Eukaryotic cells have developed specific mechanisms to overcome environmental stress. Here we show that the Src homology 2 (SH2)/SH3 domain-containing protein Nck-1 prevents the unfolded protein response normally induced by pharmacological endoplasmic reticulum (ER) stress agents. Overexpression of Nck-1 enhances protein translation, whereas it abrogates eukaryotic initiation factor 2α (eIF2α) phosphorylation and inhibition of translation in response to tunicamycin or thapsigargin treatment. Nck-1 overexpression also attenuates induction of the ER chaperone, the immunoglobulin heavy chain-binding protein (BiP), and impairs cell survival in response to thapsigargin. We provided evidence that in these conditions, the effects of Nck on the unfolded protein response (UPR) involve its second SH3 domain and a calyculin A-sensitive phosphatase activity. In addition, we demonstrated that protein translation is reduced in mouse embryonic fibroblasts lacking both Nck isoforms and is enhanced in similar cells expressing high levels of Nck-1. In these various mouse embryonic fibroblasts, we also provided evidence that Nck modulates the activation of the ER resident eIF2α kinase PERK and consequently the phosphorylation of eIF2α on Ser-51 in response to stress. Our study establishes that Nck is required for optimal protein translation and demonstrates that, in addition to its adaptor function in mediating signaling from the plasma membrane, Nck also mediates signaling from the ER membrane compartment.

The endoplasmic reticulum (ER) is the major signal-transducing organelle continuously sensing intracellular changes triggered by adverse physiological stress states. These conditions include heat shock, hypoxia, glycosylation deprivation, genetic defects that alter protein structure, and pharmacological compounds affecting protein folding. To overcome environmental stress, all eukaryotic cells have developed specific mechanisms in order to maintain ER functions and to promote cell survival. This is achieved by ER-resident molecular machines involved in the activation of at least two distinct signaling pathways that contribute to a proper folding environment in the ER (1, 2). In particular, these pathways lead to transcriptional induction of genes encoding ER chaperones and disulfide exchange proteins (3), and to inhibition of translation initiation (4) through phosphorylation of eIF2α by the ER-resident kinase PERK (5). Together, these events form what is defined as the unfolded protein response (UPR).

Accumulation of unfolded proteins in the ER lumen induces transcription of a large set of genes, many of which encode proteins that function to increase the volume and capacity for either protein folding or the degradation of misfolded proteins (6). For instance, transcription of the ER chaperone KAR2/Bip/GRP78 is a classical marker of UPR activation in yeast and mammalian cells (1). Bip interacts transiently with exposed hydrophobic patches on protein folding intermediates. This is thought to prevent their aggregation while maintaining these proteins in a folding competent state to ensure that only properly folded and assembled proteins exit the ER compartment (7). CHOP/GADD153, another UPR marker, is also activated at the transcriptional level. Its maximal induction occurs after several hours and closely parallels the time course of Bip induction (reviewed in Ref. 1). In addition, transcription of GADD34 is also enhanced under stress conditions (8), and its profile of induction is also very similar to CHOP and Bip. GADD34 is a homologue of the herpes simplex virus-encoded protein γ34.5 (9, 10), which plays an important role in the recovery of the PKR-mediated eIF2α phosphorylation on Ser-51 and shutdown of host protein synthesis in virally infected cells. This activity of γ34.5 is dependent on its ability to associate with the catalytic subunit of the protein phosphatase 1c (PP1c) and correlates with an increase in cellular phosphatase activity that dephosphorylates eIF2α (11, 12). The COOH-terminal fragment of GADD43 can substitute for the role of γ34.5 (10) as demonstrated by decreased levels of phosphorylated eIF2α in GADD43-overexpressing cells despite normal activity of the eIF2α kinases PERK and GCN2 (8).

In parallel, accumulation of unfolded proteins in the ER also leads to the inhibition of protein translation. This is due to the activation of the protein kinase PERK, which specifically phosphorylates the α subunit of eIF2 (eIF2α) on Ser-51 (5). In order to efficiently initiate protein translation, the trimeric eIF2 complex composed of the α, β, and γ subunits needs to recycle from eIF2-GDP (inactive form) to eIF2-GTP (active form), and this is regulated by the guanine nucleotide exchange factor (GEF), eIF2B (13). Phosphorylated eIF2α has a higher affinity for eIF2B and forms with it a stable complex where the bound
GDP cannot be exchanged for GTP (14). Since eIF2 is normally in excess of eIF2B, phosphorylated eIF2α in the eIF2 complex essentially sequesters the cellular eIF2B activity, and this leads to a general inhibition of translation (14).

In a previous study, we have shown that the SH2/SH3 domains-containing adaptor molecule Nck-1 modulates protein synthesis at the level of translation through its interaction with the β subunit of eIF2α (eIF2β) (15). As eIF2β appears to remain associated to eIF2α and eIF2γ throughout the translation initiation cycle (16) and that eIF2α is a major site of regulation of protein translation, we investigated the effect of Nck under ER stress conditions, where translation is normally inhibited through the phosphorylation of eIF2α. Here, we report that overexpression of Nck-1 prevents eIF2α phosphorylation and inhibition of translation in response to ER stress. This appears to depend on the integrity of the second SH3 domain of Nck and relates to a phosphatase-mediated mechanism that could modulate PERK activation. Finally, we demonstrated that protein translation is reduced in Nck-deficient cells and that in these cells translational resistance to ER stress is established by de novo re-expression of Nck-1. Our study not only reveals the importance of Nck for protein translation but also uncovers a novel role for Nck-1 in the regulation of the ER unfolded protein response.

MATERIALS AND METHODS

Cell Culture—Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). Mouse embryonic fibroblasts (MEFs), wild-type, Nck1/Nck2 knockout, and Nck1/Nck2 knockout rescued by re-expression of HA-Nck-1 were kindly provided by Dr. T. Pawson (University of Toronto, Canada) (17) and grown in Dulbecco's modified Eagle's medium high glucose (Invitrogen) containing 10% fetal bovine serum (Invitrogen).

PERK Antibodies—Nck and eIF2β antisera were described previously (15, 18). PERK antibody was purchased from Santa Cruz Biotechnology. Phospho-PERK (Thr-980), eIF2α, and Ser-51-phosphorylated eIF2α antibodies were purchased, respectively, from Cell Signal Technology and BIOSOFT International.

Western Blotting—Transfected HEK293 cells were treated with 2.5 μg ml⁻¹ tunicamycin, 1 μM thapsigargin, or 10 mM dithiothreitol (DTT) for 30 min, washed in ice-cold phosphate-buffered saline, and lysed in the lysis buffer containing 20 μM HEPE (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 100 mM NaF, 17.5 mM β-glycero- phosphatase, 1 mM phenylmethylsulfonyl fluoride, 4 μg ml⁻¹ aprotinin, and 2 μg ml⁻¹ leupeptin. The lysates were cleared by centrifugation at 14,000 rpm. Total cell lysates (50 μg of protein) were resolved by SDS-PAGE on 7.5% (PERK) or 10% (Nck, eIF2α, and eIF2β phospho-Ser-51) acrylamide gels and transferred onto nitrocellulose membranes. After incubation with appropriate primary antibodies, signals were revealed by chemiluminescence (Santa Cruz Biotechnology) using corresponding horseradish peroxidase-conjugated secondary antibodies. When MEFs were used, treatment with thapsigargin was performed at 1 μM for 30 min, and then cell lysates were prepared and probed for Western blotting as described for HEK293 cells.

Nck-1 Constructs and Transfection—Human Nck-1 constructs described previously (15) were transiently transfected in HEK293 cells by calcium phosphate precipitation. For Western blot and RNA analysis, HEK293 cells were plated at a density of 1 × 10⁶ cells in 100-mm plates and transfected with 10 μg of empty pcDNA3.1MycHis or containing various Myc-Nck-1 molecules. 24 h post-transfection, cells were subjected to the indicated treatment with ER stress agents. To assess translation, HEK293 cells were plated at 5 × 10⁴ cells/well in a 24-well plate and transfected with 0.5 μg of the luciferase reporter vector described below and either 0.5 μg of empty pcDNA3.1MycHis or encoding various Myc-Nck-1 molecules. When translation was investigated in MEFs, the cells were also plated at 5 × 10⁴ cells/well in a 24-well plate a day before being transfected by calcium phosphate with 0.5 μg of the same luciferase reporter vector described below. 24 h post-transfection, MEFs were used for treatment with ER stress agents, and as for HEK293 transfected cells, each treatment condition was analyzed in triplicate.

Luciferase Assay—Translational activity in various cell conditions was evaluated as described previously by using the bicistronic reporter plasmid pcDNA3-RLUC-POLIRES-FLUC kindly provided by Dr. N. Sonenberg (McGill University, Montreal, Canada) (19). HEK293 cells transiently co-transfected were treated as indicated with 2.5 μg ml⁻¹ tunicamycin (Sigma), 1 μM thapsigargin (Sigma), and/or 5 μM calyculin A (Upstate Biotechnology Inc.) for 30 min or with 10 mM DTT (Calbiochem) for 15 min. MEFs were treated with thapsigargin at 1 μM for 30 min. On clarified cell lysates normalized for protein content, Renilla reniformis luciferase and firefly luciferase activities were measured by using the dual-luciferase reporter assay system (Promega) and a luminometer (LUMAT).

Quantitative RNA Analysis—Transfected HEK293 cells were incubated with or without 1 μM thapsigargin for 30 min and lysed in Trizol (Invitrogen), and total RNA was prepared according to the manufacturer's recommendations. Quantitative RNA analysis was performed with BiP- and glyceraldehyde-3-phosphate dehydrogenase-specific oligonucleotides as reported previously. For the luciferase and RNAs levels, Trizol (Invitrogen)-prepared RNAs (5 μg) from HEK293 cells transiently co-transfected with the luciferase reporter vector and Myc-Nck-1 plasmid, and treated with either 2.5 μg ml⁻¹ tunicamycin, 1 μM thapsigargin, or 10 mM DTT were analyzed by semi-quantitative PCR, as described previously (15). The amounts of transcribed DNA used for all the above PCRs were in the linear range (data not shown).

Ribosomal Profile—Cell lysates from MEFs wild type (WT), Nck double knockout (DKO), or Nck-1 rescued (Nck-1R) either treated or not with thapsigargin were prepared and layered onto a 15–35% linear sucrose gradient as described (21) with minor modifications. Briefly, upon treatment, cells were washed with cold phosphate-buffered saline and buffer A (110 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 10 mM HEPE (pH 7.3)) and recovered in buffer B (10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 5 mM HEPE (pH 7.3), 10 μg/ml leupeptin, 10 μg/ml aprotinin). Cell extracts were incubated on ice for 10 min and disrupted by passages through 25-gauge needles. At this point, the KCl concentration of the cell extracts was adjusted to 100 mM. Following centrifugation at 1500 × g for 15 min, the resulting supernatant, designated as the cytoplasmic content, was layered on a 15–35% sucrose gradient and centrifuged at 4 °C in a Beckman SW40 Ti rotor at 40,000 × g for 2 h. Fractions were collected from the bottom of the gradient and the OD of each fraction measured at 254 nm represented the RNA content.

Survival Assay—Survival assays were performed as described by others (22). HEK293 cells were plated in normal media at 12,000 cells/well in 6-well dishes and grown for 16 h before being transiently transfected with either 0.5 μg of empty vector or plasmids encoding Nck-1 molecules. 36 h post-transfection, cells were treated with 0.4 μM thapsigargin (Sigma) in the presence or absence of 20 μg ml⁻¹ cycloheximide (Sigma) for the indicated period of treatment. At the end of treatment, the cells were washed and kept in normal medium. Cells remaining on the plate were stained with crystal violet 5 days later.

Overexpression of Nck-1 Prevents ER Stress-induced Inhibition of Protein Translation and Phosphorylation of eIF2α—HEK293 cells were co-transfected with a bicistronic luciferase reporter vector and either the empty pcDNA3.1MycHis vector or the same plasmid containing Nck-1 cDNA. 24–36 h post-transfection, transfected cells were treated with the ER stress agents tunicamycin or thapsigargin for 30 min or with DTT for 15 min. In this system, the luciferase activities are markers of translation, and the translation of Fluc is Cap-dependent, whereas translation of the Fluc cistron directed by the poliovirus internal ribosomal entry site is Cap-independent (19). In HEK293 cells transfected with the empty vector, Fluc activity is reduced by 25, 30, and 50% when treated with tunicamycin, thapsigargin, or DTT, respectively, compared with similar untreated cells (Fig. 1A). As reported previously (15), in cells overexpressing Nck-1, Fluc activity is increased by almost 2-fold. However, Fluc activity in these cells is not reduced by tunicamycin or thapsigargin treatment, and DTT induced only a 30% inhibition (Fig. 1A). Similar effects were observed when Fluc activity was monitored by using the same cell extracts.

a D. T. Nguyen, S. Kebache, S. Jenna, A. Fazel, A. Emaldali, H. N. Wong, J. J. M. Bergeron, R. J. Kaufman, L. Larose, and E. Chevet, submitted for publication.
suggested that the UPR largely affects initiation of protein translation (data not shown). Immunoblot analysis of total cell lysates revealed that in Nck-1-transfected cells, Nck-1 is overexpressed at the levels of endogenous Nck and that the various cell treatments did not alter the levels of endogenous or overexpressed Nck (Fig. 1B).

It is well recognized that inhibition of translation under ER stress conditions results from phosphorylation of eIF2α on Ser-51. Our results showing that overexpression of Nck-1 impairs ER stress-induced inhibition of translation prompted us to gauge eIF2α phosphorylation in cells overexpressing Nck-1 upon similar drug treatments. We found phosphorylation of eIF2α on Ser-51 increased in mock-transfected cells following thapsigargin and DTT, with the greatest increase upon DTT (Fig. 1B, middle panel). In contrast, in cells overexpressing Nck-1 treated or not with tunicamycin or thapsigargin, phosphorylation of eIF2α remained below the levels detected in untreated mock-transfected cells (Fig. 1B, middle panel, and Fig. 1C). Only cells overexpressing Nck-1 treated with DTT showed a clear increase in eIF2α Ser-51 phosphorylation but this level is still lower than the level detected in mock-transfected cells under similar conditions (Fig. 1B, middle panel and Fig. 1C). Western blot analysis for total eIF2α shows, however, comparable levels of endogenous eIF2α in all conditions (Fig. 1B, lower panel). Finally, semi-quantitative PCR performed followed by densitometric analysis of the amplified products and calculation of the ratio Rluc/18S confirmed that treatments with the ER stress pharmacological compounds did not affect luciferase mRNA levels (Fig. 1D and data not shown). This establishes that the variations in luciferase activity measured reflect translational rather than transcriptional changes. Overall, these data demonstrate that under ER stress conditions known to result from the accumulation of misfolded proteins in the ER, overexpression of Nck-1 prevents inhibition of protein translation and eIF2α phosphorylation on Ser-51.

**Effect of Nck-1 on the Translational Component of the UPR Involves Its Second SH3 Domain**—To investigate the mechanism(s) by which Nck antagonizes the translational component of the UPR, we determined the SH domain(s) that Nck-1 requires to prevent the ER stress-induced inhibition of translation. For this purpose, various Myc-tagged Nck-1 mutants deficient in specific SH domain binding abilities were co-transfected with the bicistronic luciferase reporter vector into HEK293 cells. As reported previously (15), the effect of Nck-1 on translation in serum-growing conditions is abolished by functional mutation of its first (M1), third (M3), and all SH3 (3M) domains (Fig. 2). In addition, in cells overexpressing these Nck-1 mutants (M1, M3, and 3M), thapsigargin treatment inhibits translation to the same extent as that in mock-transfected cells. As observed for the wild-type Nck-1, overexpression of Nck-1-mutated in its second SH3 domain (M2) enhances protein translation, but upon thapsigargin treatment, in contrast to wild-type Nck, this mutant allows inhibition of translation in response to ER stress conditions (Fig. 2). These results demonstrate that Nck-1 requires the functional integrity of its second SH3 domain to antagonize the translational component of the ER-induced UPR.

**Nck Is Required for Optimal Protein Translation, but Its Overexpression Antagonizes the UPR to ER Stress Conditions**—To further establish a role of Nck in modulating trans-
lation and the response of the cells to ER stress, we used mouse embryonic fibroblasts (MEFs) in which Nck-1 and Nck-2 genes have been inactivated (dKO) (17). These MEFs were transfected with the bicistronic luciferase reporter vector, and 36 h post-transfection, luciferase activity was measured following thapsigargin treatment. Compared with MEF controls (WT), Rluc activity is significantly reduced by 20% in dKO MEFs and increased by 1.3-fold in dKO MEFs rescued by the de novo expression of Nck-1 (Nck-1R) (Fig. 3A). Upon thapsigargin treatment, Rluc activity in the MEFs WT and dKO is significantly reduced by 25 and 20%, respectively, compared with reciprocal untreated cells, whereas in dKO MEFs overexpressing Nck-1 (Nck-1R), it remains unchanged (Fig. 3A). Similar data were observed when Fluc activity was monitored (data not shown). Reverse transcriptase-PCR experiments have been conducted as described above and ensured that the changes in luciferase activities observed are from changes at the translational level rather than change of transcription (data not shown). Western blot analysis showing the relative expression of Nck in the various MEFs lines revealed that the MEFs Nck-1R cell line expresses Nck-1 at 3.5–4 times more than the MEFs WT (Fig. 3B). In agreement with the data presented in Fig. 1A, the high levels of Nck-1 expression explain why in these cells translation is higher than in WT and why these cells are resistant at the translational level to thapsigargin treatment.

In order to further establish the effect of Nck on translation under ER stress conditions by using a different approach than the reporter vector, we performed analysis of ribosomal profiles prepared on sucrose gradient. It is described that agents that cause ER stress result in dissociation of polysomes into monosomes and ribosomal subunits and that this is consistent with attenuation of an early step in mRNA translation (23). Therefore, we used the ratio of monosomes to polysomes (M:P) to estimate the translational activity of the various previously described MEFs cell lines treated or not with thapsigargin (Fig. 3C and Table I). In the MEFs wild-type (WT), the M:P ratio clearly increased in response to thapsigargin treatment as the OD readout for the 80 S monosomes increased, while the polysomes decreased in this condition. In the cells lacking both Nck isoforms (dKO), the M:P ratio is already higher and increases further upon thapsigargin treatment. This is consistent with the results of Fig. 4A showing that phosphorylation of eIF2α on Ser-51 is higher in the dKO than the MEFs WT in the absence of thapsigargin and is much higher in thapsigargin conditions. Finally, in the MEFs dKO where expression of Nck is induced de novo (Nck-1R) but at much higher levels than the endogenous levels found in WT (Fig. 3B), it appears that the relatively low M:P ratio is restored in untreated conditions and that, in contrast to what occurs in MEFs WT, thapsigargin failed to increase this ratio. This is also consistent with the data showing that the Nck-1R MEFs compared with the WT shows essentially no increase in phosphorylation of eIF2α on Ser-51 (Fig. 4A). These data from ribosomal profiles not only strongly support our previous observations demonstrating that Nck antagonizes the translational component of the UPR occurring under ER stress conditions, but also that the effect of Nck on this aspect of the UPR correlates with the levels of eIF2α phosphorylation on Ser-51.

In ER stress conditions induced by the pharmacological
agents used in this study, phosphorylation of eIF2α on Ser-51 is primarily attributed to the activation of the ER resident protein kinase PERK. We then assessed the activity of PERK in the MEFs expressing different levels of Nck by using a phosphospecific (Thr-980) PERK antibody, which is recognized to detect only activated forms of PERK. As shown in Fig. 4B, thapsigargin treatment results in an apparently more robust activation of PERK in MEFs dKO compared with WT, whereas PERK appears only weakly activated in MEFs dKO rescued by expression of high levels of Nck-1 (Nck-1R). These observations revealed that under ER stress conditions, PERK activation is influenced by the levels of Nck expression. In addition, our data show a strong correlation between the state of PERK activation and the level of eIF2α phosphorylation on Ser-51.

To further characterize the effect of Nck on the UPR resulting in ER stress conditions, we analyzed the induction of BiP in HEK293 cells transfected with either empty vector or a vector

**TABLE I**

| Gradient 1 | Gradient 2 |
|------------|------------|
| MEFs WT (endogenous levels of Nck) | 3.34 | 3.57 |
| Control | 6.67 | 7.69 |
| Tg | 5.00 | 6.67 |
| MEFs dKO (deleted of both Nck isoforms) | 5.00 | 10.00 |
| Control | 7.69 | 10.00 |
| Tg | 4.20 | 3.93 |
| MEFs Nck-1R (expressing Nck-1 at 3.5–4-fold of endogenous Nck) | 4.00 | 3.13 |

Fig. 3. Nck is required to sustain optimal protein translation. A, luciferase activity of *R. reniformis* luciferase (RLUC) cistron measured in MEFs transfected with the luciferase reporter vector and treated or not for 30 min with 1 µM thapsigargin (Tg). WT genotype is Nck-1/ Nck-2, dKO is Nck-1/ Nck-2/ Nck-2, and Nck-1R denotes dKO rescued by stable expression of HA-Nck-1. Experiments were performed five times in triplicate, and the results represent the mean value ± S.E., * at least p < 0.001, as determined by Student’s t test compared with respective untreated conditions.

C, ribosomal profiles from lysates of the above MEFs treated (dashed line) or not (solid line) with 1 µM thapsigargin for 30 min. Absorbance at 254 nm was determined on each fraction collected (y axis). Ribosomal subunits position and monosomal components are indicated. Results are representative of two independent experiments.
encoding Myc Nck-1 and treated or not with thapsigargin. In these conditions where Nck-1 was overexpressed to comparable levels (data not shown) and to assess expression of BiP mRNAs, total RNAs were isolated, and semi-quantitative reverse transcriptase-PCR experiments were carried out. Thapsigargin treatment results in 1.5-fold induction of BiP in HEK293 cells transfected with an empty vector, whereas overexpression of Nck-1 greatly reduces the induction of the BiP transcript (Fig. 5A). These results demonstrate that Nck-1 also impairs BiP induction in response to ER stress conditions.

It is well characterized that long exposure to ER stress agents has a profound effect on cell survival, especially when the functions of the ER are severely impaired (reviewed in Ref. 1). Therefore, we assessed cell survival of HEK293 cells transfected with an empty vector, whereas overexpression of Nck-1 greatly reduces the induction of the BiP transcript (Fig. 5A). These results demonstrate that Nck-1 also impairs BiP induction in response to ER stress conditions.

Overall, our data established that Nck is required for optimal protein translation and that its effect could result from lowering levels of eIF2α phosphorylation on Ser-51 and higher levels of initiation of translation. Moreover, we also demonstrate that Nck participates in the regulation of numerous components of the UPR initiated by ER stress conditions. Indeed, our data proposed that by preventing PERK activation, overexpression of Nck-1 antagonizes the UPR and promotes cell death.

The Effects of Nck-1 under ER Stress Conditions Are Phosphatase-dependent—As reported previously by others, PP1c is
a phosphatase that dephosphorylates eIF2α and permits translational recovery from ER stress conditions (8, 42). To determine whether the effects of Nck-1 on components of the UPR were mediated by a phosphatase-dependent mechanism, we used calyculin A, a potent inhibitor of PP1 and PP2A phosphatases. HEK293 cells were co-transfected with the bicistronic luciferase reporter vector and either the empty pcDNA3.1MycHis vector or the vector containing the Nck-1 cDNA. These cells were treated for 30 min with calyculin A, thapsigargin, or both. As shown in Fig. 6A, calyculin A did not modify the effect of overexpression Nck-1 on protein translation in serum-growing cells. However, when combined with thapsigargin, calyculin A allows recovery of the inhibition of translation in Nck-1-overexpressing cells, whereas it did not affect the response of the mock-transfected cells to thapsigargin (Fig. 6A). Western blots demonstrated comparable levels of endogenous Nck and eIF2α and Nck-1 overexpression (Fig. 6B). Furthermore, Western blot analysis revealed that in cells treated only with calyculin A, the level of eIF2α phosphorylation on Ser-51 is increased in both control and Nck-1-overexpressing cells compared with identical cells kept untreated (Fig. 6B). In addition, when both calyculin A and thapsigargin are combined, eIF2α is phosphorylated on Ser-51 to the same extent in Nck-1-overexpressing cells and in mock-transfected cells (Fig. 6B). PERK activation detected by the presence of slower migrating bands in SDS-PAGE/Western blot analysis using an anti-PERK antibody followed a profile comparable with eIF2α phosphorylation, showing no activation in cells overexpressing Nck-1 treated with thapsigargin alone and activation in cells overexpressing Nck-1 treated with both calyculin A and thapsigargin (Fig. 6B). These data suggest that under ER stress conditions, a calyculin A-sensitive phosphatase could mediate the effects of Nck-1 on the UPR by preventing PERK activation and thus indirectly the phosphorylation of eIF2α on Ser-51 and its inhibitory effect on translation.

**DISCUSSION**

Previous studies have implicated Nck in many cellular events including cell proliferation, axonal guidance, embryonic development, cytoskeletal rearrangement, and oncogenesis (reviewed in Refs. 24–27). Recently, our group (15) has reported a novel role for Nck in modulating protein translation through its interaction with the eIF2α subunit of the eIF2α. In this study, we confirm the importance of Nck in protein translation by establishing that in serum-growing mouse embryonic fibroblasts deficient in both Nck isoforms, protein translation is decreased and correlates with higher levels of eIF2α phosphorylation on Ser-51. We also demonstrated that overexpression of Nck-1 not only enhances translation but also prevents inhibition of translation in response to pharmacological agents inducing accumulation of misfolded proteins in the ER. In these conditions, we have shown that Nck-1 abrogates PERK activation, eIF2α phosphorylation on Ser-51, BiP induction, and cell survival. All together, our findings suggest that overexpression of Nck-1...
impairs the cell response to ER stress by acting as a negative regulator of PERK activation. PERK is an ER resident transmembrane protein identified as the eIF2α/H9251 kinase responsible for the ER-induced UPR. PERK activity is repressed by the ER chaperone BiP. When unfolded proteins accumulate in the ER, BiP dissociates from PERK, resulting in PERK activation. Activated PERK phosphorylates eIF2α on Ser-51 (28), which in turn inhibits translation and alleviates the load of proteins to be processed by the ER (29, 30).

The inhibitory effect of overexpressing Nck-1 on PERK activation in ER stress conditions is also consistent with reduced BiP induction. In yeast and mammals, studies of BiP induction suggest that the signal for the UPR is not directly the accumulation of unfolded polypeptides but rather the decrease in the ER concentration of free BiP occurring when BiP is sequestered into complexes with unfolded proteins (reviewed in Ref. 31). Interestingly, this mechanism influences the transcriptional components of the UPR involving genes that are responsive to low levels of free BiP in the ER (31–34). Recent evidence showed that BiP expression is not only regulated at the level of translation but is also tightly controlled at a post-transcriptional level (35). Indeed, artificial increase in cellular BiP mRNA does not lead to increased synthesis of BiP in un-stressed cells. Under ER stress conditions, this homeostatic restriction is lost, and more BiP proteins are produced independently of gene transcription (35). In cells overexpressing Nck-1, we could propose that Nck-1 by interfering with PERK activation may prevent dissociation of BiP from PERK, therefore inhibiting UPR events triggered by both BiP transcription and translation.

It is documented that when the ER functions are severely impaired, apoptosis is induced by the transcriptional activation of the transcription factor CHOP (GADD153) downstream of the IRE-1, ATF-6, and PERK pathways (29, 36, 37) and by IRE-1-dependent activation of JNK and caspase 12 (38, 39). This correlates well with data demonstrating that overexpression of the ER protein kinase IRE-1, also mediating the UPR, induces cell death (36). Similarly, a recent study (29) also revealed that PERK-deficient cells show impaired survival to ER stress conditions. On the other hand, BiP overexpression is reported to increase cell survival to ER stress conditions (40). Our study shows that overexpression of Nck-1 has a dramatic effect on cell survival under ER stress conditions. Knowing that the two major UPR pathways are mediated by PERK and IRE-1 (reviewed in Refs. 1 and 2), and given that PERK activation and BiP induction are reduced in Nck-1-overexpressing cells, this may favor activation of the IRE-1 UPR alternative pathway and promote cell death. IRE-1 activation has been coupled to the activation of the stress-activated protein kinase JNK (38). In an independent study, we found that Nck interacts directly with IRE-1 and participates in the regulation of the ER stress-induced activation of JNK and Erk-1.2 All together, our data allow us to suggest that overexpression of Nck-1, by preventing activation of the PERK-dependent pathway, may feed back at the ER levels on the IRE-1 pathway to promote cell death in response to accumulation of misfolded proteins.

Recently, feedback inhibitory mechanisms regulating the PERK-mediated UPR at the level of eIF2α phosphorylation have been reported (8, 41). For instance, dephosphorylation of eIF2α on Ser-51 mediated by the complex GADD34 and the subunit c of the phosphatase PP1 promotes translational re-
covery from the UPR (8). Similarly, a constitutive active holo-phosphatase complex involving PP1c and its novel regulatory subunit CReP has been shown to dephosphorylate eIF2α on Ser-51 and to promote cell survival of stressed cells (42). At the level of PERK, such dephosphorylation/inactivation mechanisms, if they exist, still remain to be discovered. To date, PERK activity appears negatively regulated by a different mechanism that involves the protein p58IPK, a Hsp40 family member that has been shown to inhibit activation of PERK by directly binding PERK during ER stress (20, 41). However, our actual data allow us to suggest that in cells expressing endogenous levels of Nck, Nck could be part of a feedback loop mechanism regulating PERK activation and consequently eIF2α phosphorylation and the recovery of protein translation upon ER stress conditions. In this study, we provided evidence for the existence of a such mechanism by demonstrating that calyculin A, a potent inhibitor of the Ser/Thr protein phosphatases PP1 and PP2A, efficiently reverses the antagonistic effect of overexpressing Nck-1 on the UPR by restoring PERK activation, then allowing eIF2α phosphorylation on Ser-51 and inhibition of translation in ER stress cells. However, we cannot exclude the fact that Nck may be part of a molecular complex that also directly dephosphorylates eIF2α or regulates the activation of other eIF2α kinases (PKR and GCN2), even though activation of these kinases by the ER stress agents used here (thapsigargin and tunicamycin) is controversial.

The regulation of the activity and/or subcellular localization of a Ser/Thr phosphatase by Nck-1 may account for a novel and interesting mechanism regulating the UPR (Fig. 7). Indeed, overexpressed Nck-1 by binding and/or regulating a phosphatase may contribute to keep PERK inactivated, thus preventing phosphorylation of eIF2α on Ser-51 and then inhibition of protein translation occurring in response to ER stress. On the other hand, this could also imply that overexpression of Nck-1 favors an excess of Nck-1-eIF2 complex, which, by a mechanism that still has not been elucidated, prevents eIF2α from being phosphorylated and results in a higher translational activity (Fig. 7). Finally, the overall effects of Nck on translation in normal and stress conditions appear to play an important role in determining cell death over survival as determined by the
beneficial effect of cycloheximide on the Nck-associated decreased cell survival. Nonetheless, it remains to be determined whether in vitro Nck is in a molecular complex, including a phosphatase or regulating a phosphatase activity, and that the latter directly or indirectly can prevent PERK activation and/or enhance the efficiency of translation.

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Nck and the ER Response to Stress

9671