# Modulation of protein translation by Nck-1

**Sem Kebache*, Dongmei Zuo*, Eric Chevet†, and Louise Larose*‡**

Departments of *Medicine and †Surgery, Polypeptide Laboratory, Division of Endocrinology, McGill University, Montreal, QC, Canada H3A 2B2

Edited by Lewis Clayton Cantley, Beth Israel Deaconess Medical Center, Boston, MA, and approved February 12, 2002 (received for review September 13, 2001)

**In mammals, Nck represented by two genes, is a 47-kDa SH2/SH3 domain-containing protein lacking intrinsic enzymatic function. Here, we reported that the first and the third SH3 domains of Nck-1 interact with the C-terminal region of the $\beta$ subunit of the eukaryotic initiation factor 2 (eIF2$\beta$). Binding of eIF2$\beta$ was specific to the SH3 domains of Nck-1, and *in vivo*, the interaction Nck/eIF2$\beta$ was demonstrated by reciprocal coimmunoprecipitations. In addition, Nck was detected in a molecular complex with eIF2$\beta$ in an enriched ribosomal fraction, whereas no other SH2/SH3 domain-containing adapters were found. Cell fractionation studies demonstrated that the presence of Nck in purified ribosomal fractions was enhanced after insulin stimulation, suggesting that growth factors dynamically regulate translocation of Nck to ribosomes. In HEK293 cells, we observed that transient overexpression of Nck-1 significantly enhanced Cap-dependent and -independent protein translation. This effect of Nck-1 required the integrity of its first and third SH3 domains originally found to interact with eIF2$\beta$. Finally, *in vitro*, Nck-1 also increased protein translation, revealing a direct role for Nck-1 in this process. Our study demonstrates that in addition to mediate receptor tyrosine kinase signaling, Nck-1 modulates protein translation potentially through its direct interaction with an intrinsic component of the protein translation machinery.**

Translation initiation is a complex process in which initiator tRNA (Met-tRNA$_i$) and the 40S and 60S ribosomal subunits of initiator tRNA (Met-tRNA$_i$) are assembled into 80S ribosomes at the initiation codon of mRNA by the coordinated action of the eukaryotic initiation factors (eIFs). Thus far, signaling-dependent events regulating eIFs have involved changes in their intrinsic activity or protein-interacting properties, resulting from their phosphorylation/dephosphorylation and/or sequestration into inactive complex. For example, eIF2 is a molecular complex of three subunits ($\alpha$, $\beta$, and $\gamma$) responsible for one of the earliest steps in the initiation of protein synthesis (1). eIF2 forms a ternary complex with the Met-tRNA and GTP and, in collaboration with other initiation factors, binds the 40S ribosomal subunit to give rise to the preinitiation 43S complex (2). Inhibition of protein synthesis correlates with the phosphorylation of the eIF2$\alpha$ subunit (1) by the heme-regulated eIF2$\alpha$ kinase (HRI) (3), the IFN-inducible RNA-dependent protein kinase (PKR) (4), the serum starvation kinase (GCN2) (5), and the endoplasmic reticulum stress kinase (PERK) (6). Phosphorylation of eIF2$\alpha$ on Ser-51 by these eIF2$\alpha$ kinases inhibits the early steps of translation by blocking on eIF2 the exchange of GDP for GTP, a reaction under the control of the guanine exchange factor, eIF2B (7, 8). Thus, growth factors may enhance initiation of protein translation by preventing activation of eIF2$\alpha$ kinases or by activating specific phosphatases to maintain low levels of eIF2$\alpha$ phosphorylation. In contrast to eIF2$\alpha$, the $\beta$ and $\gamma$ subunits of eIF2 known to interact with critical components of the translational initiation machinery such as eIF2B, eIF5, mRNA, GTP, and Met-tRNA (9–11), have not yet been reported to be regulated by signaling molecules.

To date, with the exception of the p85 adapter subunit of phosphatidylinositol 3′-kinase (PI-3K), no SH2/SH3 domain-containing adapters have been directly implicated in the regulation of the initiation of protein synthesis by growth factors. Here, we demonstrate a direct interaction between the adapter protein Nck-1 and the translation initiation factor subunit eIF2$\beta$ in an enriched ribosomal fraction. Given these observations and the striking finding that the presence of Nck in ribosomes was increased upon insulin treatment, we investigated the role of Nck-1 in protein translation.

## Materials and Methods

**Antibodies.** Nck antiserum was prepared as described (12). eIF2$\beta$ antisera were obtained after rabbit immunization with a glutathione *S*-transferase (GST) chimera of the C-terminal region of mouse eIF2$\beta$ (residues 133–333). L4 antibody was provided by C. Nicchitta (Duke University Medical Center, Durham, NC). p85, CrkII, Grb2, and hemagglutinin (HA) antibodies were purchased from Santa Cruz Biotechnology.

**Cell Culture.** Transformed rat hepatocytes overexpressing the human insulin receptor (HTC-IR) cells were grown in DMEM (Invitrogen) containing 10% FBS (Invitrogen) and Geneticin (G418) at 40 $\mu$g/ml (Invitrogen). Human embryonic kidney 293 (HEK293) cells were grown in DMEM containing 10% FBS.

**Yeast Two-Hybrid Assays.** Yeast two-hybrid screen was performed as described (13). pACTII vector encoding the transcriptional activation domain of Gal4 fused to mouse T cell cDNA library fragments were introduced into *Saccharomyces cerevisiae* strains already transfected with the pASI vector encoding a fusion between the DNA-binding domain of Gal4 and the three SH3 domains of Nck-1 (residues 1–251).

**Immunoprecipitation.** Mouse tissues were homogenized in 5 mM Tris, pH 7.4/1 mM $MgCl_2$/0.25 M sucrose/2 mM $NaVO_4$/1 mM PMSF/1 mM benzamidine/1 mM NaF and centrifuged at 200,000 $\times$ *g* for 30 min. The resulting supernatants were submitted to Nck or eIF2$\beta$ immunoprecipitation. HTC-IR lysates were prepared as described (12). Clarified lysates were submitted to either Nck or eIF2$\beta$ immunoprecipitation and analyzed by immunoblotting.

**Nck-1 Constructs and Transfection.** Human Nck-1 mutants were produced by overlapping PCR with specific primers containing appropriate mutated sites (14–16), subcloned into pcDNA 3.1Myc/His plasmid (Invitrogen) and sequenced. The HA-tagged Nck-1 constructs subcloned into pRK5 were provided by W. Li (Norris Cancer Center, University of Southern California, Los Angeles). Transient transfections of HEK293 cells were performed by using calcium phosphate precipitation.

**GST Fusion Proteins.** Wild-type Nck-1 and eIF2$\beta$ cDNAs were subcloned into pGEX-2TK and GST fusion proteins were expressed, purified as recommended by the manufacturer (Amer-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: eIF, eukaryotic initiation factor; PI-3K, phosphatidylinositol 3′-kinase; SH, Src homology domain; IRES, internal ribosomal entry site; NRS, normal rabbit serum; TCL, total cell lysate; IP, immunoprecipitation; WB, immunoblotting; GST, glutathione *S*-transferase; HA, hemagglutinin.

‡To whom reprint requests should be addressed. E-mail: louise.larose@mcgill.ca.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.



www.pnas.org/cgi/doi/10.1073/pnas.082483399

sham Pharmacia), and used for binding assay experiments. GST and GST-Nck-1 were eluted from the glutathione beads before their use in overlay assays or in the *in vitro* translation system.

**Overlay Assay.** Recombinant GST and GST-Nck-1 proteins (5 $\mu$g) were spotted onto nitrocellulose and dried. Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBST) and 10% nonfat dry milk and probed overnight at 4°C with $^{32}$P-labeled GST-eIF2$\beta$. Membranes were washed in TBST and exposed for autoradiography.

**Binding Assay.** HEK293 cells transiently transfected with empty vector or plasmid encoding either HA-tagged Nck-1 wild type or mutated in its individual SH3 domains were lysed (10 mM Hepes, pH7.4/1% (vol/vol) Triton X-100/10 $\mu$g/ml aprotinin/10 $\mu$g/ml leupeptin/1 mM PMSF). After preclearing with glutathione immobilized on beads, lysates were mixed with 25 $\mu$g of GST or GST-eIF2$\beta$ recombinant protein for 2 h at 4°C. Samples were analyzed by immunoblotting with Nck (Transduction Laboratories, Lexington, KY) or HA antibodies.

**Sucrose Cushion Sedimentation.** Sucrose cushion sedimentation on confluent HTC-IR and HEK293 cells were performed as described (17). Supernatants were trichloroacetic acid-precipitated and resuspended in Laemmli buffer (18) or subjected to immunoprecipitation with either Nck, eIF2$\beta$ antibodies, or normal rabbit serum (NRS). Pellets were resuspended in Laemmli buffer or solubilized with 30 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Triton-X-100 containing protease inhibitors and heated at 65°C for 10 min to facilitate protein resuspension before immunoprecipitation. Samples were analyzed by immunoblotting with either Nck, eIF2$\beta$, p85, Grb2, CrkII, or L4 antibodies.

**Subcellular Fractionation.** Insulin-stimulated and nonstimulated HEK293 cell lysates were prepared and layered onto a 15–35% linear sucrose gradient as described (19). Fractions were analyzed by immunoblotting with either Nck, eIF2$\beta$, p85, or L4 antibodies.

**Luciferase Assay.** The bicistronic reporter plasmid pcDNA3-RLUC-POLIRES-FLUC was kindly provided by N. Sonenberg (McGill University, Montreal) (20). HEK293 cells were transiently transfected with 0.5 $\mu$g of the reporter vector and either 0.5 $\mu$g of plasmids encoding various Myc-Nck-1 molecules (Fig. 1*A*). *Renilla reniformis* luciferase (RLUC) and firefly luciferase (FLUC) activities were measured by using a dual-luciferase reporter assay system (Promega) in a luminometer (LUMAT) 36 h after transfection.

***In Vitro* Transcription and Translation Assay.** Linearized pcDNA3-RLUC-POLIRES-FLUC vector was *in vitro* transcribed and translated in the TNT-coupled wheat germ Extract System (Promega) supplemented with [$^{35}$S]methionine and increasing amounts of recombinant GST-Nck-1. Samples were prepared for [$^{35}$S]methionine incorporation assay according to the manufacturer's instructions (Promega) and quantified by scintillation counting.

**Quantitative RNA Analysis.** Trizol (Invitrogen)-prepared RNAs (5 $\mu$g) from HEK293 cells transiently cotransfected with the luciferase reporter vector and other various Myc-Nck-1 plasmids (Fig. 1*A*) were converted into single-stranded cDNAs (SuperScript II kit, Invitrogen) followed by PCR reactions with the primers FLUC: 5′-TACAATTTGGACTTTCCGCC-3′and 5′-TTCTTCGCCAAAAGCACTCT-3′, RLUC: 5′-AACGCGGCCTCTTCTTATTT-3′, and 5′-TATCAGGCCATTCATCCCAT-3′ and classic 18S internal standard primers

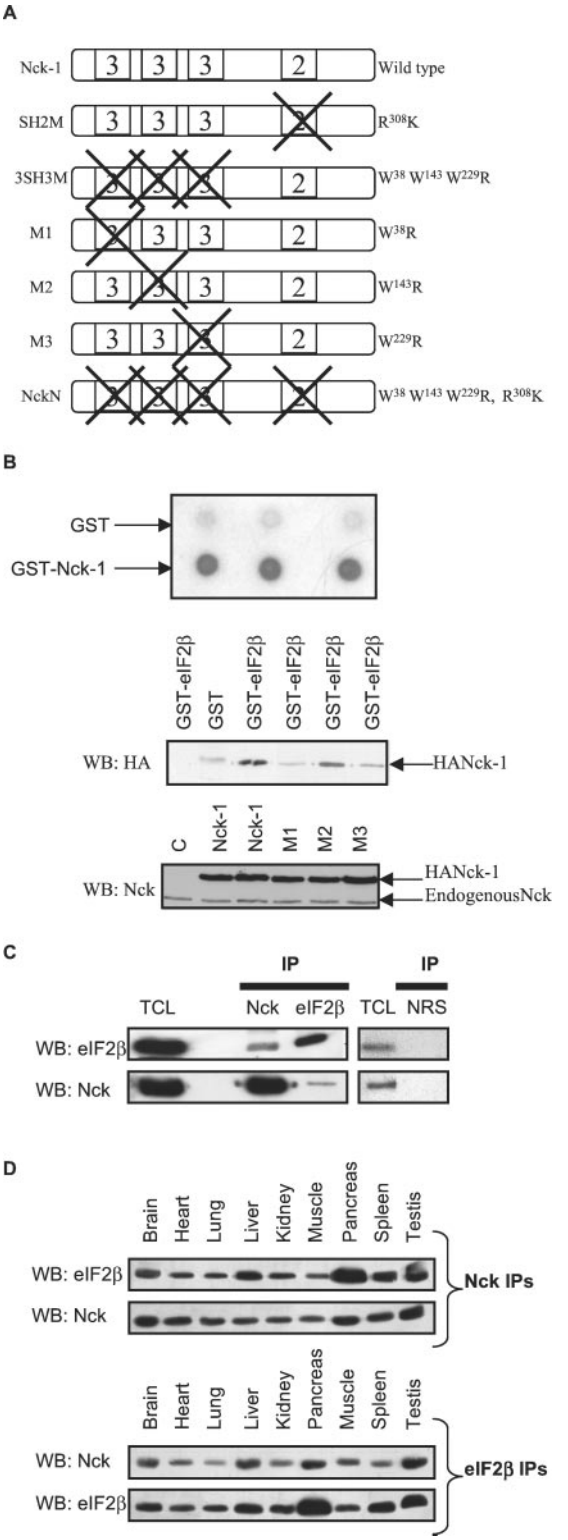


**Fig.1.** Nck and eIF2$\beta$ interaction. (*A*) Schematic representation of Nck-1 molecules. X represents functional mutation of the SH domain. (*B*) Overlay assay (*Top*). Autoradiography of GST or GST-Nck-1 (5 $\mu$g) triplicates immobilized on nitrocellulose and overlaid with $^{32}$P-labeled GST-eIF2$\beta$. Binding assay (*Middle* and *Bottom*). *In vitro* binding by GST or GST-eIF2$\beta$ (25 $\mu$g) of HA-Nck-1 molecules transiently expressed in HEK293 cells (*Middle*). Expression of endogenous and HA-tagged Nck-1 (*Bottom*). (*C*) HTC-IR cell lysates subjected to Nck, eIF2$\beta$, or NRS immunoprecipitation. (*D*) Tissue homogenates from adult male BalbC mice. Four milligrams of proteins used for coimmunoprecipitations and 1 mg for protein expression. Results are typical of two (tissues) and three (cultured cells) experiments.

**Table 1. Yeast two-hybrid assay**

| Molecules | Interaction with eIF2$\beta$ |
|---|---|
| Nck 3SH3 | + |
| Nck SH3-1 | + |
| Nck SH3-2 | − |
| Nck SH3-3 | + |
| Abl | − |
| Grb2 SH3-N | − |
| Grb2 SH3-C | − |
| CrkII SH3-C | − |
| PLC$\gamma$-1 SH3 | − |

Individual Nck SH3 domains and SH3 domains of Grb2, CrkII, p85 of PI-3K, Abl, and PLC$\gamma$-1 were used in yeast two-hybrid assays to characterize their interaction with eIF2$\beta$. The 3SH3 construct is the region of Nck comprising the three Src homology 3 domains (residues 1–251). The SH3-1 (residues 1–56), SH3-2 (residues 113–160) and SH3-3 (residues 197–251) constructs denote the first or second or third SH3 domain of Nck, respectively. SH3-C and SH3-N denote the C-terminal and N-terminal SH3 domain of Grb2, respectively.

(QuantumRNA, Ambion, Austin, TX). PCR reactions were supplemented with 10 $\mu$Ci of 3000 Ci/nM of [$\alpha$-$^{32}$P]dATP. Size markers were prepared by 5′ end-labeling of 1-kb ladder marker (Invitrogen). Samples were submitted to electrophoresis on agarose, transferred onto nylon membranes, and analyzed by autoradiography; reciprocal bands were quantified by densitometry.

## Results

**Proteins Interacting with the SH3 Domains of Nck-1.** Two clones encoding a fusion protein containing the C-terminal region of the $\beta$ subunit of the eukaryotic initiation factor 2 (eIF2$\beta$) were identified interacting with the SH3 domains of Nck-1 in the yeast two-hybrid system. Further characterization of this interaction in the same system revealed that the first and the third SH3 domains of Nck-1 were positive, but neither the second SH3 domain of Nck-1 nor the SH3 domains of Abl, Grb2, CrkII or PLC$\gamma$-1 interacted with eIF2$\beta$ (Table 1). By overlay assays, we observed a direct interaction of GST-eIF2$\beta$ with GST-Nck-1 (Fig. 1*B Top*). Furthermore, *in vitro* binding assays (Fig. 1*B Middle* and *Bottom*) also showed that Nck-1 interacts with eIF2$\beta$ and that this interaction required the first and the third SH3 domains of Nck-1.

***In Vivo* Nck/eIF2$\beta$ Interaction.** Reciprocal coimmunoprecipitations with lysates from cultured cells (Fig. 1*C*) and mouse tissue homogenates (Fig. 1*D*) were performed. As reported by others for Nck (21, 22) and expected for eIF2$\beta$, the two proteins are expressed in all tissues with high levels of expression in brain, pancreas, spleen, and testis (Fig. 1*D*). Furthermore, Nck and eIF2$\beta$ coimmunoprecipitated in HTC-IR (Fig. 1*C*), HEK293 (data not shown) cell lysates, and tissue homogenates (Fig. 1*D*), demonstrating *in vivo* their interaction.

**Nck and eIF2$\beta$ Colocalize in a Ribosome-Enriched Fraction.** By using the sucrose cushion sedimentation, a ribosome-enriched pellet (P) and supernatant containing soluble cytosolic components (S) were generated from HEK293 and HTC-IR cells. In both cell lines eIF2$\beta$, as expected, and Nck are detected in the enriched ribosomal fraction (Fig. 2*A*). Other SH2/SH3 domain-containing adapter molecules such as Grb2, CrkII (Fig. 2*A Lower*) or the p85 subunit of PI-3K (data not shown) were not detected in the ribosomal-enriched fraction. As control, immunoblot for the ribosomal protein L4 showed that the pellets were enriched in ribosomes (Fig. 2*A*).

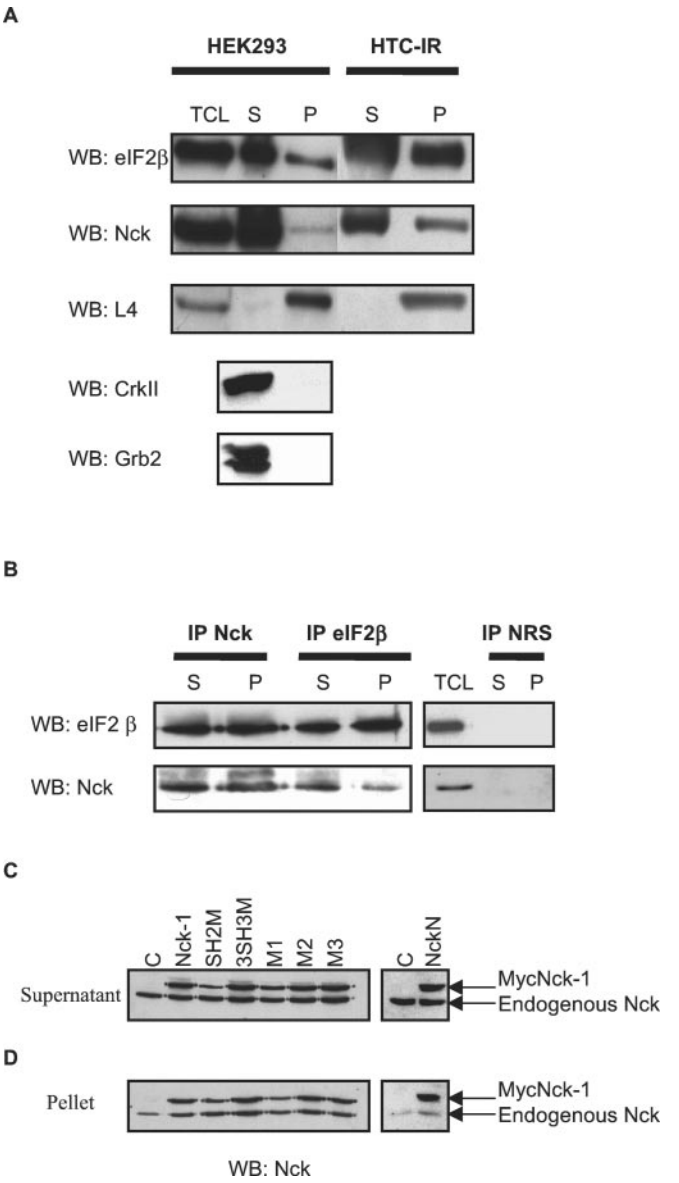


**Fig. 2.** Nck is detected and interacts with eIF2$\beta$ in an enriched ribosomal fraction, and Nck ribosomal localization is independent of its functional SH domains. Sucrose cushion sedimentation. (*A*) Immunoblot analysis of an aliquot of the supernatant (S) and the entire pellet (P). (*B*) Sucrose cushion supernatants (S) and resuspended pellets (P) were immunoprecipitated in duplicate by using Nck, eIF2$\beta$ antibodies, or NRS. One tenth of the immunoprecipitated supernatant and the entire immunoprecipitated pellet were used for immunoblotting. Results are representative of three independent experiments. (*C* and *D*) Immunoblot analysis of one tenth of the supernatants (*C*) and the total pellets (*D*) from HEK293 cells transiently transfected with various Myc-Nck-1 constructs. Results shown are typical of two independent experiments.

**Nck and eIF2$\beta$ Coimmunoprecipitate from the Ribosome-Enriched Fraction.** To determine whether Nck and eIF2$\beta$ interact in the ribosomal-enriched fraction, endogenous Nck and eIF2$\beta$ were coimmunoprecipited by using supernatant and pellet fractions after sucrose cushion sedimentation. As shown in Fig. 2*B*, Nck was detected in eIF2$\beta$ immunoprecipitates in pellet and supernatant fractions, and reciprocal results were also obtained for eIF2$\beta$ in Nck immunoprecipitates. Taken together, these results demonstrate that Nck and eIF2$\beta$ are part of the same molecular complex in this compartment.

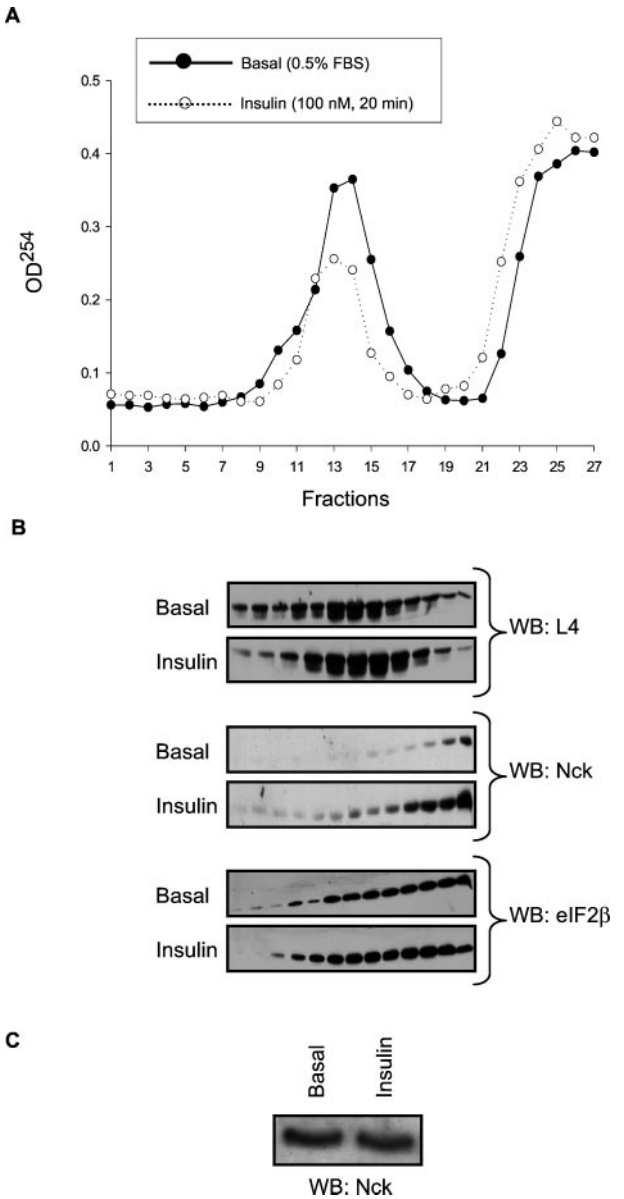


**Fig. 3.** Translocation of Nck to ribosomal fractions is enhanced upon insulin stimulation. Equal amounts of proteins from insulin-stimulated and nonstimulated HEK293 cells were separated 15–35% linear sucrose gradients. (*A*) Ribosomal profile. Optical density at 254nm representing the RNA content of each fractions. (*B*) Nck and eIF2$\beta$ detected in ribosomal fractions. Immunoblot analysis of each fraction. Membranes were cut and the lower part probed for the ribosomal protein L4, whereas the upper part was probed first for Nck, then stripped and reprobed for eIF2$\beta$. (*C*) Total Nck levels upon insulin stimulation. Nck immunoblot analysis. Results are representative of three independent experiments.

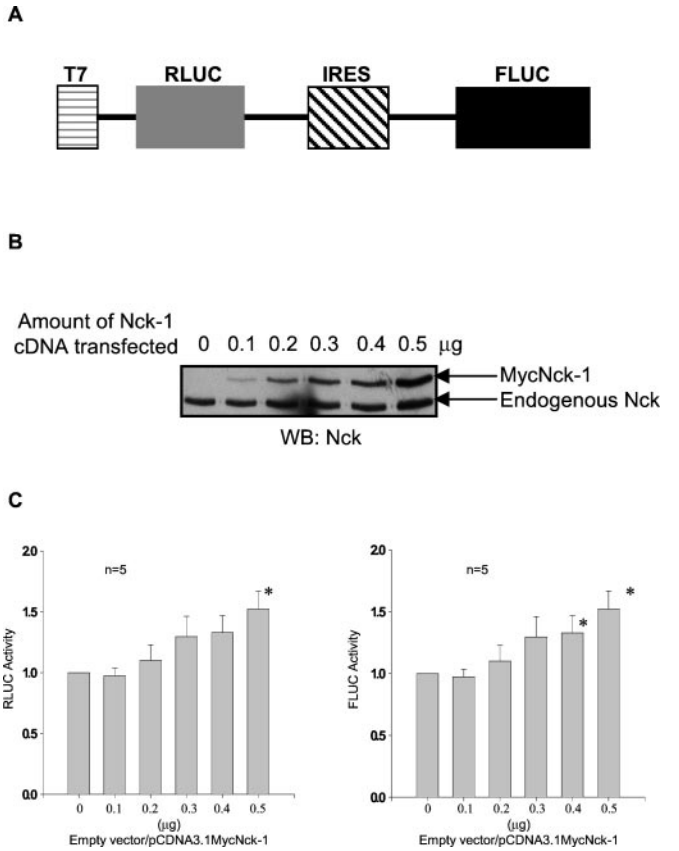


**Fig. 4.** *In vivo*, Nck-1 enhances both Cap-dependent and -independent protein translation. (*A*) Schematic representation of the pcDNA3-RLUC-POLIRES-FLUC reporter vector. (*B*) Immunoblot analysis for the presence of endogenous Nck and Myc-Nck-1 in HEK293 cells. (*C*) Luciferase activity of the FLUC cistron (*Left*) and the RLUC cistron (*Right*) measured 36 h postransfection from HEK293 cells cotransfected with reporter vector and increasing amounts of MycNck-1 constructs. Experiments were performed five times in triplicate, and the results represent the mean value ± SEM. *, At least $P < 0.001$, as determined by Student's *t* test compared with control.

**Nck Translocation to Ribosomes Is Independent of the Functional Integrity of Its SH Domains.** To determine whether the SH domains of Nck were involved in mediating its ribosomal localization, enriched ribosomal fractions were prepared from HEK293 cells transiently transfected with various Myc-Nck-1 constructs (Fig. 1*A*). All Nck-1 mutants were detected in the ribosomal-enriched fraction (Fig. 2*D*), demonstrating that the localization of Nck into ribosomes is modulated by a mechanism independent of its Src homology domains.

**The Presence of Nck in Purified Ribosomal Fractions Is Enhanced After Insulin Stimulation.** To further characterize whether Nck localization to ribosomes is modulated by growth factor stimulation, sedimentation on linear sucrose gradients of lysates from serum-starved HEK293 cells treated or not with insulin were performed. The assignment of ribosomal-enriched components were in fractions 8 to 18 as revealed by immunoblotting for the ribosomal protein L4 (Fig. 3*B Top*). These fractions were then immunoblotted for the presence of Nck (Fig. 3*B Middle*), eIF2$\beta$ (Fig. 3*B Bottom*) and p85 of PI-3K (data not shown). As reported (19), the distribution of RNA content suggested that fractions 1–8 contain the polyribosomes, whereas fractions 13–17 represent the ribosomal subunits (Fig. 3*A*). Nck association with ribosomal subunits was importantly enhanced by insulin stimulation as compared with basal state (Fig. 3*B Middle*). eIF2$\beta$ was detected in both conditions, but with a slight increase upon insulin treatment. p85 of PI-3K was not detected in the ribosomal fractions under both conditions (data not shown). Finally, the increase in the amount of Nck associated with ribosomal subunits upon insulin treatment did not result from a nonspecific effect of insulin on total Nck content (Fig. 3*C*). Taken together, these results demonstrate that Nck is dynamically translocated into ribosomal compartments upon insulin stimulation.

**Nck-1 Overexpression Increases Protein Translation in HEK293 Cells.** We studied the effects of Nck-1 on protein translation. HEK293 cells were cotransfected with a reporter plasmid (Fig. 4*A*) (20) and increasing amounts of Myc-tagged Nck-1. DNA transfected

CELL BIOLOGY

was kept constant at 1 $\mu$g by the addition of the empty vector. In this system, translation of RLUC is Cap-dependent, whereas translation of the FLUC cistron, directed by the poliovirus internal ribosomal entry site (IRES), is Cap-independent (23). Expression of Nck-1 correlated with the amount of Nck-1 cDNA transfected (Fig. 4*B*). Overexpression of Nck-1 enhanced the activity of both luciferases proportionally to its expression levels and reached a maximum of 1.5-fold increase at 0.5 $\mu$g of cDNA transfected (Fig. 4*C*). Higher concentrations of Nck-1 cDNA (1 and 2 $\mu$g) were detrimental to the cells (data not shown).

**Nck-1 Effect on Translation Depends on the Integrity of Its First and Third SH3 Domains.** We further characterized the Nck-1 effect by determining the domain(s) responsible for the enhancement of translation. Myc-tagged Nck-1 constructs cotransfected independently with the bicistronic luciferase reporter vector into HEK293 cells showed comparable expression levels (Fig. 5*A*). The stimulatory effect of Nck-1 on translation was completely abolished by functional mutation of its first, third SH3, or all SH3 domains, whereas the Nck-1 mutated in its second SH3 domain resulted in a slight increase as compared with wild-type Nck-1 (Fig. 5*B*). In the transfected cells above, we found similar amounts of both luciferase mRNAs as demonstrated by quantitative PCR and densitometric analysis (Fig. 5*C*). These data confirm that the effect of Nck-1 is on translation only and depends on the functional integrity of its first and third SH3 domains.

**Nck-1 Enhances Protein Translation *in Vitro*.** We assessed the *in vitro* translation of the luciferase mRNAs in the presence of increasing amounts of recombinant GST-Nck-1. As shown in Fig. 5*D*, GST-Nck-1 at 0.9 $\mu$g and 1.2 $\mu$g significantly enhanced overall translation of luciferase mRNAs by 1.5- to 2.0-fold. Results from *in vitro* translation strongly support a direct role for Nck-1 in the regulation of protein translation.

## Discussion

This study reports an SH3-mediated interaction between the adapter Nck-1 and eIF2$\beta$, a component of the eIF2 complex responsible for one of the earliest steps in the initiation of protein synthesis. Here, we demonstrated that Nck and eIF2$\beta$ not only interact but also colocalize in ribosomal subcellular compartments. Furthermore, we also provided evidence that in these compartments, their levels are regulated by insulin, a hormone known to stimulate protein synthesis. We observed that the ribosomal localization of Nck is independent of its Src homology domains, suggesting that a mechanism, at least independent of its interaction with eIF2$\beta$, is mediating its translocation to ribosomes. Given that Nck is reported to be phosphorylated on several residues after growth factor stimulation (24, 25), we hypothesize that such postranslational modifications may govern its translocation to ribosomal compartments.

Our study also shows that *in vivo*, Nck-1 enhances both Cap-dependent and –independent protein translation. In mammals most mRNAs are thought to be translated through a Cap-dependent mechanism involving ribosomal scanning (reviewed in ref. 26). However, a small population of mRNAs, coding for cell survival factors, cell cycle molecules, oncogenes, and viral proteins are translated by a Cap-independent mechanism mediated by direct ribosome binding to IRES elements located in the 5′-untranslated region (5′UTR) (reviewed in ref. 27). Even though this mechanism is thought not to require any translation initiation factors, assembly of the 48S complexes on these IRES elements in an *in vitro* reconstitution assay by using purified eIF2/GTP/Met-tRNA$_i$ complex is sufficient for the 40S to lock onto the initiation codon (28). Therefore Nck-1 effect on both types of translation may suggest that Nck-1, by binding eIF2$\beta$, acts at the level of the eIF2/GTP/Met-tRNA$_i$ ternary

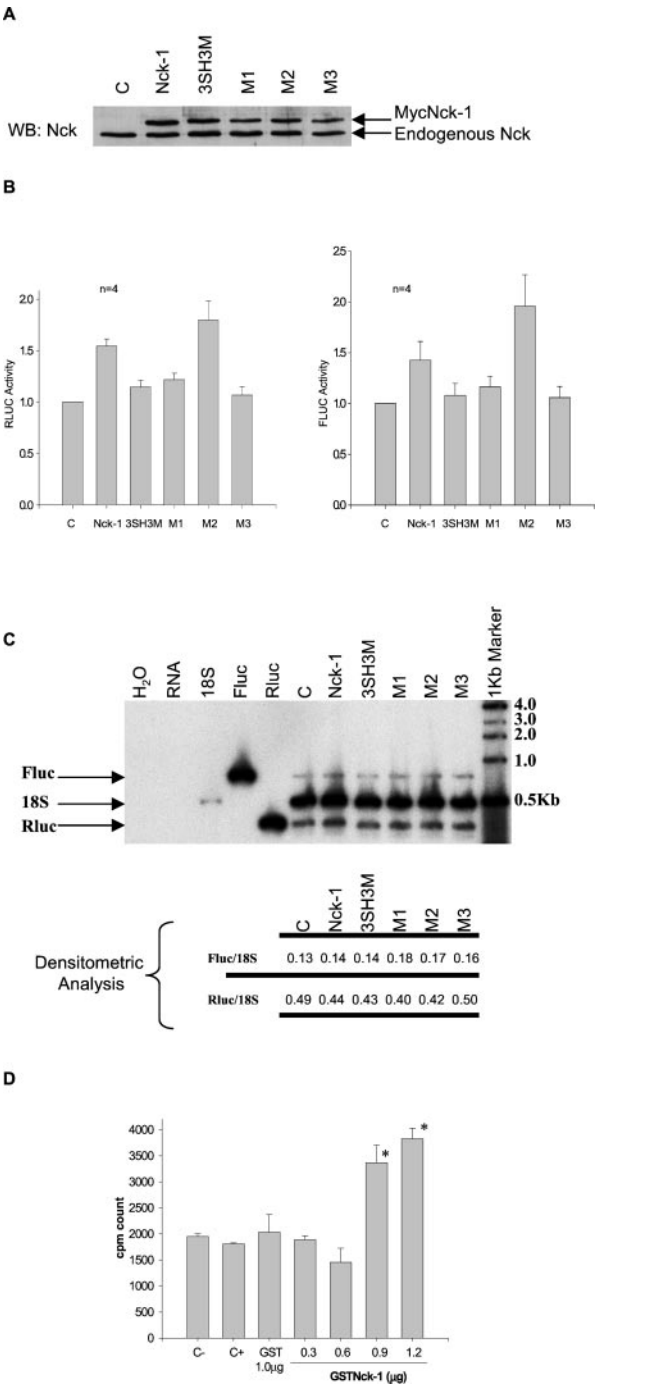


**Fig. 5.** The effect of Nck-1 on protein translation is dependent on the integrity of its first and third SH3 domains. HEK293 cells were cotransfected with the bicistronic luciferase reporter vector (0.5 $\mu$g) and either empty vector or the indicated Myc-Nck-1 constructs (0.5 $\mu$g). (*A*) Immunoblot analysis for the expression of endogenous Nck and Myc-Nck-1 constructs. (*B*) Activity of the RLUC cistron (*Left*) and FLUC cistron (*Right*). Each experiment was performed four times in triplicate, and the results represent the mean ± SEM. (*C*) Quantitative reverse transcription–PCR performed on total RNA prepared from above transfected HEK293. Water ($H_2O$) or control RNA (RNA) were used as controls. Amplified products were analyzed by densitometry for the calculation of the ratios of Fluc/18S and Rluc/18S. Results shown are representative of three experiments. (*D*) *In vitro* translation of luciferase mRNAs in wheat germ extract supplemented with increasing amounts of GST-Nck-1 and incorporation of [$^{35}$S]methionine was measured. C− and C+ denote samples without or with bicistronic luciferase vector, respectively. Results are representative of two independent experiments performed in triplicate. *, At least $P < 0.01$ as determined by Student's *t* test.

complex upstream of both Cap-dependent and independent initiation of translation. This hypothesis is supported not only by the fact that the SH3 domains of Nck-1 important for its effect on protein translation are also responsible for its interaction with eIF2$\beta$, but also by the direct effect on Nck-1 on *in vitro* protein translation.

The involvement of Nck in insulin signaling has been suggested by the fact that Nck-1 and Nck-2 have been shown to bind substrates of the insulin receptor (29–32). Therefore, the insulin receptor could regulate the activity of effector molecules associated with the SH3 domains of Nck. Within this perspective, Sam68, an RNA-binding protein (33), has been reported to interact with Nck (34) and to be a substrate of the insulin receptor (35). Moreover, Sam68 has been proposed to be a regulator of RNA metabolism and protein expression by modifying the mRNA stability and/or mRNA translation (36, 37). Therefore, Sam68 may contribute to the effect of Nck-1 on protein translation by directly targeting specific mRNA to ribosomes through the molecular complex mRNA-Sam68-Nck-1-eIF2$\beta$.

Nck-1 interacts with several protein kinases (24, 25). Among them, we have identified the isoform $\gamma$2 of the Casein Kinase I (CKI $\gamma$2) (12) and recently, several CKI substrates identified by an *in vitro* expression-cloning screening include RNA helicase, nucleolar protein hNOP56, hnRNP A1, and the ribosomal proteins L4, L8, and L13 (38), suggesting a possible involvement of CKI in RNA metabolism and protein translation.

In the past, the regulation of protein translation at the level of initiation by growth factors has been well studied (reviewed in ref. 39). Our contribution clearly establishes that Nck, an adapter protein known to mediate receptor tyrosine kinase signaling at the membrane level, also acts more downstream in the signaling pathways after its translocation to ribosomal compartments. Nck may constitute a target for tight regulation of protein translation at the initiation level through its interaction with eIF2$\beta$. This opens up a field of investigation, which will contribute to a better understanding of how growth factors regulate protein translation.

We thank the members of Dr. Andre Veillette's laboratories for expert technical advice. We also thank Drs. Nahum Sonenberg and Francis Poulin for the kind gift of the bicistronic vector and for technical support. We are also grateful to Dr. C. Nicchitta for the L4 antibody, Dr. W. Li for the HA-tagged Nck constructs, and Ali Fazel, Pamela Cameron, Gerald Baquiran, and Nathalie Bedard for valuable help. This work was supported by a grant from the Canadian Diabetes Association in memory of Aurele Labelle.

1. Hershey, J. W. (1991) *Annu. Rev. Biochem.* **60,** 717–755.
2. Pain, V. M. (1996) *Eur. J. Biochem.* **236,** 747–771.
3. Chen, J. J. & London, I. M. (1995) *Trends Biochem. Sci.* **20,** 105–108.
4. Proud, C. G. (1995) *Trends Biochem. Sci.* **20,** 241–246.
5. Sood, R., Porter, A. C., Olsen, D. A., Cavener, D. R. & Wek, R. C. (2000) *Genetics* **154,** 787–801.
6. Harding, H. P., Zhang, Y. & Ron, D. (1999) *Nature (London)* **397,** 271–274.
7. Rhoads, R. E. (1993) *J. Biol. Chem.* **268,** 3017–3020.
8. Cigan, A. M., Bushman, J. L., Boal, T. R. & Hinnebusch, A. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5350–5354.
9. Asano, K., Krishnamoorthy, T., Phan, L., Pavitt, G. D. & Hinnebusch, A. G. (1999) *EMBO J.* **18,** 1673–1688.
10. Laurino, J. P., Thompson, G. M., Pacheco, E. & Castilho, B. A. (1999) *Mol. Cell. Biol.* **19,** 173–181.
11. Gaspar, N. J., Kinzy, T. G., Scherer, B. J., Humbelin, M., Hershey, J. W. & Merrick, W. C. (1994) *J. Biol. Chem.* **269,** 3415–3422.
12. Lussier, G. & Larose, L. (1997) *J. Biol. Chem.* **272,** 2688–2694.
13. Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N. & Pawson, T. (1997) *J. Biol. Chem.* **272,** 27804–27811.
14. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77,** 51–59.
15. Tanaka, M., Gupta, R. & Mayer, B. J. (1995) *Mol. Cell. Biol.* **15,** 6829–6837.
16. Mayer, B. J., Jackson, P. K., Van Etten, R. A. & Baltimore, D. (1992) *Mol. Cell. Biol.* **12,** 609–618.
17. Chevet, E., Wong, H. N., Gerber, D., Cochet, C., Fazel, A., Cameron, P. H., Gushue, J. N., Thomas, D. Y. & Bergeron, J. J. (1999) *EMBO J.* **18,** 3655–3666.
18. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
19. Siomi, M. C., Zhang, Y., Siomi, H. & Dreyfuss, G. (1996) *Mol. Cell. Biol.* **16,** 3825–3832.
20. Poulin, F., Gingras, A. C., Olsen, H., Chevalier, S. & Sonenberg, N. (1998) *J. Biol. Chem.* **273,** 14002–14007.
21. Li, W., Hu, P., Skolnik, E. Y., Ullrich, A. & Schlessinger, J. (1992) *Mol. Cell. Biol.* **12,** 5824–5833.
22. Park, D. & Rhee, S. G. (1992) *Mol. Cell. Biol.* **12,** 5816–5823.
23. Pelletier, J. & Sonenberg, N. (1988) *Nature (London)* **334,** 320–325.
24. Buday, L. (1999) *Biochim. Biophys. Acta* **1422,** 187–204.
25. McCarty, J. H. (1998) *BioEssays* **20,** 913–921.
26. Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pilipenko, E. V., Shatsky, I. N., Agol, V. I. & Hellen, C. U. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 7029–7036.
27. Kozak, M. (2001) *Mol. Cell. Biol.* **21,** 1899–1907.
28. Pestova, T. V. & Hellen, C. U. (1999) *Virology* **258,** 249–256.
29. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G., Jr., White, M. F., Schlessinger, J. & Skolnik, E. Y. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11713–11717.
30. Xu, P., Jacobs, A. R. & Taylor, S. I. (1999) *J. Biol. Chem.* **274,** 15262–15270.
31. Tu, Y., Liang, L., Frank, S. J. & Wu, C. (2001) *Biochem. J.* **354,** 315–322.
32. Tang, J., Feng, G. S. & Li, W. (1997) *Oncogene* **15,** 1823–1832.
33. Huang, S. (2000) *J. Struct. Biol.* **129,** 233–240.
34. Lawe, D. C., Hahn, C. & Wong, A. J. (1997) *Oncogene* **14,** 223–231.
35. Sanchez-Margalet, V. & Najib, S. (1999) *FEBS Lett.* **455,** 307–310.
36. Morley, S. J. & Thomas, G. (1991) *Pharmacol. Ther.* **50,** 291–319.
37. Brawerman, G. (1989) *Cell* **57,** 9–10.
38. Gao, Z. H., Metherall, J. & Virshup, D. M. (2000) *Biochem. Biophys. Res. Commun.* **268,** 562–566.
39. Taha, C. & Klip, A. (1999) *J. Membr. Biol.* **169,** 1–12.