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Translation elongation factor-1A1 (eEF1A1) localizes to the spine by domain III

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In vertebrates, there are two variants of eukaryotic peptide elongation factor 1A (eEF1A; formerly eEF-1α), eEF1A1 and eEF1A2, which have three well-conserved domains (D_I, D_{II}, and D_{III}). In neurons, eEF1A1 is the embryonic type, which is expressed during embryonic development as well as the first two postnatal weeks. In the present study, EGFP-tagged eEF1A1 truncates were expressed in cortical neurons isolated from rat embryo (E18-19). Live cell images of transfected neurons showed that D_{III}-containing EGFP-fusion proteins (EGFP-D_{III}, -$D_{II\text{-}III}$, -$D_{I\text{-}III}$) formed clusters that were confined within somatodendritic domains, while D_{III}-missing ones (EGFP-D_I, -D_{II}, -$D_{I\text{-}II}$) and control EGFP were homogeneously dispersed throughout the neuron including axons. In dendrites, EGFP-D_{III} was targeted to the heads of spine- and filopodia-like protrusions, where it was colocalized with SynGAPα, a postsynaptic marker. Our data indicate that D_{III} of eEF1A1 mediates formation of clusters and localization to spines. [BMB reports 2012; 45(4): 227-232]

INTRODUCTION

The eukaryotic peptide elongation factor 1A (eEF1A; formerly eEF-1α) binds guanine nucleotides and, in its guanine nucleotide triphosphate (GTP)-liganded form, can interact with aminoacyl tRNA to bring it to the A-site of the ribosome (1). In addition to its canonical function in protein synthesis, eEF1A plays many noncanonical functions (2) such as the regulation of cytoskeletal dynamics by binding and bundling actin and binding microtubules (3-6), protein degradation (7), regulation of M4 muscarinic acetylcholine receptor recycling (8), and protection against apoptosis (9,10).

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eEF1A exists as two individually encoded variants in vertebrates, which are 98% similar and 92% identical to each other at the amino acid level (11,12). eEF1A1 is almost ubiquitously expressed, while eEF1A2 is normally expressed only in the heart, muscles, brain (13-16), pancreatic islet cells, and enteroendocrine cells in colon crypts (16). During postnatal development, a transition from eEF1A1 to eEF1A2 occurs. In the muscle, eEF1A1 is present throughout embryonic development but is down-regulated in neonatal muscle and ultimately shut down by 21 days after birth (15,17,18). In the brain, eEF1A1 has been shown to be expressed in glial cells (18). In neurons of mice and rats, eEF1A1 is expressed during embryonic development, as well as the first two postnatal weeks. Thereafter, eEF1A1 is replaced by its homologue, eEF1A2 (19). However, there is evidence showing that a developmental switch is not complete in neurons. For example, some nuclear staining of eEF1A1 was observed in spinal motor neurons (16). Proteomic studies have shown that both eEF1A1 (20) and eEF1A2 (21) are enriched in the postsynaptic density (PSD) isolated from adult rat brains.

Spines are small protrusions from the dendritic shaft that form most excitatory synapses. The reports so far indicate that eEF1A is associated with both excitatory and inhibitory postsynaptic sites. We reported that eEF1A was localized to the PSD of dendritic spines of rat embryonic (E18) cortical neurons in culture (14 days *in vitro*; DIV14) (22). Also, eEF1A was partially (~49%) associated with glycine receptors of DIV28 hippocampal neurons, whereas only a minority of punctuate eEF1A immunoreactivity colocalized with γ-aminobutyric acid type A receptors (23). How eEF1A is targeted to spines is not known. Expression of genes tagged with a fluorescent protein in cultured cells is a direct method to observe their subcellular distribution. In contrast to adult neurons, the embryonic neurons can be readily dissociated and maintained in culture, where they differentiate morphologically (24). Taking advantage of embryonic neuronal culture we investigated to identify the eEF1A1 domain that is responsible for its synaptic targeting. The eEF1A1 protein has three well-conserved domains (D_I, D_{II}, D_{III}) (1). In this study we expressed various EGFP-tagged eEF1A1 domains in rat embryonic cortical neurons in culture. Live cell imaging and immunocytochemistry show that the D_{III} is necessary and sufficient for targeting eEF1A to spines.

RESULTS

Exogenously expressed EGFP-tagged Domain I-III of eEF1A1 (EGFP-D_{I-III}) forms clusters in somatodendritic domains of cultured rat cortical neurons

We constructed various recombinant plasmids for expression of diverse EGFP-tagged eEF1A1 truncates (Fig. 1A), and investigated the expression profile of full-length eEF1A1 by exogenously introducing EGFP-tagged eEF1A1 (EGFP-D_{I-III}). Rat cortical cultures were transfected on DIV 8-10 with recombinant plasmid pEGFP-D_{I-III}, and live cell images were obtained after overnight expression. When observed at 12-14 h post-transfection, fluorescence clusters were evident in both dendrites (Fig. 1B, arrowhead) and soma (Fig. 1B, asterisk and *inset*). Fluorescence in axons was negligible. In contrast, the EGFP alone did not form clusters. Instead, it was dispersed throughout the neuronal structure including axons (Fig. 1C, arrow).

The domain III of eEF1A1 mediates formation of clusters in the somatodendritic domain.

The expression profiles of EGFP-eEF1A1 truncates are shown in Fig. 2. The EGFP-D_I, -D_{II}, or -D_{I-II} did not form clusters (Fig. 3, D_I, D_{II}, or D_{I-II}, respectively). Instead, they were diffused throu-

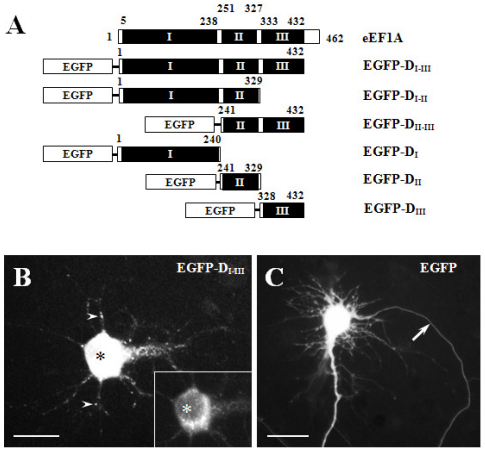

Fig. 1. The EGFP-tagged eEF1A1 forms clusters in the somatodendritic domains. (A) Schematic representation of EGFP-fusion clones containing various eEF1A1 domains. The single or various combinations of eEF1A1 domains were amplified by PCR and fused to the C-terminus of EGFP using a cloning vector pEGFP-C1 as described in detail in Materials and Methods. Filled boxes and numerals represent conserved domains and amino acid positions, respectively. (B, C) Direct visualization of EGFP-eEF1A1 clusters in the somatodendritic domain. DIV9 cortical neurons were transfected with a recombinant plasmid expressing EGFP-tagged eEF1A1 containing all three domains (EGFP-D_{I-III}) (B) or a control plasmid pEGFP (C). Live cell images were obtained at 12 h post-transfection. Exogenously expressed EGFP-eEF1A1 clusters in the soma and dendrites were marked by an asterisk and arrowheads, respectively, in (B)., An image with reduced brightness was shown in the inset of (B)., An axon was marked with an arrow in (C). Note that EGFP-D_{I-III} is not transported into axons. Scale bars, 20 μm.

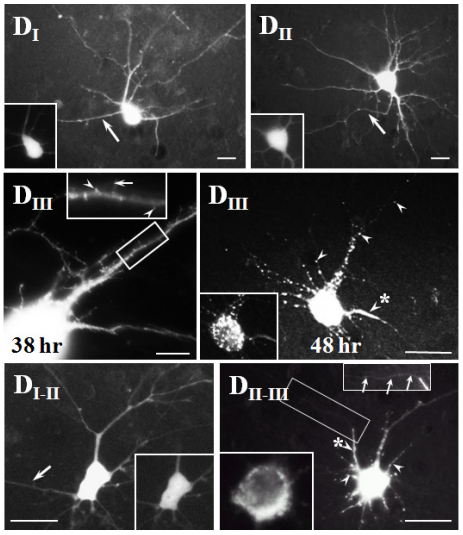

Fig. 2. Direct visualization of exogenously expressed various EGFP-tagged eEF1A1 truncates. DIV9 cortical neurons were transfected with recombinant plasmids expressing indicated EGFP-eEF1A1 domains. The *insets* show soma areas in reduced brightness. Axons are marked by arrows. Fluorescent clusters in dendrites are marked by arrowheads in neurons expressing D_{III}-containing fusion proteins. Arrowheads with an asterisk in panels D_{III} and D_{II-III} indicate AIS's. The axon extending distally from the AIS is marked by small arrows in the *inset* of panel D_{II-III}, where brightness and contrast were increased to reveal the axon. Scale bars, 20 μm.

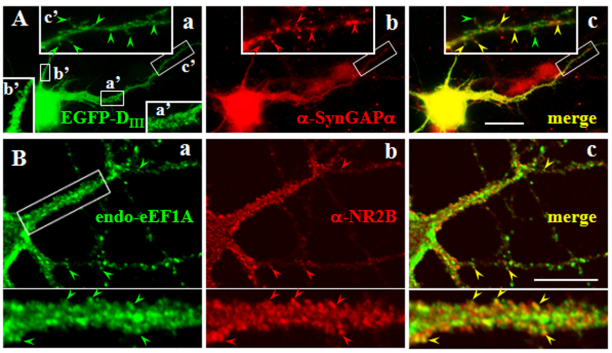

Fig. 3. Immunocytochemistry showing - that exogenously expressed EGFP-D_{III} is targeted to spines. (A) Rat hippocampal DIV9 neurons were transfected with pEGFP-D_{III} and incubated for long time (38 hr post-transfection). Cells were fixed and double-labeled with anti-GFP to visualize EGFP-D_{III} (a) and anti-SynGAPα, a PSD marker (b). EGFP-D_{III} clusters in the dendritic shaft and protrusions were shown in box a′ and b′ of panel (a). A dendritic portion (box c′) was shown enlarged in insets. Note clustering of EGFP-D_{III} (green) in the heads of dendritic protrusions (arrowheads in the inset of panel a), and some of the green clusters overlap with red ones (SynGAPα) resulting in yellow color in the merge images (arrowheads in the *inset* of panel c). (B) Expression of endogenous eEF1A. Rat hippocampal neurons (DIV 21) were double-labeled with anti-eEF1A (a) and NR2B (b), a postsynaptic marker. The boxed area of a dendrite was shown enlarged (insets) and the eEF1A clusters that overlap with those of NR2B were marked with arrowheads. Scale bar, 20 μm.

ghout neuronal structures including axons (arrows in corresponding panels). In contrast, fluorescent clusters in soma and dendrites were formed in neurons that expressed D_{III}-containing fusion proteins, i.e., EGFP-D_{III}, and -D_{II-III} (Fig. 2, D_{III} and D_{II-III}, respectively). Since the expression profile of EGFP-D_{III} is very similar to those of D_{II-III}- or D_{I-III}-GFP (Fig. 1B), these results indicate that domain III of eEF1A1 is necessary and sufficient for confining and forming clusters in the somatodendritic domain. When the expression time was extended for longer than ~40 h, the D_{III}-containing constructs were also targeted to the proximal segments of one or two processes per neuron as exemplified in Fig. 2 (arrowhead with an asterisk in D_{III}, 48 h and D_{II-III}). These processes exhibited typical features of an axon; as was exemplified in the upper inset of Fig. 2 (D_{II-III}), such processes arose from soma, did not branch immediately, and their diameters did not decrease along the path of the process. Therefore, the initial portion of these processes is the axon initial segments (AIS).These results together imply that the endogenous eEF1A1 is clustered and targeted to the somatodendritic domain and AIS by D_{III} (but see the discussion).

EGFP-D_{III} enriches in the head of dendritic filopodia and spines

Previously, we reported that the endogenous eEF1A is enriched in the spine in immunocytochemistry of cultured rat cortical neurons (22). To investigate if some of the exogenously expressed EGFP-eEF1A1 clusters represent spines, we transfected DIV9 neurons, incubated them for long time (~48 h) in order neurons to form spines, and double-stained with antibodies against GFP (to visualize EGFP-D_{III}) and SynGAPα (a PSD marker) (25, 26) antibodies. The immunocytochemical images of a typical tranfected neuron were shown in Fig. 3A-a. EGFP-D_{III} formed dense immunoreactive clusters (green) in the dendritic shaft (*inset* **a′**) and protrusions (inset **b′, c′**) as well as in the soma. SynGAPα clusters (red) were also distributed throughout somatodendritic domains (Fig. 3A-b). When the two images were merged (merge), the two proteins were colocalized at the heads of spine- and filopodia-like protrusions (Fig. 3A-c, yellow arrowheads). These results confirm that some of the EGFP-D_{III}-enriched dendritic protrusions are spines. To compare the subcellular distribution of exogenous eEF1A1 (i.e., EGFP-D_{III}) with endogenous eEF1A, cultured rat hippocampal neurons (DIV 21) were double-labeled with antibodies against eEF1A and NR2B (the 2B subunit of *N*-methyl-D-aspartate receptor, a postsynaptic marker). The expression profile of endogenous eEF1A was very similar to that of EGFP-D_{III} forming punctate clusters along dendritic shafts and protrusions (Fig. 3B-a). The image of NR2B immunostaining exhibited its typical punctate distribution (Fig. 3B-b), and many endogenous eEF1A clusters colocalized to those of NR2B (Fig. 3B-c). This result supports that the subcellular distribution of exogenous EGFP-D_{III} well represent the endogenous eEF1A. There are some endogenous eEF1A puncta that do not colocalize with NR2B (Fig. 3B-c, green puncta). These may be the eEF1A that are associated with inhibitory synapses (23) or dendritic translation complexes.

DISCUSSION

In this study we have shown that the D_{III} plays a critical role in clustering, confining in the somatodendritic domain, and localizing eEF1A1 to the spine. In terms of peptide elongation, the D_I functions as a GTP binding domain, while D_{II} and D_{III} are beta barrel domains that are involved in binding to charged tRNA (27). In addition to its canonical function in protein synthesis, eEF1A has a spectrum of noncanonical functions. One well-documented noncanonical function of eEF1A is the regulation of cytoskeletal dynamics by binding actin/microtubule and bundling actin (3, 6). Interaction of eEF1A with actin filament was first reported in *Dictyostelium* amoebae (28), and its actin-binding and bundling properties are conserved from yeast to mammals (5, 29). In *Dictyostelium*, >60% of eEF1A is estimated to be associated with actin cytoskeleton (30) indicating that actin-binding activity is a universal property of eEF1A. eEF1A is highly concentrated in cellular protrusions such as filopodia and other cortical regions that contain actin filament bundles (28). Neuronal dendritic spines are small protrusions of the dendritic membrane and are highly enriched with actin filaments (31). In the previous report, we have shown by biochemical and immunocytochemical studies that eEF1A is highly enriched in the neuronal spines in association with the postsynaptic density (PSD) (22). Proteomic studies also proved the presence of both eEF1A1 (20) and eEF1A2 (21) in PSDs from adult rat brains. In this study we found that eEF1A is targeted to the spine by its domain III. Both domain D_I and D_{III} can bind to F-actin, but D_{III} is the dominant actin-binding domain of eEF1A (32). When the interaction of each domain with F-actin was investigated using three glutathione-S-transferase (GST)-domain fusion proteins, only GST-D_{III} bound to F-actin (33) and to itself (33, our unpublished observation). Since actin-binding activity of eEF1A is a universal property, it is likely that that eEF1A interacts with the actin filaments in the spine through domain III.

At present, the function of the spine-associated eEF1A1 is not known. The PSD-proteome studies have shown that both eEF1A1 (20) and eEF1A2 (21) are present in the spine. eEF1A1 and eEF1A2 appear to function with equivalent properties in terms of peptide elongation (11). However, it is as yet unclear to what extent eEF1A2 shares the noncanonical functions including cytoskeletal modification. Developmental switch from eEF1A1 to eEF1A2 indicates functional differences. Indeed, forced expression of eEF1A2 can transform cells and give rise to tumors in nude mice (34). It is noteworthy that the cell types that switch off eEF1A1 tend to be those that have a strong, stable cytoskeletal organization, such as neurons and muscle. Therefore, Abbott *et al*. (35) raised a hypothesis that these cell types need to switch off eEF1A1 to prevent or modify the cytoskeletal rearranging properties, but for the obvious need to maintain protein synthesis, they use eEF1A2. Supporting this hypothesis, muscle cells switch eEF1A1 back on in response to

denervation or toxic injury, reverting back to high levels of eEF1A2 after recovery (36, 37). A similar hypothesis could be raised. During embryonic and early postnatal periods, neurons undergo extensive development of dendritic and axonal arborization. Spine formation, maturation, and its structural plasticity heavily depend on the actin cytoskeleton remodeling (38). Therefore, in this highly dynamic period the eEF1A1 would serve better because this embryonic type has a cytoskeletal rearranging property. As neurons mature, it would be necessary to stabilize the differentiated morphology so that there would be a switch into eEF1A2, which supposedly exhibits little activity in cytoskeletal remodeling. In addition to the developmental period, dendritic spines in the adult brain are still highly dynamic structures undergoing rapid morphological changes depending on synaptic activities [see a recent paper and references therein by Tropea *et al*. (39)]. To subserve this morphological dynamics eEF1A1 variant may remain in spines, even long after eEF1A2 is predominantly expressed in other parts of the neuron. Therefore, it is tempting to hypothesize that the spine-associated eEF1A1 and eEF1A2 orchestrate together for the activity-dependent local protein synthesis, synaptic remodeling, and/or other signal transductions.

We also found that EGFP-eEF1A is targeted to the AIS by domain III. Chronologically, EGFP-D_{III} localized to AIS lastly after soma and dendrites. In our hands, endogenous eEF1A was never localized to the AIS except in one case, where the cells were cultured for 6 weeks (Supplementary Fig. S1). Most neurons in this particular culture had one or two processes, of which proximal portions were highly enriched with eEF1A. This portion, however, was devoid of *N*-acetylglucosamine kinase (Supplementary Fig. S1), which is not present in the axon (manuscript in preparation). The morphology of cells in this culture implied that they were not healthy. To test the possibility that endogenous eEF1A is targeted to the AIS when neurons are cultured *in vitro* for a very long time, we have grown the hippocampal dissociate neurons for DIV 48. Immunocytochemistry of this culture did not show accumulation of eEF1A in the AIS (data not shown).Therefore, it must be concluded that targeting of exogenous EGFP-D_{III} to the AIS does not represent the *in vivo* phenomenon. Overexpression in the case of exogenous EGFP-D_{III} and damages in the diffusion barrier in the case of endogenous eEF1A in long-cultured unhealthy neurons may have caused overflow into the AIS. Our work provides a platform for functional analysis for synaptic eEF1A.

MATERIALS AND METHODS

Construction of expression plasmids for EGFP-eEF1A1 domain fusion proteins

The following polymerase chain reaction (PCR) primers were used for amplification of various domains of rat eEF1A1 gene (GenBank NM_175838; forward primer/reverse primer in 5' to 3' direction). D_I (aa 1 to 240): CGAGATCTTTCAAAGCAAAA ATGGGA/CCGGTACCACGAGTTGGCGGCAGAAT; D_{II} (aa 241 to 329): CGAGATCTCCAACTGACAAGCCTCTG/CCGGT ACCGCTGTCCCCAGCAACATT; D_{III} (aa 328 to 432): CGAGA TCTGACAGCAAAAATGACCCA/CCGGTACCTGTCTGCCTCA TGTCACG; D_{I-II} (aa 1 to 329): CGAGATCTTTCAAAGCAAA AATGGGA/CCGGTACCGCTGTCCCCAGCAACATT; D_{II-III} (aa 241 to 432): CGAGATCTCCAACTGACAAGCCTCTG/CCGGT ACCTGTCTGCCTCATGTCACG. The PCR products were inserted in-frame into the *BglII* and *KpnI* sites of the C-terminus of pEGFP-C1 (Clontech, Palo Alto, CA). All clones were confirmed by DNA sequencing.

Dissociated neuronal culture and transfection

Cortical (for transfection) and hippocampal (for immunocytochemistry) cells of embryonic day 18 (E18) fetal rat (Sprague-Dawley) brains were isolated and plated onto 12 mm diameter poly-DL-lysine/laminin-coated glass coverslips at a density of ~150 (for ICC) and ~2,500 cells/mm^2 (for transfection), and grown in astrocyte-conditioned Neurobasal media as described (40, 41). Neurons were transfected using Lipofectamine^{TM} 2000 reagents according to manufacturer's instruction (Invitrogen, Carlsbad, CA).

Immunocytochemistry (ICC)

Culture coverslips were rinsed briefly in Dulbecco's phosphate-buffered saline (Invitrogen) and fixed by a sequential paraformaldehyde/methanol fixation procedure [incubation in 4% paraformaldehyde in PBS (20 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl) at RT for 10 min, followed by incubation in methanol at -20°C for 20 min] (42). Cells were blocked overnight at 4°C in preblocking buffer (5% normal goat serum, 0.05% Triton X-100, and 450 mM NaCl in 20 mM $NaPO_4$ buffer, pH 7.4). Primary antibodies [mouse monoclonal anti-eEF1A and rabbit polyclonal anti-SynGAPα, each 1 : 1,000 (Upstate Biotechnology Inc., Lake Placid, NY); mouse monoclonal anti-GFP (1 : 1,000) were added to the coverslips and incubated overnight at 4°C. Coverslips were rinsed (3 × 15 min in preblocking buffer) and incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 568-conjugated goat anti-rabbit IgG, each 1 : 1,000; Invitrogen) at RT for 1-2 h. Coverslips were rinsed once in preblocking buffer for 15 min, twice in PBS, and mounted on slides with 4% n-propylgallate in 90% glycerol and 10% sodium carbonate buffer (pH~8.7).

Image acquisition

Live images were acquired using 40X or 100X objective lens on a Leica Research Microscope DM IRE (Leica Microsystems AG, Wetzlar, Germany) with a high-resolution CoolSNAP^{TM} CCD camera (Photometrics Inc., Germany) under the control of a computer using Leica FW4000 software. Confocal images of ICC (1024 × 1024 pixels) were acquired using 100X oil-immersion lens on the Leica TCS SP2 confocal system with laser lines at 488, 543, and 633 nm. Image contrast and brightness were optimized in Adobe Systems Photoshop 5.0 software.

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