Nck in a Complex Containing the Catalytic Subunit of Protein Phosphatase 1 Regulates Eukaryotic Initiation Factor 2α Signaling and Cell Survival to Endoplasmic Reticulum Stress*

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Stress imposed on the endoplasmic reticulum (ER) induces the phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF2) on Ser31. This results in transient inhibition of general translation initiation while concomitantly activating a signaling pathway that promotes the expression of genes whose products improve ER function. Conversely, dephosphorylation of eIF2αSer31 is accomplished by protein phosphatase 1 (PP1c) complexes containing either the protein CReP or GADD34, which target PP1c to eIF2. Here, we demonstrate that the Src homology (SH) domain-containing adaptor Nck is a key component of a molecular complex that controls eIF2α phosphorylation and signaling in response to ER stress. We show that overexpression of Nck decreases basal and ER stress-induced eIF2α phosphorylation and the attendant induction of ATF4 and CHOP. In contrast, we demonstrate that the mouse embryonic fibroblasts lacking both isoforms of Nck (Nck1−/−Nck2−/−) show higher levels of eIF2α phosphorylation and premature induction of ATF4, CHOP, and GADD34 in response to ER stress and finally, are more resistant to cell death induced by prolonged ER stress conditions. We establish that a significant amount of Nck protein localizes at the ER and is in a complex with eIF2 subunits. Furthermore, analysis of this complex revealed that it also contains the Ser/Thr phosphatase PP1c, its regulatory subunit CREP, and dephosphorylates eIF2α on Ser51 in vitro. Overall, we demonstrate that Nck as a component of the CREP/PP1c holophosphatase complex contributes to maintain eIF2α in a hypophosphorylated state. In this manner, Nck modulates translation and eIF2α signaling in response to ER stress.

Nck is part of a family of adaptor proteins composed almost exclusively of a Src homology 2 (SH2) and 3 (SH3) domains (1). Like other members of this family, Nck is believed to couple activated receptor tyrosine kinases at the plasma membrane and/or their substrates to downstream effectors through its various SH domains (2). In the past decade, identification of molecules interacting with the different SH domains of Nck has mainly implicated this adaptor in signaling processes regulating actin cytoskeleton reorganization (2–4). In mammals, separate genes encode two Nck molecules (Nck-1 and Nck-2) with 68% amino acid identity (5–7). Although we cannot totally exclude specific roles for each Nck isoform, their functional redundancy was demonstrated by the knock-out of either Nck in mice, which did not present any particular phenotype (8). Nonetheless, early embryonic lethality (9.5 days) of the double Nck knock-out has revealed a crucial role for this adaptor during development (8).

In a previous study, we uncovered a novel function for Nck in modulating mRNA translation at the level of initiation through its direct interaction with the β-subunit of the eukaryotic initiation factor 2 (eIF2) (9). eIF2 is a heterotrimeric complex (α-, β-, and γ-subunit) that in part drives the initiation of mRNA translation by carrying out the delivery of the methionyl-initiator tRNA to the 40 S ribosomal subunit (10). Various cellular insults are known to reduce protein synthesis at the level of initiation by inhibiting the activity of eIF2 through the phosphorylation of its α-subunit on Ser51 by a family of Ser/Thr kinases, so called eIF2α kinases (11). This prevents recycling of eIF2 into its active GTP-bound form by the nucleotide exchange factor eIF2B, thereby transiently inhibiting general mRNA translation (12). To date, four eIF2α kinases have been identified: 1) HRI (heme-regulated inhibitor), which couples mRNA translation with heme availability in erythroid cells (13), 2) GCN2 (general control non-derepressible-2), which is activated in response to amino acid deprivation (14), 3) PKR (double-stranded RNA-activated protein kinase), a component of the antiviral response activated by double-strand RNA (15), and 4) PERK (PKR-like endoplasmic reticulum kinase), a type 1 transmembrane protein resident of the endoplasmic reticulum (ER) and activated upon accumulation of improperly folded secretory proteins (referred as to ER stress) (16, 17). In this latter condition, attenuation of translation due to eIF2α phosphorylation on Ser51 limits the influx of new proteins into the ER and prevents further buildup of unfolded proteins. On the other hand, reduced eIF2 activity allows the selective translational up-regulation of the mRNA encoding the stress-regulated tran-

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The abbreviations used are: SH, Src homology; eIF2, eukaryotic initiation factor 2; PERK, PKR-like endoplasmic reticulum kinase; ER, endoplasmic reticulum; Tg, thapsigargin; TRITC, tetramethylrhodamine isothiocya-

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scription factor ATF4 (16, 18) via a mechanism involving initiation at upstream open reading frames and reinitiation at the downstream ATF4 start codon (19, 20). ATF4 controls a transcriptional program that accounts for the increase in amino acid transporter and redox protein expression observed during ER stress. In this manner, ATF4 contributes to preserve ER homeostasis in stress conditions (21, 22). Among other genes regulated by ATF4 is GADD34, which coordinates the recovery of protein synthesis by interacting with the catalytic subunit of protein phosphatase 1 (PP1c) and targeting PP1c toward eIF2 to promote eIF2α dephosphorylation (23). On the other hand, prolonged ER stress conditions were found to induce programmed cell death (apoptosis) in part through the UPR-mediated induction of the CHOP/GADD153 gene, a transcriptional activator of the C/EBP protein family with pro-apoptotic properties (for review, see Ref. 24). CHOP is believed to promote the death of stress cells by increasing the load and oxidation of client proteins in the ER through the up-regulation of GADD34 and ERO1α, respectively (25). Together these events downstream of eIF2α phosphorylation occur in response to a broad range of stress conditions and therefore, are referred as the integrated stress response (ISR).

In concert with activation of PERK, ER stress also activates two other ER-resident transmembrane proteins, IRE1 (26) and ATF6 (27). IRE1 is a type-I transmembrane Ser/Thr protein kinase with ribonuclease activity that splices the XBP-1 mRNA (28). The spliced XBP-1 mRNA encodes a transcription factor that migrates to the nucleus where it up-regulates a subset of genes that contribute to overcome damage at the ER (29). On the other hand, ATF6 activation occurs via proteolytic cleavage, which like XBP-1 splicing, generates a functional transcriptional regulator (30, 31). Together, IRE1 and ATF6 transduce signals leading to the up-regulation of genes encoding ER chaperones such as BiP/Grp78 and degrading enzymes, which enhance ER function and alleviate ER stress. Collectively, these events are integrated in a cellular response called the unfolded protein response (UPR), which protects cells against the deleterious effects of proteotoxicity in the ER. Functional defects in some players of the UPR have been associated with metabolic or neurologic pathological manifestations. For example, patients affected by the Wolcott-Rallison syndrome, which results in neonatal insulin-dependent diabetes, show mutations in the PERK/EIF2AK3 gene (32). Similarly, mice lacking expression of PERK (33) or harboring an eIF2α escape phosphorylation (eIF2αSer51) for Ala (18) further illustrate a central role for PERK and eIF2α phosphorylation in pancreatic β-cells function.

We recently provided strong evidence that Nck has an essential function in regulating the UPR (34, 35). We demonstrated that overexpression of Nck strongly impairs cell survival to thapsigargin (Tg), a pharmacological inducer of ER stress, by preventing phosphorylation of eIF2αSer51 and attenuation of translation that normally occur in these conditions (34). In parallel, we also showed that Nck regulates IRE1-mediated ERK-1 activation in response to the protein misfolding inducer azetidine-2-carboxylic acid (35). In the present study we further exemplify the concept that Nck regulates signaling from the ER by showing that mouse embryonic fibroblasts (MEFs) deleted of Nck (Nck1−/−Nck2−/−) present increased eIF2α phosphorylation levels and expression of numerous stress-induced genes. We observed that these cells induce faster and to a higher extent various components of the eIF2α-dependent arm of the UPR and cope better with ER stress. We provide various lines of evidence demonstrating that Nck, as an adaptor protein, assembles a molecular complex containing the Ser/Thr phosphatase PP1c, its regulatory subunit CReP, and components of eIF2. From these, we conclude that Nck, by being a component of an eIF2α holophosphatase complex, significantly contributes to accurate control of eIF2α phosphorylation and eIF2-dependent signaling in response to ER stress.

Finally, our study sheds light on a potential mechanism by which Nck adaptors regulate initiation of translation.

EXPERIMENTAL PROCEDURES

Cells—HeLa cells were cultured at 37 °C in 5% CO2 in minimal essential Eagle’s medium (Sigma) containing 10% fetal bovine serum (Invitrogen), whereas Dulbecco’s modified Eagle’s medium (Invitrogen) was used for MEFs and HEK293 cells. Tg (Sigma), tunicamycin (Tn, Sigma), or hydrogen peroxide (H2O2, Sigma) treatments were as indicated.

Antibodies—Protein A-purified pan-Nck (1793) and eIF2β (2087) antibodies were previously described (9, 36). Nck (C-19), eIF2α (F-315), eIF2β (P-3), CREB2/ATF4 (C-20), GADD34 (H-193), Gadd153/CHOP (F-168), and PP1 (E-9) antibodies were from Santa Cruz. Ser(P)51 eIF2α antibody was from BioSource International. The KDEL antibody (10C3) that detects Grp94 and BiP was from Stressgen. Calnexin (clone 37) antibody was from BD Transduction Laboratories. Caspase-3 (9662) and poly(ADP-ribose) polymerase (9542) antibodies were from Cell Signaling. β-Actin antibody (AC-74) and anti-rabbit IgG fluorescein isothiocyanate-conjugated and antmouse IgG TRITC-conjugated antibodies were from Sigma.

Constructs, Transfection, and Luciferase Assays—Human HA-tagged Nck1 and Nck2 constructs generously provided by Dr. Wei Li were described previously (37). FLAG-tagged mCREP (amino acids 24–698) (38), mGADD34 (39), and the ATF4-Luc reporter gene (21) were from Dr. David Ron. Green fluorescent protein-tagged human Nck1 and Nck2 constructs were generated by subcloning the respective Nck cDNAs into pEGFP-C1 (Clontech) using appropriate enzyme restriction digestions. For transfection, cells plated in 60-mm dishes (3 × 10⁶ cells) or 100-mm dishes (1 × 10⁶ cells) were transiently transfected the following day using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. The amount of plasmid used was normalized with corresponding empty plasmid. For luciferase assays, 0.5 μg of the ATF4-Luc reporter gene was used in transient transfection of MEFs. The following day, cell lysates prepared in passive lysis buffer (Promega) and normalized for protein content were used (in triplicates) to assess luciferase activity using the luciferase assay system (Promega).

Western Blot—MEFs plated in 100-mm dishes (1 × 10⁶ cells) and treated the next day with ER stressors at 37 °C were washed in ice-cold phosphate-buffered saline (PBS) and lysed in radiolabeled precipitation assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% SDS, 1% sodium...
deoxycholate, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 17.5 mM β-glycerophosphate) containing protease inhibitors (2 μg ml⁻¹ leupeptin, 4 μg ml⁻¹ aprotinin, 1 mM benzamidine, 100 μg ml⁻¹ Pefabloc SC PLUS). Cell lysates were passed through a 26-gauge syringe and clarified by centrifugation. For immunoprecipitation, cell lysates were prepared in lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 1% Triton X-100, and 1 mM dithiothreitol, supplemented with protease inhibitors). 30–50 μg of proteins from clarified cell lysates were resolved on 10% SDS-PAGE, except for GADD34, where 8% gels were used. Proteins transferred onto polyvinylidene difluoride membranes were subjected to Western blots using specific antibodies and signals detected with enhanced chemiluminescence according to the manufacturer’s specification.

**Immunofluorescence**—HeLa cells plated (2.5 x 10⁴) on coverslips were transfected with 50 ng of pEGFP-C1-Nck1 or -Nck2 plasmid. 18 h post transfection, cells were washed twice in PBS, fixed in 3% para-formaldehyde, PBS for 10 min, permeabilized for 5 min with 0.2% Triton X-100 PBS, and blocked in 1% bovine serum albumin PBS for 30 min. Primary antibodies (anti-Nck 1793, anti-calnexin) diluted in PBS were added for 1 h at 37 °C. After washing with T-TBS (5 times) and PBS (3 times), cells were incubated with secondary antibodies in PBS for 1 h at room temperature. Cells were washed, and DNA was stained with 4,6-diamidino-2-phenylindole (Sigma) for 5 min in PBS at room temperature. Coverslips mounted in MOWIOL were air-dried for 18 h at room temperature and examined on a Zeiss Axiosvert 135 fluorescence microscope (63×). Images were recorded using a digital camera (DVC), analyzed with Northern Eclipse software (Empix Imaging Inc.), and processed using Adobe Photoshop 7.0.

**Cell Survival**—MEFs were plated (3 x 10⁵ cells) in 60-mm dishes in triplicate and treated with 50 μM Tris-HCl, pH 7.4, 50 mM KCl, 2 mM MgCl₂, 1% Triton X-100, and 1 mM dithiothreitol in PBS supplemented with protease inhibitors. Cells were washed and DNA was stained with 4,6-diamidino-2-phenylindole (Sigma) for 5 min in PBS at room temperature. Coverslips mounted in MOWIOL were air-dried for 18 h at room temperature and examined on a Zeiss Axiosvert 135 fluorescence microscope (63×). Images were recorded using a digital camera (DVC), analyzed with Northern Eclipse software (Empix Imaging Inc.), and processed using Adobe Photoshop 7.0.

**Phosphatase Assay**—HeLa cells were plated (2.5 x 10⁵ cells) in 60-mm dishes in triplicate, and phosphatase activity was determined the next day using a Ser/Thr phosphatase kit (Upstate) according to manufacturer’s instructions. Briefly, cells were washed twice in ice-cold phosphate-free buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, and 1 mM EGTA) and lysed in the same buffer supplemented with 1% Triton-X100 and protease inhibitors. Equal amounts of proteins (1–2 μg) from clarified cell lysates were processed in triplicate in a reaction containing the phosphorylating peptide (KRpTIRR; pT is phosphothreonine) in a 25-μl final volume of phosphate buffer (19.5 mM Tris-HCl, pH 7.4, 39 μM CaCl₂). After 10 min at 30 °C, the reaction was stopped by adding 100 μl of STOP buffer provided by the manufacturer. Samples were transferred into a 96-well plate, and optical density was read at 620 nm. Optical density of the blank was subtracted from the optical density of each sample.

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**In Vitro eIF2α Dephosphorylation Assay—**In vitro eIF2α dephosphorylation assays were performed using recombinant His-eIF2α-32P-labeled by GST-PP1c as previously described (40). Free [γ-32P]ATP was removed by gel filtration using Probe Quant G-50 microcolumns (Amersham Biosciences). Endogenous Nck (C-19) and PP1 (E-9) immunoprecipitates prepared from HEK293 cell lysates (1–1.5 mg of total protein) were incubated at 30 °C for 30 min with radiolabeled His-eIF2α as previously reported (39). At the end of the reaction, levels of 32P-labeled eIF2α were determined by submitting 30% of the reaction mixture to SDS-PAGE followed by autoradiography and densitometry for quantitation. Alternatively, in vitro eIF2α phosphatase assays were performed on crude detergent cell lysates following the approach described by David Ron’s laboratory (17, 38, 39). Briefly, recombinant His-eIF2α (10–50 ng) was radiolabeled in reticulocyte lysates using 50 ng of recombinant bacterially expressed GST-PERK. After gel filtration to get rid of the free [γ-32P]ATP, 2 μl of the radiolabeled proteins were incubated at 30 °C for 30 min with 5 μg of proteins (5 μl) from crude detergent lysates of 293 mock-transfected cells, 293 cells transiently transfected with plasmids encoding HA-Nck-1, FLAG-CReP, or FLAG-GADD34 in a final reaction volume of 10 μl (dephosphorylation buffer, 20 mM Tris-HCl, pH 7.4, 50 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.8 mM ATP). The reactions were stopped by adding 2 μl of 6× Laemmli buffer, then boiled and resolved on 10% SDS-PAGE before being exposed to autoradiography. Crude detergent cell lysates were obtained by solubilizing the cells in a lysis buffer containing 20 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 10% glycerol, 0.1 mM EDTA, aprotinin/leupeptin at 10 μg ml⁻¹, and phenylmethylsulfonyl fluoride at 100 μM.

**RESULTS**

**Nck Overexpression Down-regulates eIF2α Phosphorylation-dependent Signaling**—To determine whether Nck isoforms are functionally redundant in preventing eIF2α phosphorylation in response to ER stress, we overexpressed these proteins in HeLa cells. As we previously reported in HEK293 cells (34), transient overexpression of HA-Nck1 in HeLa cells completely blocked eIF2αSer⁵¹ phosphorylation induced by cell treatment with Tn, an inhibitor of N-linked glycosylation perturbing ER function (Fig. 1A, upper panels compare lane 4 to 2). We further observed that high levels of Nck1 yield lower basal levels of eIF2α phosphorylated on Ser⁵¹ (Fig. 1A, upper panels, compare lanes 3 to 1). Likewise, overexpression of Nck2 also resulted in down-regulation of both basal and stress-induced eIF2αSer⁵¹ phosphorylation (Fig. 1A, upper panels, compare lanes 5 and 6 to lanes 1 and 2). Furthermore, similar effects were observed in various cell lines (HeLa, HEK293, N1E-115, NIH 3T3), suggesting that the regulation of eIF2α phosphorylation by Nck is rather ubiquitous than cell-specific (data not shown). Interestingly, the effects of overexpressing Nck on eIF2α phosphorylation resemble those observed after CReP and GADD34 overexpression (Fig. 1A, bottom panels), two related PP1c regulatory subunits found in holophosphatase complexes that dephosphorylate eIF2αSer⁵¹ in non-stressed (38) and ER-stressed cells.
Reduced in Nck1-overexpressing cells (Fig. 1D). Taken together, these results establish Nck as a regulator of the ER stress-induced eIF2αSer51 phosphorylation-dependent ISR.

**Nck Localizes at the ER**—Because phosphorylation of eIF2α by PERK occurs on the cytoplasmic side of the ER and Nck antagonizes PERK-mediated eIF2α phosphorylation, we then used indirect immunofluorescence to determine whether Nck could be detected in the ER area in HeLa cells. Cell staining using a pan-Nck antibody (1793) that recognizes both Nck isoforms revealed a specific and intense reticular signal around the nucleus reminiscent of ER localization (Fig. 2A). Consistent with a role for Nck in receptor-tyrosine kinase and integrin signaling, this antiserum also showed enriched local plasma membrane staining (Fig. 2A, arrowheads). Similarly, after transient expression of green fluorescent protein-tagged Nck1 or Nck2 at low levels in HeLa cells, we detected both Nck proteins at the ER (green) as judged by their extensive colocalization with calnexin (red), a specific ER marker (Fig. 2B). Because neither Nck1 nor Nck2 amino acid sequence predicts a receptor signaling motif, and thus, are not expected to be synthesized and translocated across the ER, these results establish Nck as a regulator of the ER stress-induced eIF2αSer51 phosphorylation-dependent ISR.

**Genetic Inactivation of Nck Expression Establishes Spontaneous eIF2α-dependent Signaling Events**—Having shown that overexpression of Nck profoundly impairs phosphorylation of eIF2α and expression of ER stress-related genes, we wished to determine whether deletion of Nck expression promotes activation of components of the ISR. Supported by our previous observation that deletion of Nck in MEFs results in increased phosphorylation of eIF2αSer51 (34), we addressed eIF2α-dependent signaling events in these cells first in unstressed conditions. As presented in Fig. 3, mutant MEFs presented spontaneous increased levels of ATF4 along with increased induction of its target gene GADD34 (triplicate plates of each cell line). In addition, Grp94 is also extensively induced in these cells. Although the mutant cells show a primed eIF2α-dependent sig-
naling in the absence of exogenous stress, we excluded the possibility that these continuously experience ER stress because BiP, a marker of the UPR downstream of IRE1 and ATF6 activation, is expressed at comparable levels in both cell lines (Fig. 3). This supports our previous observations reporting no constitutive IRE1-dependent events (XBP-1 mRNA levels and splicing) leading to BiP induction in untreated MEFs lacking Nck (35). Overall, our results show that the spontaneous increased levels of eIF2α phosphorylation that we previously reported in cells genetically deleted of Nck (34) correlates with up-regulation of ATF4 and activation of IRS target genes.

Genetic Inactivation of Nck Expression Results in Higher Levels of ER Stress-induced eIF2α Phosphorylation and Premature Induction of eIF2α-dependent Signaling Events and Protects Cells from ER Stress—

We then postulated that Nck adaptors, by regulating the phosphorylation of eIF2α Ser51, participate in the accurate control of the timing and intensity of the ISR upon ER stress. To explore this hypothesis, MEFs WT or lacking Nck were subjected to Tn treatment and analyzed for eIF2α phosphorylation (Fig. 4).

Western blot analysis revealed greater levels of phosphorylated eIF2α Ser51 in MEFs devoid of Nck, both in a dose- (Fig. 4A) and time-dependent (Fig. 4B) manner compared with MEFs WT. At 2.5 µg/ml Tn, eIF2α phosphorylation was induced by 3.1-fold in MEFs lacking Nck compared with 1.7-fold in MEFs WT (Fig. 4A). After 90 min of treatment with Tn, eIF2α phosphorylation was induced by 2.1-fold in mutant MEFs while being increased by only 1.6-fold in MEFs WT (Fig. 4B). These results suggest that the mechanism regulating the phosphorylation of eIF2αSer51 in response to ER stress are defective in MEFs lacking Nck.

We next examined the kinetics of induction of eIF2α-dependent signaling events in WT and mutant MEFs subjected to ER stress. As shown in Fig. 5, MEFs lacking Nck are more responsive to ER stress. First, both ATF4 and CHOP are readily induced at very low concentrations of Tn in mutant MEFs but are detected only at much higher concentrations in WT MEFs (Fig. 5A). Second, using an ATF4-Luc reporter vector that is a measure of ATF4 translation, we observed that Tg treatment for 6 h resulted in a 5-fold induction of the ATF4-Luc reporter in mutant MEFs but only 2-fold in WT MEFs (Fig. 5B). Finally, the induction of ISR effectors downstream of eIF2α occurs much faster and to a higher extent in mutant MEFs compared with WT (Fig. 5C and D). In particular, ATF4 and CHOP proteins appear within 4–6 h upon treatment with Tg in control cells but are easily detected between 1 and 2 h in mutant cells (Fig. 5C).

As shown in Fig. 5D, this prominent signaling persists up to 24 h after initiating ER stress. β-Actin levels were very similar among the different MEFs lysates, showing that equivalent amounts of total proteins were analyzed in each con-

FIGURE 2. Nck adaptors are detected at the ER. A, indirect immunofluorescence in HeLa cells using a pan-Nck antibody (1793) or normal rabbit serum (control). The third panel on the right represents higher magnification of the area selected in the second panel. Bar, 10 µm. B, HeLa cells transiently transfected with 50 ng of pEGFP-Nck1 or -Nck2 (green) were monitored for Nck expression by direct fluorescence (green) and processed for calnexin detection by indirect immunofluorescence (red). Nucleus staining with 4′,6-diamidino-2-phenylindole (DAPI; blue) is shown. GFP, green fluorescent protein.

FIGURE 3. Genetic inactivation of Nck expression results in spontaneous induction of components of the ISR in unstressed cells. A, equal amount of cell lysate proteins prepared from three separate dishes of early passages of MEFs were analyzed by Western blot for indicated proteins using commercial specific antibodies. +/+ MEFs WT; −/− MEFs Nck1−/− Nck2−/−. p, phosphorylated.

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A

|       | +/+  | -/- |
|-------|------|-----|
| p-eIF2α |      |     |
| eIF2α   |      |     |
| Ratio   |      |     |

B

|       | +/+  | -/- |
|-------|------|-----|
| p-eIF2α |      |     |
| eIF2α   |      |     |
| Ratio   |      |     |

FIGURE 4. Genetic inactivation of Nck expression enhances eIF2αSer⁵¹ phosphorylation (p) in response to ER stress. A, dose response to Tn. MEFs were treated with indicated concentrations of Tn for 90 min. Cell lysates normalized for protein content were analyzed by Western blot with specific Ser(P)⁵¹ and total eIF2α antibodies. B, time course of eIF2αSer⁵¹ phosphorylation to Tn (2.5 mg ml⁻¹) in MEFs. Upon treatment, cell lysates were processed and analyzed as described in A.

dition. Noteworthy, no change in Nck levels was observed upon exposure of WT MEFs to either Tn or Tg (Fig. 5, C and D). Moreover, we noticed that BiP and Grp94 are induced at to higher extent in mutant MEFs and in a manner very similar to effectors of the ISR (Fig. 5, C and D). Because normal XBP-1 mRNA splicing has been reported in ER-stressed MEFs lacking Nck (35), possible cross-talks between PERK and IRE1 and/or ATF6 branches of the UPR can account for the rapid and robust BiP and Grp94 induction we observed in mutant compared with WT MEFs. From these results, we conclude that Nck adaptors modulate the kinetic and robustness of eIF2α-dependent events in response to ER stress.

Phosphorylation of eIF2α on Ser⁵¹ serves as a protective mechanism for cells to survive diverse stressful situations including ER stress (38, 41). In opposition, the inability to overcome stress leads to apoptosis through the activation of caspases (42). To directly address the role of Nck in apoptosis induced by chronic ER stress, we treated MEFs WT or deleted of Nck with Tg or Tn for 24 h and analyzed their morphology and survival at the end of the treatment. Under phase-contrast microscopy, we observed that WT MEFs initially round up and ultimately detached from the surface of the culture dish (Fig. 6A). In contrast, this behavior is less pronounced in cells lacking Nck adaptors, suggesting that these cells are protected from ER stress. This was demonstrated by determining that only 26% of WT MEFs survived to 24 h treatment with Tg, whereas this percentage significantly increased to 57% in mutant MEFs (Fig. 6B). Treatment with Tn yielded similar results, whereas hydrogen peroxide exposure at doses that had no effect on eIF2α phosphorylation (43) induced much higher levels of cell death in MEFs lacking Nck compared with WT (Fig. 6B). These data demonstrate that the absence of Nck specifically protects cells from ER stress.

To further substantiate our findings, we treated MEFs with different concentrations of Tg or Tn for 24 h and measured the number of apoptotic cells by counting nuclei with condensed chromatin as revealed by 4',6-diamidino-2-phenylindole staining (Fig. 6C). We found that the number of apoptotic cells was significantly reduced in mutant MEFs as compared with WT in both Tn- and Tg-treated conditions, confirming that the MEFs lacking Nck are protected from ER stress-induced cell death. We then assessed caspase-3 and poly(ADP-ribose) polymerase processing, which normally occur during programmed cell death. As expected, both proteins were found to be preferentially cleaved in a time-dependent manner in WT but not in mutant MEFs (Fig. 6D). Similar results were obtained upon 24 h of treatment with Tn (Fig. 6E). In contrast, cleavage of poly(ADP-ribose) polymerase was more pronounced in MEFs lacking Nck, in agreement with their higher sensitivity to cell death induced by H₂O₂ (Fig. 6F). From these results, we concluded that Nck proteins are required to initiate the events leading to caspases/poly(ADP-ribose) polymerase cleavage and apoptosis in cells experiencing prolonged ER stress.

Nck Is Part of a Molecular Complex That Controls Dephosphorylation of eIF2α—The results presented above suggest that Nck adaptors determine the kinetics of eIF2α phosphorylation and the attendant ISR activation in cells undergoing ER stress by being at the center of a mechanism that antagonizes the phosphorylation of eIF2α. However, how Nck down-regulates the phosphorylation of eIF2α still remains to be defined. Work from our laboratory demonstrated that Nck regulates translation through its interaction with the β-subunit of the eIF2 complex (9). This present study suggests that Nck acts on translation most probably by lowering cellular levels of eIF2α phosphorylation. In agreement with this hypothesis, we previously reported that calyculin A, a potent inhibitor of PP1/PP2A activity, prevented Nck from down-regulating eIF2α phosphorylation in response to ER stress (34), suggesting that a phosphatase activity mediates the effects of Nck overexpression on eIF2α phosphorylation. To elucidate the mechanism by which Nck controls the phosphorylation state of eIF2α, we initiated a series of experiments aimed to determine whether a protein Ser/Thr phosphatase interacts with Nck. First, using a commercial kit that monitors in vitro dephosphorylation of a phosphopeptide by PP1 and PP2A phosphatase activity (KRPtIRR), we detected a Ser/Thr phosphatase activity of this type in Nck immunoprecipitates prepared from 293 cells (Fig. 7A). Moreover, we found that this phosphatase activity correlated with the presence of PP1c in Nck immunoprecipitates isolated from 293 (Fig. 7B) and HeLa cells (data not shown), whereas PP2A could not be detected (data not shown). In contrast, PP1c was not detected in normal rabbit IgG immunoprecipitates (Fig. 7C). To confirm this finding, we quantified the phosphatase activity in Nck immunoprecipitates as described in Materials and Methods. From these results, we concluded that Nck proteins are required to down-regulate the phosphorylation of eIF2α in response to ER stress by being at the center of a mechanism that antagonizes the phosphorylation of eIF2α.

We then assessed caspase-3 and poly(ADP-ribose) polymerase processing, which normally occur during programmed cell death. As expected, both proteins were found to be preferentially cleaved in a time-dependent manner in WT but not in mutant MEFs (Fig. 6D). Similar results were obtained upon 24 h of treatment with Tn (Fig. 6E). In contrast, cleavage of poly(ADP-ribose) polymerase was more pronounced in MEFs lacking Nck, in agreement with their higher sensitivity to cell death induced by H₂O₂ (Fig. 6F). From these results, we concluded that Nck proteins are required to initiate the events leading to caspases/poly(ADP-ribose) polymerase cleavage and apoptosis in cells experiencing prolonged ER stress.

Nck Is Part of a Molecular Complex That Controls Dephosphorylation of eIF2α—The results presented above suggest that Nck adaptors determine the kinetics of eIF2α phosphorylation and the attendant ISR activation in cells undergoing ER stress by being at the center of a mechanism that antagonizes the phosphorylation of eIF2α. However, how Nck down-regulates the phosphorylation of eIF2α still remains to be defined. Work from our laboratory demonstrated that Nck regulates translation through its interaction with the β-subunit of the eIF2 complex (9). This present study suggests that Nck acts on translation most probably by lowering cellular levels of eIF2α phosphorylation. In agreement with this hypothesis, we previously reported that calyculin A, a potent inhibitor of PP1/PP2A activity, prevented Nck from down-regulating eIF2α phosphorylation in response to ER stress (34), suggesting that a phosphatase activity mediates the effects of Nck overexpression on eIF2α phosphorylation. To elucidate the mechanism by which Nck controls the phosphorylation state of eIF2α, we initiated a series of experiments aimed to determine whether a protein Ser/Thr phosphatase interacts with Nck. First, using a commercial kit that monitors in vitro dephosphorylation of a phosphopeptide by PP1 and PP2A phosphatase activity (KRPtIRR), we detected a Ser/Thr phosphatase activity of this type in Nck immunoprecipitates prepared from 293 cells (Fig. 7A). Moreover, we found that this phosphatase activity correlated with the presence of PP1c in Nck immunoprecipitates isolated from 293 (Fig. 7B) and HeLa cells (data not shown), whereas PP2A could not be detected (data not shown). In contrast, PP1c was not detected in normal rabbit IgG immunoprecipitates (Fig. 7C). To confirm this finding, we quantified the phosphatase activity in Nck immunoprecipitates as described in Materials and Methods. From these results, we concluded that Nck proteins are required to down-regulate the phosphorylation of eIF2α in response to ER stress by being at the center of a mechanism that antagonizes the phosphorylation of eIF2α.
pressing a FLAG-tagged version of CReP, we detected CReP, PP1c, and elf2β in Nck immunoprecipitates (Fig. 7D). To clearly establish that Nck is part of an actual elf2α phosphatase complex that dephosphorylates elf2αSer51, we performed in vitro elf2α dephosphorylating assays using lysates obtained from Nck-overexpressing cells. From these assays we noticed that crude cell lysates prepared from Nck1-overexpressing cells always present higher elf2α dephosphorylating activity than equivalent mock-transfected cell lysates (Fig. 7E, left panel) and was comparable with the dephosphorylating activity detected in CReP or GADD34 transiently overexpressing cell lysates (Fig. 7E, middle panel). Finally, we found that Nck immunoprecipitate contains an activity that dephosphorylates elf2αSer51, as revealed by the decrease of radioactivity associated with elf2α in Nck immunoprecipitates compared with normal IgGs immunoprecipitates (Fig. 7E, right panel). In these assays, we were surprised to find that the PP1c immunoprecipitates that we used as a positive control did not show a robust elf2α dephosphorylating activity. We propose that the antibody we used to immunoprecipitate PP1c possibly interferes with the active catalytic site of PP1c, thus reducing PP1c phosphatase activity. On the other hand, we believe the elf2α dephosphorylating activity detected in Nck immunoprecipitates to be significant because the Nck molecular complex containing PP1c probably represents only a minor fraction of the cellular complexes assembled by Nck adaptors.

Last, we reasoned that recruitment of PP1c and elf2 components in a common complex might be perturbed in the absence of Nck proteins. In determining the amount of PP1c detected in elf2β immunoprecipitates, we observed that it was reduced by ~70% in MEFs lacking Nck (Fig. 8). This suggests that Nck plays an important role in maintaining a significant amount of PP1c in the vicinity of elf2, allowing elf2α dephosphorylation. All together, our results support the idea that Nck assembles a molecular complex that contains PP1c, CReP, and components of elf2 and in this manner controls the dephosphorylation of elf2α. Importantly, a defect in this Nck-dependent regulatory mechanism has clear impacts on the cellular response to stress.

7B), further underscoring the specificity of the Nck-PP1c coimmunoprecipitation. As expected, Nck immunoprecipitates contain elf2β and, most importantly, elf2α (Fig. 7B). We confirmed reciprocal coimmunoprecipitations by showing that Nck, PP1c, and elf2β are all found in an elf2α immune complex (Fig. 7C) but not in normal rabbit IgG immunoprecipitates (data not shown). Together, these results demonstrate that Nck, PP1c, and components of elf2 are in a common complex.

We further investigate this molecular complex by determining whether CReP, a PP1c interacting protein regulating the dephosphorylation of elf2α on Ser51, could be found in Nck immune complex. The lack of potent CReP antibody prevented us from monitoring the presence of endogenous CReP in Nck immunoprecipitates. However, in cells overexpressing a FLAG-tagged version of CReP, we detected CReP, PP1c, and elf2β in Nck immunoprecipitates (Fig. 7D). To clearly establish that Nck is part of an actual elf2α phosphatase complex that dephosphorylates elf2αSer51, we performed in vitro elf2α dephosphorylating assays using lysates obtained from Nck-overexpressing cells. From these assays we noticed that crude cell lysates prepared from Nck1-overexpressing cells always present higher elf2α dephosphorylating activity than equivalent mock-transfected cell lysates (Fig. 7E, left panel) and was comparable with the dephosphorylating activity detected in CReP or GADD34 transiently overexpressing cell lysates (Fig. 7E, middle panel). Finally, we found that Nck immunoprecipitate contains an activity that dephosphorylates elf2αSer51, as revealed by the decrease of radioactivity associated with elf2α in Nck immunoprecipitates compared with normal IgGs immunoprecipitates (Fig. 7E, right panel). In these assays, we were surprised to find that the PP1c immunoprecipitates that we used as a positive control did not show a robust elf2α dephosphorylating activity. We propose that the antibody we used to immunoprecipitate PP1c possibly interferes with the active catalytic site of PP1c, thus reducing PP1c phosphatase activity. On the other hand, we believe the elf2α dephosphorylating activity detected in Nck immunoprecipitates to be significant because the Nck molecular complex containing PP1c probably represents only a minor fraction of the cellular complexes assembled by Nck adaptors.

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that converge on the phosphorylation of eIF2α as seen in MEFs lacking Nck.

**DISCUSSION**

Research over the past years has revealed that a number of molecules involved in signaling pathways initiated at the plasma membrane also transit to endomembrane compartments to transduce signals into different cellular outcomes (44, 45). Our study supports this concept by showing that the SH2/SH3 domain-containing Nck adaptors not only transduce signal from receptor-tyrosine kinases at the plasma membrane but also localize at the ER where they participate in ER signaling regulating cellular response to stress. Thus, Nck adaptors are used by different cellular compartments in various cell conditions to generate specific cellular responses.

It is well documented that other conditions beside ER stress, such as amino acid deprivation (14), viral infection (15), or heme deficiency (13), activate distinct eIF2α kinases that also phosphorylate eIF2α on Ser51, resulting in inhibition of mRNA translation. In contrast, in the past years the identification of complexes regulating the dephosphorylation of eIF2αSer51 revealed the importance of appropriate control of eIF2α-dependent signaling in response to stress. However, our knowledge of the mechanisms regulating eIF2αSer51 dephosphorylation is still limited to proteins targeting the Ser/Thr phosphatase PP1c to eIF2α and to the molecular determinants underlying their interaction with PP1c. For example, CReP, the constitutive repressor of eIF2α phosphorylation, is expressed in unstressed cells where it keeps eIF2α hypophosphorylated through its interaction with PP1c (38). Similarly, GADD34 is also known as a PP1c interacting protein that controls dephosphorylation of eIF2α (47). In contrast to CReP, GADD34 expression is induced upon stress and mediates the translational recovery phase of the UPR (39, 47, 48). In addition, some viruses encode products with high homology to GADD34. For example, herpes simplex virus encodes the ICP34.5 protein, which by interacting with PP1c, promotes eIF2α dephosphorylation and allows the virus to escape inhibition of protein synthesis occurring upon infection (49, 50). For the most part, PP1c regulatory proteins involved in eIF2α dephosphorylation contain a KVX motif that was found to mediate their interaction with PP1c (51). Although we begin to appreciate the physiological importance of these regulatory check points, other protein components of eIF2α phosphatase complexes remain to be identified. In this study we report that the Nck adaptor is part of a CReP-PP1c-containing molecular complex that dephosphorylates eIF2α and controls the temporal activation of the ISR and cell survival to ER stress.
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We established that both Nck1 and Nck2 redundantly modulate eIF2αSer51 phosphorylation (Fig. 1A). Particularly, we demonstrated that like CREP and GADD34, overexpression of either Nck down-regulates both basal and stress-induced eIF2αSer51 phosphorylation (Fig. 1, A and B). In contrast, in MEFs lacking Nck, we previously reported that eIF2α phosphorylation was increased (34), and our present data show that this correlates with a primed eIF2α signaling (Fig. 3). We believe that this directly impacts on the kinetic and intensity of the ISR (Figs. 4 and 5) and improves cell survival rates to ER stress (Fig. 6). We uncovered that Nck is part a large molecular complex containing CREP, PP1c, and subunits of eIF2 that dephosphorylate eIF2α on Ser51 (Fig. 7). This complex is required for proper control of eIF2α phosphorylation on Ser51, eIF2α-dependent signaling, and an appropriate cell response to ER stress. Importantly, the amount of PP1c found in this complex is dependent on the presence of Nck because it is greatly reduced in MEFs deleted of Nck (Fig. 8). Based on these results, we propose that Nck significantly contributes to the organization and/or stability of this CREP/PP1c-containing complex, regulating the amount of PP1c recruited in close proximity to eIF2 and in this way regulates eIF2α dephosphorylation on Ser51 (Fig. 9). Additional experiments are required to dissect the molecular interactions underlying the formation of this complex and to determine its structural organization. Given that the recruitment of PP1c in this complex is perturbed in Nck-deficient MEFs, one possibility is that Nck directly interacts with either PP1c and/or CREP and in this way contributes to the stability of the complex. In agreement with this is the presence of a potential PP1c binding site (XVXF) (51) in both Nck isoforms and conserved from Caenorhabditis elegans to humans. Alternatively, based on its modular structure, Nck, by mediating proline and/or phosphorytrosine-based protein interactions via its SH3 and SH2 domains, could be responsible for proper assembly of this molecular complex. Experiments are now being conducted to gain insights into the possible interplay existing between Nck and other components of this eIF2α holophosphatase complex.

As mentioned above, GADD34 has been identified as a
regulatory subunit that also targets PP1c to eIF2β, and the GADD34-PP1c complex has been shown to act in a coordinated and timely manner to dephosphorylate eIF2β(H9251) (39, 48). Although we found CReP and Nck in a common complex, it is possible that Nck could also be part of the GADD34 and ICP34.5 eIF2α holophosphatase complex. It is still to be addressed whether Nck could represent a critical component of more than one molecular complex regulating the dephosphorylation of eIF2α in response to diverse stresses. Interestingly, recent data from our group suggest that Nck shows preference or specificity toward stresses activating eIF2α/β kinases converging to the phosphorylation of eIF2α/β, suggesting that its effect is not general to any eIF2α/β kinase. Last, in a manner very homologous to its adaptor role in translocating specific effectors to activated receptor kinases at the plasma membrane, Nck could target the holophosphatase complexes to specific subcellular compartments to properly control localized mRNA translation and/or eIF2α/β-dependent signaling. This was proposed in a recent study demonstrating that ER-bound ribosomes are still translationally active during the UPR albeit up-regulated eIF2αSer51 phosphorylation (52), suggesting that spatially restricted mechanisms exist that allow translation of a limited number of mRNA by locally preventing phosphorylation and/or promoting dephosphorylation of eIF2α.

Numerous genes are up-regulated by ER stress after phosphorylation of eIF2α. However, how these late events are coordinated among each other over time remains to be determined. Here, we show that MEFs lacking Nck show spontaneous induction of ATF4 and GADD34 (Fig. 3) in addition to primed UPR in response to pharmacological inducers of ER stress (Figs. 4 and 5). Therefore, the Nck-containing eIF2α holophosphatase complex represents an essential regulatory mechanism that restricts signaling from eIF2 in unstressed cells and determines the kinetics and the intensity of this signaling in ER stress conditions. Although we anticipated that effectors of the ISR would be modulated by Nck proteins levels, we were surprised to find changes in BiP and Grp94 induction in response to Tg in MEFs lacking Nck (Fig. 5). First, transactivation of the BiP promoter, which has been highly investigated, is known to be under the control of XBP-1 and ATF6 (29, 53, 54). Second, no change in IRE1-dependent splicing of XBP-1 or BiP mRNA levels were found in azetidine-2-carboxylic acid-treated MEFs lacking Nck compared with WT (35). Conversely, up-regulation of BiP mRNA levels was reduced in MEFs expressing a nonphosphorylated form of eIF2α (eIF2αSer51 for Ala) (18), and ATF4 overexpression leads to transactivation of the BiP promoter (55). Therefore, increased BiP levels in MEFs lacking Nck could be partially attributed to the higher basal and stress-induced levels of ATF4. Based on these observations, cross-talks between PERK and IRE1 pathways leading to BiP and Grp94 induction likely exist. Alternatively, the discordance in BiP induction upon azetidine-2-carboxylic acid (35) and Tg (this study) could account on the fact that these drugs do no recapitulate identical ER signaling.

4 E. Cardin and L. Larose, unpublished results.
The Nck-dependent regulatory mechanism described in this study appears of major importance as we confirmed that the ability of cells to survive to ER stress inversely correlates with expression levels of Nck (Fig. 6) (34). We found that MEFs lacking Nck show prominent ER stress signaling from eIF2α (Fig. 5). This allows these cells to rapidly clear stress in the ER and escape apoptotic programs (Fig. 6). Noticeably, this closely resembles the behavior of the CREP RNA interference cells, which show spontaneous induction of the CHOP promoter and resistance to stresses that cause eIF2αSer51 phosphorylation (38). However, we cannot exclude that other ER-induced signaling pathways such as IRE1-mediated ERK1/2 activation also contribute to improve cell survival in absence of Nck (35). Collectively, our results are in accordance with the protective function of eIF2αSer51 phosphorylation (56) and in agreement with reports demonstrating that preconditioning of cells to stress targeting eIF2 establishes a resistant state toward subsequent challenges (41, 57). It is believed that the temporal induction of the ER stress response strongly influences cellular outcomes and could underlie the difference in sensitivity of various cell types to ER stress. Our finding that Nck expression levels markedly influence signaling and cell survival to ER stress reveals the interest in searching for defects in Nck expression in proteotoxic diseases where uncontrolled ER stress-induced signaling is linked to pathogenesis (58 – 60).

Results presented here reinforce the concept that Nck is a bona fide player in such a fundamental cellular process that is mRNA translation and highlights the versatile properties of this protein. More importantly, this study sheds light on the potential mechanism by which Nck adaptors regulate mRNA translation. This could change our understanding of essential biological processes such as development of the central nervous system. Significant evidence has accumulated in recent years showing that local translation in axons is integral for growth cone navigation in response to environmental cues (61). Concomitantly, Nck has been implicated in photoreceptor cell axon guidance in Drosophila (62, 63) and in signaling downstream of various mammalian axon guidance receptors (64, 65). It is, thus, possible that Nck might regulate neural network formation by coupling local protein synthesis with guidance receptors activation at the growth cone. Therefore, our study emphasizes the importance of evaluating the role of Nck on translation in biological processes depending on local protein synthesis and simultaneously opens up new research avenues meant to examine the in vivo significance of this novel function of Nck adaptors.

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