Functional and direct interaction between the RNA binding protein HuD and active Akt1

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Received June 29, 2011; Revised and Accepted October 14, 2011

ABSTRACT

The RNA binding protein HuD plays essential roles in neuronal development and plasticity. We have previously shown that HuD stimulates translation. Key for this enhancer function is the linker region and the poly(A) binding domain of HuD that are also critical for its function in neurite outgrowth. Here, we further explored the underlying molecular interactions and found that HuD but not the ubiquitously expressed HuR interacts directly with active Akt1. We identify that the linker region of HuD is required for this interaction. We also show by using chimeric mutants of HuD and HuR, which contain the reciprocal linker between RNA-binding domain 2 (RBD2) and RBD3, respectively, and by overexpressing a dominant negative mutant of Akt1 that the HuD–Akt1 interaction is functionally important, as it is required for the induction of neurite outgrowth in PC12 cells. These results suggest the model whereby RNA-bound HuD functions as an adapter to recruit Akt1 to trigger neurite outgrowth. These data might also help to explain how HuD enhances translation of mRNAs that encode proteins involved in neuronal development.

INTRODUCTION

RNA binding proteins (RBPs) are key mediators of post-transcriptional control mechanisms, including the control of mRNA translation (1,2). This cytoplasmic regulatory mechanism also plays crucial roles in neuronal development (2). Several RBPs are specifically expressed in neurons such as the neuronal Hu proteins, which are essential for proper neuronal development and plasticity (3). The neuronal Hu family consists of three members, HuB, HuC and HuD. Hu proteins contain three RNA-binding domains (RBDs) and a linker region between RBD2 and RBD3 (3). The biological functions of Hu proteins result from their ability to bind to target mRNAs. Hu proteins stabilize adenine/uridine-rich element (ARE)-containing transcripts by binding to AREs via RBD1 and RBD2 and also affect translation (3). We have recently shown that HuD upregulates cap- and poly(A)-dependent translation through a direct interaction with eukaryotic initiation factor 4A (eIF4A) via its linker region (4). This enhancer function also involves the poly(A) binding activity of HuD via RBD3 (4). Interestingly, the linker region and RBD3 are also crucial for the stimulatory effect of HuD on neurite outgrowth, revealing a posttranscriptional role in neuronal development and plasticity (4). However, the underlying molecular mechanism(s) and interactions are poorly understood. In
MATERIALS AND METHODS

Plasmids

Plasmids encoding T7-tagged mouse HuD proteins and glutathione-S-transferase (GST)-HuD fusion proteins were described previously (7,8). To generate the chimeric constructs FLAG-HuR-DL and FLAG-HuD-RL, the HuD- or HuR-linker, respectively, was inserted into the pFLAG HuR and HuD constructs. pFLAG-Akt1 dn was generated from pFLAG-Akt1 by replacing Lys179, Thr308 and Ser473 with Ala, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) (9,10).

Recombinant proteins

Hu proteins expressed in Escherichia coli as GST-fusions were purified as described (7,8). Recombinant active and inactive Akt1 were purchased from Upstate (Millipore).

Purification of FLAG-Akt1 and FLAG-eIF4B

HeLa cells were transfected with the expression plasmid coding for FLAG-tagged Akt1 or FLAG-tagged eIF4B and cultured for 48 h at 37°C. The purification procedures were carried out on ice. The extract from HeLa cells (1 x 10^7 cells) was lysed in lysis buffer (20 mM Tris–HCl, pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4 and 50 μg/ml phenylmethylsulfonylfluoride), treated with benzozene and centrifuged at 18 000g for 10 min. The supernatant was incubated with FLAG M2 affinity gel (Sigma) equilibrated with lysis buffer for 3 h. After washing resin, FLAG-Akt was eluted with 100μl of elution buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, 50 μg/ml phenylmethylsulfonylfluoride and 100 μg/ml 3x FLAG-peptide) (11).

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).

Immunoprecipitation

HeLa cells that have been cotransfected with the constructs coding for T7-HuD or T7-GFP and FLAG-tagged Akt1 were lysed in TNE buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The extracts were then used for immunoprecipitation. Anti-T7 monoclonal antibody (Novagen) or anti-FLAG polyclonal 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 anti-T7 monoclonal antibody and anti-FLAG polyclonal antibody.

In vitro binding experiments

GST pull-down assays were performed in TNE buffer (composition see above) as described previously (8). Bound proteins were separated by SDS–PAGE. Immunoblotting (IB) was performed with anti-FLAG, anti-Akt1 polyclonal antibodies and anti-GST monoclonal antibody (Sigma).

Determination of neurite-inducing activity in PC12 cells

After transfection of PC12 cells with constructs coding for T7-tagged or FLAG-tagged proteins, cells were cultured for 3 days and immunostained with anti-T7 polyclonal antibody (Bethyl) and anti-β-tubulin monoclonal antibody (Sigma). To address the role of Akt1 on the neurite-inducing activity of HuD, PC12 cells were cotransfected with T7-HuD or HuR-RL and either Akt1 dn or wild-type Akt1 and incubated for 3 days at 37°C. Immunostaining was then performed with anti-T7 polyclonal antibody and anti-FLAG monoclonal antibody (Sigma). Alexa 488 anti-rabbit IgG and Alexa 546 anti-mouse IgG were used as secondary antibodies. 1:1000 dilution (Invitrogen). Confocal analysis was performed using a confocal laser-scanning microscope (Zeiss LSM5 Pascal).

GSK-3β protein kinase assay

The enzyme activity of Akt1 was assayed by measuring the incorporation of radioactivity from [γ-32P] ATP to glycogen synthase kinase 3β (GSK-3β) fusion protein (cell signaling), a synthetic substrate specific to Akt. Active Akt1 (upstate) was incubated with purified GST-HuD, GST-HuR or GST. Next, poly(U) beads were added to the mixture and incubated for 120 min. The poly(U) beads were collected by centrifugation and washed three times with TNE buffer. Before the kinase assay, the collected precipitates were washed at 0–4°C with 20 mM Tris–HCl pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 150 mM NaCl and 50 μg/ml phenylmethylsulfonyl fluoride to remove NP-40. The reaction mixture (25 μl) containing 20 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 20 mM ATP, 15–50 kBq of [γ-32P] ATP and 100 mg/ml GSK-3β fusion
protein was then added to the precipitates and incubated for 30 min at 30°C. After boiling in SDS sample buffer, phosphorylated proteins were separated by SDS-PAGE. The radioactivity of the GSK-3β fusion protein band was analyzed by Bio-imaging Analyzer BAS2500 (Fujix).

In vitro kinase reaction using GST-HuD, FLAG-eIF4B or MBP-FOXO4

GST-HuD, FLAG-eIF4B or maltose-binding protein (MBP)-FOXO4 (Forkhead box protein O4) were incubated with and without active Akt1 in 20 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 20 μM ATP and [γ-³²P] ATP at 30°C for 30 min. After boiling in SDS-sample buffer, proteins were separated by SDS-PAGE. ³²P-labeled proteins were visualized using Bio-imaging Analyzer, BAS2500(Fuji) after Coomassie Brilliant Blue (CBB) staining (12).

RESULTS

HuD directly interacts with active Akt1

To examine the mechanism of how HuD induces neurite outgrowth, we used PC12 cells, which are an established model system for studying neuronal differentiation (13,14). PC12 cells can be induced to form neurites by overexpression of HuD (15). Using this system, we have recently shown that the eIF4A- and poly(A)-binding domains of HuD contribute to its neurite-inducing activity (4). We have also shown that these activities are critical for stimulating cap-dependent translation. Here, we further investigated the underlying molecular interactions.

Activation of the mTOR pathway by PI3K-Akt signaling is key for stimulating translation. Akt signaling promotes growth and proliferation including NGF-mediated neurite outgrowth induction (6). We hypothesized that (i) Akt1 function might be critical for the neurite-inducing activity of HuD and (ii) Akt1 might directly interact with HuD to fulfill this function. To examine this possibility, we performed first immunoprecipitation assays using HeLa cell lysates expressing FLAG-tagged Akt1 and T7-tagged HuD or GFP (Figure 1). Indeed, HuD, but not the negative control GFP, copurifies with FLAG-Akt1 (Figure 1A, left panel) and vice versa Akt1 copurifies with T7-HuD, but not with the control T7-GFP (Figure 1A, right panel).

To further confirm the interaction between endogenous Akt1 and HuD, we performed immunoprecipitation assays using PC12 cell lysates expressing T7-tagged HuD or GFP (Figure 1B) and examined coprecipitation of endogenous Akt1 with T7-tagged HuD or GFP. We found that endogenous Akt1 coimmunoprecipitated with T7-HuD but not with T7-GFP (Figure 1B).

Next, we tested whether the interaction is direct and specific or mediated by bridging RNA. To that end, we performed GST pull-down assays using purified recombinant GST-HuD and FLAG-tagged Akt1, which has been purified via FLAG M2 affinity gel (see ‘Materials and Methods’ section for further details) from benzonase-treated HeLa extracts before the GST pull-down assays (Figure 2A). We have used the endonuclease benzonase because it works well also at low temperatures and degrades all forms of RNA importantly also poly(A) RNA which is not digested by RNase A. We found that FLAG-Akt1 copurifies with GST-HuD but not with the negative control GST (Figure 2A). Thus, the interaction between Akt1 and HuD is direct and specific.

To test whether active (phosphorylated) or inactive Akt1 interacts with HuD, we used commercially available

Figure 1. Protein–protein interaction by HuD and Akt1. (A) Specific coimmunoprecipitation of Akt1 with HuD. HeLa cells were transfected with T7-HuD or T7-GFP and FLAG-Akt1-coding plasmids. HuD was immunoprecipitated with anti-T7 antibody (right panel). Akt1 was immunoprecipitated with anti-FLAG antibody (left panel). Coimmunoprecipitation was monitored by IB. GFP is a negative control. (B) Specific coimmunoprecipitation of endogenous Akt1 with HuD. PC12 cells were transfected with T7-HuD- or T7-GFP-coding plasmids. HuD was immunoprecipitated with anti-T7 antibody. Coimmunoprecipitation of endogenous Akt1 was monitored by IB using anti-Akt1 antibodies.
recombinant active and inactive Akt1 and performed GST pull-down assays using purified recombinant GST-HuD and active or inactive Akt1. Interestingly, we find that the phosphorylated form of Akt1 (active Akt1) interacts with HuD, as active, but not inactive Akt1 copurifies with GST-HuD (Figure 2B). To further confirm the interaction between HuD and active Akt1, we treated the samples with lambda protein phosphatase before the pull-down assays, which lead as expected to a strong reduction of phosphorylated Akt1 within the input samples and in line with the data shown above to a strong reduction of Akt1 in GST pull-down eluates (Figure 2C). Thus, we conclude that HuD binds directly and specifically to active/phosphorylated Akt1. In good agreement with these data, we also find that HuD colocalizes with active Akt1 in PC12 cells (Supplementary Figure S1).

**Linker region of HuD is required for the interaction of HuD with Akt1**

Next, we delineated the HuD sequence elements that are important for its interaction with Akt1 (Figure 3A). Using GST-HuD deletion mutants, we find that the linker region between amino acids 250 and 302 is required for the optimal interaction of HuD with Akt1 (Figure 3A). Interestingly, the overall amino acid sequence of Hu proteins is well conserved (4). However, the linker region of Hu proteins differs between the neuronal Hu proteins HuB, HuC and HuD and the ubiquitously expressed HuR (Supplementary Figure S2). HuR lacks a short sequence within the linker region, which is found in all neuronal Hu proteins (Supplementary Figure S2). This has prompted us to determine whether Akt1 interacts specifically with the neuronal Hu proteins but not with HuR. Indeed, Akt1 interacts with HuB, HuC and HuD but not with HuR (Figure 3B). To confirm the importance of the linker region in Akt1 binding, we generated chimeric mutants of HuD and HuR, which contain the reciprocal linker between RBD2 and RBD3, respectively (Figure 3C). As predicted, HuR carrying the HuD linker region gains the ability to bind Akt1 whereas HuD, containing the HuR linker regions looses its ability to interact with Akt1 (Figure 3C).

**Role of Akt1 in HuD-induced neurite outgrowth in PC12 cells**

We next wished to test whether the HuD–Akt1 interaction is functionally important. To address this question, we...
used an established cell-based model system for studying neuronal differentiation, which is based on PC12 cells (13,14). If the HuD–Akt1 interaction is contributing significantly to the function of HuD, then our data predict that the chimeric HuR mutant should gain and the chimeric HuD mutant should lose its ability to induce neurite outgrowth. To test this prediction, we assayed the ability of the different HuD and HuR mutants described above to induce outgrowth in PC12 cells. As shown in Figure 4A and in concert with the data described above, wild-type HuD and the chimeric mutant HuR but not wild-type HuR or the chimeric HuD mutant transfected into PC12 cells can induce neurite outgrowth. These results suggest that binding of HuD to Akt1 is critical for the neurite-inducing activity of HuD. We note that wild-type HuD and the chimeric mutant HuR-DL that promotes neurite outgrowth are cytoplasmic, while those proteins that are not able to do so (wild-type HuR and the chimeric HuD mutant) are nuclear. To confirm that the Akt1 interaction with HuD and not just the cytoplasmic

Figure 3. The linker region between RBD2 and RBD3 is required for HuD to interact with Akt1. (A) Akt1 was incubated with the indicated GST-HuD proteins. GST pull-downs were examined for copurification of Akt1 by IB (upper panel) and for pull-down efficiency by CBB staining (lower panel). The white rectangles represent the RBDs of HuD. (B) Akt1 specifically interacts with neuronal Hu proteins. Akt1 was incubated with the indicated proteins. (C) HuD binds Akt1 via the linker region of HuD. Akt1 was incubated with the indicated proteins.
location is the relevant component, we have further mapped linker region of HuD to identify the critical interaction domain. We find that the HuD mutant 277-385 containing a truncated linker region (277-302) and RBD3 is still capable of (i) interacting with Akt1 (Figure 5B) and (ii) inducing neurite outgrowth in PC12 cells (Figure 5C). Strikingly, we find that the truncated HuR mutant 219-327 containing an almost identical linker sequence (see scheme in Figure 5A for comparison) and RBD3 cannot interact with Akt1 (Figure 5B) and cannot induce outgrowth although it localizes to the cytoplasm (Figure 5C). Thus, we are confident to conclude that the interaction of HuD with Akt1 is the relevant component and not just the cytoplasmic localization.

To further test whether the induction of outgrowth correlates with Akt1 binding and function, we used a dominant negative mutant of Akt1 that is defective in ATP binding and has a phosphorylation site defect within the kinase domain (see ‘Materials and Methods’ section for further details). Binding of this inactive mutant to HuD is strongly reduced (Supplementary Figure S3). The results shown in Figure 4B further confirm the importance of Akt1 in HuD-induced neurite outgrowth as overexpression of dominant-negative Akt1 but not wild-type Akt1 inhibits neurite outgrowth induced by both HuD and the chimeric mutant HuR-DL.

**RNA-bound HuD interacts with active Akt1**

As all of the biological functions of HuD are believed to be a result of its ability to bind to RNA, we tested whether RNA-bound HuD can associate with Akt1. To address this question, we performed pull-down assays using poly(U) sepharose beads. Indeed, active but not inactive Akt1 associates with RNA-bound HuD (Figure 6A). This binding is specific, because it is not observed with GST or GST-HuR (Figure 6A). Importantly, active Akt1 associated with RNA-bound HuD retains its kinase activity (Figure 6B).

**HuD itself is not the substrate for Akt1**

We finally asked whether HuD is itself substrate for Akt1 phosphorylation or whether HuD recruits the kinase to a different substrate. To address this question, we have...
performed an in vitro kinase reaction using GST-HuD and active Akt1 (see ‘Materials and Methods’ section for further details). The data shown in Figure 7 reveal that HuD is not phosphorylated by Akt1, whereas the positive control FOXO4 is. On the basis of these results, we conclude that HuD itself is not the target of Akt1. Taken together, with the results described above, our data suggest the model whereby RNA-bound HuD recruits Akt1 to activate neurite outgrowth.

**DISCUSSION**

Neuronal Hu proteins are RBPs that are critical for neuronal development (16). However, the underlying molecular mechanisms how neuronal Hu proteins exert their function are poorly understood. We have recently shown that HuD, a family member of neuronal proteins, stimulates cap- and poly(A)-dependent translation and found that the eIF4A and poly(A) binding
domains which are key in this process also contribute to its neurite inducing activity (4). Here, we further investigated the underlying molecular interactions and show that HuD directly and specifically interacts with active Akt1. This interaction is RNA independent, mediated by the linker region between RBD2 and RBD3 and required for its neurite inducing activity (Figures 2–5). We also tested for the interaction of HuD with downstream targets of Akt1 such as FLAG-mTOR or FLAG-S6K1 in HuD immunoprecipitates. However, we could not detect these downstream targets in the eluates (data not shown). Therefore, it appears that HuD interacts with Akt1 specifically and this interaction does not extend to other closely related AGC family kinases.

The strategy of an RBP to interact with a signaling pathway component has some similarity to that of the splicing factor 2 (SF2)/ASF which recruits mTOR or protein phosphatase 2A to activate translation of a subset of mRNAs (17). Interestingly, the finding that the eIF4A and poly(A) domains of HuD are not only critical for HuD’s enhancer function in translation but also contribute to its neurite-inducing activity, strongly suggests that stimulation of translation is a prerequisite for the neurite inducing activity of HuD. This raises the possibility that the HuD–Akt1 interaction might underlie a function in regulating translation. eIF4A, which interacts with HuD (4), may help to recruit the HuD/Akt1 dimer into messenger ribonucleoprotein (mRNP) complexes to phosphorylate target molecules to stimulate translation.

What are the targets of Akt1 in such HuD-containing mRNP complexes? We found that HuD itself is not a direct target of Akt1 (Figure 7). A prime candidate target is eIF4B, which can be phosphorylated by Akt1 (Supplementary Figure S4) (18–20) and can stimulate the helicase activity of eIF4A (21,22) and as a consequence translation. Thus, eIF4A activity might indirectly be enhanced by the action of Akt1 kinase via phosphorylation of an activator of eIF4A (e.g. eIF4B) rather than directly, as eIF4A is known not to be phosphorylated. Future experiments will aim to directly address the question of whether tethered HuD enhances cap-dependent translation via regulating the phosphorylation status of eIF4B by recruiting Akt1 into translation mRNP complexes.

Taken together, our study uncovers a functional interaction between a key RBP involved in neuronal development and a key signaling molecule, which is a master modulator of translation. To our knowledge, this is the first demonstration of a signaling pathway kinase that is specifically recruited by an RBP to trigger neurite outgrowth. In addition, these data might help to explain how HuD enhances translation of mRNAs that encode proteins involved in neuronal development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures S1–S4.

ACKNOWLEDGEMENTS

We thank Sven Danckwardt, Kent Duncan and Toshifumi Inada for comments on the manuscript and Yukiko Gotoh for kindly providing the Akt1 expression plasmid.
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FUNDING

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.F. and H.S.); Foundation NAGASE Science Technology Development (to T.F.). C.T. is a recipient of a Heisenberg-Fellowship from the Deutsche Forschungsgemeinschaft (DFG) (TH788/2-1 and TH788/2-2, to C.T.). Funding for open access charge: Intramural funding.

Conflict of interest statement. None declared.

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