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Role of mitogen-activated protein kinase (MAPK) docking sites on Staufen2 protein in dendritic mRNA transport

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ABSTRACT

Although transport and subsequent translation of dendritic mRNA play an important role in neuronal synaptic plasticity, the underlying mechanisms for modulating dendritic mRNA transport are almost completely unknown. In this study, we identified and characterized an interaction between Staufen2 and mitogen-activated protein kinase (MAPK) with co-immunoprecipitation assays. Staufen2 utilized a docking (D) site to interact with ERK1/2; deleting the D-site decreased colocalization of Staufen2 with immunoreactive ERK1/2 in the cell body regions of cultured hippocampal neurons, and it reduced the amount of Staufen2-containing RNP complexes in the distal dendrites. In addition, the deletion completely abolished the depolarization-induced increase of Staufen2-containing RNP complexes. These results suggest that the MAPK pathway could modulate dendritic mRNA transport through its interaction with Staufen2.

In neurons, dendritic transport of a subset of mRNAs and the subsequent local translation is critical for several types of synaptic plasticity [1–3]. Recent studies suggested that a highly regulated mechanism, rather than a simple diffusion, mediates this dendritic mRNA transport [4–7]. Although much is known about the mRNA transporting module in dendrites [8,9], little is known about the mechanism underlying this transport or its regulation.

Staufen, a double-stranded RNA-binding protein participates in the localization of specific mRNAs during *Drosophila* oocytes [10] and embryonic neuroblast [11] differentiation, and more recent work has suggested that Staufen has a key role in the dendritic mRNA transport of mammalian neurons as well [12,13] . In mammals, Staufen consists of two genes, *Staufen1* (*STAU1*), which is expressed ubiquitously, and *Staufen2* (*STAU2*), which is specific for neurons [14]. Since some reports have indicated that Staufen exists in a variety of ribonucleoprotein (RNP) complexes in neurons, this protein may function as a core component in these mRNA transporting modules [9,15–19], making it an ideal marker protein for dendritic mRNA transport. Additionally, Staufen1 mediates mRNA decay, together with a nonsense-mediated mRNA decay factor, Upf1 [20].

In addition to dendritic mRNA transport, signaling extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase (MAPK), also influence a wide variety of synaptic plasticity types [21,22]. ERK transduces signals with high efficiency and specificity through its ability to "dock" with upstream or downstream kinases and substrates [23–26].

A recent study suggested that the MAPK pathway modulated the transport of Staufen2-containing RNP complexes in dendrites [27]. In order to clarify how the MAPK pathway participates in the dendritic mRNA transport, we examined the interaction between Staufen2 and ERK using co-immunoprecipitation (co-IP) analyses. Our results showed that ERK2 interacted with Staufen2 through RNA-binding domains (RBD) in its N-terminal regions. Deletion of the ERK docking (D) site on Staufen2 decreased its interaction and colocalization with ERK2. In addition, the deletion decreased the amounts of Staufen2-containing RNP complexes and abolished the depolarization-induced increase in distal dendrites. Taken together, our results suggest the MAPK pathway modulated the dendritic mRNA transport through its interaction with Staufen2.

Materials and methods

Reagents. Unless stated otherwise, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human embryonic kidney (HEK) 293T cells were maintained in DMEM media containing 10% FBS (Invitrogen, Carlsbad, CA).

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Preparation of constructs. To generates the pCMV-Myc-Stau2 (full-length Staufen2: 62 kDa), pCMV-Myc-RBD (Staufen2 RNA-binding domain), and pCMV-Myc-MTA (Staufen2 microtubule-association domain) constructs, each region was isolated by PCR from pSinRep5-GFP-Stau2 [7,13,27], and inserted to the pCMV-Myc vector (Invitrogen). To construct the Staufen2 deletion (Δ15, Lys^{290}-Gly^{304}) and point mutants ($296^{Ile \rightarrow Gly}$: I296G), a series of recombinant PCRs were performed, and the products were inserted to pCMV-Myc for transfection or pSinRep5 (Invitrogen) for Sindbis viral expression. For pCFP-ERK2, mouse ERK2 was isolated by PCR and inserted pCFP-C1 vector (Clontech Laboratories, Mountain View, CA). The methods used to construction and infect neurons with Sindbis virus are identical with those described before [27].

Preparation of hippocampal neuron culture. Dissociated hippocampal neuron cultures were prepared from post-natal 1-day-old rat pups, as described elsewhere [27]. The neurons were plated onto poly-D-lysine-coated coverslips at a density of 10,000–20,000 cells/cm^2 for low-density culture. The cultures were maintained and allowed to mature in growth medium (Neurobasal-A supplemented with B-27 and Glutamax-1; Invitrogen) for 12 days prior to use.

Co-immunoprecipitation. Myc-tagged Staufen2 proteins were immunoprecipitated from HEK 293T cells lysates with 3 μg of monoclonal anti-Myc antibody (Clone 9E10; Sigma–Aldrich) and 50 μl of 50% protein A-Sepharose (Amersham Biosciences, Tokyo, Japan). The immunoprecipitates were washed three times with 1 ml of ice-cold lysis buffer [150 mM NaCl, 1% IGEPAL® CA-630, 50 mM Tris·Cl (pH 8.0)] and once with 1 ml of 50 mM Tris·Cl (pH 8.0). Immunoprecipitated proteins were separated by 8% SDS–PAGE and subjected to Western blotting analysis, during which blots were incubated with anti-MAPK antibody (1:2000; Cell Signaling Technology, Danvers, MA). As a control, the blot was washed with stripping buffer (Thermo Scientific, Rockford, IL) and reprobed with anti-Myc antibody.

Immunocytochemistry. For immunostaining, the neurons were fixed with 4% paraformaldehyde and 4% sucrose on ice for 20 min. Cell membranes were permeabilized by washing the fixed cultures with PBT (1× PBS, 0.1% BSA, 0.1% Triton X-100) for 15 min. The cultures were subsequently pre-blocked (1× PBS, 2% BSA, 0.08% Triton X-100) for 2 h at room temperature. The primary antibody, anti-MAPK (Cell Signaling Technologies) was added to the pre-block solution, and the cells were incubated overnight at 4 °C. After washing with PBT, a secondary antibody, Alexa Fluor® 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) was added to the culture and incubated for 2 h at room temperature, followed by stringent washing in PBT and PBS. The immunostained cultures were imaged in PBS as described in the imaging section below.

Potassium chloride (KCl) stimulation. The neuronal cultures were infected with Sindbis virus, incubated for 12 h, and subsequently exchanged in Hepes-buffered saline (HBS), containing 60 mM KCl (high-K^+ HBS). After for a 10-min stimulation, the high-K^+ HBS was immediately exchanged with normal HBS (5.4 mM KCl). The treated neurons were further incubated in 5% CO_2, at 37 °C for 2 h 50 min, for a total experimental time of 3 h.

Imaging and image analysis. Images of fixed or immunostained neurons were acquired by confocal microscopy (TCS-SP2 AOBS; Leica Microsystems, Heidelberg, Germany) and saved in a 8-bit grayscale image (0–255). The images were analyzed by using the "straighten" mode of the NIH image analysis program (ImageJ), in order to measure the transport of Staufen-containing RNP complexes, or in the "colocalization threshold" mode to measure the colocalization of Staufen2 with ERK1/2. Neurons with a similar morphology (according to phase contrast microscopy and/or fluorescence imaging) were selected, and a single primary dendrite >100 μm in length was scored for each. The relative fluorescent intensity was obtained by normalizing the mean to that of the control group (wild type; no KCl stimulation). In the colocalization analyses, to avoid saturated images, GFP images were acquired using low laser power and gain values. A Student's *t*-test was employed to determine the statistical difference between the groups. All the image acquisitions and analyses were performed in blind experiments.

Results and discussion

In order to examine whether Staufen2 interacted with ERK, we co-expressed Myc-tagged Staufen2 (Myc-Stau) and CFP-tagged ERK2 (CFP-ERK2) in 293T cells. We subsequently immunoprecipitated Myc-Stau and any interacting proteins with anti-Myc antibody, separated the immunoprecipitated proteins by SDS–PAGE, and subjected to Western blotting using a MAPK antibody (Fig. 1A). The results revealed that Myc-Stau associated not only with CFP-ERK2, but also with endogenous ERK1 and ERK2.

In order to identify which region of Staufen2 interacted with ERK, we co-expressed Myc-tagged full-length Staufen2 (wild type, WT), the isolated RNA-binding domain regions (RBD), or the isolated microtubule-associated region (MTA) with CFP-tagged ERK2 in 293T cells. We identified an ERK interaction for the wild type and RBD forms of Staufen2 (Fig. 1B).

The high fidelity and specificity of MAPK signal transduction depends on docking interactions with substrates, and upstream or downstream kinases [23–26]. To investigate whether Staufen2 contained a docking site for ERK, we performed a motif search on the protein using Scansite 2.0 [28]. We identified two canonical ERK2-docking (D) sites for MAP kinase kinase1 (MEK1), one using a high-stringency filter (Lys^{290}-Gly^{304}) and the other (Lys^{280}-Phe^{294}) using a medium-stringency filter. These D-sites, which contained clusters of basic amino acids followed by hydrophobic amino acids, mediate the interaction between ERK2 and MEK1, which is critical for ERK2 phosphorylation [24,29,30].

In order to examine whether ERK2 could utilize the canonical D-site for a docking interaction with Staufen2, we deleted the Lys^{290}-Gly^{304} region to produce the Δ15 mutant, which we subsequently used in a co-IP assay. Deletion of the D-site dramatically reduced the interaction of ERK2 (Fig. 1D), but it did not completely block the interaction, suggesting that the other D-site might be compensating for the loss.

To examine whether deleting the D-site hampered colocalization of Staufen2 with MAPKs, we expressed GFP-tagged full-length (wild type, WT) or Δ15 (deletion of Lys^{290}-Gly^{304}) Staufen2 in cultured hippocampal neurons using a Sindbis viral expression system. After a 12-h infection, we stained the neurons with MAPK antibody, and look for colocalization in the somatic regions between the GFP-tagged Staufen2 proteins and MAPKs. Deleting the canonical D-site decreased the Staufen2/MAPK colocalization by 40% compare to the wild type control [WT, 69.65 ± 2.973% ($N = 20$); Δ15, 41.84 ± 2.941% ($N = 15$); $^{***}p < 0.001$] (Fig. 2). Because we acquired the GFP images under low laser power and gain values to avoid saturation, we could not detect GFP fluorescence in the dendrites.

In the following experiments, we examined whether eliminating the D-site affected the transport of Staufen2-containing RNP complexes, which exhibit properties similar to endogenous RNA granules [27]. We infected cultured hippocampal neurons with Sindbis viruses encoding GFP-tagged Staufen2 (WT), the GFP-tagged deletion mutant of Staufen2 (Δ15), the GFP-tagged Staufen2 point mutant ($Ile^{296} \rightarrow Gly^{296}$, I296G), or an isolated GFP-tagged RBD (the isolated RNA-binding domains without the microtubule-association domain). Because the $isoleucine^{296}$ is both the most central and hydrophobic residue in the D-site, we selected it for point mutation. After a 12-h infection, we quantified the Staufen2-containing RNP complexes by image analysis. Interest-