE1α or ΔN-TRAF2 were generated by transfection with IRE1α-K599A-pCR3.1 or ΔN-TRAF2-

* To whom correspondence should be addressed. e-mail: nomura@pharm.hokudai.ac.jp © 2003 Pharmaceutical Society of Japan

pcDNA6 using the standard calcium-phosphate method, and then selected in medium containing 5 μg/ml of blasticidin (Invitrogen) or 0.8 mg/ml of G-418 (Invitrogen).

Electrophoretic Mobility Shift Assays (EMSA) Nuclear extracts were prepared using previously described methods.[16,17] Oligonucleotide probe for NF-κB was purchased from Santa Cruz (U.S.A.): it has a NF-κB-binding site (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; the core recognition sequence of this oligonucleotide is underlined). For supershift assay, the nuclear extracts were incubated with 1 μg of specific antibodies (anti-NF-κB p65, A, Santa Cruz; anti-NF-κB p50, NLS, Santa Cruz) against each NF-κB component for 2 h at 4 °C before the addition of the oligonucleotide probe.

Western Blotting and Immunoprecipitation Preparation of whole cell lysate and western were performed as previously described.[16,17] 293 cells were lysed in lysis buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 10% glycerol and 0.5% Triton X-100) with complete protease inhibitors (Roche Diagnostics, Germany) at 4 °C for 20 min, and then lysates were centrifuged. The supernatant was immunoprecipitated using anti-Myc mAb and protein G-Sepharose (Amersham Pharmacia Biotech, U.K.). The whole cell lysate were subjected to Western blot analysis. The antibodies used here were anti-IκBα polyclonal antibody (pAb; C-21; Santa Cruz), anti-phospho-IκBα pAb (Ser-32; Cell Signaling), anti-HA pAb (Y-11; Santa Cruz), or anti-Myc monoclonal (mAb; 9E10; Oncogene).

RESULTS

Activation of NF-κB by ER Stress ER stress has been reported to lead to the activation of NF-κB. However, the concentration of ER stress-inducing agents, such as thapsigargin (5 μM, sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor) and tunicamycin (50 μg/ml, N-glycosylation inhibitor), used was too high to appropriately evaluate specific effects on the ER.[11,12] In the UPR studies, thapsigargin and tunicamycin are generally used at lower concentrations, 0.1—1 μM or 1—10 μg/ml, respectively.[4—10] To reconfirm with appropriate concentrations the effect of ER stress-inducing agents on NF-κB activation, we performed EMSA on nuclear extracts prepared from 293 cells treated with thapsigargin (1 μM) or tunicamycin (10 μg/ml). A time-dependent increase-complex was observed in 293 cells treated with both ER stress-inducing agents (Fig. 1A, lane 1—8). An excess of non-labeled probe for NF-κB effectively competed with the labeled probe for binding to the complex (Fig. 1A, lane 10). Moreover, addition of anti-p50 and p65 antibody to the mixture resulted in slow migrating complexes. These results indicate that the complex is NF-κB containing p65 and p50 (Fig. 1A, lane 11, 12). Phosphorylation and subsequent ubiquitination-mediated proteolysis of IκBα, an inhibitory subunit of NF-κB, are necessary for translocation of the NF-κB dimers to the nucleus. Western blot analysis showed that phosphorylation and degradation of IκBα were observed in 293 cells treated with thapsigargin (Fig. 1B). The reason for a delay in the phosphorylation of IκBα seems to be due to a small amount of gradual phosphorylation and degradation of IκBα by ER stress.

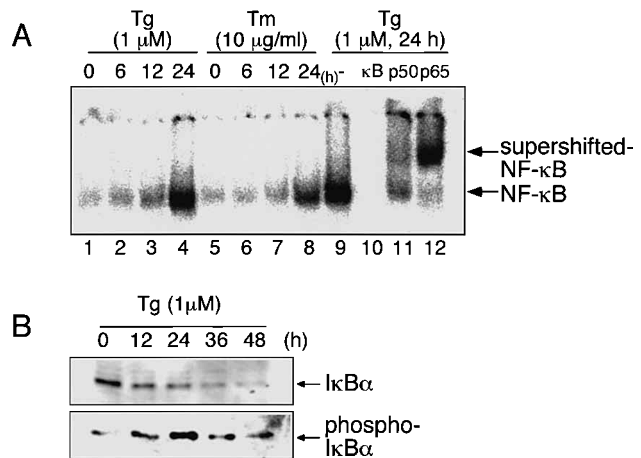

Fig. 1. Activation of NF-κB by ER Stress-Inducing Agents

(A) Time course of the binding activity of NF-κB. 293 cells were treated for the periods indicated with 1 μM of thapsigargin (Tg) and 10 μg/ml of tunicamycin (Tm). Nuclear extracts were prepared and subjected to EMSA using a ^{32}P-labeled oligonucleotide including κB sites. Competition assay was performed with a 1000-fold molar excess of unlabelled probe (lane 10). For supershift assay, the nuclear extracts were incubated with 1 μg of specific antibodies against each NF-κB component (lane 11, 12). (B) Phosphorylation and degradation of IκBα caused by ER stress. Cells were treated with thapsigargin for the periods indicated. The whole cell lysates were subjected to Western blot analysis using anti-IκBα (top) or anti-phospho-specific (Ser-32) IκBα (bottom) antibodies.

Involvement of IRE1 in ER Stress-Induced NF-κB Activation JNK is activated through interaction with IRE1s and TRAF2 in response to ER stress. On the other hand, it has been demonstrated that activation of NF-κB caused by ER stress is attributed to the EOR that causes release of calcium from the ER and generation of ROS.[12] However, NF-κB might be activated by IRE1s through a TRAF2-mediated pathway, since the activation of JNK and NF-κB by TNF-α involves a common pathway, including TRAF2 and upstream molecules.[18,19] To determine whether ER stress-induced NF-κB activation is dependent on IRE1 activation, we investigated the effect of a dominant negative IRE1 on the activation of NF-κB. A kinase-defective mutant IRE1α (K599A) was constructed in which the conserved lysine at residue 599 of the ATP binding site was substituted with alanine in the kinase domain. This mutant exerts a dominant negative effect by forming kinase-inactive heterodimers with wild-type IRE1.[5] Indeed, expression of CHOP, an IRE1-mediated ER stress response gene, independent of NF-κB, was partially suppressed in 293 cells stably expressing K599A-IRE1α as compared to normal 293 cells, indicating that this transfectant certainly has a dominant negative effect (Fig. 2B, top). Under these conditions, ER stress-induced NF-κB activation was effectively suppressed by the expression of K599A-IRE1α, as compared to that in normal 293 cells (Fig. 2A). On the other hand, TNF-α-induced NF-κB activation was not affected by K599A-IRE1α (Fig. 2C). These results indicate that the ER stress-induced NF-κB activation is regulated *via* activation of IRE1α.

Interaction of IRE1α with TRAF2 The cytosolic effector domain of IRE1 has been reported to interact with the C-terminus of TRAF2, depending on IRE1 kinase activity.[15] To examine the interaction of IRE1α with TRAF2, myc-tagged TRAF2 and HA-tagged IRE1α, including the wild-type (wt), a kinase-defective mutant (K599A) or a RNase-deletion mutant (ΔRN), were coexpressed in 293 cells and coimmunoprecipitated with anti-myc antibody to the epitope-tagged

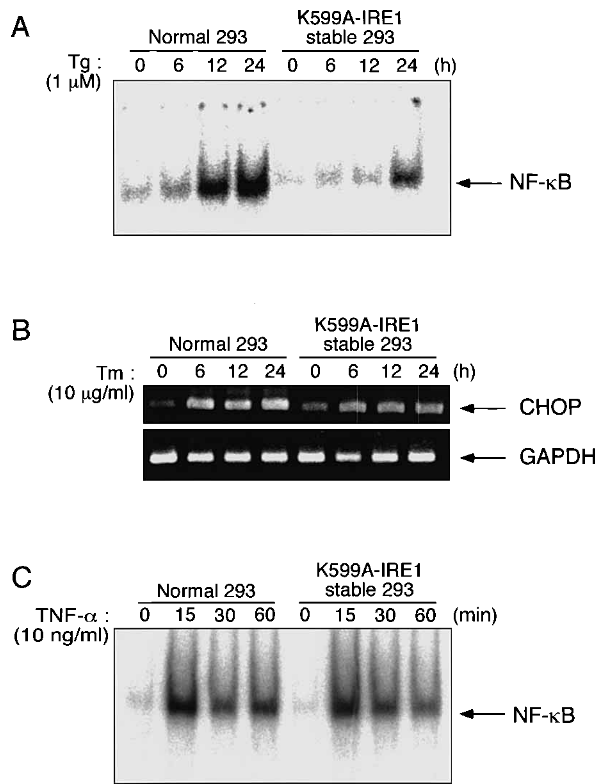

Fig. 2. A Dominant-Negative IRE1 Mutant Inhibits ER Stress-Induced NF-κB Activation

(A) Effect of the dominant-negative K599A-IRE1 mutant on NF-κB activation in response to ER stress. 293 cells and those stably expressing K599A-IRE1 were treated with 1 μM of thapsigargin (Tg) for the periods indicated. Nuclear extracts were prepared and subjected to EMSA. (B) Dominant negative IRE1 suppresses the UPR. Normal 293 cells and those stably expressing K599A-IRE1 were treated with 10 μg/ml of tunicamycin for the periods indicated. Total RNA was prepared and subjected to RT-PCR using specific primers (CHOP, top; GAPDH, bottom). (C) K599A-IRE1 has no effect on TNF-α-induced NF-κB activation. 293 cells and those stably expressing K599A-IRE1 were treated with 10 ng/ml of TNF-α for the periods indicated. Nuclear extracts were prepared and subjected to EMSA.

TRAF2. Although the wt- and K599A-mutant IRE1α were coimmunoprecipitated with TRAF2 (Fig. 3, lane 3, 4), the ΔRN-mutant IRE1 was not recovered in the TRAF2 immunoprecipitates (Fig. 3, lane 5). This result is not consistent with the report that TRAF2 was able to interact with wt- or ΔRN-IRE1, not K599A-IRE1.[15] Therefore, our result indicates that the RNase domain of IRE1 in the C-terminus is necessary for the binding of IRE1 with TRAF2, and that ER stress-induced NF-κB activation is dependent on not only the interaction between IRE1 and TRAF2, but also the kinase activity of IRE1.

Involvement of TRAF2 in ER Stress-Induced NF-κB Activation TRAF2 truncated in its N-terminal activation domain acts as a dominant negative inhibitor of TRAF2-mediated signaling pathways, including TNF-α-induced NF-κB activation and ER stress-induced JNK activation.[15,18,19] To investigate the involvement of TRAF2 in ER stress-induced NF-κB activation, we performed EMSA with 293 cells stably expressing the dominant-negative TRAF2. The dominant-negative mutant of TRAF2 effectively suppressed activation of NF-κB by ER stress as well as that of TNF-α, as compared with normal 293 cells (Figs. 4A, B). These results indicate that TRAF2 is necessary for the activation of NF-κB in response to ER stress, similar to that of JNK.

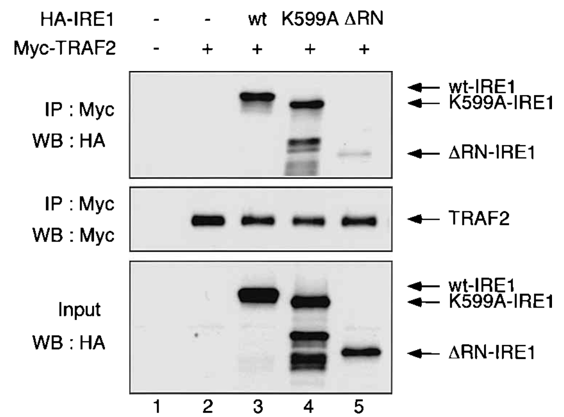

Fig. 3. Interaction of IRE1 with TRAF2

293 cells were cotransfected with the expression vectors encoding Myc-tagged TRAF2 and HA-tagged IRE1s, including wild-type IRE1α (wt, lane 3), a kinase-inactive mutant (K599A, lane 4) or a deletion mutant lacking the ribonuclease domain (ΔRN, lane 5). The whole cell lysates were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were then subjected to Western blotting using anti-HA antibody (upper) or anti-Myc antibody (middle). The lysates used for immunoprecipitation were immunoblotted with anti-HA antibody (bottom).

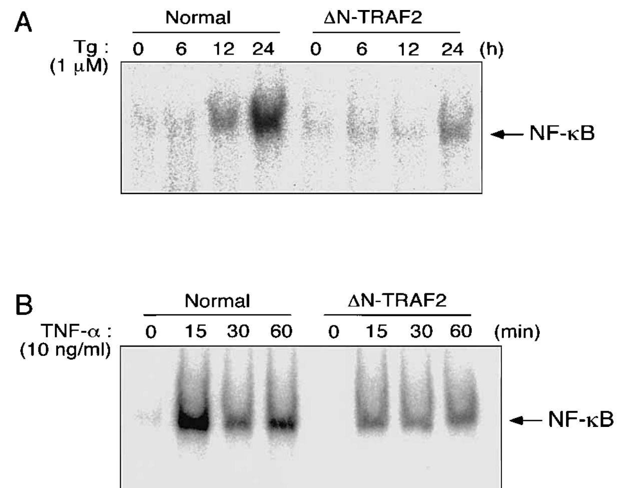

Fig. 4. The Involvement of TRAF2 in ER-Stress Induced NF-κB Activation

(A) Dominant-negative TRAF2 mutant inhibits the activation of NF-κB induced by ER stress. 293 cells and those stably expressing ΔN-TRAF2 were treated with 1 μM of thapsigargin (Tg) for the periods indicated. Nuclear extracts were prepared and subjected to EMSA. (B) Dominant-negative TRAF2 suppresses TNF-α-induced NF-κB activation. 293 cells and those stably expressing ΔN-TRAF2 were treated with 10 ng/ml of TNF-α for the periods indicated. Nuclear extracts were prepared and subjected to EMSA.

DISCUSSION

In this study, we have shown that IRE1 and TRAF2 regulate the activation signaling of NF-κB in response to ER stress, like the case of JNK.

A number of stimuli that disrupt ER functions cause accumulations of proteins in the ER, resulting in activation of both the UPR and the EOR. The signal transduction pathway of the UPR is dependent on ER stress transducers, including IRE1, ATF6 and PERK (PKR-ER-related kinase), that sense the protein accumulation in the ER.[5—7,20—22] It has yet to be determined whether the signal-transducing molecule(s) for the EOR depend on the ER stress transducers, while the EOR induces calcium release from the ER and generation of

ROS.[11,12] In the present paper, we demonstrated that IRE1 is involved in the activation of NF-κB in response to ER stress. In addition, induction of GRP78 expression by ER stress, similar to activation of NF-κB, has been reported to be inhibited by treatment with an intracellular calcium chelator, such as BAPTA, though the target of calcium in the UPR remains to be determined.[23] Furthermore, it is known that the binding activity of NF-κB is directly impaired by the antioxidants that inhibit the EOR-induced NF-κB activation.[24] Thus, there is little, if any, contribution of the EOR to activation of NF-κB. Taken together, these findings suggest that the signal for NF-κB activation from the ER is transduced by IRE1.

Activation signals for NF-κB and JNK triggered by ER stress endogenously generated from the ER are transduced *via* IRE1 activated by dimerization and autophosphorylation. Likewise, extracellular signals initiated by TNF-α induce oligomerization and phosphorylation of TNF receptors that interact with TRAF2.[25] In the case of both IRE1 and TNF receptors, TRAF2 binds with its C-terminus and is clustered, resulting in the recruitment of its downstream components.[15,26] On the other hand, very recently, Leonardi *et al.* have also demonstrated the involvement of TRAF2 in ER stress-induced NF-κB activation using FRTL-5 thyroid cells, though that of IRE1 has yet to be determined.[27] Therefore, TRAF2 is also required for the activation of NF-κB in response to ER stress. Interestingly, the responses leading to the activation of NF-κB and JNK seem to be a mirror symmetric signal on both sides of the cytoplasm. In other words, IRE1 plays a role as a receptor that senses and transduces the signals of intracellular stress from the ER lumen to the nucleus.

A number of apoptosis signals from the ER have recently been identified. Aggregates of polyglutamine (polyQ) proteins in polyQ diseases, including Huntington's disease, accumulate in the cytosol and/or nucleus, not in the ER, and inhibit the ER-associated degradation (ERAD) system by which proteins are eliminated by proteasomes using retrograde transport from the ER back to the cytosol.[27] Under these conditions, activation of JNK leading to apoptosis is induced by ASK1, which recruits the complex of IRE1 and TRAF2. Caspase-12 is known to be essential for apoptosis induced by ER stress including Aβ.[29] Procaspase-12 probably forms a complex with IRE1 and TRAF2 and then autoactivates itself by oligomerization during ER stress.[30] In contrast, the activation of NF-κB induces the expression of genes, such as XIAP and Bcl-X_L, that provide protection against apoptosis.[31,32] However, it remains to be elucidated whether IRE1 is able to induce the expression of such genes in response to ER stress.

It has recently been reported that dysfunction of the UPR is associated with neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and some polyQ diseases.[28,33—35] For instance, mutations of presenilin-1 (PS-1) in familial Alzheimer's disease cause attenuation of the UPR due to inhibition of the ER stress transducers, IRE1, ATF6 and PERK by the PS-1 mutants.[33,34] The disturbed activation of the components leads to a decreased gene expression of ER chaperones, eventually initiating neuronal cell death, due to increased vulnerability to ER stress. It is possible, based upon these new findings, that suppression of IRE1 activation by the PS-1 mutations might cause the attenuation of NF-κB activation and the induction of ER chaperones in response to ER stress, and augment susceptibility to apoptosis.

In conclusion, we propose the model of another signal transduction pathway transduced by IRE1 and TRAF2, from the ER to NF-κB, in addition to a pathway from extracellular signals.

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