Zinc as a translation regulator in neurons: implications for P-body aggregation

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Summary
Post-transcriptional mechanisms of gene expression in neuronal cells include mRNA transport and local protein synthesis, which play a vital role in the control of polarity, synaptic plasticity and growth cone motility. RNA-binding proteins, which form the transported ribonucleoparticle (RNP), control mRNA stability and local translation. Recently, the existence of processing bodies (P-bodies), in which mRNA decapping and degradation take place, was revealed in neurons. It was suggested that P-bodies serve as a transient storage compartment for mRNAs, which can be released and, upon inhibition of protein synthesis by puromycin, which causes disruption of polysomes, P-bodies increase in size and, upon stimulation, resume translation. In this study, we focused on the localization of the Dcp1a protein, which serves as a P-body marker, in PC12 growth cones and P19 neuronal cells and its association with the tau mRNA-binding protein HuD. We found that stimulation of neurons by zinc, which is stored and released from synaptic vesicles, caused a disruption of polysomes into monosomes, whereas HuD protein distribution in sucrose gradient fractions remained unaffected. In addition, zinc application caused an aggregation of Dcp1a protein in an RNA-dependent manner. These findings suggest a role for zinc in translation regulation via disruption of polysomes, aggregation of P-bodies in neurons and impairment of the RNP-polysome interaction.

Key words: Dcp1a, HuD, P-bodies, Neurons, Translation

Introduction
Processing bodies (P-bodies) were first described as cytoplasmic sites of mRNA decapping and degradation. They were shown to contain various enzymes that are engaged in mRNA decay, such as: Dcp1a, Dcp2 and Xrn1 (Sheth and Parker, 2003; Hillebrand et al., 2007). Immunostaining of cells using antibodies for Dcp1a and Dcp2, which are key markers for P-bodies, revealed P-bodies to be large macroscopic cytoplasmic granules (Teixeira et al., 2005). In addition P-bodies contain Ago1 and Ago2 (Argonaut 1 and Argonaut 2), which form part of the RISC complex, suggesting a role for P-bodies in mRNA/siRNA gene silencing (Behm-Ansmant et al., 2006). P-bodies and polysomes exist in a dynamic equilibrium and, upon inhibition of protein synthesis by puromycin, which causes disruption of polysomes, P-bodies increase in size and number. When polysomes are stabilized by cycloheximide treatment, P-bodies shrink or even disappear (Cougot et al., 2004). Other results suggest that, in response to modulation of translation efficiency, mRNAs can be stored in P-bodies and are released when translation is restored; thus the P-bodies act as a storage unit (Brengues et al., 2005). Much less is known about the physiological role of P-bodies in neurons. In Drosophila neurons, staufen and dFMR1 ribonucleoparticles (RNPs) contain somatic P-body enzymes, such as Dcp and pacman proteins, which are the Drosophila homologues for Dcp1a and Xrn1, respectively (Barbee et al., 2006). Previous data demonstrated the presence of Dcp containing neuronal granules in dendrites (Vessey et al., 2006).

In the brain, zinc was first observed in mossy fibres of the hippocampus that connect the dentate gyrus to Ammon’s horn (McLardy, 1976). The only neurons that have vesicular zinc in the brain are glutamatergic, but not all glutamatergic neurons contain zinc (Beaulieu et al., 1992). Zinc is implicated as a factor in Alzheimer disease and in excitotoxicity cases, such as brain injury, ischemia and seizures (Choi and Koh, 1998). In Alzheimer disease, it was shown that β-amyloid peptide (Aβ) binds to zinc in vitro, and that amyloid plaques are enriched with zinc (Bush et al., 1994b; Bush et al., 1994a; Lovell et al., 1998). In addition, zinc acts as a neurotransmitter, in the sense that it is released to the synapse in response to extracellular cues, and can affect the postsynaptic cell. Using neuronal cell lines, it was demonstrated that zinc binds Trk receptors and can phosphorylate GSK3β and P70S6K, thus affecting tau protein phosphorylation and synthesis (An et al., 2005; Hwang et al., 2005). Zinc can also inhibit translation in neuronal cells via phosphorylation of initiation factor elf-2 (Ahirezaei et al., 1999).

Various mRNAs localize within neurons to dendrites and axons in the form of large RNPs. Localization of mRNA molecules serves as a mechanism to control local protein synthesis and the synaptic plasticity of the neurons (Kiebler and Bassell, 2006). We have shown that tau mRNA localization to axons is dependent on a specific sequence, or ‘zip code’, found in its 3’UTR, that is bound by the ELAV homologue and RNA-binding protein, HuD (ELAV4) (Aronov et al., 1999; Aronov et al., 2002). A screen for revealing HuD partners in tau RNPs showed that HuD binds the insulin-like growth factor mRNA-binding protein IMP1 (IF2B1) and the Ras regulatory protein G3BP in P19 neurons. Subsequently, we found that IMP1 and HuD are found in association with polysomes in neurons (Atlas et al., 2004).

In this work, we studied the effect of zinc on polysomes and the association of HuD and IMP1 RNA-binding proteins with polysomes. Previous studies have shown that polysomes and P-bodies are dynamically linked (Cougot et al., 2004; Brengues et al., 2005) and therefore we were interested in following the effect of zinc treatment on Dcp1a and P-body formation. The effects of Dcp1a overexpression and of the induction of P-bodies on polysome formation were also studied. We further investigated colocalization...
and association between HuD and Dcp1a granules in neuronal processes and growth cones.

**Results**

**Zinc application causes disruption of polysomes**

It was previously shown that tau RNP includes HuD and IMP1 RNA-binding proteins, which are found in association with polysomes. To further characterize this association, we used zinc, an endogenous neuronal modulator, which affects protein synthesis. Addition of extracellular zinc to cultured neurons inhibits the initiation of protein translation by increasing the phosphorylation of elf-2 (Alirezaei et al., 1999).

The distribution of polysomes in response to zinc chloride treatment was analysed by their migration in 15-45% sucrose gradients (Fig. 1A). In control cells, the distribution of ribosomal proteins, as a marker of ribosomes, was detected in the dense sucrose fractions (8-10) where polysomes migrate, whereas a relative small amount of protein was detected in the light fraction (fraction 5), which correlates with the migration of monosomes in the gradient. Following treatment of the cells with 100 μM zinc for 1 hour, a marked decrease was observed in the migration of the polysomes to the dense fractions. Simultaneously, we noticed an increase in the amount of monosomes in fractions 5-6. An increasing shift in ribosomal protein distribution, from polysomes to monosomes, was also observed after 2 hours and 4 hours of zinc treatment. We monitored the distribution of rRNA in the sucrose gradient by ethidium bromide staining. In control cells, rRNA was concentrated in fractions 8 and 9 of the gradient, which was similar to the distribution of ribosomal protein. Upon 4 hours of zinc treatment, a shift in rRNA staining towards less dense fractions was observed (Fig. 1B). Statistical analysis of the shift from polysomes to monosomes indicates that zinc treatment caused a 50% increase in the amount of ribosomal protein accumulating in light fractions 4-6. In parallel, a similar decrease in the amount of ribosomal protein was observed in the heavy fractions 7-10. These results indicate that zinc treatment causes a shift in the distribution of heavy polysomes towards the lighter monosomes fraction, which indicates the disruption of polysomes and an affect on protein synthesis.

**Zinc treatment impairs the interaction between polysomes, IMP1 and HuD RNA-binding proteins**

In light of the above results, we further explored the effect of zinc treatment on the association of the tau RNP proteins, IMP1 and HuD, with polysomes. We monitored the migration of both proteins in 15-45% sucrose gradients, and found that zinc treatment had no effect on the distribution of IMP1 and HuD (Fig. 2, fractions 7-9), which were found in the dense fractions of the gradient, both in control cells and in cells treated with zinc, although disruption of polysomes was observed. These observations suggest that, upon disruption of polysomes, HuD and IMP1 are found in large complexes that sediment in the dense sucrose fractions, independently of their association with polysomes.

**Zinc treatment of P19 neurons induces aggregation of Dcp1a**

The distribution of Dcp1a protein in response to the zinc treatment of P19 neurons was monitored using differential centrifugation (Fig. 3). We noticed that, in response to zinc application, the relative amount of Dcp1a protein decreased in the 20K supernatant (Fig. 3A,C), whereas it simultaneously increased in the 20K pellet (Fig. 3B,D). We further monitored Dcp1a distribution in sucrose gradient
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fractions and found that, although the majority of Dcp1a protein was found in the light fractions of the gradient (1-6), in zinc-treated cells, a shift in Dcp1a distribution to the dense sucrose fractions was detected. This increase was observed at all time points tested following zinc application to the cells (Fig. 4A). The increase of total Dcp1a content in fractions 6-10 was statistically analysed and the data showed more than a sixfold increase in Dcp1a protein levels in fractions 6-10, compared with control cells (Fig. 4B).

Aggregation of Dcp1a following zinc treatment is RNA dependent

A previous study demonstrated that P-bodies are sensitive to RNase A treatment (Teixeira et al., 2005). Consequently, we treated lysates of P19 neurons, which had been previously stimulated with zinc, with RNase A prior to loading them onto the sucrose gradient. We monitored Dcp1a distribution in the sucrose gradient fractions and found that treatment with RNase A induced a breakdown of Dcp1a aggregates and therefore Dcp1a protein accumulated in the light fractions of the gradient (1-3) (Fig. 5A). As a control, we monitored the effect of the RNase A treatment on HuD protein distribution. A similar shift of HuD protein towards lighter gradient fractions was observed (Fig. 5B). These observations led us to conclude that Dcp1a aggregation in response to the zinc treatment of P19 neurons is solely RNA dependent, and is not the result of interactions between Dcp1a protein and cellular organelles/components.

Fig. 2. Zinc treatment impairs the association between IMP1 and HuD with polysomes. P19 neurons were treated with 100 M zinc for the indicated times. The cell lysates were loaded on a 15-45% linear sucrose gradient. Gradient fractions were collected and equal volume samples were separated and analysed on SDS-PAGE using IMP1, HuD and ribosomal proteins antibodies.

Fig. 3. Zinc application induces the aggregation of Dcp1a protein. P19 neurons were treated with 100 M zinc for 1 hour. Cells were lysed and extracts were centrifuged for 10 minutes at 1000 g. Supernatants with an equal amount of protein were centrifuged for 10 minutes at 20,000 g. (A) Dcp1a protein levels in 20K supernatants in lysates of zinc-treated cells (Z1-Z3) compared with control cell lysates (C1-C3). (B) Dcp1a protein levels in 20K pellets (resuspended in equal volumes of lysis buffer) of zinc-treated cells (Z1-Z3) compared with control cell pellets (C1-C3). (C) Statistical analysis of Dcp1a protein levels in 20K supernatants of zinc-treated cell lysates and control cell lysates, normalized to -actin protein levels. Bars represent mean ± s.e.m., n = 3. *P<0.01, Student’s t-test. (D) Statistical analysis of Dcp1a protein levels in 20K pellets of zinc-treated cells and in control cells, normalized to -actin protein levels. Bars represent mean ± s.e.m., n = 3. *P<0.01, Student’s t-test.
Dcp1a overexpression does not affect polysomes formation

It was previously demonstrated that mRNA molecules shuttle between translating polysomes and cytoplasmic P-bodies in response to treatments/stimulations that affect translation (Brengues et al., 2005). Recent studies showed that stabilizing (freezing) polysomes by treatment with cycloheximide protein synthesis inhibitor, resulted in the disappearance of P-bodies, whereas treatment with puromycin, which causes premature termination of translation, induced an increase in the number and size of P-bodies (Teixeira et al., 2005). We tested whether the induction of P-body formation by overexpression of Dcp1a interferes with the distribution of polysomes. We overexpressed GFP-Dcp1a and GFP alone (control) in HEK293 cells, and used a Dcp1a antibody to mark the presence of P-bodies in the cells (Fig. 6A). In cells transfected with the control GFP vector, only a few small P-bodies were stained. In comparison, cells that were transfected with GFP-Dcp1a, exhibited the formation of many large P-bodies. We further analysed the cell extracts for ribosomal proteins and Dcp1a protein distribution in the fractions of the sucrose gradient. In control cells (overexpressing GFP), endogenous Dcp1a was found in the light fractions (fractions 1-6) of the gradient (Fig. 6B). In cells overexpressing GFP-Dcp1a, the protein was distributed throughout all the gradient fractions, including the dense sucrose fractions (Fig. 6B). In control cells, ribosomal proteins were accumulated in dense sucrose fractions where polysomes migrate. A similar ribosomal protein distribution was observed in cells overexpressing GFP-Dcp1a. These results demonstrate that overexpression of Dcp1a does not interfere with polysomes, which remain intact. Our findings suggest that overexpression of Dcp1a, which competes with ribosomes on the cap structure of mRNAs, is not sufficient to disrupt polysomes and to shift the translation/degradation balance towards degradation.

Fig. 6. Dcp1a overexpression does not affect polysomes formation.

Dcp1a aggregation in response to zinc treatment is RNA dependent.

Colocalization of HuD with Dcp1a in 293 HEK cells

Previous studies showed the colocalization of eIF4E binding protein 4E-T, and proteins GW182, Staufen, FMRP and RAP55 with P-bodies. Accordingly, we showed that tau mRNA binds HuD and IMP1 RNA-binding protein to form a large RNP complex (Atlas et al., 2004). We overexpressed HuD and IMP1 in HEK293 cells to test for the colocalization of HuD and IMP1 RBPs with P-bodies. From Fig. 7A, it appears that HuD colocalizes with endogenous Dcp1a in the large cytoplasmic granules, whereas IMP1 is absent from P-bodies. To further analyse the interaction between HuD and Dcp1a, we used a GST pull-down assay. Our results demonstrate that GST-HuD binds the GFP-Dcp1a protein overexpressed in HEK293 cells (Fig. 7B). These observations suggest a possible association between Dcp1a and HuD proteins.

Fig. 7. HuD and Dcp1a colocalize in neuronal cells

Very few data have been compiled on the role of Dcp1a in neuronal cells. It has been demonstrated that Dcp1a granules exist in neurons, specifically in dendrites (Barbee et al., 2006; Vessey et al., 2006). We further investigated the possible colocalization of endogenous HuD and Dcp1a in neuronal cells, and particularly in neuronal processes and growth cones. In these experiments, antibodies were used to visualize HuD and Dcp1a proteins in neuronal P19 and PC12 cells. We detected colocalization of both proteins in large granules in the cell bodies and in the neuronal processes of P19 neurons (Fig. 8). In differentiated PC12 cells, colocalization of both proteins was visualized in the cell body and neurites. In addition, the presence of Dcp1a granules and their colocalization with HuD was clearly observed in the neuronal-like growth cone structure of differentiated PC12 cells (Fig. 9).
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Discussion

In the brain, zinc is found concentrated specifically in the mossy fibres of the hippocampus (McLardy, 1976). Studies of patients with Alzheimer disease, epilepsy and brain injury suggest that zinc contributes to their pathological effects (Bush et al., 1994b; Bush et al., 1994a; Choi and Koh, 1998). Zinc levels are increased in the brain area severely affected by Alzheimer disease, and zinc can activate signal transduction pathways and phosphorylate P70S6K and GSK3β (Danscher et al., 1997; An et al., 2005). In this work, we have demonstrated how zinc application to differentiated P19 neurons leads to the disruption of polysomes, and to impairment of the interaction between IMP1 and HuD RNA-binding proteins with polysomes. In parallel, we followed the changes in P-body formation by differential centrifugation, and by monitoring their migration in sucrose gradients. Using these biochemical methods, we were able to observe Dcp1a protein aggregation in response to zinc treatment in an RNA-dependent manner. In addition, we could show that HuD RNA-binding protein colocalizes with P-bodies in differentiated P19 neurons, in PC12 neuronal processes and in growth-cone-like structures.

We investigated the effect of zinc on the distribution of polysomes by monitoring the migration of ribosomal RNA and protein in sucrose gradient fractions based on an earlier study describing the effect of zinc as a translation inhibitor via reduction of polysomes in the cell (Alirezaei et al., 1999). We found that, in untreated cells, ribosomal protein and RNA were located in dense sucrose fractions together with polysomes (fractions 8-10). Upon stimulation with zinc, a shift in both ribosomal protein and RNA towards less dense fractions was observed (Fig. 1). These observations suggest that extracellular zinc disrupts polysomes, and therefore inhibits translation in neurons. We have previously shown that the RNA-binding proteins HuD and IMP1, which bind tau mRNA and regulate its translation, are found in association with polysomes (Atlas et al., 2004; Atlas et al., 2007), therefore, the effect of zinc treatment on this association was tested. P19 neurons were treated with zinc, and the distribution of HuD and IMP1 RNA-binding proteins along the sucrose gradient fractions was monitored. Similarly to our previous results, we found that, upon zinc treatment, ribosomal protein distribution shifted towards lighter fractions of the gradient, accumulating in fractions 4-6. However, the distribution of HuD and IMP1 proteins was similar to that found for control cells, meaning that zinc treatment did not have any effect on their distribution (Fig. 2). These results suggest that the disruption of polysomes by zinc treatment impairs the interaction between neuronal RNPs and polysomes, thus leading to formation of mRNA storage compartments or to RNA degradation.

We further tested P-body aggregation in neurons treated with zinc, compared with untreated cells, using differential centrifugation. Following zinc treatment of the cells, we noticed a decrease in the amount of Dcp1a protein in the 20K supernatant together with a simultaneous and significant increase in the amount of the protein in the 20K pellet (Fig. 3A,B). In addition, we followed the distribution of Dcp1a in 15-45% sucrose gradient fractions. We noticed that, upon zinc treatment, Dcp1a protein accumulated in...
the dense sucrose fractions, compared with control experiments, where Dcp1a protein was found in the light fractions of the gradient (Fig. 4). These aggregates were found to be RNA dependent (Fig. 5). Based on our experiments, we suggest that P-bodies are structures that are sensitive to vigorous centrifugation assays, therefore they are disrupted and their components are found in light sucrose fractions. We therefore propose the use of a sucrose gradient centrifugation assay as a biochemical tool to monitor and quantify small changes in P-body formation. This method is complementary to the differential centrifugation assay we used (Fig. 3), which enables us to monitor changes in crude extracts. Several groups have demonstrated a dynamic correlation between P-bodies and polysomes. Stabilization of polysomes by cycloheximide treatment led to the disappearance of P-bodies, whereas the disruption of polysomes using puromycin results in P-bodies increasing in both size and number (Cougot et al., 2004). These observations, which demonstrated that modulation of translation leads to changes in P-body formation, encouraged us to ask whether inducing the formation of P-bodies could influence polyribosomal organization. Overexpression of GFP-Dcp1a (a known P-body marker) in HEK293 cells, induced the formation of P-bodies (Fig. 6A). Induction of large P-bodies was also demonstrated when overexpressing either mRFP-Dcp1a or YFP-Dcp1A (data not shown). In HEK293 cells transfected with GFP, endogenous Dcp1a protein was found in light fractions of the gradient, whereas in GFP-Dcp1a transfected cells, GFP-Dcp1a was found in all gradient fraction, including dense sucrose fractions (Fig. 6B). GFP-Dcp1a distribution in heavy fractions of the polysomes, as can be seen in Fig. 6C, is probably due to its enhanced aggregation with P-body components, as seen in Fig. 6A. In control cells, the majority of ribosomal proteins were found in the dense sucrose fractions that correlate with polysomes (Fig. 6C). This distribution is similar to that observed in GFP-Dcp1a-transfected cells. This observation suggests that, although stabilization of polysomes leads to disassembly of P-bodies, induction of P-bodies by overexpression of Dcp1a is not sufficient for the disassembly of polysomes.

We further investigated the interaction between Dcp1a and HuD in HEK293 cells. By overexpressing GFP-HuD and immunostaining with anti-Dcp1a antibodies, we were able to visualize colocalization of HuD with endogenous Dcp1a in specific P-body granules (Fig. 7A). In previous studies, we have shown a strong association between HuD and the RNA-binding protein IMP1 with tau mRNA (Atlas et al., 2004), therefore we investigated whether IMP1 is also localized to P-bodies. We followed the same procedure using GFP-IMP1 and we noticed that IMP1 is excluded from Dcp1a-containing granules (Fig. 7A). For further validation of the association between HuD and Dcp1a, a GST pull-down assay was exploited, using GST-HuD as bait in the presence of GFP-Dcp1a protein. Using this approach, we were able to show a strong interaction between HuD and Dcp1a (Fig. 7B). Taken together, these results demonstrate a strong association between HuD and Dcp1a, whereas IMP1 was not found to colocalize with Dcp1a and P-bodies.

Previous studies demonstrated the presence of P-bodies in neurons, specifically in dendrites (Barbee et al., 2006; Vessey et al., 2006). In this study, we examined the endogenous colocalization of HuD RNA-binding protein with Dcp1a protein in two neuronal cells: P19 neurons and NGF differentiated PC12 cells. In P19

Fig. 8. Endogenous colocalization of HuD with Dcp1a in P19 neurons. Day 8 (post retinoic acid) P19 neurons were immunostained using antibodies against HuD and Dcp1a proteins. Fluorescein isothiocyanate (FITC)- and Cy3-conjugated secondary antibodies were used to label (A) Dcp1a and (B) HuD, respectively. (C) Merged image of HuD and Dcp1a. Arrows indicate colocalization of both proteins (D) 3D reconstruction of the merged image, produced with the LSM 510 software. Scale bars: 1 μm.

Fig. 9. Endogenous colocalization of HuD and Dcp1a in growth-cone-like structures of differentiated PC12 cells. PC12 cells were neuronally differentiated by incubation with 50 ng/ml NGF for 4 days. Cells were then fixed and immunostained using antibodies against HuD and Dcp1a. Fluorescein isothiocyanate (FITC)- and Cy3-conjugated secondary antibodies were used to label (A) Dcp1a and (B) HuD, respectively. (C) Merged image of both Dcp1a and HuD. Arrows indicate colocalization of both proteins. (D) Phase image of differentiated PC12 cells.
neurons, we detected colocalization of both proteins along the neuronal processes. The colocalization of HuD with P-bodies is clearly visible in both merged and 3D reconstructions (Fig. 8). In NGF-differentiated PC12 cells, which develop structures similar to neuronal growth cones at the end of the neuronal process, we saw the colocalization of HuD with Dcp1a in P-bodies in the cell body, neuronal process and in the growth cones (Fig. 9). The presence of P-bodies in association with HuD in neuronal processes and growth cones may suggest regulation of translation in these domains. Translation regulation of localized mRNA molecules in neuronal growth cones was shown to regulate growth cone steering and synapse formation. Processing bodies can, therefore, attenuate this translation process, by serving as sites for either mRNA degradation or storage.

The effect of zinc as a neuromodulator, with an emphasis on its role as a neuronal translation regulator, was tested. We found that application of zinc reduces the translation efficiency in the cell, by dissociating polysomes and allowing only a few ribosomes to be engaged in an active translation process. RNA-binding proteins, such as HuD and IMP1, which were previously shown to associate with polysomes and thus regulate translation, are released from this association by the administration of zinc, and may serve as large storage compartments for the released mRNA molecules. In addition, we found that zinc induced the aggregation of Dcp1a to form large P-bodies. These results demonstrate the existence of a physiological mechanism that regulates protein synthesis by inhibition of translation and aggregation of P-bodies.

Materials and Methods

Plasmids, antibodies and zinc treatment

Polyclonal antibody against Dcp1a was a generous gift from Jens Lykke-Anderson and Bertrand Seraphin (Couget et al., 2004). GFPpX2-Dcp1a construct was a gift from Jingdong Liu (Liu et al., 2005). Polyclonal antibody for proteins found in the large subunit 60S of the ribosome was a generous gift from J. Hesketh (Horne and Hesketh, 1990). Anti-HuD antibody is a high-titer polyclonal human antiserum (1:1000), which specifically recognize ELAV-like HuD proteins (GenBank accession no. M62843) (Szabo et al., 1991; Aranda-Abreu et al., 1999). EGFP-HuD was constructed by inserting HuD-coding cDNA in-frame into EGFP-C1 vector (Clontech, Palo Alto, CA). EGFP-IMPI was a gift from Jan Christensen and Finn Nielsen (Nielsen et al., 2002). GST-HuD vector was constructed as described previously (Atlas et al., 2004). Cells were treated with 100 μM zinc chloride (Sigma-Aldrich) for the indicated times.

Cell culture, neuronal differentiation and transfection

HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO-BRL) supplemented with 10% heat-inactivated foetal calf serum (FCS) in a 5% CO2 incubator at 37°C. P19 embryonic carcinoma cells were grown in minimal essential medium (MEM, Beit-Haemek, Israel) supplemented with 5% heat-inactivated FCS in a 5% CO2 incubator at 37°C. Cells were induced to differentiate by addition of 1 M retinoic acid (RA), as previously described (Aronov et al., 2001). PC12 cells were grown in DMEM medium supplemented with 10% FCS + 5% horse serum. PC12 cells were induced to neurally differentiate by incubation for 48 hours with 50 ng/ml NGF. HEK293 cells were transfected using the calcium phosphate precipitation method as described previously (Aronov et al., 2001). Transfections into PC12 cells were achieved using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol in 35 mm plates with 10 μg DNA.

Differential centrifugation and sucrose gradient analysis

Cell extracts were prepared from neurally differentiated P19 cells (8 days post RA treatment). Cells were washed with ice-cold PBS and scraped with cold lysis buffer (140 mM KCl, 20 mM HEPES pH 7.4, 3 mM MgCl2, 1% NP40) supplemented with protease inhibitors and RNase inhibitor (Takara, 200 μl in sucrose gradient analysis only). In differential centrifugation experiments, cell extracts were centrifuged for 10 minutes at 10000 g to obtain post-nuclear supernatant. Lysates containing equal amounts of protein were centrifuged for 20 minutes at 200000 g to obtain P-body-enriched pellets. The 20K pellets were resuspended in 20 l lysis buffer, supplemented with SDS-sample buffer and boiled. Supernatants of 20K centrifugation were collected, supplemented with SDS-sample buffer and boiled. Both pellets and supernatants were analysed using SDS-PAGE with the indicated antibodies. In the sucrose gradient experiments, cell extracts were centrifuged for 10 minutes at 120000 g. Supernatants were loaded on linear 15-45% (w/w) sucrose gradients (140 mM KCl, 20 mM HEPES pH 7.4, 3 mM MgCl2) and centrifuged for 1 hour at 40,000 r.p.m. using an AH-650 Servor rotor. Fractions were collected from the top of the gradient with SDS-sample buffer and analysed using SDS-PAGE analysis. Band analysis and statistical quantification were performed using the image J software (rsb.info.nih.gov/ji/) and Microsoft Excel.

GST pull-down assay

HuD cDNA was cloned at the 3’ end of the GST coding sequence in pGEX-4T-1 (Pharmacia, Milton Keynes, UK). Exponentially growing bacterial cultures were induced to express GST-HuD or GST proteins using 1 mM IPTG. Bacteria were lysed in GST lysis buffer (50 mm HEPES pH 7.5, 350 mM KCl, 5 mM EDTA, 1 mm dithiothreitol) containing lysozyme and protease inhibitors. The cleared lysate was incubated with glutathione beads, and washed under stringent conditions to remove nonspecifically bound bacterial proteins. HEK293 cells transfected with GFP-Dcp1a protein, were extracted in a BKCl buffer (20 mm HEPES pH 7.5, 150 mm KCl, 1 mm MgCl2, 1% glycerol, 15 mm EDTA, 1% NP-40, 1 mM DTT) that included an RNase inhibitor (Porcine ribonuclease inhibitor, Takara) and protease inhibitors. Cytoplasmic extracts were incubated with GST or GST-HuD bound beads for 4 hours at 4°C in the presence of RNasin and protease inhibitors. Beads were washed in BKCl buffer to remove nonspecifically bound proteins. Specifically bound proteins were eluted from the beads by 1 mM KCl wash at room temperature. The eluate containing the proteins was boiled for 5 minutes, and the products were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Immunostaining of fixed cells

Cells were grown on coated coverslips with poly-l-lysine or collagen. Cells were fixed with 4% paraformaldehyde in 4°C for 20 minutes at room temperature as previously described (Aranda-Abreu et al., 1999). Following permeabilization with 0.3% Triton X-100 for 3 minutes, and blocking with 5% goat serum in phosphate-buffered saline (PBS), the slides were incubated with primary antibodies for 1.5 hours at room temperature, washed and reacted with a secondary antibody conjugated to fluorescein isothiocyanate (FITC) or Cy3 as necessary. The slides were visualized by confocal laser microscopy using 488 nm and 545 nm laser excitations, for FITC and Cy3, respectively.

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