The Protein Kinase/Endoribonuclease IRE1α That Signals the Unfolded Protein Response Has a Luminal N-terminal Ligand-independent Dimerization Domain*

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In response to accumulation of unfolded proteins in the endoplasmic reticulum (ER), cells activate an intracellular signal transduction pathway called the unfolded protein response (UPR). IRE and PERK are the two type-I ER transmembrane protein kinase receptors that signal the UPR. The N-terminal luminal domains (NLDs) of IRE1 and PERK sense ER stress conditions by a common mechanism and transmit the signal to regulate the cytoplasmic domains of these receptors. To provide an experimental system amenable to detailed biochemical and structural analysis to elucidate the mechanism of ER-transmembrane signaling mechanism mediated by the NLD, we overexpressed the soluble luminal domain of human IRE1α in COS-1 cells by transient DNA transfection. Here we report the expression, purification, and characterization of the soluble NLD. The biological function of the NLD was confirmed by its ability to associate with itself and to interact with both the membrane-bound full-length IRE1α receptor and the ER chaperone BiP. Functional and spectral studies suggested that the highly conserved N-linked glycosylation site is not required for proper protein folding and self-association. Interestingly, we demonstrated that the NLD forms stable dimers linked by intermolecular disulfide bridges. Our data support that the luminal domain represents a novel ligand-independent dimerization domain.

In response to accumulation of unfolded proteins in the endoplasmic reticulum (ER),1 cells activate an intracellular signal transduction pathway called the unfolded protein response (UPR). Eukaryotic cells have evolved three different mechanisms that mediate the UPR: 1) transcriptional induction of nuclear genes encoding ER resident folding catalysts and chaperones to increase the folding capacity of the ER; 2) translational attenuation to minimize the amount and/or concentration of unfolded proteins in the ER; and 3) ER-associated degradation for rapid clearing of unfolded proteins from the ER (1, 2). IRE1 and PERK are two type I transmembrane serine/threonine protein kinase receptors that are activated in response to unfolded proteins in the ER to signal adaptive responses, transcriptional induction, and translational attenuation, respectively.

The yeast UPR is an elegant linear pathway. Activated Ire1p in Saccharomyces cerevisiae functions as a site-specific endoribonuclease (RNase) that splices HAC1 mRNA encoding Hac1p, a basic leucine zipper (bZIP) transcription factor, that binds to the unfolded protein response element to activate the transcription of target genes, including KAR2 (encoding yeast BiP/GRP78) (3). The UPR in metazoan cells is, however, more diverged in both the key players in the pathway and its outputs. Two mammalian homologs, IRE1α and IRE1β, were identified in the human and murine and both exhibit endoribonuclease activities (4–6). In addition to their roles to induce transcription of target genes, IRE1 activation in response to ER stress also leads to activation of caspase-7 and caspase-12, resulting in apoptosis (7, 8), and the stimulation of c-Jun phosphorilation through the c-Jun N-terminal kinase (JNK) pathway (9). In addition, ATF6 is an ER membrane-localized stress-specific transcription factor, and its activation is regulated by ER stress-induced proteolysis (10–12). Because deletion of Ire1α and/or Ire1β did not interfere with BiP induction in response to ER stress, it remains unknown if IRE1 plays a significant role in this process. PERK, although not present in S. cerevisiae, is a protein kinase that is activated by ER stress to phosphorylate the eukaryotic translation initiation factor 2α (eIF2α). Phosphorylated eIF2α inhibits protein synthesis at the level of translation initiation, thereby limiting the amount of proteins that require folding (13–15). The translation of cyclin D1 was decreased in response to prolonged ER stress, causing a cell cycle arrest in G1 phase (16, 17). The importance of IRE1 and PERK signaling was further demonstrated by studies at the organismal level through gene knock-out and knock-in approaches. Ire1α is an essential gene that is required for normal embryogenesis in early development (9). Although knock-in mice with a homozygous mutation at the PERK eIF2α phosphorylation site (Ser51Ala) die within 18 h after birth due to severe hypoglycemia, PERK knock-out homozygous mice developed hyperglycemia (18, 19). Therefore, at a minimum, translational control through PERK is linked to in vivo glucose homeostasis.

Although studies of the UPR in recent years have generated

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1 The abbreviations used are: ER, endoplasmic reticulum; NLD, N-terminal luminal domain; UPR, unfolded protein response; BiP, immunoglobulin heavy chain-binding protein; NLD-S, an S-tagged NLD protein; ΔC, cytoplasmic domain deletion mutant of human IRE1α; CD, circular dichroism; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NEM, N-ethylmaleimide; DSS, disuccinimidyl suberate; JNK, c-Jun N-terminal kinase; eIF2α, α subunit of eukaryotic translation initiation factor 2; Ni-NTA, nickel-nitrotriacetic acid; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DTT, dithiothreitol; SA, sinapinic acid; Endo H, endonuclease H.
a growing body of exciting results, one basic question remains as to how ER stress activates IRE1 and PERK. The UPR represents a novel intracellular ER transmembrane signaling pathway. IRE1 and PERK are structurally similar to type I growth factor receptors that are activated by ligand-induced dimerization. Whereas the activation mechanism of growth factor receptors is well understood, the mechanism of IRE1 and PERK activation remains largely unknown. Both IRE1 and PERK contain a remarkably large N-terminal luminal domain (NLD) residing in the ER. Although the primary amino acid sequences of the NLDs show limited homology and have diverged among species, we demonstrated that stress-induced oligomerization of the NLD is sufficient for IRE1 or PERK activation (20). We showed that the NLDs of IRE1 and PERK are required for and functionally interchangeable in the transcriptional induction of KAR2 upon ER stress (20). In addition, replacement of Ire1p NLD with a functional leucine zipper dimerization motif yielded an active kinase that, surprisingly, was activated by ER stress (20). These findings support a ligand-independent negative regulatory model for IRE1 and PERK activation. Recent studies have demonstrated a direct interaction between BiP and IRE1 or PERK (21, 22) (results from this study). Therefore, BiP was proposed to bind the NLD by direct protein interaction and repress IRE1 and PERK signaling through maintaining them in a monomeric state. Upon ER stress, unfolded proteins relieve the repression by triggering the release of BiP from the NLDs of IRE1 and PERK (20, 21). Although the proposed model may be oversimplified, it is generally agreed that the NLDs of IRE1 and PERK sense conditions in the ER lumen using a similar mechanism and transmit stress signals to regulate their cytoplasmic domains. We sought to illustrate the activation mechanism by focusing our studies on the NLD of human IRE1.

To provide an experimental system amenable to a detailed biochemical and structural investigation of the receptor activation mechanism, a sufficient amount of pure protein is needed. However, at present, it is extremely difficult to solubilize and purify receptor proteins from membrane preparations. Our initial attempts to express the NLD in bacteria and in yeast were not satisfactory. In the present studies, we have developed a method to express the soluble NLD protein in COS-1 cells by transient DNA transfection. Here we describe the expression, purification, and characterization of the soluble N-terminal luminal domain (NLD) of human IRE1α. This is the first demonstration that the NLD of IRE1α forms stable dimers and functions as a novel ligand-independent dimerization domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—HIT-T15 β cells and COS-1 cells were from the American Type Culture Collection (ATCC); disuccinimidyl suberate (DSS) was from Pierce; α-KDEL and α-BiP antibodies were from Stressgen; α-NLD antibody raised against the NLD and α-CD antibody against the cytoplasmic domain of human IRE1α were previously described (4); Ni-NTA resin was purchased from Qiagen; S-protein-agarose beads were from Novagen; all salts and buffer reagents were from Sigma, Fisher, or Calbiochem.

**Construction of Expression Vectors**—The DNA fragment corresponding to the soluble NLD was amplified using Vent DNA polymerase (New England BioLabs) and inserted into pED vector at the XbaI/EcoRI sites. The final expression construct was designated pED-NLD or pED-NLD-His (Hisα tag). The two oligonucleotides designed to amplify the NLD region of IRE1α cDNA were derived from sequences encoding the signal sequence (MPARRLL) and the N terminus of the transmembrane segment (APVDSML). The antisense primer allows for the addition of a Hisα tag to the C terminus followed by an ERKDEL sequence. Similarly, pED-NLD-S tag was constructed in which the Hisα tag in NLD-His was replaced with a DNA sequence corresponding to the S tag (KETAAAK-FERQHMDMS). The mutant N176Q was constructed by PCR-based overlapping site-directed mutagenesis so that the Aap-176 (AAT) was mutated to Gln (CAG). In addition, pED-NLD with no tag was also constructed in a similar manner. PCR-amplified DNA was verified by DNA sequencing. Plasmid DNA used for COS-1 cell transfection was prepared using plasmid purification kits (Qiagen).

**COS-1 Cell Transfection, Lysate Preparation, and Analysis**—COS-1 cells were cultured, and DNA transfection was performed as described previously (4). At 60 h post-transfection, cells were harvested and lysed in Nonidet P-40 lysis buffer (25 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) plus protease inhibitors. Cell lysates were centrifuged at 20,000 × g at 4 °C for 15 min, and cell extracts were used for protein purification.

**In vivo cross-linking**—Cells were washed three times with cold PBS and incubated with gentle shaking for 30 min on ice with the thiol-non-cleavable cross-linker DSS at 0.3 mM diluted in PBS from a 30 mM stock in Me2SO. The cells were washed with Tris-buffered saline to terminate the reaction. Cross-linked protein complexes were analyzed by Western blotting. Their molecular masses were determined from the logarithmic plot of molecular masses of known protein standards versus their relative electrophoretic mobilities on SDS-PAGE. To induce ER stress, sub-confluent HIT-T15 cells were either left untreated or treated with ER stress reagents for the indicated periods of time.

To detect the formation of intermolecular disulfide bridges, transfected cells were incubated in PBS with or without N-ethylmaleimide (NEM) (10–20 mM) at room temperature for 1 h. Protein samples were processed in the continued presence of NEM starting from the point of gel electrophoresis, including gel electrophoresis and protein purification steps. NEM was also included in the loading buffer in the presence or absence of 100 mM DTT.

**NLD Protein Purification**—The Ni-NTA-agarose resin was first equilibrated with 1× protein binding buffer (50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 5 mM imidazole) and pre-equilibrated cell protein extract was added. The mixture was incubated at 4 °C with gentle vertical rotating for 30 min and poured onto a column. The resin was washed extensively with 1× binding buffer and then with 1× wash buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole). The NLD protein was eluted with 100 mM imidazole. The eluted protein was concentrated in a filter apparatus (Millipore). 1 ml of protein solution was then loaded onto a Superdex 200 HR 10/30 gel filtration FPLC column (Amersham Biosciences, Inc.). The column was pre-equilibrated with PBS buffer (10 mM NaH2PO4, pH 8.0, 150 mM NaCl), and calibrated with thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B-12 (1.35 kDa) (Bio-Rad). Protein was eluted with PBS buffer at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected, and the position of NLD proteins in the elution profile was determined. Protein fractions corresponding to major peaks were collected and concentrated.

Concentrations of cell protein extracts were determined photometrically by the Bradford assay method at 595 nm using BSA as a standard (Bio-Rad protein assay kit). The concentration of purified protein was determined photometrically according to ε (1 mg/ml) 280 nm = 1.3. Its extinction coefficient at 280 nm ε280 = 62990 m² cm⁻¹) was calculated from amino acid composition.

For enzymatic deglycosylation analysis, the high mannose-containing oligosaccharides were removed by digestion with endoglycosidase H (Roche Molecular Biochemicals) as previously described (23).

**Immunoprecipitation, Gel Electrophoresis, and Western Blot Analysis**—For immunoprecipitation, cell extracts were pre-absorbed with protein A-Sepharose beads. Precleared cell extracts were subsequently incubated with specific antibodies in Nonidet P-40 lysis buffer at 4 °C for 2–4 h, and then the immuno-complexes were absorbed to protein A-Sepharose beads for 1–2 h. The beads were thoroughly washed, and immuno-complexes were eluted by boiling in standard sample buffer and separated by SDS-PAGE.

After electrophoresis, gels were either stained with Coomassie Brilliant Blue R-250 or with silver nitrate. For detection by Western blotting, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with 5% (v/v) gelatin in PBS buffer containing 0.2% Tween 20 (PBST), washed with PBST, and incubated with the primary antibody in PBS containing 3% gelatin for 2 h at room temperature. After washes with PBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody in PBS containing 5% gelatin for 1 h at room temperature, and bands were detected using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Inc.).

For native PAGE analysis, protein samples were mixed with sample buffer free of SDS and reducing agent and run in Tris-glycine buffer at 4 °C for overnight. After gel electrophoresis, the gels were placed in...
IRE1α Luminus Is a Ligand-independent Dimerization Domain

**RESULTS**

**NLD Is Highly Expressed in COS-1 Cells by Transient DNA Transfection**—Our initial attempts at expressing the NLD in *Escherichia coli* and in *Pichia pastoris* were unsatisfactory, because the recombinant NLD formed inclusion bodies in bacteria and its expression level was extremely low in yeast. Therefore, we produced the soluble NLD in a eukaryotic expression system to enhance correct folding and post-translational processing and modification. The nucleotide sequences corresponding to IRE1α from the signal peptide to the N terminus of the transmembrane segment were cloned into the mammalian expression vector pEED. The expressed NLD protein was designed to contain a His$_6$ tag at the C terminus, by which the recombinant NLD formed inclusion bodies in bacteria and its expression level was extremely low in yeast.

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**Transfer buffer** (25 mM Tris, 190 mM glycine, 0.1% SDS, pH 8.3) at room temperature for 15 min with shaking, and proteins were transferred onto nitrocellulose. Native protein markers used were: chicken egg albumin (45 kDa), BSA (monomer 66 kDa and dimer 132 kDa), jack bean urease (trimer 272 kDa and hexamer 545 kDa) (Sigma). For non-reducing PAGE analysis, 100 mM DTT or 2% of β-mercaptoethanol was omitted from the sample buffer. Proteins were boiled in the presence of 2% SDS and analyzed by SDS-PAGE.

**Protein Binding Assay Using S-protein Agarose**—Protein extracts (0.9 ml/10/cm plate) were equilibrated with 100 µl of 10× bind/wash buffer (200 mM Tris-Cl, pH 8.0, 1.5 mM NaCl, 1% Triton X-100) prior to the addition of S-protein-agarose beads (Novagen). The reaction was allowed to proceed at 4 °C with gentle vertical rotating for 1 h. Protein complexes were washed three times with 1× bind/wash buffer and resuspended in 200 µl of sample buffer. The eluted sample (10 µl) was analyzed by SDS-PAGE (10%) followed by silver staining.

**Mass Spectrometry**—Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) was run on the PE Biosystems Voyager DE-STR in linear mode with 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid, SA) (Aldrich) as the matrix. Samples were diluted with water and added 50:50 (v:v) to matrix solution (10 mg of sinapinic acid in 1 ml of 60% water containing 0.1% trifluoroacetic acid and 40% acetonitrile). Calibration was done externally using bovine serum albumin as a calibrant.

**Circular Dichroism Measurements**—CD measurements were performed on a Jasco J-500 CD spectropolarimeter at 25°C using 1-mm light path-length for far UV spectra and 1 cm for near UV spectra while flushing the cuvette chamber with nitrogen gas. The protein concentration was 0.5 mg/ml in 10 mM NaH$_2$PO$_4$, pH 8.0. All the solutions were centrifuged before measurement. CD spectra represent the average of three runs and have buffer blanks subtracted. Mean residue mass ellipticities $\theta$ (degree cm$^2$dmol$^{-1}$) were calculated based on 111 as the average molecular mass per residue.

**Steady-state Tryptophan Fluorescence Decay Measurement**—The steady-state tryptophan fluorescence was measured in 1-cm path-length cells in a Shimadzu fluorescence spectrophotometer at an excitation wavelength of 280 nm. Emission spectra were taken from 300 to 450 nm. The bandwidth was 5 nm for excitation as well as emission. The protein concentration was ~0.25 mg/ml in PBS buffer. To denature the protein, guanidine hydrochloride was added to a final concentration of 6 M. Appropriate buffer blanks were subtracted from each spectrum. All fluorescence spectra were expressed in arbitrary units.

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**FIG. 1.** Expression and purification of NLD from transfected COS-1 cells. A, a schematic diagram of IRE1α structure is shown at the top. The arrow indicates signal peptide cleavage site as determined by N-terminal sequencing. The N-terminal methionine, the N-linked glycosylation site (Asp-176), the last residue of the NLD (Leu-441), and the C-terminal leucine of the KDEL sequence in the recombinant NLD are numbered. The positions of the three Cys residues (Cys-109, Cys-148, and Cys-332) are numbered, and the two conserved Cys residues (Cys-109 and Cys-148) among the IRE1 proteins are underlined. An His$_6$ tag was inserted between the C terminus of the NLD and the KDEL ER retention sequence. The sequences of the mature NLD are in boldface. The cytoplasmic domain contains a kinase domain and an RNase domain. TM, transmembrane segment. B, SDS-PAGE (10%) analysis by Coomassie Blue staining of total protein lysates from COS-1 cells transfected with pEED vector, pED-NLD, or pEED-N176Q. C, NLD overexpression in COS-1 cells did not significantly induce BiP transcription. Northern blot analysis was performed using total RNA (5 µg) from transfected cells 24 or 48 h post-transfection. Mock, transcription without plasmid. Control, non-transfected cells. µ, hamster immunoglobulin heavy chain.Δµ, mutant µ with the signal peptide deleted. D, SDS-PAGE (7.5%) analysis of purified NLD or N176Q with ~10 µg of each protein/lane. E, SDS-PAGE (10%) analysis of purified NLD after cleavage with Endo H under denaturing conditions to yield a deglycosylated form. Endo H (1 or 5 µl of 0.01 unit/µl) was used to digest 5–10 µg of NLD for 30 min at 37 °C. Lane 2 shows an incomplete reaction.
a mutant μ heavy chain deleted of the signal peptide did not induce the UPR.

**NLD Protein Can Be Purified for Biochemical Characterization**—The NLD protein was purified to homogeneity by a two-step purification procedure. The recombinant soluble NLD protein in cell extract was initially purified by affinity chromatography on Ni-NTA-agarose beads, and the eluted NLD was further fractionated by FPLC size-exclusion chromatography. The fractions corresponding to the major peak in the chromatogram were collected. The integrity and purity of the purified NLD was further fractionated by FPLC size-exclusion chromatography on Ni-NTA-agarose beads, and the eluted NLD and N176Q have identical near UV absorption spectra (220–350 nm). A and C, typical Far UV (180–250 nm) and near UV (250–320 nm) CD spectra characteristic of the soluble NLD and N176Q. Ellipticity values (θ) are shown in degree-cm²-dmol⁻¹. D, steady-state Trp fluorescence emission spectra (300–350 nm) for excitation at 280 nm of soluble NLD (solid line), N176Q (dotted line), and guanidine hydrochloride (6 M) denatured NLD (dashed line).

**Spectroscopic Studies Support that Glycosylation Is Not Required for Protein Folding**—Because the NLD represents a novel protein domain with no homologous proteins in the data base and no structural information available, we decided to determine its signature spectra. The NLD and N176Q proteins showed identical absorption spectra (220–350 nm) (Fig. 2A) with no absorbance from 310 to 340 nm, supporting the absence of large proteins or aggregates in the solution. For spectroscopic studies, all the proteins were dissolved in 10 mM NaH₂PO₄, pH 8.0. Elimination of NaCl did not cause significant protein precipitation in the phosphate buffer. The far UV and near UV CD spectra of the NLD and N176Q were compared as an indicator of the secondary structures of the two species. Measurements made on the same material at a later date and on different preparations were very similar, suggesting that the protein was stable and that there was little variation between individual preparations. The far UV spectra of NLD and N176Q were characterized by two extrema, a minimum at 208 nm and a maximum at 202 nm, and a characteristic shoulder at 230 nm (Fig. 2B). The near UV spectrum was negative throughout, with two minima at 286 nm and 292 nm, and the minimum at 286 nm had a larger intensity than that at 292 nm (Fig. 2C). The CD spectra of the NLD and N176Q were identical both in shape and intensity or the molecular ellipticity values, demonstrating that the NLD and N176Q assume identical conformations. In addition, the CD spectra also suggested that the native NLD protein contains α-helices and β-sheets, and it is not predominated by either structure.

The intrinsic tryptophan fluorescence of a protein is a sensitive indicator of the local environment of its tryptophan residues. The NLD contains 7 tryptophan residues. The fluorescence emission spectra of the NLD, N176Q, and denatured NLD were analyzed at an excitation wavelength of 280 nm (Fig. 2D). The emission maxima of the NLD and N176Q were identical at 352 nm whereas that of the denatured protein was at 363 nm. This red shift, indicative of fully exposed Trp residues, suggests that the secondary structure of the protein was characteristic and was lost upon denaturation. The emission intensity difference between NLD and N176Q represents the difference in protein concentration used in the analysis. We extended the steady-state experiment to time-resolved fluorescence. A comparison of relative amplitudes and lifetimes indicates that the NLD and N176Q assume identical folding and conformation (data not shown).

In support of the biophysical conclusion that the NLD and N176Q assume identical conformations, functional protein binding assays showed that N176Q was capable of interacting with S-tagged NLD (Fig. 3B). Together with results from func-
Next we tested whether the soluble NLD can functionally interact with membrane-bound IRE1α receptor proteins. A kinase-defective human IRE1α mutant (K599A), an RNase-defective mutant (K907A), and a cytoplasmic domain deletion mutant (ΔC) were previously described (4, 25). Immunofluorescence studies demonstrated that K599A, K907A, and ΔC are preferentially localized close to the nuclear membrane, a sub-cellular localization similar to wild-type IRE1α (4). The soluble NLD protein is expected to be in the ER lumen due to the addition of a KDEL sequence at its C terminus. We therefore proposed that the NLD, if folded properly and localized correctly in the ER lumen, should be able to associate with wild-type, K599A, and ΔC in vivo. To test this hypothesis, NLD-His was co-transfected into COS-1 cells with pED vector, K599A, or ΔC. Protein association was detected by immunoprecipitation using α-KDEL antibody, followed by immunoblotting with α-NLD antibody. Immunoprecipitation of NLD with α-KDEL antibody coprecipitated K599A only from NLD- and K599A-co-transfected cells. Likewise, immunoprecipitation of NLD coprecipitated ΔC only from NLD- and ΔC-co-transfected cells (Fig. 4C, lanes 2 and 6). In addition, overexposure of the blot with the α-NLD antibody also detected K599A and ΔC in the absence of NLD expression (data not shown). This is likely due to the interaction of K599A and ΔC with endogenous BiP, which is recognized by α-KDEL antibody in immunoprecipitation.

The Soluble NLD Directly Interacts with the ER Protein Chaperone, BiP—Previous studies suggested that BiP serves as a negative regulator of IRE1 and PERK activation (20, 26). To test whether this results from a direct interaction, we first examined by co-immunoprecipitation and Western blot analysis the behavior of endogenous IRE1α and BiP in HIT-T15 pancreatic β cells. BiP was detected in the IRE1α-immunocomplex through its interaction with IRE1α from untransfected cells, but not in a control IgG immuno-complex (Fig. 4A, lanes 1 and 2). This observation is in agreement with results obtained in mammalian cells (21) and in yeast (22). However, we were not able to show a consistent dissociation of the BiP-IRE1α complex in this cell line (Fig. 4A). The IRE1α and BiP interaction was further examined in C1 cells, a Chinese hamster ovary cell line (CHO) in which BiP is stably overexpressed. Overexpressed BiP in C1 cells interacted with endogenous BiP, which is associated with BiP in C1 cells to a much greater extent than in control CHO cells, although its association did not detectably change upon tunicamycin treatment (Fig. 4B). Addition of 5 mM Mg2+ ATP released BiP from the BiP-IRE1α immuno-complex, suggesting the interaction is typical of other BiP-substrate interactions (Fig. 4B).

We further purified the NLD-BiP complex from NLD and BiP co-transfected cells using an Ni-NTA column. Silver staining of purified NLD revealed endogenous BiP associated with overexpressed NLD (Fig. 4C, lane 2). Although overexpressed BiP alone was not eluted after extensive washes (Fig. 4C, lane 3), a significant amount of BiP was co-eluted from the column through its association with His6-tagged NLD in co-transfected cells (Fig. 4C, lane 4). Only a fraction of the overexpressed BiP formed complexes with NLD (Fig. 4C, lane 4). This suggests that the affinity for NLD self-association is much higher than for NLD and BiP interaction.

In summary, the self-association of the NLD and its intrinsic ability to interact with membrane-bound IRE1α receptor proteins supported that the overexpressed NLD in the ER lumen was folded properly in a manner resembling the natural NLD of the native full-length IRE1α.

The Soluble NLD Forms a Stable Dimer Independent of a Ligand—Analysis of purified NLD by native PAGE showed the

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2 C. Y. Liu and R. J. Kaufman, unpublished data.
NLD exists as a stable homo-oligomeric complex. The only major form ran at ~145 kDa, a position definitely larger than a monomer (Fig. 5A). The migration position of the NLD oligomeric form was further confirmed by analyzing transfected cell lysate on a native gel followed by transfer onto nitrocellulose and immunoblotting (Fig. 5B). The migration behavior of the purified NLD and the NLD present in protein extract was identical. Upon analytical gel filtration, the only major peak in both spectra was characterized by three peaks corresponding to their molecular masses definitely larger than a monomer but smaller than the dimeric NLD predominates in the spectra (Fig. 7). The determined molecular weight of the monomeric N176Q was identical to the calculated mass. The detection of dimeric and tetrameric species from the mass spectrometry confirmed the results obtained from native gel analysis, gel filtration, and cross-linking experiments. In addition, the exact molecular mass determination excluded the possibility that there may be additional small protein molecules in the homodimers, such as a ligand.

MALDI-TOF mass spectrometry analysis of the purified NLD and N176Q proteins in sinapinic acid (SA) matrix was characterized by three peaks corresponding to their monomeric, dimeric, and tetrameric forms: m/z ~48.2 kDa, 96.4 kDa, and 192.6 kDa for N176Q; ~50.2 kDa, 100.4 kDa, and 200.8 kDa for NLD. Cell lysates were analyzed by MALDI-TOF mass spectrometry. The NLD forms stable dimers in a ligand-independent manner. The results showed that dimeric complexes were detected in all these different IRE1α proteins (Fig. 6, A and B). However, due to the low cross-linking efficiency of DSS, only a fraction of NLD and ΔC were observed as cross-linked complexes (Fig. 6A, lanes 1 and 2). In addition, tunicamycin treatment prior to cell harvesting did not alter the outcome of cross-linking analysis (data not shown). This may be due to the high level of protein expression and the high efficiency of dimer formation mediated by the NLD.

To test the physiological significance of this finding, we investigated the endogenous IRE1α in HIT-T15 pancreatic β cells. The β cell extract was first immunoprecipitated with α-CD antibody, and immune-complexes were then transferred onto nitrocellulose membranes followed by immunoblotting with α-CD and α-NLD antibodies. Similarly, DSS was used to cross-link the endogenous IRE1α complexes in intact β cells. As expected, the cross-linked complex migrated at a position corresponding to its dimeric form (Fig. 6C). IRE1α formed a dimeric complex under non-stress conditions, suggesting that the basal UPR is high in these cells. The level of stress-induced dimer formation in our cross-linking analysis is probably an underestimate of the actual extent of stress-induced dimerization, because the covalent cross-linking reaction has a low efficiency and the transfer efficiency of the complex is low.

MALDI-TOF MS Analysis Demonstrates That the NLD Forms Dimers—MALDI-TOF mass spectrometry analysis of the purified NLD and N176Q proteins in sinapinic acid (SA) matrix was characterized by three peaks corresponding to their monomeric, dimeric, and tetrameric forms: m/z ~48.2 kDa, 96.4 kDa, and 192.6 kDa for N176Q; ~50.2 kDa, 100.4 kDa, and 200.8 kDa for NLD. The determined molecular weight of the monomeric N176Q was identical to the calculated mass. The detection of dimeric and tetrameric species from the mass spectrometry confirmed the results obtained from native gel analysis, gel filtration, and cross-linking experiments. In addition, the exact molecular mass determination excluded the possibility that there may be additional small protein molecules in the homodimers, such as a ligand.

To our surprise, however, the major peaks in both spectra were dimers rather than monomers. The protein samples for MALDI-TOF were mixed with an equivalent volume of sinapinic acid (SA) matrix and a buffer containing acetonitrile and water/0.1% trifluoroacetic acid. The acidic nature of these conditions is generally considered to be sufficient to disrupt quaternary structures in most circumstances. The fact that the dimeric NLD predominates in the spectra reinforced the concept that the NLD forms stable dimers in a ligand-independent fashion. These data shed light on the possible mechanism for the stable homodimer formation. Although the possible existence of a large contact surface area between the two interacting subunits may be a contributing factor, covalent bonding between the two subunits in the dimer could play a critical role.

The NLD Forms Intermolecular Disulfide Bridges—In eukaryotic cells, the redox state of the ER is more oxidizing than that of the cytosol. Protein disulfide bond formation occurs in the lumen of the ER, where protein oxidation initiates upon the translocation of nascent peptide chains into the ER lumen. It is noted that there are three cysteine residues (Cys-109, Cys-148, and Cys-332) in the NLD of human IRE1α with Cys-109 and Cys-148 being highly conserved among IRE1 proteins (Fig. 1). To test whether intermolecular disulfide bonds occur in the NLD, we performed SDS-PAGE analysis in the presence and absence of reducing agents. Western blot analyses of cell lysates transfected with NLD or N176Q showed single bands on...
the gel under reducing conditions (Fig. 8A, lanes 1 and 2). Under non-reducing conditions, both bands, comprising the majority of the NLD or N176Q proteins, shifted to positions of approximately twice the size of the bands observed under reducing conditions, suggesting dimer formation (Fig. 8A, lanes 3 and 4). Higher molecular mass species corresponding to tetramers were also observed (Fig. 8A, lanes 3 and 4). SDS-PAGE analysis under non-reducing conditions of the purified N176Q present in the major peak in gel filtration showed similar results, except that the higher molecular mass species disappeared (Fig. 8B, lanes 3 and 4), further confirming that the major peak in gel filtration analysis corresponds to dimers. Non-reduced NLD migrates as a clearly resolved doublet or triplet. Although the origin of the doublet is unclear at this time, these findings support the concept that the NLD forms disulfide-linked dimers.

To exclude the possibility that the disulfide-linked dimers were artifacts due to disulfide bond exchange reactions during
sample preparation, transfected cells were incubated with 10–20 mM NEM, the membrane-permeable SH group alkylating agent, for 1 h at room temperature immediately before harvesting. NEM (10 mM) was continually present in all the buffers, including loading buffer thereafter. NEM treatment had no effect on the observed dimer formation under non-reducing conditions (Fig. 8B). This result indicated that the disulfide-linked NLD dimer pre-existed in COS-1 cells prior to processing of cells and samples for SDS-PAGE analysis.

The dimeric NLD complex formed by DSS cross-linking and the dimer formed via disulfide bonding are both covalently-linked complexes. To further confirm their identities, the DSS-cross-linked complex was analyzed by SDS-PAGE under reducing conditions along with the disulfide-linked dimer under non-reducing conditions. Western blot analysis showed that these covalently linked complexes migrated at the same position on SDS-PAGE, suggesting that they represent the same complex (Fig. 8C).

We next applied our analysis to wild-type IRE1α, K599A, and ΔC proteins in transfected cells. Our results showed that these proteins migrate at positions larger than monomers, most likely dimers, under non-reducing conditions (data not shown). This result suggests that the intermolecular disulfide bond formation occurs also in full-length IRE1α proteins.

DISCUSSION

A Soluble Form of the NLD Was Overexpressed in Mammalian Cells by Transient DNA Transfection—The unfolded protein response has become an exciting research area in recent years. IRE1 and PERK were identified to be the key proximal sensors to transmit ER stress to the cytoplasm and the nucleus. The two receptors contain a very large luminal domain. Although the luminal domains share very limited homology in amino acid sequences among the IRE1 and PERK proteins from different origins, chimeric receptor studies showed that they are functionally interchangeable in inducing the ER stress response (20). To elucidate the molecular mechanism of receptor activation regulated by the NLD, biochemical and structural studies are needed. The goal of this study was to produce a functional N-terminal luminal domain of human IRE1α. We have succeeded in producing a soluble form of the NLD without the ER transmembrane domain by transient DNA transfection in COS-1 cells (Fig. 1). We believe that a eukaryotic expression system provides the best chance for correct folding and post-translational modification. The NLD protein was expressed in COS-1 cells to an extremely high level, to our surprise. In contrast to many other proteins expressed at high levels (29), a high level of expression of the NLD did not induce the UPR significantly. We propose that the high level expression of the NLD is attributed to its inhibitory effect on UPR activation and translational attenuation mediated by PERK. Future studies are required to test this hypothesis.

N-Linked Glycosylation Is Not Essential for the NLD Protein Folding or Its Function—The N-linked glycosylation site is highly conserved among the NLDs of known IRE1 and PERK proteins. However, the conserved glycosylation site is not essential for sensing ER stress to induce the UPR as demonstrated in yeast (20). In this report, we have provided biochemical basis for this observation. We showed that N-linked glycosylation is not required for protein folding and self-association. In addition, non-denatured NLD was not processed in vitro by Endo H (data not shown), suggesting that the mannose oligosaccharide group is not exposed. Because the presence or absence of glycosylation did not affect protein conformation or its function, it is reasonable to speculate that the glycosylation site is not located near the NLD dimer interface and therefore does not interfere with the NLD complex formation.

The Soluble NLD Is a Functional Ligand-independent Dimerization Domain—We have provided compelling evidence that the soluble NLD is a functional protein. The NLD associates with itself as demonstrated by protein binding assay. It interacts with membrane-bound IRE1α receptor proteins, including the full-length IRE1α, a kinase mutant, and a cytoplasmic domain-deletion mutant. It also interacts with BiP, an ER chaperone protein that serves as a negative regulator of IRE1 activation. These functional analyses encouraged us to purify the soluble NLD to homogeneity for characterization.

A general mechanism for the activation of cell membrane growth factor receptors and receptor tyrosine kinases has been established (30), in which ligand binding to the extracellular domain induces receptor dimerization. Receptor dimerization in turn leads to activation and autophosphorylation of the catalytic kinase domain. The interactions between the ectodomains of many classes of receptors and their corresponding ligand(s) have been extensively studied biochemically and structurally. Although IRE1 and PERK are structurally similar to type I growth factor receptors, their dimerization is ligand-independent. We showed here that the NLD is present in solution in dimeric form in a ligand-independent manner. It
should be noted that our data demonstrated the intrinsic properties of the NLD in forming tight dimers. Tunicamycin treatment did not dramatically affect the dimeric status of the NLD upon its high level overexpression in transfected cells (data not shown). In these transfected cells, the amount of NLD was considerably greater than the level of endogenous BiP. In non-transfected cells, however, the endogenous BiP is in great excess of the endogenous IRE1 protein. In these cells, endogenous IRE1 will be kept monomeric through its interaction with abundant endogenous BiP. We also showed that the NLD indeed interacts with endogenous BiP. The high affinity selfassociation of the NLD (discussion below) provides a driving force for receptor dimerization and activation under conditions of ER stress. In support of our observation, the formation of homodimeric IRE1 upon ER stress has been reported through glycerol-gradient sedimentation studies (21). The biochemical data presented here conform to a previously proposed ligand-independent negative regulatory model for receptor activation (20, 21). The activation mechanism of this class of receptors represents another paradigm for receptor dimerization and activation. In this theme, the end product of a signal transduction pathway, BiP in this case, serves as a negative regulator, and its release from the receptor complex leads to receptor dimerization in a ligand-independent manner.

Although we demonstrated a physical association between BiP and NLD, we have not been able to determine the molecular nature of the BiP interaction. Although ATP dissociated the complex, we do not know if this interaction represents a typical BiP-peptide ligand interaction or whether the NLD and BiP interaction is unique. In addition, we do not know the stoichiometry of the NLD/BiP complex. This requires an accurate determination of the molecular weight of the NLD-BiP complex. Glycerol gradient sedimentation (21) and chemical cross-linking with SDS-PAGE (data not shown) suggested a stoichiometry of one to one for the IRE1-BiP complex, however, more accurate molecular weight determination of the complex is required to address this question.

Previously, BiP interaction with IRE1 was detected in a pancreatic acinar cell line under non-stressed conditions. Upon exposure to ER stress, the complex dissociated (21). Similar results were reported with Kar2p and Ire1p in yeast (22). However, using HIT-T15, a pancreatic β cell line, we did not observe a consistent dissociation of the IRE1α-BiP complex upon ER stress. Furthermore, cross-linking experiments dem-

![MALDI-TOF mass spectra of the purified N176Q (top) and NLD (bottom) identifies IRE1α dimers.](http://www.jbc.org/Downloaded from)
non-reducing conditions, followed by silver staining. Protein samples were transferred onto nitrocellulose and detected with α-NLD antibody. B, aliquots (1 μg) of purified proteins were subjected to 7.5% SDS-PAGE under reducing conditions with 100 mM DTT and under non-reducing conditions, followed by silver staining. Protein samples were prepared either in the presence or absence of 10 mM NEM as described under "Experimental Procedures." C, the disulfide-linked dimer under non-reducing conditions (lane 1) and the DSS-linked protein complex under reducing conditions (lane 2) showed identical mobility by SDS-PAGE and Western blot analysis. Protein samples were prepared as in Figs. 8A and 6A, and were run side by side on the same gel.

A.

B.

C.

FIG. 8. The NLD forms intermolecular disulfide bridges. A, total lysate (1 μl) was loaded on 7.5% SDS-PAGE under reducing conditions with 100 mM DTT or under non-reducing conditions. Proteins were transferred onto nitrocellulose and detected with α-NLD antibody. B, aliquots (1 μg) of purified proteins were subjected to 7.5% SDS-PAGE under reducing conditions with 100 mM DTT and under non-reducing conditions, followed by silver staining. Protein samples were prepared either in the presence or absence of 10 mM NEM as described under "Experimental Procedures." C, the disulfide-linked dimer under non-reducing conditions (lane 1) and the DSS-linked protein complex under reducing conditions (lane 2) showed identical mobility by SDS-PAGE and Western blot analysis. Protein samples were prepared as in Figs. 8A and 6A, and were run side by side on the same gel.

Oxidation of NLD/H18528 in vitro purified proteins (data not shown). Finally, in vitro transactivation of NLD-BiP association using purified BiP and NLD has also been successful (data not shown). These results suggest that the dissociation rate of the NLD dimer is very slow under the tested conditions. In other words, its dissociation rate constant is extremely low.

We next explored the possible mechanism for the tight dimer quaternary structure. We demonstrated that the NLD forms intermolecular disulfide bridges. Although the real role for the three cysteines in the NLD of human IRE1α remains to be determined, it is speculated that the intermolecular disulfide bonding contributes to the stability of the dimer. It should be noted that the amino acid sequence alignment showed that there are two highly conserved cysteine residues among IRE1 proteins, and two among PERK proteins. Interestingly, the positions of the conserved cysteines between IRE1 and PERK have diverged. In support of this divergence, IRE1 and PERK transactivation has not been reported. The unique redox environment in the ER may provide a mechanism to catalyze the oxidation and reduction of disulfide bonds depending on the redox status in the ER upon stress. It is worth mentioning that ERP57, ERP72 and protein disulfide isomerase (PDI), all having disulfide bond isomerase activities, are induced upon activation of the UPR (2). It is possible that the status of disulfide bonding may contribute to the mechanism of IRE1 activation. It is also possible that, rather than being required for IRE1 function, the disulfide bridges provide covalent forces to fully stabilize the conformation of the dimeric receptor. Clearly these possibilities need to be tested.

In summary, we have developed an expression system for the production of the soluble NLD. The recombinant NLD is functional and represents a novel ligand-independent dimerization domain. The solubility and availability of the purified NLD should permit further studies to probe its modular or subdomain organization, to identify intermolecular disulfides involved in dimer formation, to define the roles of intermolecular disulfide bonds, and ultimately, to define its three-dimensional structure. The structural determination will allow for identification of potential protein binding interfaces for high affinity self-association and for interaction with other proteins such as BiP. This protein should prove useful for examining the mechanism of how ER stress leads to receptor activation.

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IRE1α Luminus Is a Ligand-independent Dimerization Domain

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