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3′ Untranslated Region in a Light Neurofilament (NF-L) mRNA Triggers Aggregation of NF-L and Mutant Superoxide Dismutase 1 Proteins in Neuronal Cells

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The pathogenesis of neurodegenerative diseases is believed to involve abnormal aggregation of proteins, but the mechanisms initiating protein aggregation are unclear. Here we report a novel phenomenon that could be instrumental in triggering protein aggregation in neurodegenerative diseases. We show that the 3′ untranslated region (3′UTR) of a light neurofilament (NF-L) transcript enhances the reactivity of its own translated product and leads to loss of solubility and aggregation of NF-L protein and to coaggregation of mutant superoxide dismutase 1 (SOD1) protein. Full-length mouse NF-L cDNAs, with and without NF-L 3′UTR, were fused to the C terminus of a green fluorescent protein (GFP) reporter gene, and the GFP-tagged NF-L proteins were examined in transfected Neuro2a cells. The GFP-tagged NF-L protein expressed from the transgene containing NF-L 3′UTR, but not from the transgene lacking NF-L 3′UTR, colocalizes with endogenous heavy neurofilament protein and, at high-level expression, leads to loss of solubility and aggregation of GFP-tagged NF-L protein. Aggregation of GFP-tagged NF-L protein triggers coaggregation and loss of solubility of coexpressed DsRed-tagged mutant (G93A) SOD1 protein but not wild-type SOD1 protein. Deletional mutagenesis maps the RNA sequence causing aggregation of GFP-tagged NF-L protein to the proximal 45 nucleotides of NF-L 3′UTR. This is the site of a major destabilizing element in NF-L RNA and binding site for RNA-binding proteins. Our findings support a working model whereby NF-L RNA, or cognate RNA-binding factors, enhances the reactivity of NF-L protein and provides a triggering mechanism leading to aggregation of NF-L and other proteins in neurodegenerative diseases.

Key words: **neurofilament; RNA; mutant SOD1 protein; protein aggregation; ubiquitin; heat shock proteins; neuronal inclusions; neurodegeneration; polyglutamine expansion**

Introduction

Accumulations of insoluble protein aggregates as intracellular inclusions or extracellular amyloid deposits are hallmark features of neurodegenerative diseases. They are landmarks for classifying diseases as well as end products of neurodegenerative change. There is also growing consensus that protein aggregation is neurotoxic (Hardy and Selkoe, 2002; Kirkitadze et al., 2002; Taylor et al., 2002; Berke and Paulson, 2003; Caughey and Lansbury, 2003; Ross et al., 2003; Soto, 2003) and that neurotoxic effects arise during early stages of protein aggregation before formation of highly insoluble fibrillary forms (Conway et al., 1998, ,2000; Harper et al., 1999; Hartley et al., 1999; Walsh et al., 1999, 2002; Sanchez et al., 2003). Mechanisms initiating protein aggregation may therefore be particularly relevant in the pathogenesis of neurodegenerative diseases.

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Protein aggregation is also strongly implicated in the pathogenesis of motor neuron degeneration attributable to a mutant Cu/Zn superoxide dismutase (SOD1) protein (Lindberg et al., 2002; Rakhit et al., 2002; Rodriguez et al., 2002; DiDonato et al., 2003; Elam et al., 2003; Khare et al., 2003; Son et al., 2003; Valentine and Hart, 2003). Aggregates of mutant SOD1 protein in degenerating human motor neurons (Shibata et al., 1996) are reproduced in transgenic models (Bruijn et al., 1997, 1998; Johnston et al., 2000; Watanabe et al., 2001; Wang et al., 2002a,b, 2003) and in primary motor neuron cultures (Durham et al., 1997). Neuropathic aggregates are enhanced by proteosome inhibitors (Johnston et al., 2000; Urushitani et al., 2002) and alleviated by coexpression of heat shock proteins (HSPs) (Bruening et al., 1999; Shinder et al., 2001; Takeuchi et al., 2002), ubiquitin (Urushitani et al., 2002), or an E3 ligase that ubiquitinates SOD1 protein (Niwa et al., 2002).

Abnormal neurofilament (NF) aggregates are common pathological features of sporadic and mutant SOD1-linked amyotrophic lateral sclerosis (ALS). However, the role of NF aggregation in motor neuron degeneration is unclear. Altering levels of light NF (NF-L) or heavy NF (NF-H) expression reduces neurotoxicity of mutant SOD1 in transgenic mice (Couillard-Despres et al., 1998; Williamson et al., 1998; Kong and Xu, 2000) but can also have neuropathic effects on motor neurons attribut-

able to high-level expression of an NF transgene (Cote et al., 1993; Xu et al., 1993; Sosa et al., 2003) or low-level expression of NF-L transgenes with a mutant RNA-binding site in the transcript (Lee et al., 1994; Canete-Soler et al., 1999). Expression of NF-L RNA gives rise to a motor neuron phenotype in transgenic mice (Nie et al., 2002) and has neuropathic effects on primary motor neurons in culture (Lin et al., 2003). However, the mechanisms underlying RNA-induced neuropathic effects are still poorly understood.

In the present study, we describe an RNA-induced phenomenon leading to aggregation of NF-L and mutant SOD1 proteins. We report that NF-L sequence in the 3′ untranslated region (3′UTR) of an NF-L transgene has profound effects on the properties of translated protein, causing loss of solubility and aggregation of NF-L protein and of coexpressed mutant (G93A) SOD1 proteins. We further map the RNA sequence causing protein aggregation to the proximal 45 nucleotides of NF-L 3′UTR. The ability of untranslated RNA to trigger aggregation of translated protein is a novel phenomenon with important implications for the pathogenesis of neurodegenerative diseases.

Materials and Methods

Expression vectors. Full-length NF-L coding region and 3′UTR or NF-L coding region lacking NF-L 3′UTR were amplified by PCR from a mouse brain cDNA library (Stratagene, La Jolla, CA) and fused in frame to the C terminus of enhanced green fluorescent protein (EGFP) coding region in pEGFP/C2 expression vector (BD Biosciences Clontech, Palo Alto, CA) to generate pGFP/NF+3′UTR and pGFP/NF−3′UTR transgenes, as shown schematically in Figure 1*A*. Deletion mutants lacking distal 3′UTR were also constructed by PCR to generate transgenes with full length 442 [pGFP/NF+3′UTR(1–442)] or the proximal 345 [pGFP/NF+3′UTR(1–345)], 245 [pGFP/NF+3′UTR(1–245)], 145 [pGFP/NF+3′UTR(1–145)], and 45 [pGFP/NF+3′UTR(1–45)] nucleotides (nt) of NF-L 3′UTR, as shown schematically in Figure 5*A*. The vector with proximal 45 nt of NF-L 3′UTR [pGFP/NF+3′UTR(1–45)] was further mutated by placing the 45 nt of NF-L 3′UTR in the antisense orientation [pGFP/NF+3′UTR(45–1)]. A GFP reporter transgene with NF-L sequence in 3′UTR (pEGFP/NF-L RNA) was described previously (Lin et al., 2003). GFP-tagged NF-L protein was expressed as a 97 kDa protein in transfected cells (see Figs. 1*J*, 2*L*, 5*F*).

HSP40 and HSP70 were amplified by PCR from a mouse spinal cord cDNA library (Stratagene) and cloned into pDsRed1C1 (BD Biosciences Clontech) or pHM6 (Roche Applied Science, Indianapolis, IN) vectors. cDNA encoding human SOD1 protein was amplified from total RNA of HeLa cells by reverse transcription-PCR and fused in frame to the C terminus of DsRed1 cDNA in pDsRed1C1. G93A point mutation was constructed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the instructions of the manufacturer. DsRed-tagged wild-type (wt) and mutant proteins were expressed as 45 kDa proteins in transfected cells (see Fig. 6*M*). Q112AR, obtained from Dr. Andrew Lieberman (University of Michigan Medical School, Ann Arbor, MI), was fused in frame to the C terminus of DsRed2 in pDsRed2C1 (Clontech). Androgen receptor (AR) peptides with smaller expansions were obtained from contractions during amplification of the parent clone. The integrity of all DNA constructs were verified by sequencing.

Transfection of neuronal cultures. Mouse neuroblastoma Neuro2a cells maintained in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal calf serum were transfected (2 μg/well, 10 μg/dish) with FuGENE6 (Roche Applied Science), according to the instructions of the manufacturer. Cotransfection studies were done with 1:1 ratio of each vector unless otherwise specified. Cells were collected for Western blots at 24 or 48 hr after transfection.

Immunocytochemistry and confocal microscopy. Cells were fixed in 4% paraformaldehyde for 1 hr at 4°C or in acetone–methanol for 3–5 min at room temperature and permeabilized in 0.5% Triton X-100. Fixed cells were incubated overnight at 4°C with the following primary antibodies: goat polyclonal anti-NF-L (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-NF-H (1:100 dilution; Santa Cruz Biotechnology), goat polyclonal anti-HSP70 antibody (1:100 dilution; Santa Cruz Biotechnology), or rabbit polyclonal anti-ubiquitin (1:500 dilution; Dako, Carpinteria, CA). Secondary antibody incubations were conducted with Alexa Fluor 594 chicken anti-rabbit IgG or Alexa Fluor 594 donkey anti-goat IgG (Molecular Probes, Eugene, OR), and coverslips were mounted in Vectashield mounting medium with 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). GFP fluorescence, DsRed fluorescence, anti-NF-L, anti-NF-H, anti-HSP70, and anti-ubiquitin immunofluorescence were visualized in DAPI-stained cells by multi-track sequential scanning using a Zeiss (Oberkochen, Germany) LSM510 META laser scanning confocal microscope.

Western blot analyses of protein solubility. Neuro2a cells were lysed on ice in lysis buffer containing 10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1× proteinase inhibitor mixture (Sigma, St. Louis, MO), and 1% Triton X-100. Total lysates were fractionated by centrifugation at 13,500 × *g* to obtain detergent-soluble supernatants and detergent-insoluble pellets. To further assess protein solubilities, detergent-insoluble pellets were washed with lysis buffer, solubilized (by vortexing and sonification) in Tris buffer (in mM: 10 Tris-HCl, pH 7.5, 125 NaCl, and 10 EDTA) containing 2 M urea (2 M urea buffer), and centrifuged at 13,500 × *g* to obtain supernatant fractions of proteins solubilized in 2 M urea. Pellets were washed with 2 M urea buffer, solubilized (by vortexing and sonification) in Tris buffer containing 4 M urea (4 M urea buffer), and centrifuged at 13,500 × *g* to obtain supernatant fractions of proteins solubilized in 4 M urea. Pellets were then washed with 4 M urea buffer and solubilized in 1× SDS-PAGE sample buffer (by sonification and boiling) to obtain fractions of proteins solubilized in 2% SDS. Aliquots of proteins in supernatant fractions were resolved on denaturing 4–12% NuPAGE gel (Invitrogen) and transferred to Immobilon-P (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C in TBST–milk (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20, and 5% nonfat milk powder) and incubated sequentially in primary and secondary antibodies (anti-rabbit or anti-goat IgG conjugated with HRP) for 1 hr at room temperature. Labeled proteins were detected using Western blot chemiluminescence reagent (Roche Applied Science). Amounts of protein in each fraction were assessed by densitometric scanning and normalized to the respective dilution factors.

Results

NF-L 3′UTR triggers aggregation and loss of solubility of GFP-tagged NF-L protein in Neuro2a cells

To examine the effects of 3′ untranslated region in NF-L mRNA on the expression of NF-L protein, full-length NF-L coding region with NF-L 3′UTR (pGFP/NF+3′UTR) or NF-L coding region without NF-L 3′UTR (pGFP/NF−3′UTR) was fused in frame to the C terminus of GFP, as shown in Figure 1*A*. Transgenes were constructed with the same coding sequence and stop codon to generate identical GFP-tagged NF-L proteins in transfected cells. Expressed GFP-tagged NF-L proteins had the same 97 kDa electrophoretic mobility on SDS gels (Fig. 1*J*).

Translated proteins were readily visualized by GFP fluorescence (Fig. 1*B*,*D*,*F*,*H*) and confirmed by red anti-NF-L immunofluorescence (Fig. 1*C*,*E*,*G*,*I*). Low- and high-level expression of NF-L transgene lacking NF-L 3′UTR (pGFP/NF−3′UTR) leads to diffuse distribution of NF-L protein throughout perikarya and neuritic processes (Fig. 1*B*,*C*), whereas expression of the same protein from a transgene with NF-L 3′UTR (pGFP/NF+3′UTR) markedly alters the disposition of translated protein (Fig. 1*D–I*). At low-level expression, GFP-tagged NF-L protein appears as short, curvilinear, filamentous arrays (Fig. 1*D*,*E*). At higher levels of expression, GFP-tagged NF-L protein undergoes progressive aggregation, appearing initially as small punctate aggregates (Fig. 1*F*,*G*) that enlarge and coalesce into large irregular juxtanuclear inclusions (Fig.

1*H*,*I*). Hence, expression of transgene with NF-L 3′UTR (pGFP/NF+3′UTR) leads to a concentration-dependent aggregation of GFP-tagged NF-L protein and redistribution of GFP-tagged NF-L protein from filamentous to aggregative forms.

To examine the effects of NF-L 3′UTR on the solubility of GFP-tagged NF-L protein, lysates of Neuro2a cells transfected with pGFP/NF−3′UTR or pGFP/NF+3′UTR were fractionated by centrifugation in buffer containing 1% Triton X-100, and Triton X-100-insoluble pellets were solubilized in a series of buffers with increasing solvent strength. Immunoblot analyses of proteins in total lysates, in Triton X-100-soluble fractions, and in Triton X-100-insoluble fractions solubilized in 2 M urea, 4 M urea, and 2% SDS show that the solubility of GFP-tagged NF-L protein is markedly diminished when expressed from a transgene containing NF-L 3′UTR (+) compared with GFP-tagged NF-L protein expressed from a transgene lacking NF-L 3′UTR (−) (Fig. 1*J*). GFP-tagged NF-L protein expressed from a transgene with NF-L 3′UTR (filled columns) has a >100-fold loss of the Triton X-100-soluble fraction and a 22-fold increase of the Triton X-100-insoluble fraction solubilized in 2% SDS compared with the same protein expressed from a transgene lacking NF-L 3′UTR (open columns) (Fig. 1*K*). The findings indicate that the presence of NF-L 3′UTR in the transgene causes loss of solubility as well as aggregation of GFP-tagged NF-L protein in transfected cells.

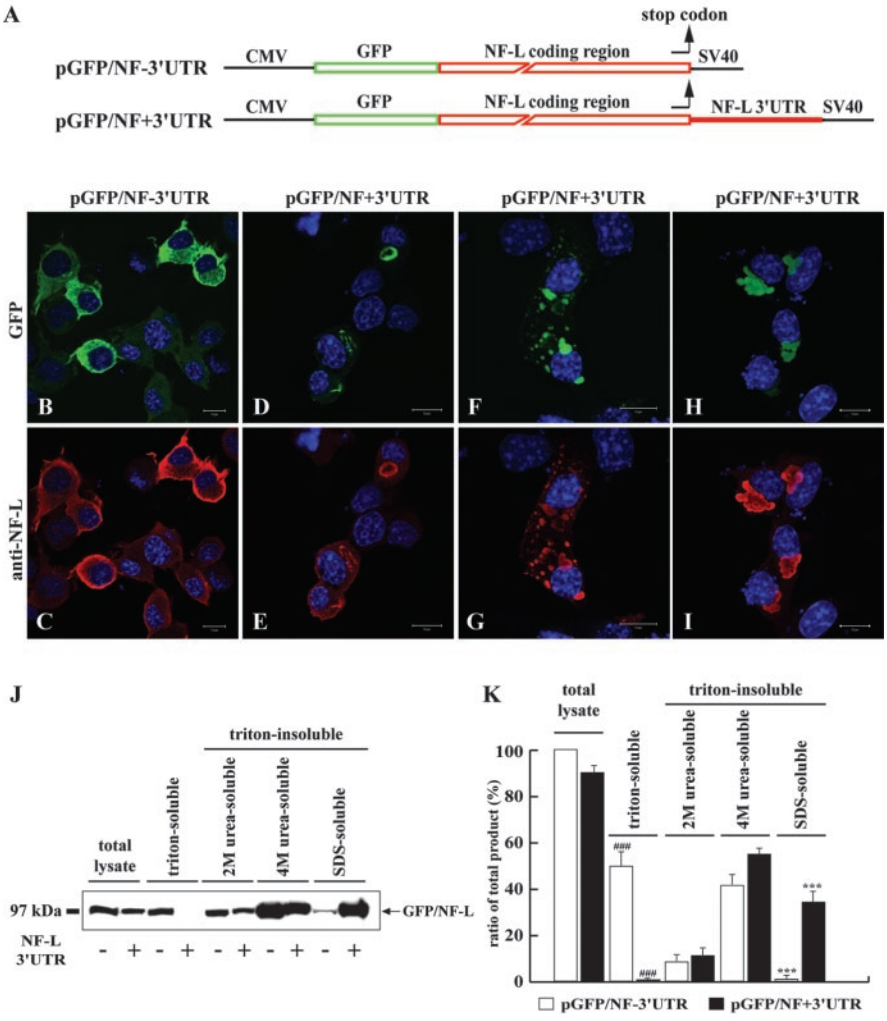

Figure 1. NF-L sequence in 3′UTR of transgene affects the disposition of GFP-tagged NF-L protein in transfected Neuro2a cells. *A*, Schematic diagram of transgenes with (pGFP/NF+3′UTR) or without (pGFP/NF−3′UTR) NF-L 3′UTR sequence (thick red line) in the 3′UTR of pEGFP/C2 expression vector. Transgenes contain identical GFP/NF-L coding sequence (open green–red lines), stop codon (angled arrows), and flanking 5′UTR and 3′UTR sequences (thin lines). *B*, *C*, Confocal microscopy of GFP fluorescence (*B*) and anti-NF-L reactivity (*C*) showing diffuse distribution of GFP-tagged NF-L protein expressed from pGFP/NF−3′UTR transgene. *D*, *E*, GFP-tagged NF-L protein in short, curvilinear filamentous arrays from low-level expression of pGFP/NF+3′UTR. *F*, *G*, GFP-tagged NF-L protein in multiple punctate aggregates from higher-level expression of pGFP/NF+3′UTR. *H*, *I*, GFP-tagged NF-L protein in large juxtanuclear aggregates from high level of pGFP/NF+3′UTR expression. *J*, Anti-GFP immunoblot of GFP-tagged NF-L protein (97 kDa) from cells transfected with pGFP/NF−3′UTR (−) or pGFP/NF+3′UTR (+) in total lysates (total lysate) or lysates separated by centrifugation into supernatant and pelleted fractions showing proteins in pelleted fractions solubilized in buffers of increasing solvent strength, including 1% Triton X-100 (triton-soluble), 2 M urea (2M urea-soluble), 4 M urea (4M urea-soluble), and 2% SDS (SDS-soluble). Immunoblots were conducted on 1:30 aliquots of Triton X-100-soluble fractions, 1:5 aliquots of 2 and 4 M urea-soluble fractions, and 1:2 aliquots of SDS-soluble fractions. *K*, Quantitation of anti-GFP immunoblot of from cells transfected with pGFP/NF−3′UTR (open bars) or pGFP/NF+3′UTR (filled bars) in total lysates (total lysate) or in fractions solubilized in 1% Triton X-100 (triton-soluble), 2 M urea (2M urea-soluble), 4 M urea (4M urea-soluble), and 2% SDS (SDS-soluble). *t* test; $^{***,\#\#\#}p < 0.001$. Scale bars, 10 μm.

NF-L 3′UTR promotes colocalization of NF-L protein with endogenous NF-H protein in Neuro2a cells

Endogenous NF-H immunoreactivity in Neuro2a cells occurs in short, twisted, curvilinear, filamentous structures (Fig. 2*A*,*B*) and in Triton X-100-insoluble fractions (Fig. 2*L*), consistent with a filamentous assembly in Neuro2a cells (Yabe et al., 2003). GFP-tagged NF-L protein expressed from transgenes lacking NF-L 3′UTR (pGFP/NF−3′UTR) does not colocalize with endogenous NF-H but is diffusely distributed throughout the perikarya and neurites at low and high levels of transgene expression (Fig. 2*C–E*). GFP-tagged NF-L protein from a transgene with NF-L 3′UTR (pGFP/NF+3′UTR) colocalizes with endogenous NF-H in the above-described filamentous structures at low-level expression (Fig. 2*F–H*). At high levels of expression, GFP-tagged NF-L protein from pGFP/NF+ 3′UTR undergoes aggregation and no longer colocalizes with NF-H in the filamentous arrays (Fig. 2*I–K*). At the same time, NF-H remains localized in filamentous structures (Fig. 2*D*,*G*,*J*). Immunoblot analyses show that the solubility of endogenous NF-H is not diminished in cells transfected with pGFP/NF+3′UTR compared with cells transfected with pGFP/NF−3′UTR (Fig. 2*L*), whereas the solubility of GFP-tagged NF-L protein is markedly reduced in the same cells transfected with pGFP/NF+3′UTR (Fig. 2*L*). The findings indicate that NF-L 3′UTR promotes interactions of NF-L with NF-H at low-level expression but causes preferential self-interaction and aggregation at higher levels of expression. Moreover, aggregation of GFP-tagged NF-L protein does not trigger coaggregation and loss of solubility of endogenous NF-H protein.

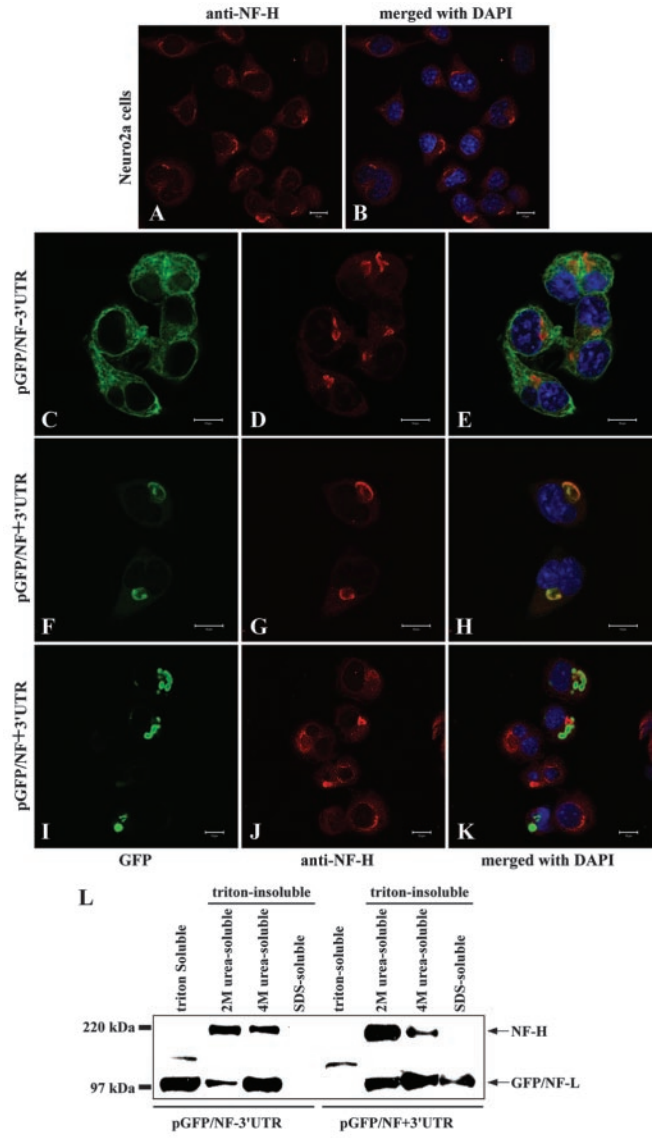

Figure 2. NF-L 3′UTR promotes colocalization of NF-L with endogenous NF-H at low-level transgene expression. *A*, *B*, Endogenous NF-H forms short curvilinear filamentous arrays in untransfected Neuro2a cells. *C–E*, Confocal microscopy of GFP fluorescence (*C*), anti-NF-H reactivity (*D*), and merged images (*E*) showing separate localizations of GFP-tagged NF-L and NF-H in cells transfected with pGFP/NF−3′UTR. *F–H*, GFP-tagged NF-L protein (*F*) colocalize with NF-H (*G*) and in merged image (*H*) of filamentous arrays at low-level pGFP/NF+3′UTR expression. *I–K*, NF-H (*J*) in filamentous arrays does not colocalize with NF-L aggregates (*I*) in merged image (*K*) at high-level pGFP/NF+3′UTR expression. *L*, Anti-GFP and anti-NF-H immunoblots of cell lysates showing loss of solubility of GFP-tagged NF-L (97 kDa) but not loss of endogenous NF-H (220 kDa) solubility in cells transfected with pGFP/NF+3′UTR when lysates were fractionated as described in Figure 1*J*. Scale bars, 10 μm.

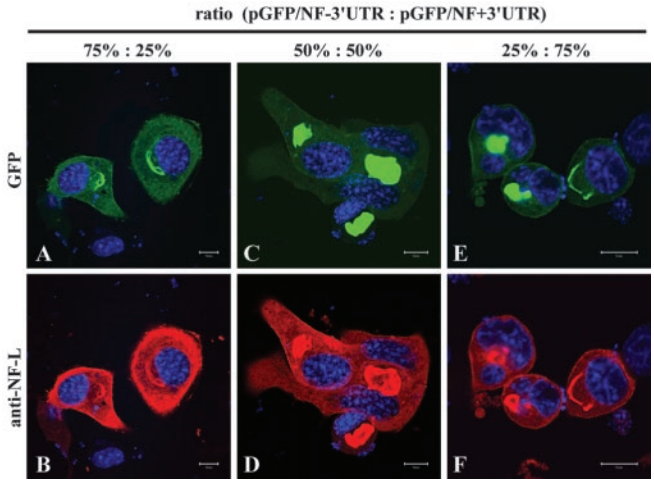

Figure 3. NF-L 3′UTR expressed from pGFP/NF+3′UTR has limited trans-acting aggregative effect on GFP-tagged NF-L protein from pGFP/NF−3′UTR. *A–F*, Confocal microscopy of GFP fluorescence (*A*, *C*, *E*) and anti-NF-L reactivity (*B*, *D*, *F*) of GFP-tagged NF-L protein in cells cotransfected with pGFP/NF−3′UTR and pGFP/NF+3′UTR at 75:25% (*A*, *B*), 50:50% (*C*, *D*), and 25:75% (*E*, *F*) ratios of the respective transgenes. Differing admixtures of diffusely distributed and filamentous or aggregative forms of GFP-tagged NF-L protein correspond to the relative amounts of transfected transgenes. Scale bars, 10 μm.

The ability of NF-L 3′UTR to trigger aggregation of NF-L protein has limited trans-acting effect on the aggregation of NF-L protein expressed from transgene lacking NF-L 3′UTR

To determine whether NF-L 3′UTR-induced aggregative properties on NF-L protein could be conferred to NF-L protein expressed from transgene lacking NF-L 3′UTR, Neuro2a cells were cotransfected with equal amounts of GFP-tagged NF-L transgenes but with varying (75:25%, 50:50%, and 25:75%) ratios of pGFP/NF−3′UTR:pGFP/NF+3′UTR transgenes. We found that the diffusely distributed and the filamentous or aggregated forms of NF-L protein coexist to extents that approximately correspond to the amounts of cotransfected pGFP/NF−3′UTR and pGFP/NF+3′UTR, respectively (Fig. 3). At 75% pGFP/NF−3′UTR, the diffusly distributed form is prominent and admixed with small amounts of filamentous forms (Fig. 3*A*,*B*). As pGFP/NF−3′UTR was reduced to 50% (Fig. 3*C*,*D*) and 25% (Fig. 3*E*,*F*), there is reduction, but not extinction, of diffusely distributed forms of GFP-tagged NF-L. At the same time, there are prominent aggregates of GFP-tagged NF-L protein. The findings indicate that NF-L 3′UTR has limited trans-acting aggregative effects on GFP-tagged NF-L protein expressed from transcripts lacking NF-L 3′UTR. Because the aggregation of NF-L proteins only occurs when NF-L protein is expressed from transcripts containing NF-L 3′UTR, the aggregative effects may be preferentially conferred during or shortly after translation of nascent NF-L protein.

Aggregates of GFP-tagged NF-L protein are ubiquitinated and colocalize with heat shock proteins

The widespread and diffuse expression of GFP-tagged NF-L protein from pGFP/NF−3′UTR does not alter anti-ubiquitin immunohistochemical distribution in Neuro2a cells (Figs. 4*A–C*). However, aggregates of GFP-tagged NF-L protein from pGFP/NF+3′UTR (Fig. 4*D*) colocalize with anti-ubiquitin reactivity (Fig. 4*D–F*). Anti-ubiquitin reactivity was readily seen in large juxtanuclear NF-L protein aggregates but was less apparent in the multiple small punctate aggregates during early stages in NF-L protein aggregation. Aggregates of GFP-tagged NF-L protein also colocalize with hemagglutinin-tagged HSP70 (Fig. 4*G–I*) and DsRed-tagged HSP40 (Fig. 4*J–L*) and alter the fluorescence color of merged images when pGFP/NF+3′UTR is cotransfected with pHM6/HSP70 or pDsRed1/HSP40. However, cotransfection of HSP70 and HSP40 could not prevent protein aggregation triggered by NF-L 3′UTR (data not shown).

A 45 nt sequence of NF-L 3′UTR corresponding to a major destabilizing element of the transcript is sufficient to cause aggregation and insolubilization of GFP-tagged NF-L protein

To map the sequence in NF-L 3′UTR conferring aggregation of NF-L protein, chimeric GFP-tagged NF-L transgenes were con-

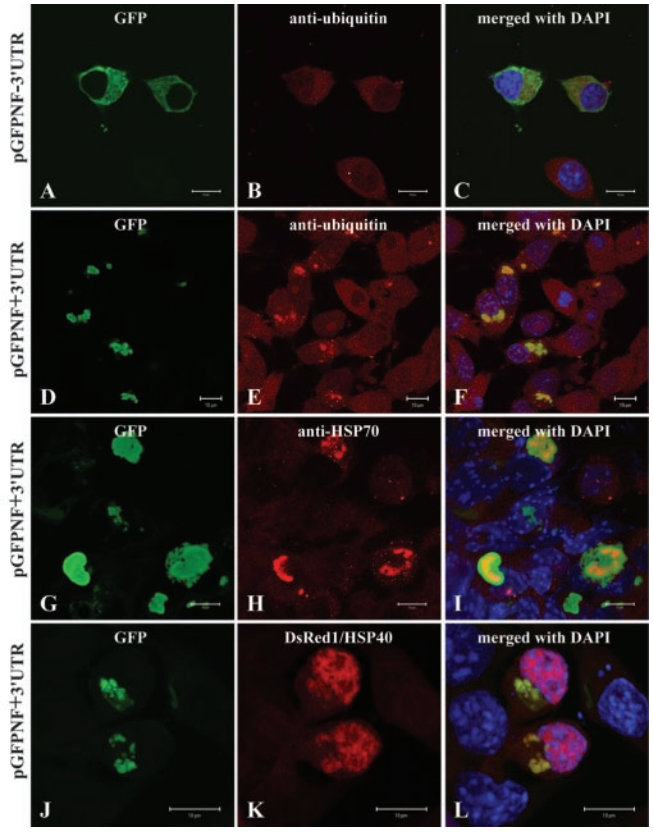

Figure 4. GFP-tagged NF-L protein aggregates colocalize with ubiquitin and heat shock proteins. *A–C*, Confocal microscopy showing similar anti-ubiquitin reactivity (*B*) in untransfected cell and two cells transfected with pGFP/NF−3′UTR. *D–F*, Aggregates of GFP-tagged NF-L protein (*D*) in cells transfected with pGFP/NF+3′UTR colocalize with anti-ubiquitin (*E*) and alter the tincture of the merged image (*F*). *G–I*, Aggregates of GFP-tagged NF-L protein (*G*) colocalize with anti-HSP70 in cells cotransfected with pGFP/NF+3′UTR and pHM6/HSP70 (*H*, *I*). *J–L*, Aggregates of GFP-tagged NF-L protein (*J*) also colocalize with DsRed-tagged HSP40 in cells cotransfected with pGFP/NF+3′UTR and pDsRed1/HSP40 (*K*, *L*). Scale bars, 10 μm.

structed with increasing 3′ deletions of NF-L 3′UTR (Fig. 5*A*). Extensive deletions of NF-L 3′UTR did not abolish RNA-induced aggregation of GFP-tagged NF-L protein (Fig. 5*A*). The aggregative effects of NF-L 3′UTR were reproduced by GFP-tagged NF-L transgenes containing only the proximal 45 nt sequence of NF-L 3′UTR [pGFP/NF+3′UTR(1–45)] (Fig. 5*B*,*C*). The sequence specificity of the proximal 45 nt of NF-L 3′UTR in conferring aggregation of GFP-tagged NF-L protein was demonstrated by showing the aggregative effects are lost when the 45 nt sequence is placed in an antisense orientation [pGFP/NF+3′UTR(45–1)] (Fig. 5*D*,*E*). The ability of the proximal 45 nt of NF-L 3′UTR in the sense orientation [pGFP/NF+3′UTR(1–45)], but not in the antisense orientation [pGFP/NF+3′UTR(45–1)], to cause protein aggregation was also associated with loss of solubility of GFP-tagged NF-L protein (Fig. 5*F*). Because the proximal 45 nt of NF-L 3′UTR coincides with a major destabilizing element of NF-L mRNA and binding site for RNA-binding proteins (Canete-Soler et al., 1998a), the findings strongly suggest that the aggregative properties of NF-L protein conferred by NF-L 3′UTR are triggered by interactions of RNA sequence in the destabilizing element or by interactions of cognate RNA-binding proteins.

RNA-induced NF-L aggregation leads to loss of solubility and aggregation of mutant SOD1 protein

To explore the effects of NF-L 3′UTR on the aggregation of SOD1 protein, wild-type and mutant (G93A) SOD1 proteins were fused in frame to the C terminus of a DsRed-tagged expression vector and cotransfected with GFP-tagged NF-L transgenes with (pGFP/NF+3′UTR) or without (pGFP/NF−3′UTR) NF-L 3′UTR. Cotransfection of pDsRed1/wtSOD1 and pGFP/NF−3′UTR leads to a diffuse distribution of DsRed-tagged wild-type SOD1 in transfected cells (Fig. 6*A–C*). Cotransfection of pDsRed1/SOD1-G93A with pGFP/NF−3′UTR also results in a diffuse distribution of DsRed-tagged mutant SOD1 protein (Fig. 6*D–F*). Cotransfection of pGFP/NF+3′UTR with pDsRed1/wtSOD1 transgene leads to aggregation of GFP-tagged NF-L protein but does not alter the distribution of DsRed-tagged wild-type SOD1 protein (Fig. 6*G–I*). However, cotransfection of pGFP/NF + 3′UTR and pDsRed1/SOD1-G93A leads to aggregation of mutant SOD1 protein and colocalization with GFP-tagged NF-L in protein aggregates (Fig. 6*J–L*). Hence, RNA-induced aggregation of GFP-tagged NF-L protein causes aggregation of mutant, but not wild-type, SOD1 protein in cotransfected cells.

To determine whether the coaggregation and colocalization of GFP-tagged NF-L and mutant SOD1 proteins is associated with altered solubility of SOD1 protein, lysates of cotransfected cells were immunoblotted for anti-SOD1 activity after separation into detergent-soluble and detergent-insoluble fractions. Whereas solubility of 45 kDa DsRed-tagged SOD1 protein is not altered when wild-type SOD1 protein (pDsRed1/wtSOD1) is coexpressed with GFP-tagged NF-L protein from pGFP/NF−3′UTR (−) or pGFP/NF+3′UTR (+) (Fig. 6*M*, 4 left-hand lanes), a fourfold to fivefold increase in Triton X-100-insoluble fraction occurs when mutant SOD1 protein (pDsRed1/SOD1-G93A) is coexpressed with pGFP/NF+3′UTR (+) compared with coexpression with pGFP/NF−3′UTR (−) (Fig. 6*M*, 4 right-hand lanes). Together, the findings indicate the NF-L 3′UTR-induced aggregation of GFP-tagged NF-L protein leads to loss of solubility and coaggregation of mutant SOD1 protein in neuronal cells.

Aggregation of DsRed-tagged mutant SOD1 protein is not induced by NF-L sequence in the 3′UTR of a GFP reporter gene

To determine whether NF-L RNA alone could induce aggregation of DsRed-tagged mutant SOD1 protein, a 680 nt segment comprising the distal coding sequence and entire 3′UTR of mouse NF-L cDNA was placed in the 3′UTR of a GFP reported gene (pGFP/NF-L RNA), as described previously (Lin et al., 2003), and cotransfected with pDsRed1/wtSOD1 (Fig. 7*A–C*) or pDsRed1SOD1-G93A (Fig. 7*D–F*). GFP (Fig. 7*A*,*D*) and DsRed-tagged SOD1 proteins (Fig. 7*B*,*E*) are widely and diffusely distributed in cotransfected cells at low and high levels of transgene expression. The inability of NF-L sequence in the 3′UTR of a GFP reporter gene to induce aggregation of DsRed-tagged mutant SOD1 protein indicates that expression of NF-L RNA sequence, apart from NF-L protein, is insufficient to promote aggregation of mutant SOD1 protein.

RNA-induced aggregation of GFP-tagged NF-L protein does not enhance aggregation of peptide with polyglutamine expansion

To examine the effects of RNA-induced aggregation of GFP-tagged NF-L protein on protein aggregates attributable to expanded polyglutamine repeats, an androgen receptor peptide with expanded polyglutamine repeats [Q112AR (Thomas et al., 2004)] was fused in frame to the C terminal of a DsRed2 reporter protein (pDsRed2-Q112AR) and cotransfected with GFP-tagged NF-L transgenes lacking (pGFP/NF−3′UTR) or containing (pGFP/NF+3′UTR) NF-L 3′UTR. Cotransfection of DsRed-

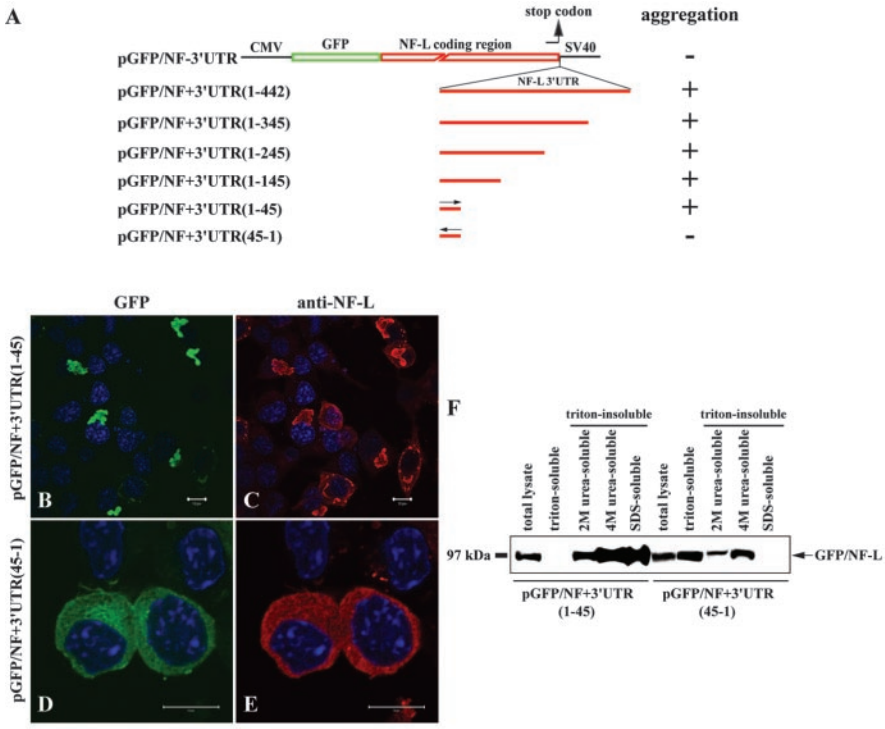

Figure 5. Protein-aggregative properties of NF-L 3′UTR are localized to the proximal 45 nt sequence. *A*, Schematic diagram of GFP-tagged NF-L transgene in pEGFP/C2 vector (pGFP/NF−3′UTR) with NF-L 3′UTR sequence inserted into the proximal 3′UTR. Inserted sequence includes full-length NF-L 3′UTR (1–442) and increasing 3′ deleted sequence in sense (1–345, 1–245, 1–145, and 1–45) or antisense (45–1) orientations. The effects of transgenes on GFP-tagged NF-L protein aggregation are listed. *B*, *C*, Confocal microscopy of GFP fluorescence (*B*) and anti-NF-L reactivity (*C*) showing aggregation of GFP-tagged NF-L protein expressed from pGFP/NF+3′UTR(1–45) transgene. *D*, *E*, GFP-tagged protein from pGFP/NF+3′UTR(45–1) transgene is diffusely distributed and does not undergo aggregation. *F*, Anti-GFP immunoblot of GFP-tagged NF-L protein (97 kDa) from cells transfected with pGFP/NF+3′UTR(1–45) or pGFP/NF+3′UTR(45–1) in total lysates (total lysate) and lysates separated into soluble supernatant and insoluble pelleted fractions, as described in Figure 1*J*. Loss of GFP-tagged NF-L protein solubility occurs in cells transfected with pGFP/NF+3′UTR(1–45) but not in cells transfected with pGFP/NF+3′UTR(45–1). Scale bars, 10 μm.

tagged pDsRed2-Q112AR with pGFP/NF−3′UTR results in aggregates of DsRed-tagged Q112AR (Fig. 7*H*) surrounded by enhanced rims of GFP-tagged NF-L protein (Fig. 7*G*,*I*), whereas most of the GFP-tagged NF-L protein remains diffusely distributed (Fig. 7*G*). When DsRed-tagged pDsRed2-Q112AR is cotransfected with pGFP/NF+3′UTR (Fig. 7*J–L*), aggregates of DsRed-tagged Q112AR (Fig. 7*K*) become closely associated with aggregates of GFP-tagged NF-L (Fig. 7*J*), but the respective fluorescent-tagged proteins remain separate and do not colocalize with each other.

Aggregation of DsRed-tagged Q112AR occurs when pDsRed2-Q112AR is transfected alone (data not shown) or when cotransfected with pGFP/NF−3′UTR (Fig. 7*H*), and there is not appreciable enhancement of aggregation when cotransfected with pGFP/NF+3′UTR (Fig. 7*K*). The findings suggest that aggregates of GFP-tagged NF-L have a much stronger synergistic effects on the aggregation of DsRed-tagged mutant SOD1 protein than on the aggregation of a DsRed-tagged androgen receptor peptide with expanded polyglutamine repeats.

Discussion

RNA-induced protein aggregation and neurodegeneration

This study demonstrates novel effects of untranslated sequence in an NF-L transcript on the reactivity and disposition of its own translated protein. The findings open up new perspectives on the interactions between RNA and protein with important implications for the pathogenesis of neurodegenerative diseases. Previous examples of RNA-mediated neurotoxicity are based on diseases arising from trinucleotide expansions in noncoding regions of mRNA (Ranum and Day, 2002), including the Fragile X-associated tremor/ataxia syndrome attributable to premutation (PM) expansions of CGG repeats in the 5′ untranslated region (5′UTR) of the *Fmr1* transcript (Hagerman and Hagerman, 2002). Whereas large CGG repeats (>200) are associated with methylation and silencing of the *Fmr1* gene resulting in the Fragile X syndrome, expression of *Fmr1* RNA with PM expansions (55–200 CGGs) leads to elevated *Fmr1* mRNA levels and neurodegenerative changes with prominent intranuclear inclusions in neurons and astrocytes (Greco et al., 2002; Hagerman and Hagerman, 2002; Oostra and Willemsen, 2003). Expression of the *Fmr1* gene with a targeted PM expansion reproduces the elevated *Fmr1* mRNA levels, neurodegenerative changes, and intranuclear inclusions in mice (Willemsen et al., 2003). Ectopic expression of expanded CGG repeats in the 5′UTR of a GFP reporter gene leads to dose-, repeat length-, and age-dependent neurodegeneration with formation of ubiquitinated inclusions in *Drosophila* neurons expressing the reporter transgene (Jin et al., 2003). Moreover, overexpression of HSP70 suppresses neurodegeneration, suggesting a linkage between neurodegeneration and formation of ubiquitinated inclusions. Presently, the composition of the neuronal inclusions remain unknown.

Our study shows that protein alterations can be initiated by wild-type RNA sequence in the 3′UTR of a neuronal transcript, that the resulting protein alterations can lead to loss of solubility and aggregation of translated protein, and that ensuing protein aggregates can have synergistic effects on the aggregation of other proteins in the cell. To our knowledge, the demonstration of RNA-induced loss of solubility and aggregation of translated proteins in living cells is an unexpected and novel phenomenon. We attribute the RNA-induced changes in NF-L protein to enhanced protein reactivity that is manifested by colocalization with endogenous NF-H at low-level transgene expression and by aggregation and loss of solubility of NF-L protein at high-level expression. Whereas aggregation and loss of solubility are attributable to overexpression of protein in transfected cells, additional factors or conditions may promote similar phenomena in tissues. Interactions of RNA, or cognate RNA-binding proteins, on translated protein in neurons-at-risk could be a triggering mechanism leading to abnormal protein aggregation in neurodegenerative diseases.

The ability of NF-L 3′UTR in an NF-L mRNA to provoke aggregation of NF-L protein and coaggregation of mutant SOD1 protein provides novel perspectives into the role of NF-L expression in mutant SOD1-mediated motor neuron degeneration. For example, a lack of NF-L expression would eliminate the possibility of RNA-mediated aggregation of NF-L protein as well as any synergistic effects on the coaggregation of mutant SOD1 protein

and could explain why deletion of endogenous NF-L reduces the neuropathic effects and prolongs survival of mice bearing a mutant SOD1 transgene (Williamson et al., 1998). Evidence that NF-L oligomerization–aggregation may contribute to neurotoxicity has also been observed in G86R mutant SOD1 transgenic mice (Morrison et al., 2000). Examination of NF subunit disposition in neurons-at-risk in presymptomatic mice shows that there are initial aggregations of NF-L protein, lacking medium NF (NF-M) or NF-H proteins, in perikarya of motor neurons before the development of more severe neurodegenerative changes. NF-L protein has been identified in aggregates of mutant SOD1 protein (Hyun et al., 2003). Moreover, synergistic effects of NF-L on mutant SOD1 protein aggregation could also account for the preferential degeneration of NF-rich motor neurons in mutant SOD1 transgenic mice (Morrison et al., 1996, 1998). Why NF-L transgene expression does not exaccerbate mutant SOD1 neurotoxicity (Kong and Xu, 2000; Nguyen et al., 2000) is unclear. Compensatory mechanisms from transgene expression in transgenic mice may be able to counter the synergistic neurotoxic effects of low-level NF-L expression on mutant SOD1 protein.

RNA-induced aggregation of NF-L protein may help to explain the neurotoxic effects of high-level expression of wild-type NF-L transgene (Xu et al., 1993), low-level expression of mutant NF-L transgenes (Lee et al., 1994; Canete-Soler et al., 1999), expression of a GFP transgene with NF-L sequence in the 3′UTR (Nie et al., 2002) on motor neurons of transgenic mice as well as the dose-dependent, neuron-specific degenerative effects of NF-L RNA expression on cultured motor neurons (Lin et al., 2003). In the latter instance, direct examination of neuropathic changes showed that the neurotoxic effects of NF-L RNA are associated with accumulations of ubiquitinated protein aggregates in degenerating motor neurons. The present study suggests that aggregation and ubiquitination of proteins in degenerating motor neurons may be initiated by RNA-induced aggregation of NF-L protein with synergistic or seeding-like effects on the aggregation and ubiquitination of other neuronal proteins. Mutations of NF-L may also be a predisposing factor in protein aggregation. For example, the missense mutations that disrupt NF-L assembly (Brownlees et al., 2002; Perez-Olle et al., 2002) may also destabilize and promote protein aggregation and motor neuron degeneration in Charcot-Marie-Tooth disease (Mersiyanova et al., 2000; De Jonghe et al., 2001; Yoshihara et al., 2002). Mutations in NF-H (Figlewicz et al., 1994; Tomkins et al., 1998) and NF-M (Kruger et al., 2003) have also been implicated in neurodegenerative diseases.

The role of protein aggregation in the pathogenesis of neurodegenerative disease derives primarily from studies of mutant proteins in rare familial forms of disease. There is limited insight into the causal events triggering protein aggregations in the common sporadic forms of neurodegenerative diseases in which insoluble aggregates arise from abnormal processing of wild-type endogenous gene products. The present study raises the possibility that RNA-induced aggregations of NF protein may be a contributing factor. Aggregations of NF proteins are commonly seen in degenerating neurons-at-risk, especially in motor neurons, and have been identified recently as the distinguishing feature in a subgroup of patients with dementia (Cairns and Armstrong, 2003; Josephs et al., 2003). A causal link to NF expression is suggested by the predilection of neurodegenerative changes in

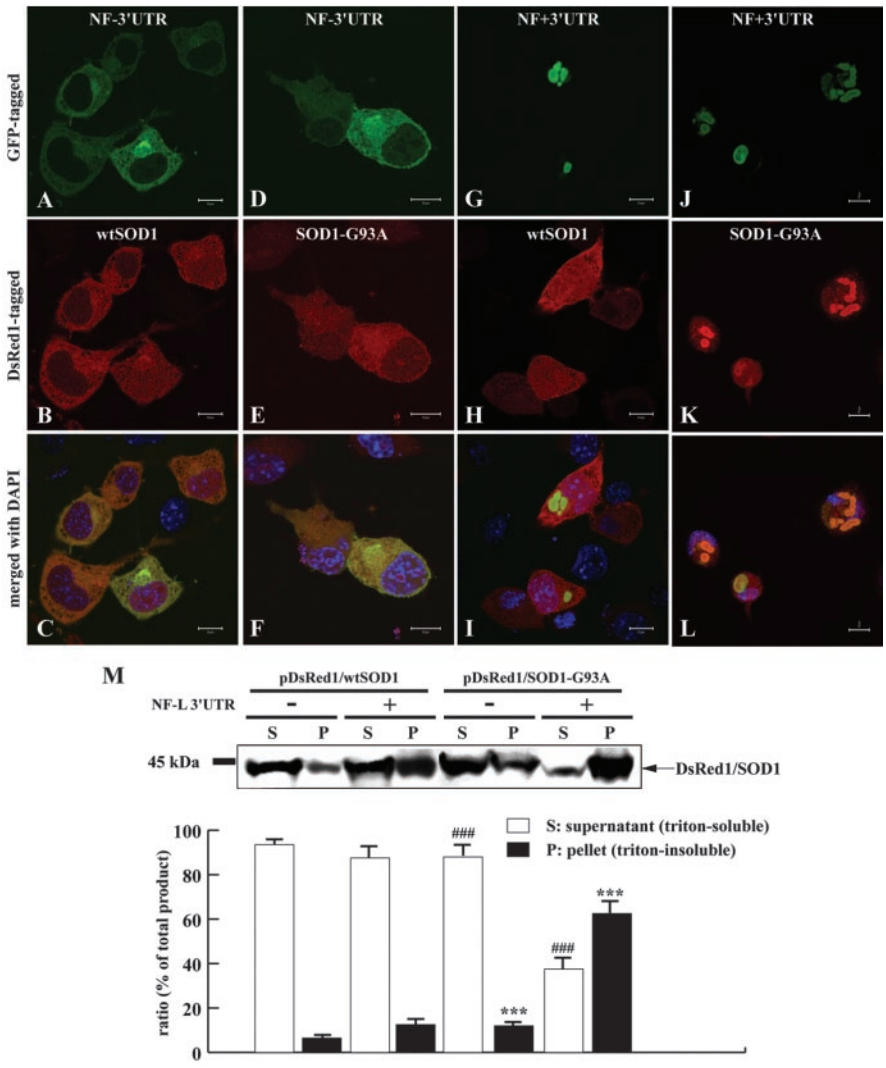

Figure 6. RNA-induced aggregation of NF-L leads to loss of solubility and aggregation of mutant, but not wild-type, SOD1 protein. *A–C*, Confocal microscopy of Neuro2a cells cotransfected with pGFP/NF−3′UTR and pDsRed1/wtSOD1 transgenes showing GFP fluorescence of GFP-tagged NF-L protein (*A*), DsRed1 fluorescence of DsRed-tagged wild-type SOD1 transgene (*B*), and merged image (*C*). *D–F*, GFP fluorescence from pGFP/NF−3′UTR expression (*D*) and DsRed1 fluorescence from pDsRed1/SOD1-G93A expression (*E*) and merged image (*F*). *G–I*, GFP fluorescence from pGFP/NF+3′UTR expression (*G*) and DsRed1 fluorescence from pDsRed1/wtSOD1 expression (*H*) and merged image (*I*). *J–L*, GFP fluorescence from pGFP/NF+3′UTR expression (*J*) and DsRed1 fluorescence from pDsRed1/SOD1-G93A transgene (*K*) and the merged image (*L*). *M*, Anti-SOD1 immunoblot of DsRed-tagged SOD1 protein (45 kDa) in supernatant (*S*) and pelleted (*P*) fractions of cell lysates solubilized in 1% Triton X-100 from cells cotransfected with pGFP/NF−3′UTR (−) or pGFP/NF+3′UTR (+) and pDsRed1/wtSOD1 or pDsRed1/SOD1-G93A transgenes. Graph shows relative amounts of DsRed-tagged SODI protein in Triton X-100-soluble supernatants (open bars) and Triton X-100-insoluble pellets (filled bars). Immunoblots were conducted on 1:30 aliquots of supernatant fractions and 1:5 aliquots of pelleted fractions. *t* test: $^{***,\#\#\#}p < 0.001$. Scale bars, 10 μm.

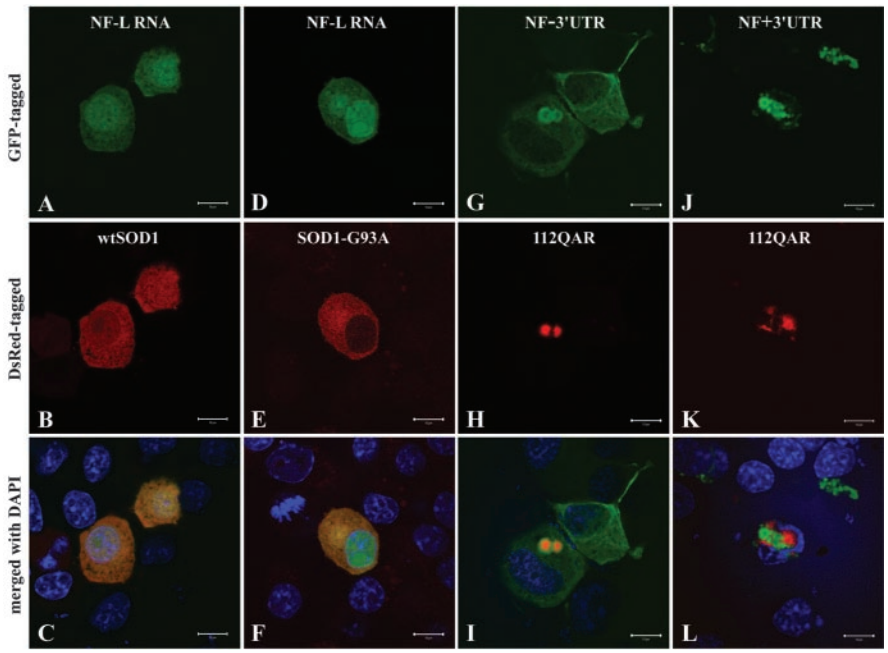

Figure 7. RNA-induced coaggregation of mutant SOD1 requires aggregation of NF-L protein and differs from the coaggregation of NF-L protein and polyglutamine repeat peptide. *A–F*, GFP fluorescence from GFP reporter gene with NF-L sequence in 3′UTR (*A*, *D*), DsRed fluorescence from DsRed-tagged wild-type (*B*) and mutant (*E*) SOD1 proteins, and merged images (*C*, *F*). NF-L sequence in the 3′UTR of GFP reporter gene does not induce protein aggregation. *G–L*, DsRed fluorescence from pDsRed/Q112AR reveals polyglutamine-induced aggregation (*H*, *K*) that does not colocalize with GFP fluorescence from pGFP/NF−3′UTR (*G*) or pGFP/NF+3′UTR (*J*). Merged images (*I*, *L*) reveal a rim of concentrated NF-L protein around the DsRed/Q112AR aggregate (*I*) and close association, but not colocalization, of DsRed-tagged Q112AR and GFP-tagged NF-L protein aggregates. Scale bar, 10 μm.

NF-rich neurons in the prefrontal cortex in Alzheimer's disease (Bussiere et al., 2003). Involvement of NF-L in the pathogenesis of neuronal diseases with cognitive impairment is supported by the attenuation of neurodegenerative changes after deleting the NF-L gene in mice expressing a mutant tau transgene (Ishihara et al., 2001). The same phenomena, including a prominent involvement of motor neurons, occurs in mice expressing a mutant SOD1 transgene (Williamson et al., 1998), raising the prospects that motor neurons may be particularly susceptible to abnormal processing of NF-L. The potential toxic effects of NF-L protein aggregation in neurodegeneration, especially in motor neuron diseases, warrants additional study.

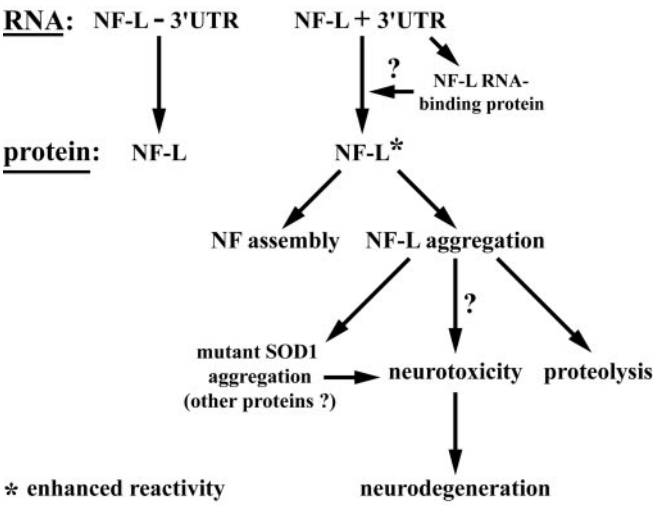

Figure 8. A working model of RNA-induced protein aggregation and neurodegeneration. We propose that RNA sequence in the 3′UTR of NF-L mRNA or cognate binding factors confer enhanced reactivity (*) to translated NF-L protein and that enhanced reactivity promotes NF assembly or aggregation of NF-L protein. The latter phenomenon could promote aggregation of mutant SOD1 proteins or other proteins with neurotoxic effects on neurons-at-risk in neurodegenerative diseases.

The destabilizing element in NF-L mRNA and protein aggregation

The nature of RNA-induced protein aggregation is further suggested by the localization of the protein aggregative effects to the proximal 45 nt in NF-L 3′UTR. This is the site of a major regulatory element controlling stability of the NF-L transcript (Canete-Soler et al., 1998a,b). Mutation of this regulatory element alters the stability of the transcript and creates a mutant NF-L transgene with potent neuropathic effects on motor neurons of transgenic mice (Canete-Soler et al., 1999). It is likely that cognate RNA-binding proteins (or cofactors) are responsible for the function and dysfunction of the regulatory element, including the regulation of NF-L expression levels as well as the neurotoxic effects of overexpression. RNA-binding factors may therefore be involved in the selective alteration of NF-L expression and motor neuron degeneration in sporadic and familial ALS (Bergeron et al., 1994; Wong et al., 2000; Menzies et al., 2002; Ge et al., 2003), transgenic mice bearing G86A (Morrison et al., 2000) and G93A (Menzies et al., 2002), SOD1 transgenes, and hereditary canine spinal muscular atrophy (Muma and Cork, 1993). Altered expression of other intermediate filaments is also associated with neurodegeneration (Beaulieu et al., 1999, 2000; Ching et al., 1999; Pernas-Alonso et al., 2001; Beaulieu and Julien, 2003; Sosa et al., 2003). In each instance, linkage between altered gene expression and neurodegeneration could reflect the strategic positioning of RNA regulatory elements in pathways coordinating cytoskeletal expression. Feedback mechanisms regulating levels of cytoskeletal protein expression are complex and poorly understood but represent pathways that maintain cytoskeletal integrity as well as neuronal homeostasis. Alterations of RNA–protein interactions in feedback pathways could be instrumental in triggering adverse effects in neurodegenerative diseases.

Interactions between RNA and proteins have primarily been viewed from the perspective of RNA metabolism. Our studies now indicate a novel role of RNA or RNA-binding proteins on the processing of proteins. The ability of NF-L RNA sequence to effect the reactivity of NF-L protein could be attributable to direct or indirect interactions of NF-L protein with RNA sequence or with cognate RNA-binding factors. Intermediate filaments, including NF-L protein, have nucleic acid binding properties that map to the head and rod domains of the proteins (Traub et al., 1983; Wang et al., 2001). It is also possible that cognate binding factors on NF-L RNA interact with NF-L proteins and confer chaperone-like activities on nascent NF-L protein. Cognate RNA-binding factors may be dislodged from the transcript during passage through the ribosome (Weiss and Liebhaber, 1994)

and would be located in the immediate vicinity of nascent NF-L protein. Chaperone-like activities could then result from direct interactions with NF-L protein or by recruitment of chaperone proteins. We favor the view that enhanced NF-L protein reactivity is mediated by cognate binding proteins (Fig. 8). Interactive factors linking NF-L RNA with aggregation of NF-L and mutant SOD1 proteins are currently under study.

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