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The ELAV Protein HuD Stimulates Cap-Dependent Translation in a Poly(A)- and eIF4A-Dependent Manner

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SUMMARY

The RNA-binding protein HuD promotes neuronal differentiation by an unknown mechanism. Here we identify an enhancer function of HuD in translation. Translation stimulation by HuD requires both a 3′ poly(A) tail and a 5′ m^7G cap structure. We also show that HuD directly interacts with eIF4A. This interaction and the poly(A)-binding activity of HuD are critical for its translational enhancer function because HuD-eIF4A- and HuD-poly(A)-binding mutants fail to stimulate translation. We show that translation of HCV IRES mRNA, which is eIF4A independent, is not stimulated by HuD. We also find that the eIF4A and poly(A)-binding activities of HuD are not only important for stimulating translation but also are essential for HuD-induced neurite outgrowth in PC12 cells. This example of cap-dependent translational regulation might explain at least in part how HuD triggers the induction of neuronal differentiation.

INTRODUCTION

Translational control is critical for cell proliferation, development, and differentiation. RNA-binding proteins (RBPs) are key mediators of translational control and function by binding to sequences within the 5′, 3′, or both UTRs (Mazumder et al., 2003; Beckmann et al., 2005; Bhattacharyya et al., 2006; Abaza and Gebauer, 2008; Sonenberg and Hinnebusch, 2009). Nevertheless, little is known about how RBPs regulate translation. Translation initiation is the rate-limiting step of translation and is the main target of translational control mechanisms (Gingras et al., 1999; Gebauer and Hentze, 2004). In the initiation of translation, a set of eukaryotic translation initiation factors (eIFs) are required for (1) recruitment of the 43S preinitiation complex (a complex of the 40S ribosomal subunit, the initiator tRNA, GTP, and initiation factors) to the mRNA, (2) scanning along the 5′ untranslated region by this complex to recognize the start codon, (3) joining of the 60S ribosomal subunit, and (4) formation of a translation-competent 80S ribosome (Pestova et al., 2007).

The 5′ cap structure and the 3′ poly(A) tail, which are bound by the eIF4F complex and the poly(A)-binding protein (PABP), respectively, act synergistically to enhance translation (Gingras et al., 1999; Kahvejian et al., 2001). eIF4F consists of the cap-binding protein eIF4E, the RNA helicase eIF4A, and the large scaffolding protein eIF4G. eIF4G binds eIF4A and eIF3 and interacts with eIF4E and PABP to promote circularization of the mRNA (closed loop model) (Wells et al., 1998), which is thought to underlie the translational synergy between the cap structure and the poly(A) tail. Mammalian eIF3 binds to the small ribosomal subunit, providing a key link between the circularized mRNA and the ribosome (Sachs et al., 1997; Gingras et al., 1999). eIF4A is an ATP-dependent RNA helicase (Gingras et al., 1999) and plays a key role in initiation by unwinding of RNA secondary structure in the 5′UTR and by promoting ribosomal scanning. Despite eIF4A's central role in translation, only few functional binding partners have been described (Craig et al., 1998; Oberer et al., 2005).

The neuronal members of the Hu family, HuB, HuC, and HuD, are specifically expressed in neurons and are critical for neuronal differentiation. Hu proteins contain three RNA-binding domains (RBDs) of the RNP-consensus sequence and a linker region that separates the two N-terminal RBDs (RBD1 and RBD2) from the C-terminal RBD (RBD3) (Hinman and Lou, 2008). As they share homology with the *Drosophila* ELAV protein, they are referred to as the ELAV family. Overexpression of HuD accelerates neurite outgrowth in E19 rat cortical neurons, PC12 cells, and retinoic acid-induced embryonic stem cells (Kasashima et al., 1999; Anderson et al., 2000, 2001). It has been proposed that the biological function of Hu proteins results from their ability to bind to target mRNAs. Indeed, Hu proteins stabilize target

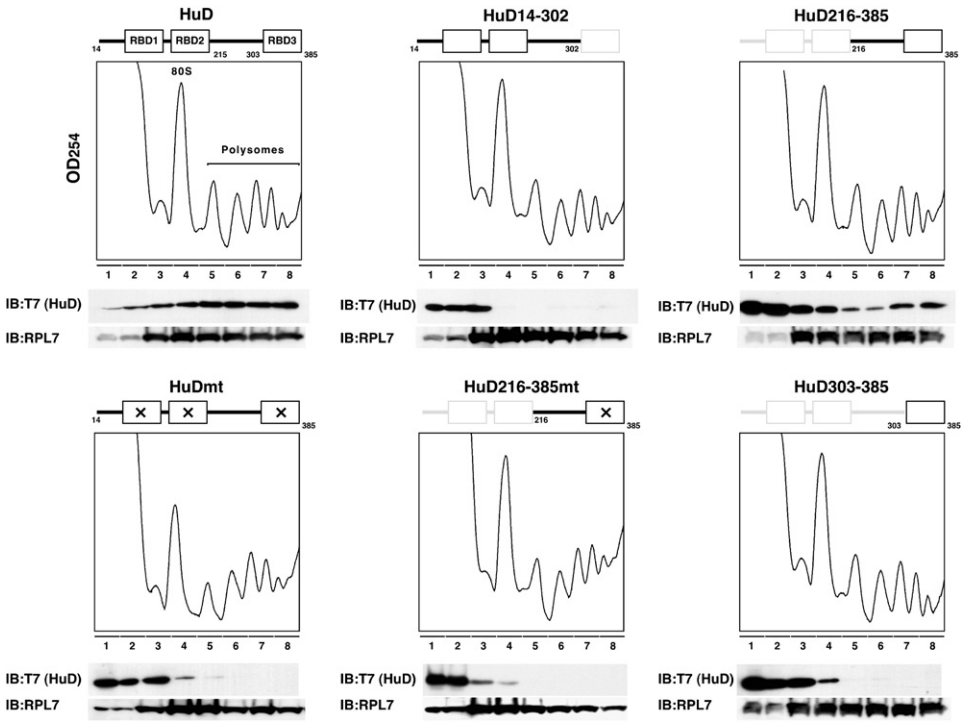

Figure 1. RBD3 Is Critical for Polysome Association of HuD

Distribution of the indicated T7-tagged HuD proteins expressed in PC12 cells was monitored by immunoblotting. RPL7 was used as a positive control. The lane numbers correspond to the fraction numbers in the polysome profiles. Amino acid substitutions in the RNP1 motif of the respective RBDs that abrogate RNA-binding activity are indicated by X.

mRNAs by binding to adenine/uridine-rich elements (AREs) in their 3′UTR via RBD1 and RBD2 (Chung et al., 1996; Hinman and Lou, 2008). In line with these reports, we showed by performing SELEX-binding studies that HuB and HuC bind to ARE-rich sequences (Abe et al., 1996a, 1996b) and identified for HuB the c-*fos* ARE as a target (Abe et al., 1996b).

Moreover, Hu proteins bind to the 5′UTR or 3′UTR of target mRNAs to enhance or suppress translation (Antic et al., 1999; Kullmann et al., 2002; Galban et al., 2008; Hinman and Lou, 2008). However, very little is known about the mechanism(s) by which Hu proteins effect translation.

Here, we report that HuD stimulates translation in a cap- and poly(A)-dependent way, and we elucidate the underlying mechanism. We demonstrate that HuD specifically and directly interacts with eIF4A. We map the binding site in HuD and show that this interaction and the poly(A)-binding activity of HuD via RBD3 are critical for HuD's enhancer function. We also show that the stimulatory effect of HuD on neurite outgrowth depends on these molecular interactions.

RESULTS

The Poly(A)-Binding Domain RBD3 and the Linker Region between RBD2 and RBD3 Are Important for HuD to Associate with Polysomes

Previous work showed that HuD associates with polysomes (Atlas et al., 2007), implying that HuD interacts with actively translating mRNAs. To first dissect the requirements for polysome association, we generated mutant forms of HuD (Figure 1), expressed them in PC12 cells, and subjected extracts from these cells to sucrose density gradient analyses. Although no significant difference between wild-type HuD and its mutants is observed in the overall polysome profiles (Figure 1), immunoblot analyses reveal that the distribution pattern of HuD proteins differs dramatically. Wild-type HuD and HuD216-385 lacking the ARE-binding domain (RBD1 and RBD2) (Figure 1) cosediment with heavy polysomes. In contrast, HuD14-302 lacking the poly(A)-binding domain RBD3 (Figure 1) and HuDmt, which contains amino acid substitutions in the RNP1 motif of all RBDs and consequently lacks any RNA-binding activity (Kasashima et al., 2002), do not cosediment with polysomes but are present in lighter fractions (Figure 1). These results suggest that RBD3 (i.e., poly[A]-binding activity) is required for HuD to associate with polysomes. To test this hypothesis, we constructed the HuD216-385mt by introducing amino acid substitutions in the RNP1 motif of RBD3 to eliminate its poly(A)-binding activity. While wild-type HuD binds specifically and directly to poly(A) (Figure 2A), HuD216-385mt fails to bind to poly(A) RNA (Figure 2B). Interestingly, no polysome association is detected with HuD216-385mt (Figure 1). This confirms our hypothesis that RBD3 is critical for HuD association with polysomes. However, RBD3 is not sufficient, as removal of the linker region between RBD2 and RBD3 (HuD303-385) also abolishes the ability of HuD to associate with polysomes (Figure 1). Thus the

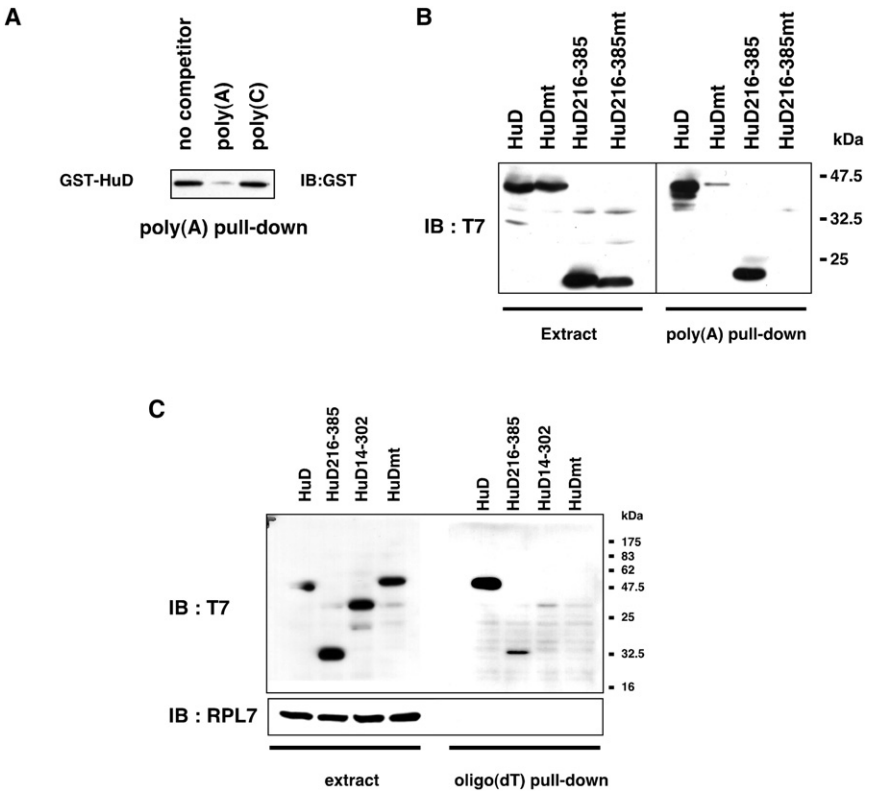

Figure 2. HuD Binds to Poly(A) RNA and Associates with Actively Translating mRNAs via RBD3

(A) Specific binding of HuD to poly(A) RNA. GST-HuD was precipitated with poly(A) agarose and analyzed by immunoblotting. Poly(A) was used as a specific competitor and poly(C) as a nonspecific competitor.

(B) RBD3 is required for binding to poly(A). The indicated HuD proteins were expressed in HeLa cells. Extracts were micrococcal nuclease treated. T7-tagged HuD proteins were precipitated with poly(A) agarose from these extracts and detected with anti-T7 antibody.

(C) Polysomal mRNPs (fractions 6–8; see Figure 1) were pooled, subjected to ultracentrifugation, and subsequently EDTA treated. HuD proteins and RPL7 were monitored by immunoblotting.

linker and RBD3 are important to promote polysome association.

Next, we tested whether HuD is bound to actively translating mRNAs. To address this question, we purified mRNPs from polysome gradient fractions using oligo(dT)-cellulose beads. In concert with the data described above, both wild-type HuD and HuD216-385 were identified in eluates from oligo(dT)-cellulose (Figure 2C), indicating that HuD binds to actively translating mRNAs via its poly(A)-binding domain rather than binding via the ARE-binding domain. The absence of ribosomal protein L7 (RPL7) (Figure 2C) indicates that the eluates are indeed ribosome free. Notably, binding of HuD216-385 is less efficient in comparison to wild-type HuD. This could reflect a stabilizing function of RBD1 and RBD2 in the binding of HuD to RNA. To confirm that HuD association with polysomes is indeed due to RNA binding, we performed pull-down assays from the RNase-treated extracts. HuD association with polysomes was abrogated by RNase treatment (data not shown). We conclude that HuD can bind to actively translating mRNAs, and hypothesize that this interaction might underlie a function of HuD in translation.

HuD Associates with the Cap-Binding Complex

We reasoned that HuD might associate with the translation initiation machinery. To examine this possibility, we first performed cap pull-down assays using 7-methylguanosine (m^7GTP) Sepharose beads and HeLa cell lysates expressing T7-tagged HuD proteins or GFP (Figure 3). Indeed, HuD associates with the m^7GTP cap-binding complex (CBC) (Figure 3A). This binding is specific, because it is inhibited by adding m^7GTP (Figure 3B) and is not impaired by addition of GTP (Figure 3B). We next delineated the HuD sequence elements that are important for its association with the CBC and find that, interestingly, RBD3 and the linker between RBD2 and RBD3 are also required for CBC association (Figure 3C). The same results were obtained in RNase-treated extracts (data not shown). These data point to an interaction of HuD with the eIF4F complex.

HuD Directly Interacts with eIF4A

To test whether HuD directly interacts with components of the eIF4F complex, we performed immunoprecipitation (IP) assays using HeLa cell lysates expressing T7-tagged HuD or GFP and anti T7-antibodies. eIF4G, eIF4A, eIF4E, and PABP copurify with T7-HuD, but not with the control GFP (Figure 4A, left panel, −RNase). To test whether the interaction is specific or mediated by bridging RNA, we performed IP assays from RNase-treated extracts (Figure 4A, right panel, +RNase). We observe a specific interaction of HuD with eIF4A (Figure 4A, right panel) but not with eIF4G, eIF4E, or PABP. To confirm our results, we included La protein as an additional negative control (see Figure S1 available online). La protein is an unrelated RBP commonly used as a control protein in IP assays (Svitkin et al., 1996; Imataka et al., 1997). We conclude that HuD does not bind to eIF4F, but rather only to eIF4A. Since eIF4A is present both in the eIF4F complex and as a free form, it appears that it is the free form of eIF4A that interacts with HuD. To confirm the HuD-eIF4A interaction, we performed IP assays using anti-eIF4A antibodies and found that HuD copurifies with endogenous eIF4A (Figure 4B). Moreover, we performed GST pull-down assays using purified recombinant GST-HuD and purified eIF4A (Figure 4C) and found that eIF4A copurifies with GST-HuD, but not with the negative control GST (Figure 4C). Thus, the interaction between eIF4A and HuD is direct. Next we wished to identify the region in HuD that mediates the eIF4A interaction. Using

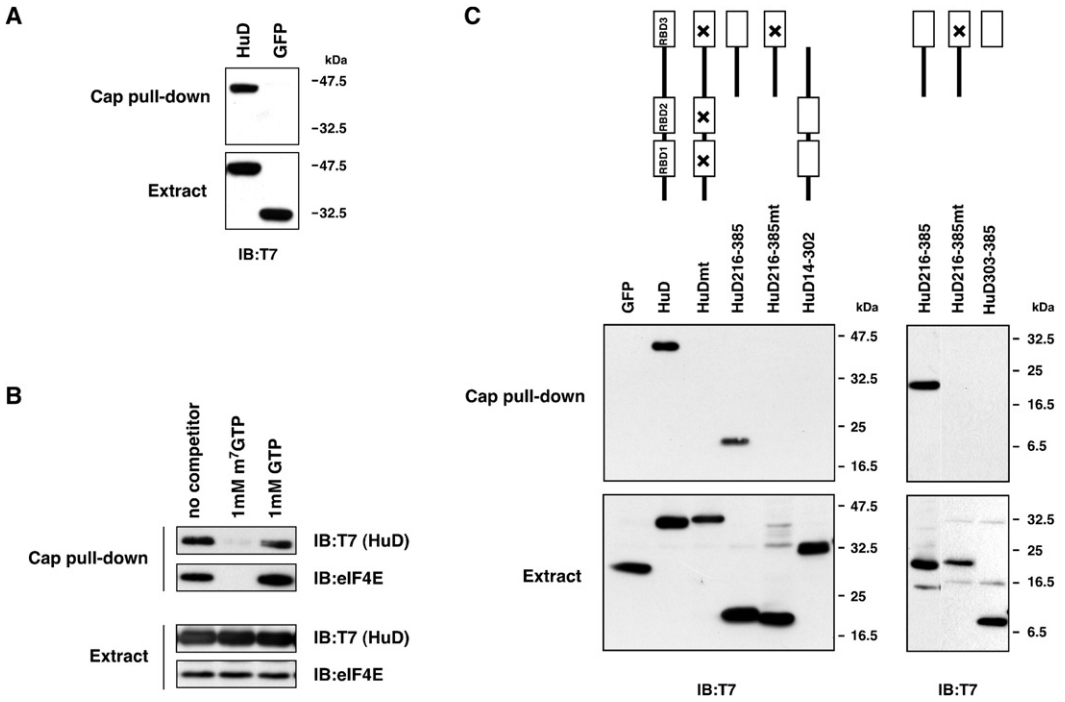

Figure 3. Association of HuD with the CBC

(A) Purification of HuD via m^{7}GTP affinity chromatography. HeLa cells were transfected with T7-HuD or T7-GFP-coding plasmids. Cytoplasmic extracts from transfected cells were subjected to m^{7}GTP affinity chromatography. Eluates were monitored by immunoblotting.

(B) Specific association of HuD with the CBC. HeLa cells were transfected with a T7-HuD containing plasmid. Extracts were subjected to m^{7}GTP affinity chromatography in the presence of no, specific (m^{7}GTP), or unspecific (GTP) competitor. Eluates were monitored by immunoblotting. eIF4E was used as a positive control.

(C) RBD3 and the linker region between RBD2 and RBD3 are required for HuD to associate with the CBC. HeLa cells were transfected with the indicated mRNAs. Extracts from these cells were subjected to m^{7}GTP affinity chromatography. Eluates were monitored by immunoblotting. GFP is a negative control. T7-tagged HuD reporter constructs are also shown above. Amino acid substitutions in the RNP1 motif of the respective RBD that abrogate RNA-binding activity are indicated by X.

GST-HuD deletion mutants, we find that the linker region between aa 250 and 302 is required for HuD's interaction with eIF4A (Figure 4C). To further map the interaction domain embedded within the linker region, we generated point mutants (R277A, F278A, S279A, and P280A) where the respective amino acid at positions 277, 278, 279, and 280 is changed into alanine (Figures 4D and 4E). This approach allowed the assignment of the eIF4A-binding site to position 278, because F278A, but not R277A, S279A, or P280A, fails to interact with eIF4A (Figure 4E).

In this context we also wanted to test whether the incorporation of HuD into the CBC depends on the HuD-eIF4A interaction. As shown in Figure 4F, HuD F278A association with the CBC is indeed impaired. In sum, we conclude that HuD's association with the CBC involves the interaction of HuD with eIF4A and poly(A), with both being required.

The Interactions of HuD with eIF4A and Poly(A) Are Important for Enhancement of Cap-Dependent Translation

The data presented above provide evidence for physical interactions between HuD with both eIF4A and poly(A), pointing to a potential role of HuD in translation. To assess this possibility directly, we explored the influence of HuD on the activity of translation utilizing a HeLa-derived cell-free translation system that reconstitutes the synergism between the 5′ cap structure and the 3′ poly(A) tail (Figure S2 and Bergamini et al., 2000).

Reporter mRNAs are incubated in micrococcal nuclease-treated HeLa translation extracts that are supplemented with HuD or GFP (see the Experimental Procedures). Interestingly, we observe significant stimulation of translation (approximately 2-fold) by HuD in comparison to the negative control GFP when translation occurs on transcripts that contain both a physiological m^{7}G cap structure and a poly(A) tail of 98 residues (Figures 5A and 5B). The mRNAs were of equal stability at the end of the incubation time (Figures 5A and 5B). Thus, changes in mRNA stability are not responsible for the observed stimulatory effect of HuD. The stimulatory effect of HuD on translation is also observed with capped and polyadenylated mRNAs that contain the c-*fos* 3′UTR, which has been shown to interact with HuD (Wang and Tanaka Hall, 2001) (Figure 5A). However, stimulation of translation by HuD is strictly cap and poly(A) dependent, as it is not observed with (1) polyadenylated mRNAs containing a nonphysiological ApppG-cap structure which cannot bind to eIF4E, or (2) transcripts with a m^{7}G cap structure that lack a poly(A) tail (Figure 5A). Moreover, we find that the eIF4A and poly(A)-binding activity of HuD is critical to enhance

translation, as both the HuD14-302 mutant, which lacks RBD3 and thus cannot bind to poly(A), and the HuD/F278A mutant, which cannot interact with eIF4A, fail to stimulate translation (Figure 5B). Having identified the importance of the HuD-eIF4A and the HuD-poly(A) interactions in translation, we validated our finding by monitoring translation of the HCV IRES mRNA, which is naturally nonpolyadenylated and which does not require the function of the RNA helicase eIF4A (Pestova et al., 1998). In contrast to cap-dependent translation, the HCV IRES is unaffected by addition of wild-type HuD (Figure 5C). This result strongly supports our hypothesis that HuD stimulates translation via binding to eIF4A and poly(A). Interestingly, HuD216-385 displays only a moderate stimulatory effect on translation in comparison to wild-type HuD (Figure 5B), which is mirrored by its reduced association with polysomes and actively translating mRNAs (Figures 1 and 2C). This implies that RBD1 and RBD2 may exert a stabilizing function in the binding of HuD to RNA.

The eIF4A and Poly(A)-Binding Domains of HuD Contribute to Its Neurite-Inducing Activity

It is well appreciated that neuronal Hu proteins, but not the ubiquitously expressed Hu protein HuR, can promote neuronal differentiation without neurotrophin (Akamatsu et al., 1999; Kasashima et al., 1999). PC12 cells are an established model system for studying neuronal differentiation (Greene and Tischler, 1976; Vaudry et al., 2002) and can be induced to form neurites by overexpression of HuD (Anderson et al., 2000). The results shown above have prompted us to test whether the interaction of HuD with poly(A) and eIF4A to stimulate translation underlies its ability to induce neurite outgrowth in PC12 cells. To address this question directly, we assayed the ability of different HuD mutants to induce outgrowth in these cells. To that end, PC12 cells were transfected with wild-type HuD or HuD mutant constructs as well with constructs coding for GFP or HuR, which serve as negative controls. As shown in Figure 6A, wild-type HuD and HuD 216–385, both capable of CBC association, can induce neurite outgrowth in PC12 cells. In sharp contrast, the induction of neurite outgrowth is impaired when PC12 cells are transfected with constructs lacking RBD3. Similarly, no outgrowth is seen when the linker is absent, underscoring the importance of both RBD3 and the linker in inducing outgrowth. Next, we tested whether the induction of outgrowth correlates with eIF4A binding and eIF4A function. The results shown in Figures 6B and 6C highlight the importance of eIF4A in this process, as neurite outgrowth is strongly reduced in PC12 cells that have been transfected with the eIF4A-binding mutant F278A (Figure 6B). Furthermore, neurite outgrowth is completely repressed if the dominant-negative mutant DQAD of eIF4A is cotransfected with HuD (Figure 6C). On the basis of these results, we suggest that CBC association of HuD is a prerequisite for the neurite-inducing activity of HuD.

DISCUSSION

Neuronal Hu proteins play an essential role in neuronal development. However, the underlying molecular mechanisms are poorly understood. It is known that Hu proteins bind to mRNAs (Hinman and Lou, 2008). Here we have explored the role of HuD, a family member of neuronal proteins, on translation and found that it stimulates cap- and poly(A)-dependent translation. We investigated the underlying molecular interactions and demonstrate that HuD interacts with eIF4A (Figure 4 and Figure S1). This interaction is direct and RNA independent and is mediated by the linker domain of HuD (critical aa, 278) (Figures 4C and 4E). We also find that the poly(A)-binding domain RBD3 of HuD is required for its enhancer function (Figure 5B). Mutating the eIF4A and/or the poly(A)-binding domain in HuD abolishes HuD-mediated translational stimulation (Figure 5B).

The strategy of HuD to interact with eIF4A bears some similarity to that of the PABP-interacting protein (PAIP-1), which also binds eIF4A and stimulates cap-dependent translation (Craig et al., 1998; Martineau et al., 2008). The similar qualitative effects of HuD and PAIP1 could suggest that the underlying molecular interactions are also the same. Beside eIF4A, PAIP1 binds to PABP and eIF3 and forms ternary complexes composed of PAIP1-PABP-eIF4G and PAIP1-eIF3-eIF4G. These complexes are believed to stabilize the interaction between eIF4G and PABP (Martineau et al., 2008) and as a consequence enhance translation. Our data reveal that HuD does not interact directly with PABP (Figure 4A) but rather with poly(A) (Figures 2A and 2B), suggesting that HuD contributes to cap-dependent translation in a distinct way and not by stabilizing the interaction between eIF4G and PABP. Interestingly, we find that HuD binds to eIF3 via the eIF3b subunit, which belongs to the functional core of mammalian eIF3 (T.F. and C.T., unpublished data; Masutani et al., 2007). This interaction appears to be at variance with the PAIP1-eIF3 interaction, which is mediated by the eIF3g subunit (Martineau et al., 2008), which is not a component of the functional core of mammalian eIF3. Thus, the resulting functional consequences from these interactions might also be distinct. The functional relevance of the HuD-eIF3b interaction remains to be studied.

Translational activation of luciferase reporter mRNAs by HuD requires the presence of a cap structure and a poly(A) tail (Figure 5A). HuD binds to the CBC at the 5′ end of the mRNA via eIF4A and to poly(A) at the 3′ end of the mRNA. We suggest that HuD could enable bridging to occur between the mRNA 5′ and 3′ ends (Figure 7). Such an interaction may permit additional levels of translational regulation and may lead to the stabilization of the HuD-containing mRNP. Translation stimulation may occur by directly enhancing eIF4A activity. It is possible that HuD/eIF4A interactions create a platform for the recruitment of distinct translational activators. Another intriguing possiblity is that HuD regulates eIF4A function via activating eIF4B, as it has been shown that eIF4B can stimulate the helicase activity of eIF4A (Rozen et al., 1990; Altmann et al., 1993). As recently demonstrated, eIF4B can be phosphorylated (Raught et al., 2004; Shahbazian et al., 2006; van Gorp et al., 2009). This raises the question of whether HuD can enhance translation via regulating the phosphorylation status of eIF4B. Future experiments will aim to distinguish between these possibilities.

At which step does HuD enhance cap-dependent translation? The requirement for eIF4A suggests that HuD stimulates the initiation step of translation as eIF4A triggers the unwinding of RNA secondary structures and promotes scanning of the 43S

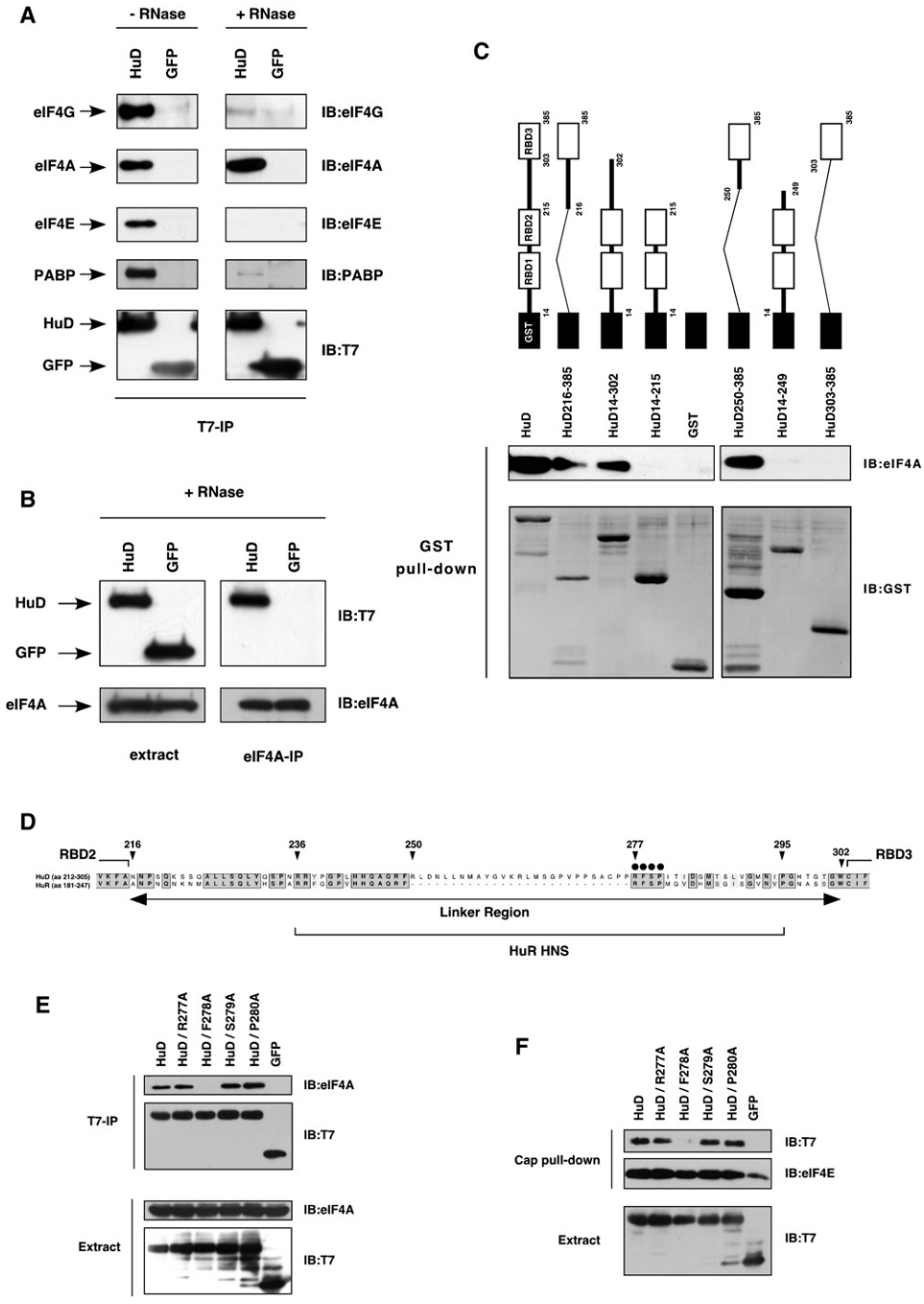

Figure 4. Direct Protein-Protein Interaction by HuD and eIF4A

(A) Specific coimmunoprecipitation of eIF4A with HuD. HeLa cells were transfected with T7-HuD or T7-GFP-coding plasmids. Cytoplasmic extracts from transfected cells were micrococcus nuclease treated (right panel) or nontreated (left panel). HuD was immunoprecipitated with anti-T7 antibody. Coimmunoprecipitation was monitored by immunoblotting. GFP is a negative control.

(B) Specific coimmunoprecipitation of HuD with eIF4A. HeLa cells were transfected with T7-HuD or T7-GFP-coding plasmids. Cytoplasmic extracts from transfected cells were micrococcal nuclease treated. Endogenous eIF4A was immunoprecipitated with anti-eIF4A antibody. Coimmunoprecipitation was monitored by immunoblotting. GFP is a negative control.

(C) RNA-independent interaction between HuD and eIF4A. Recombinant eIF4A was incubated with the indicated GST-HuD proteins. GST pull-downs were examined for copurification of eIF4A by immunoblotting (upper panel) and for pull-down efficiency by immunoblotting with anti-GST antibody (lower panel).

(D) Amino acid alignment of the linker region of HuD and HuR. The amino acid sequence of mouse HuD is compared to that of human HuR. Identical residues are shown in gray. The shuttling signal of HuR (HNS) is indicated. Black filled circles indicate the positions of alanine substitutions.

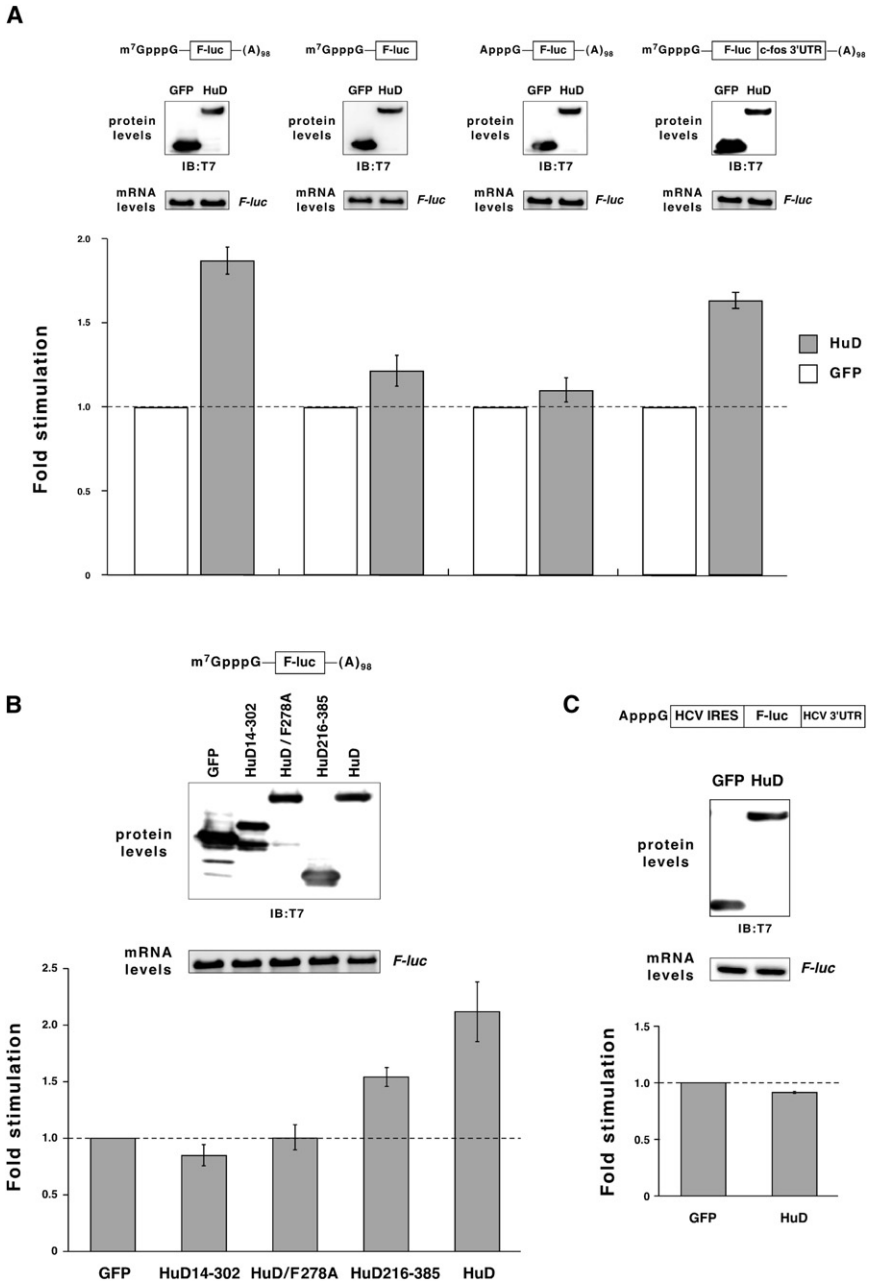

Figure 5. Role of HuD in Translation

(A) The poly(A) tail and the cap structure are important for translation stimulation by HuD. Micrococcal nuclease-treated HeLa translation extracts were programmed with the indicated firefly luciferase reporter mRNAs in the presence of equal amounts of wild-type HuD or GFP (for details, see the Experimental Procedures). Fold stimulation by HuD was calculated by dividing firefly luciferase counts obtained in HuD-containing translation reactions by those in GFP-containing translation reactions, which are set as 1 (symbolized as dotted line). Error bars reflect the standard deviation of values in at least three independent experiments. Input levels of GFP or HuD were monitored by immunoblotting. Physical stabilities of the mRNAs at the end of the incubation time are analyzed by northern blotting.

(B and C) Translation stimulation by HuD is eIF4A dependent. Micrococcal nuclease-treated HeLa translation extracts were programmed with capped and polyadenylated (98 poly[A] residues) firefly luciferase reporter mRNAs (B) or HCV IRES mRNAs (C) in the presence of equal amounts of the indicated proteins. Fold stimulation was calculated by dividing firefly luciferase counts obtained in the respective HuD-containing translation reactions by those in GFP-containing translation reactions, which are set as 1 (symbolized as dotted line). Error bars reflect the standard deviation of values in at least three independent experiments. Input levels of GFP, HuD mutants, or HuD were monitored by immunoblotting. Physical stabilities of the mRNAs at the end of the incubation time are analyzed by northern blotting.

preinitiation complex to the start codon. Moreover, the interaction of HuD with eIF3b (T.F. and C.T., unpublished data) also points to a role of HuD in regulating translation initiation as eIF3b promotes ribosome binding to the mRNA (Masutani et al., 2007). However, the present results are equally consistent with a postinitiation step being targeted, in particular, as recent data suggest that eIF3 also functions downstream of initiation (Nielsen et al., 2004; Kolupaeva et al., 2005). The HuD-induced proximity of the mRNA ends may promote recycling of terminating ribosomes on the mRNA, thus enhancing translation. Sucrose density gradient experiments will have to be performed to address this question.

We note a reduced association of HuD216-385 with polysomes (Figure 1) and actively translating mRNAs (Figure 2C) in comparison to wild-type HuD. Moreover, we observe that wild-type HuD is more active than HuD216-385 in the translation assay (Figure 5B, left panel). This is interesting but not unexpected, given the importance of RBD1 and RBD2 in the binding of HuD to RNA (Deschenes-Furry et al., 2006). Possibly, binding of HuD to mRNAs is weakened in the absence of RBD1 and RBD2. This might also explain why Atlas and colleagues did not detect a truncated form of HuD lacking RBD1 and RBD2 in

(E) The interaction between HuD and eIF4A is mediated by the phenylalanine-278 residue in HuD. HeLa cells were transfected with the indicated plasmids. Cytoplasmic extracts from transfected cells were micrococcal nuclease treated. HuD proteins were immunoprecipitated with anti-T7 antibody. Coimmunoprecipitation of eIF4A was monitored by immunoblotting.

(F) Association of HuD with the CBC depends on the HuD-eIF4A interaction. HeLa cells were transfected with the indicated plasmids. Cytoplasmic extracts from transfected cells were subjected to m^7GTP affinity chromatography. Eluates were monitored by immunoblotting. eIF4E is a positive control.

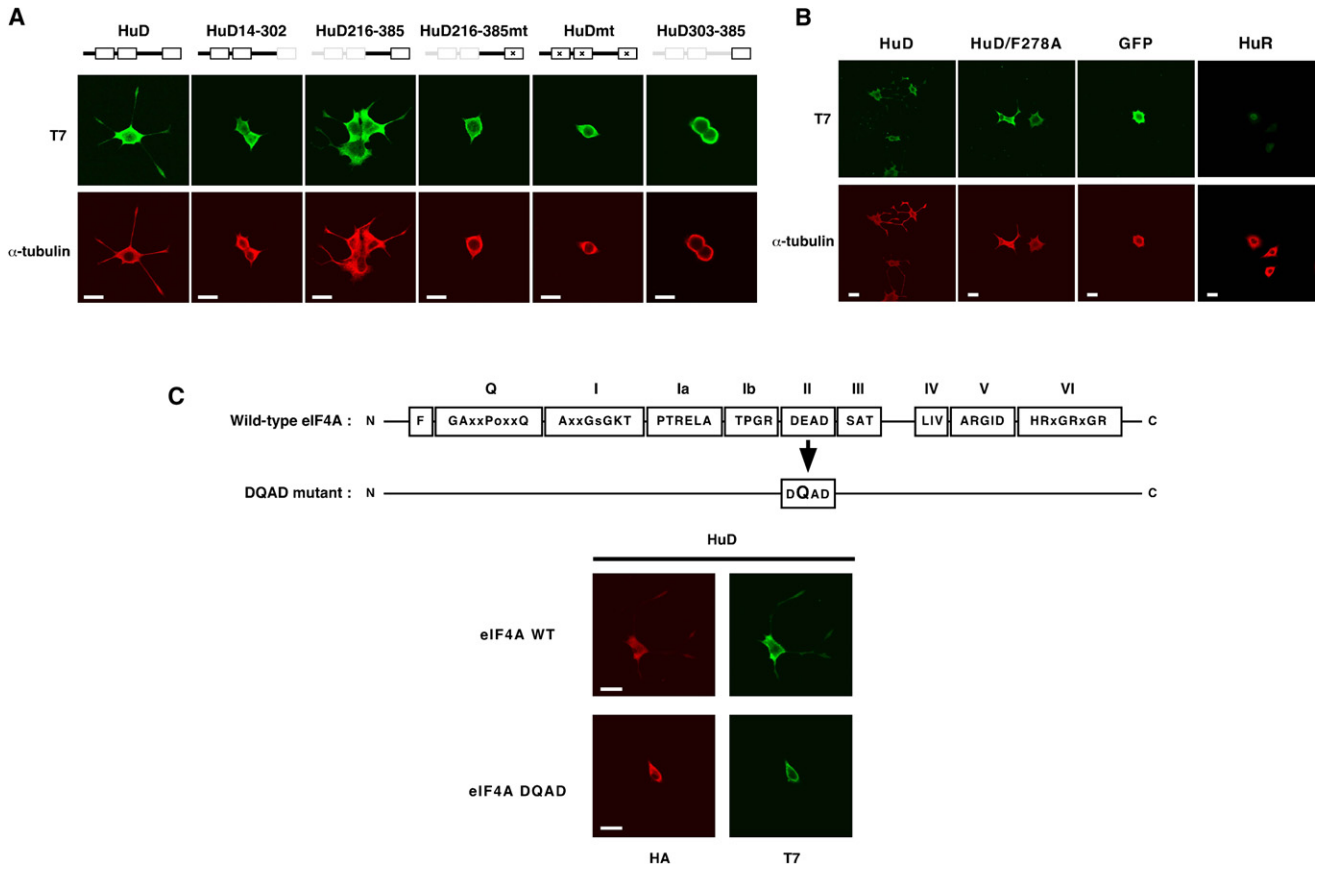

Figure 6. Role of HuD in Induction of Neurite Outgrowth

(A) RBD3 and the linker region between RBD2 and RBD3 are required for HuD to induce neurite outgrowth. Shown is confocal analysis of PC12 cells that were transfected with the indicated constructs. Cells were costained with anti-T7 (green) and with anti-α-tubulin antibody (red). The same results were obtained in at least three independent experiments (mt = mutant). Scale bar, 20 μm.

(B) The eIF4A-binding activity of HuD is critical for neurite outgrowth induction. Confocal analysis of PC12 cells that were transfected with constructs coding for wild-type HuD, HuD/F278A mutant, GFP, or HuR. Cells were costained with anti-T7 (green) and with anti-α-tubulin antibody (red) The same results were obtained in at least three independent experiments. Scale bar, 20 μm.

(C) HuD-mediated induction of neurite outgrowth requires eIF4A. (Upper panel) Schematic representation of eIF4A. The conserved motifs of the DEAD box protein are indicated. The mutations in DQAD are in bold. (Lower panel) HuD-induced neuronal differentiation is inhibited by the dominant-negative eIF4A mutant DQAD. Confocal analysis of PC12 cells that were cotransfected with HA-tagged wild-type eIF4A or the DQAD mutant of eIF4A and T7-tagged HuD. Cells were costained with anti-HA monoclonal antibody (a-HA) followed by Alexa 546-conjugated anti-mouse IgG (red) and with anti-T7 polyclonal antibody (a-T7) followed by Alexa 488-conjugated anti-rabbit IgG (green). Scale bar, 20 μm.

polysome-enriched pellets of transfected cells when using different and more stringent conditions (Atlas et al., 2007).

In summary, we have presented data that uncover an enhancer function of HuD in cap-dependent translation. Interestingly, there is a correlation between the domains that are required for translation activation and neurite outgrowth induction (Figures 5B and 6). This correlation suggests that HuD's ability to stimulate translation may be a prerequisite for its neurite-inducing activity.

EXPERIMENTAL PROCEDURES

Plasmids

Plasmids encoding T7-tagged mouse HuD proteins, T7-tagged GFP, T7-tagged HuR, and GST-HuD fusion proteins were described previously (Kasashima et al., 2002; Saito et al., 2004). T7-tagged HuD/R277A, HuD/F278A, HuD/S279A, HuD/P280A mutants were generated from pBOS-T7-HuD by replacing Arg277, Phe278, Ser279, and Pro280 with Ala, respectively, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). To obtain the FLAG-La protein construct, the La protein-coding sequence of pcDNA3-HA-La, which has been described previously (Imataka et al., 1997), was inserted into the p-FLAG construct. All constructs were verified by DNA sequencing. The plasmids for HA-tagged wild-type eIF4A and a dominant-negative mutant of eIF4A (eIF4A/DQAD) were described previously (Pause et al., 1994). Plasmids encoding firefly luciferase, pT3LUC, and pT3LUC(pA) were described previously (Iizuka et al., 1994). pHCV-IRES-luc was described recently (Thoma et al., 2004). pFLAG-HuD was described previously (Kasashima et al., 1999).

Recombinant Proteins

Hu proteins expressed in *E. coli* as GST fusions were purified as described (Kasashima et al., 2002; Saito et al., 2004). Recombinant eIF4A and mutants were expressed in *E. coli* and purified as described previously (Mikami et al., 2008).

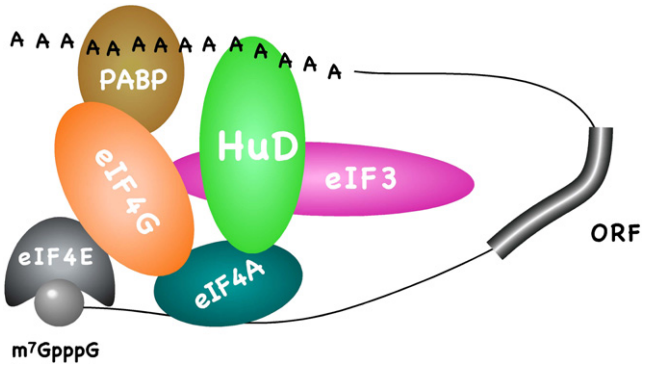

Figure 7. HuD-eIF4A Translation Stimulation Model
Physical interactions occur between HuD and eIF4A on one side and HuD and poly(A) on the other.

Cell Culture and Transfection

PC12 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum and 5% horse serum (for PC12 cells) or 10% fetal bovine serum (for HeLa cells), respectively. Cells were transiently transfected using the Lipofectamine 2000 transfection reagent (Invitrogen).

PC12 cells were transiently transfected with 8 μg of plasmid DNA and 10 μl of Lipofectamine 2000 (Invitrogen) per 6 cm dish for polysome analyses and with 1 μg of plasmid DNA and 2 μl Lipofectamine 2000 for determination of neurite-inducing activity. The cells were harvested for polysome analyses 44 hr after transfection and for determination of neurite-inducing activity 72 hr after transfection. HeLa cells were transiently transfected with 1.5 μg of plasmid DNA and 6 μl of PolyFect Transfection Reagent (QIAGEN) per 3.5 cm dish. The cells were harvested for biochemical analyses 24 hr after transfection.

m^7GTP Sepharose Pull-Down Assay

m^7GTP Sepharose pull-down assays were carried out as described previously (Yoder-Hill et al., 1993). Briefly, extracts of HeLa cells that had been transfected with constructs coding for T7-HuD WT or mutants or T7-GFP were incubated with m^7GTP Sepharose beads in TNE buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) for 2 hr at 4°C. Beads were washed five times in TNE buffer, and bound proteins were eluted with SDS-PAGE loading buffer and subjected to 5%–20% linear gradient SDS-PAGE and western blotting using monoclonal anti-T7 antibody, 1:5000 dilution (Novagen). Competition experiments were performed with 1 mM m^7GpppG cap analog (Sigma) or 1 mM GTP (Sigma).

Immunoprecipitation

HeLa cells that have been transfected with constructs coding for T7-HuD, T7-GFP, FLAG-HuD, or FLAG-La were lysed in TNE buffer. The extracts were then used for IP. Where indicated, extracts were subjected to micrococcal nuclease treatment (Takara). Monoclonal anti-T7 antibody (Novagen), polyclonal anti-eIF4A antibody (Abcam), or monoclonal anti-Flag M2 antibody (Sigma) was added to the extracts together with protein G Sepharose beads. Bound proteins were eluted with SDS-PAGE loading buffer and subjected to SDS-PAGE and western blotting using monoclonal anti-T7 antibody, 1:5000 dilution (Novagen); polyclonal anti-eIF4G antibody, 1:1000 dilution (Santa Cruz Biotechnology); polyclonal anti-eIF4AI antibody, 1:1000 dilution (Abcam); polyclonal anti-eIF4E antibody, 1:1000 dilution (Santa Cruz Biotechnology); and polyclonal anti-PABP1 antibody, 1:2000 dilution (Imataka et al., 1998).

Determination of Neurite-Inducing Activity in PC12 Cells

After transfection of PC12 cells with constructs coding for T7-tagged HuD proteins or T7-tagged GFP, cells were cultured for 3 days and immunostained with polyclonal anti-T7 antibody, 1:2000 dilution (Bethyl), and monoclonal anti-α-tubulin antibody, 1:5000 dilution (Sigma). To address the role of eIF4A on the neurite-inducing activity of HuD, PC12 cells were cotransfected with T7-HuD and either HA-eIF4A wild-type or HA-eIF4A/DQAD mutant and incubated for 3 days at 37°C. Immunostaining was then performed with polyclonal anti-T7 antibody, 1:2000 dilution (Bethyl); and monoclonal anti-HA antibody, 1:1000 dilution (Sigma). Alexa 488 anti-mouse IgG and Alexa 546 anti-rabbit IgG were used as secondary antibodies, 1:1000 dilution (Invitrogen). Confocal analysis was performed using a confocal laser-scanning microscope (Zeiss LSM5 Pascal).

In Vitro Binding Experiments

GST pull-down assays were performed as described previously (Saito et al., 2004). Bound proteins were separated by SDS-PAGE. Immunoblotting was performed with polyclonal anti-eIF4A1 antibody, 1:1000 dilution (Abcam); and monoclonal anti-GST antibody, 1:5000 dilution (Sigma).

Poly(A)-Binding Assay

The poly(A)-binding assay was performed with 1 μg of recombinant GST-HuD. GST-HuD in binding buffer (20 mM Tris-HCl [pH 7.5], 150 mM KCl, 3 mM MgCl2, 0.1% Triton X-100) was incubated with 10 mg of poly(A) Sepharose beads (Pharmacia) for 2 hr at 4°C. For competition analysis, an excess amount of poly(A) or poly(C) (Pharmacia) was preincubated with GST-HuD for 30 min on ice, followed by the addition of poly(A) Sepharose beads. After washing five times with binding buffer, proteins were eluted with SDS-PAGE loading buffer and subjected to SDS-PAGE. Immunoblotting was performed by using monoclonal anti-GST antibody, 1:5000 dilution (Sigma).

To assay the poly(A)-binding activity of HuD mutant proteins, the extracts from HeLa cells transfected with constructs coding for T7-HuD, HuDmt, HuD216-385, and HuD216-385mt were analyzed. Extracts were treated with micrococcal nuclease (Takara) in the presence of 1 mM calcium acetate for 6 min at 26°C. Micrococcal nuclease was inactivated by adding EGTA to a final concentration of 2 mM. The supernatants were centrifuged for 2 min at 13,000 g at 4°C and analyzed as described above. Immunoblotting was performed with monoclonal anti-T7 antibody, 1:5000 dilution (Novagen).

Sucrose Gradient Centrifugation

PC12 cells transfected with the indicated reporter constructs (Figure 1) were lysed in polysome buffer (20 mM HEPES-KOH [pH 7.5], 100 mM KCl, 10 mM $MgCl_2$, 0.25% NP-40, 10 mg/ml cycloheximide, 100 units/ml RNase inhibitor, protease inhibitor cocktail). Lysates were loaded on top of a linear 10%–45% sucrose gradient. After centrifugation at 40,000 rpm for 60 min at 4°C in a Beckman MLS50 rotor, fractions were collected from the top of the gradient and subjected to UV-densitometric analysis. Next, each fraction was subjected to TCA precipitation and SDS-PAGE. Immunoblotting was performed using a monoclonal anti-T7 antibody, 1:5000 dilution (Novagen); and a polyclonal anti-RPL7 antibody, 1:2000 dilution (Abcam).

Oligo(dT)-Cellulose-Binding Assay

Polysome fractions were collected and centrifuged again by using a Beckman TLA-100 rotor at 75,000 rpm for 120 min at 4°C. The polysome pellet was resuspended in buffer containing 20 mM HEPES (pH 7.5), 100 mM KCl, 25 mM EDTA, 100 units/ml RNase inhibitor, and protease inhibitor cocktail to dissociate polysomes and to release poly(A) mRNA-mRNP complexes according to the methods described (Lindberg and Sundquist, 1974). The suspensions were centrifuged at 12,000 × g for 2 min, and the supernatants were mixed with prewashed oligo(dT)-cellulose (Ambion). After 60 min of constant rotation at 4°C, the resins were washed five times and analyzed for the presence of T7-tagged HuD proteins by immunoblotting using monoclonal anti-T7 antibody, 1:5000 dilution (Novagen); and polyclonal anti-RPL7 antibody, 1:2000 dilution (Abcam).

In Vitro Translation and In Vitro Transcription

In vitro transcription of mRNAs in the presence of either ^{7}mGpppG or ApppG, the preparation of HeLa cell extracts, the micrococcal nuclease treatment, and in vitro translation assays were described previously (Bergamini et al., 2000; Thoma et al., 2004). The concentration of exogenous mRNA was 10 ng/μl for

reporter luc mRNAs. Translation reactions were incubated for 60 min at 37°C. For analyzing the effect of HuD on translation, extracts from HeLa cells that have been transfected with HuD, HuD mutants, or GFP-expressing constructs were first micrococcal nuclease treated and then added to in vitro translation reactions. Equal amounts of HuD, HuD mutants, or GFP were added as confirmed by immunoblotting using a monoclonal anti-T7 antibody, 1:5000 dilution (Novagen).

Northern Blot Analysis

Total RNA was extracted from translational reactions using a High Pure RNA Isolation Kit (Roche) as recommended by the manufacturer. Samples were separated in a 1.0% formaldehyde-containing agarose gel and transferred onto a nylon membrane (Roche Diagnostics). Membranes were hybridized with DIG-labeled antisense RNA probes corresponding to the luciferase ORF and subjected to analysis with the LAS-4000 image analyzer (Fuji).

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00827-2.

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