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Identification of tomoregulin-1 as a novel addicsin-associated factor

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ABSTRACT

Addicsin is a novel factor encoding a 23-kDa hydrophobic protein that is highly upregulated in the amygdala nuclei of morphine-administered mice. It is a murine homolog of human JWA and rat glutamate transporter-associated protein 3-18 (GTRAP3-18), a negative modulator of the neural glutamate transporter excitatory amino acid carrier 1 (EAAC1). Recent findings demonstrated that addicsin participates in various physiological processes by forming hetero- or homomultimeric complexes. However, the binding targets and molecular functions of addicsin remain largely unknown. To identify potential factors that associate with mouse addicsin, we performed a yeast two-hybrid screen using a 17-day-old mouse whole embryo cDNA library. We identified tomoregulin-1 (TR1) as a novel addicsin-associated factor. TR1, a type I transmembrane protein containing two follistatin-like modules and an epidermal growth factor-like domain, participates in nodal and bone morphogenetic protein signaling. Immunoprecipitation assays demonstrated that TR1 bound to addicsin, and that amino acids 145–188 of addicsin and amino acids 228–266 of TR1 were important for the formation of the addicsin-TR1 heterocomplex. The double-fluorescent immunohistochemical analysis revealed that addicsin and TR1 were coexpressed in neurons in the mature mouse brain regions tested. Moreover, TR1 showed a punctuate pattern throughout the cell, with preferential expression on the cell surface when expressed alone. However, TR1 predominantly redistributed to the endoplasmic reticulum (ER) when coexpressed with addicsin. Furthermore, coexpression of an addicsin mutant that lacked TR1 binding ability had little effect on the distribution of TR1. Biotinylation assays showed that coexpression of addicsin with TR1 suppressed the cell surface expression of TR1. Wound-healing assays demonstrated that the interaction of addicsin with TR1 had a significant effect on cell migration. These findings demonstrate that addicsin in the ER controls intracellular TR1 trafficking from the ER to plasma membrane and regulates cell migration through its interaction with TR1.

1. Introduction

We previously identified a novel mRNA encoding a 23-kDa hydrophobic protein designated "addicsin" that is highly upregulated in the amygdala nuclei of repeated morphine-administered mice (Ikemoto et al., 2002). Computational homology analysis revealed that addicsin is a murine homolog of rat glutamate transporter-associated protein 3-18 (GTRAP3-18), a negative modulator of Na^+-dependent glutamate transporter excitatory amino acid carrier 1 (EAAC1); human JWA, a vitamin A-responsive factor; and prenylated Rab acceptor 1 domain family member 3 (PRAF3), a microtubule associated protein containing a prenylated Rab acceptor motif (Butchbach et al., 2002; Ikemoto et al., 2002; Lin et al., 2001). In recent papers, the protein described as addicsin, GTRAP3-18, JWA, or PRAF3 has been renamed ADP ribosylation factor-like 6 interacting protein 5.

Addicsin is ubiquitously present in various tissues, such as the brain (Butchbach et al., 2002; Ikemoto et al., 2002; Lin et al., 2001), and is predominantly expressed in the principal neurons in the central nervous system (CNS) (Akiduki et al., 2007; Inoue et al., 2005). Recent studies demonstrated that addicsin regulates

Abbreviations: Arl6ip1, ADP ribosylation factor-like 6 interacting protein 1; BMP, bone morphogenetic protein; CNS, central nervous system; CREB, cAMP response element binding protein; EAAC1, excitatory amino acid carrier 1; EGF, epidermal growth factor; ER, endoplasmic reticulum; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GTRAP3-18, glutamate transporter-associated protein 3-18; HRP, horseradish peroxidase; IC, immunocytochemistry; IHC, immunohistochemistry; MAP2, microtuble associated protein 2; NIH, National Institutes of Health; ORF, open reading frame; PRAF3, prenylated Rab acceptor 1 domain family member 3; RT, reverse transcription; TR1, tomoregulin-1; WB, Western blot.

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the physiological functions of EAAC1 (Ikemoto and Arano, 2012). Addicsin negatively modulates glutamate uptake through direct interaction with EAAC1 in a protein kinase C (PKC)-dependent manner (Akiduki and Ikemoto, 2008; Lin et al., 2001). Furthermore, the PKC-dependent interaction of addicsin with ADP-ribosylation factor-like 6 interacting protein 1 (Arl6ip1) reduces the amount of addicsin available to bind EAAC1, thus promoting EAAC1-mediated glutamate transport (Akiduki and Ikemoto, 2008). Additionally, addicsin negatively regulates neural glutathione (GSH) content through its direct interaction with EAAC1 (Aoyama et al., 2012b; Watabe et al., 2007, 2008) because EAAC1 also transports cysteine as a substrate for GSH synthesis (Chen and Swanson, 2003; Himi et al., 2003; Zerangue and Kavanaugh, 1996). Interestingly, addicsin-deficient mice showed increased neural GSH levels and neuroprotection against oxidative stress in the CNS (Aoyama et al., 2012a).

Besides regulating the activity of EAAC1, addicsin participates in various physiological and pathophysiology processes (Ikemoto and Arano, 2012). *In vitro* data showed that addicsin is involved in intracellular trafficking. For instance, addicsin regulates the intracellular trafficking of EAAC1 in the endoplasmic reticulum (ER) (Ruggiero et al., 2008) and negatively regulates Rab1, a small Rab GTPase, to delay the exit of EAAC1 from the ER (Maier et al., 2009). Furthermore, addicsin participates in apoptosis and suppresses migration in human esophageal squamous cells (Shi et al., 2012). Addicsin also responds to diverse stimuli, including oxidative stress, differentiation, and heat shock (Zhu et al., 2005; Chen et al., 2007b). Meanwhile, *in vivo* data indicate that addicsin may contribute to the development of various neurological diseases. In humans, addicsin expression increases in the thalamus and cingulate cortex in schizophrenia (Bauer et al., 2008; Huerta et al., 2006). In rats, addicsin affects the formation of chronic morphine dependence through its interaction with δ-opioid receptor (DOR) (Wu et al., 2011) and participates in epilepsy induced by levetiracetam (Ueda et al., 2007). Moreover, addicsin knockout mice have improved motor learning and spatial memory compared with wild-type mice (Aoyama et al., 2012a) and show resistance to the development of skin papillomas induced by phorbol ester (Gong et al., 2012). In *Drosophila*, addicsin is necessary for the acquisition of ethanol tolerance (Li et al., 2008). These findings indicate that addicsin has critical roles in various physiological and pathological processes in the CNS. However, the precise molecular functions of addicsin remain largely unknown.

Addicsin is widely distributed in various tissues, including the brain (Akiduki et al., 2007; Butchbach et al., 2002; Ikemoto et al., 2002; Inoue et al., 2005; Lin et al., 2001). Cumulative evidence suggests that addicsin participates in various physiological functions by forming hetero- or homomultimeric complexes (Akiduki and Ikemoto, 2008; Aoyama et al., 2012b; Ikemoto and Arano, 2012; Ikemoto et al., 2002; Lin et al., 2001; Maier et al., 2009; Ruggiero et al., 2008; Wu et al., 2011). Thus, the identification of novel addicsin associating partners may elucidate the physiological functions of addicsin. Therefore, we screened for addicsin-associating factors using a yeast two-hybrid assay. Here, we demonstrate that addicsin associates with tomoregulin-1 (TR1), controls the ER localization of TR1, and regulates cell migration through protein–protein interaction.

2. Materials and methods

2.1. Animals

Male ddY mice (6 weeks old; 25–30 g) were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained in individual cages (12-h light–dark cycle; 23–24 °C). Animals were used in experiments after decapitation. All efforts were made to minimize suffering. Animals were cared for in accordance with Law No. 105 and Notification No. 6 of the Japanese Government, and all animal experiments were conducted with permission of the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology (AIST) (Permit No. 2013-030).

2.2. Antibodies

The monoclonal anti-FLAG M2 IgG antibody (4.9 mg/ml, 1:3000 for Western blot (WB), 1:500 for immunocytochemistry (IC)) and monoclonal anti-actin IgG antibody (1.0 mg/ml, 1:400 for WB) were purchased form Sigma (St. Louis, MO, USA). The monoclonal anti-c-myc IgG antibody (clone 9E10; 5.0 mg/ml, 1:500 for WB, 1:100 for IC) was obtained from Roche Diagnostics (Mannheim, Germany). The monoclonal anti-V5 IgG antibody (0.8 mg/ml, 1:5000 for WB, 1:400 for IC) was purchased from Invitrogen (Carlsbad, CA, USA). The polyclonal rabbit anti-calnexin IgG antibody (1:500 for WB, 1:200 for IC) was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). The polyclonal rabbit anti-addicsin IgG antibody (1.2 mg/ml, 1:200 for WB, 1:100 for IC, 1:100 for immunohistochemistry (IHC)) was a generous gift of Transgenic Co., Ltd., (Kumamoto, Japan). The polyclonal rabbit anti-TR1 IgG antibody (0.2 mg/ml, 1:200 for WB, 1:100 for IHC) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal mouse anti-TR1 IgG antibody (0.2 mg/ml, 1:50 for IHC) was obtained from Santa Cruz Biotechnology. The polyclonal rabbit anti-CD9 IgG antibody (0.2 mg/ml, 1:100 for WB) was purchased from Santa Cruz Biotechnology. The polyclonal rabbit anti-CREB IgG antibody (0.1 mg/ml, 1:100 for WB) was obtained from Santa Cruz Biotechnology. The monoclonal mouse anti-MAP2 (2a + 2b) IgG antibody (0.13 mg/ml, 1:250 for IHC) was purchased from Sigma. The polyclonal rabbit anti-GFAP IgG antibody (12.8 mg/ml, 1:200 for IHC) was purchased from Sigma. The horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG secondary antibody (5.7 mg/ml, 1:2000 for WB) was obtained from Chemicon International Inc., (Temecula, CA, USA). Tv (Solon, OH, USA). The Alexa Fluor 488 goat anti-mouse IgG secondary antibody (2.0 mg/ml, 1:500 for IC) and Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (2.0 mg/ml, 1:500 for IC) were obtained from Molecular Probes (Eugene, OR, USA). The FITC goat anti-rabbit IgG DS Grade antibody (0.6 mg/ml, 1:500 for IHC) was purchased from Zymed Laboratorie, Inc., (San Francisco, CA, USA).

2.3. Construction of expression plasmids

Expression plasmids for the open reading frame (ORF) of mouse addicsin and various mouse addicsin deletion mutants were subcloned into the pcDNA3.1/Myc-His (+) A vector (Invitrogen) as described in our previous study (Aoyama et al., 2008). To construct wild-type mouse TR1, the ORF of TR1 was amplified by PCR using full-length mouse TR1 cDNA as a template (DDBJ ID: BC057598, cDNA clone MGC67249; Invitrogen). The primers for TR1 included primer 1 (5′-AAA GGT ACC ATG GGC GCC CAG GCG CCG CTC-3′) and primer 2 (5′-AAA GGG CCC GAC CAT TCT GGA TGA AGT GTC-3′). To construct the ΔC truncation, PCR was performed using primer 1 and primer 7 (5′-AAA GGG CCC GCA TTT CCT TGT TAT GCA CAT-3′). These two amplified cDNAs were subcloned into the KpnI and ApaI sites of the pcDNA3.1/FLAG-His (+) A vector (modified from pcDNA3.1/Myc-His (+) A by M.J. Ikemoto). The various TR1 deletion mutants, except for those with ΔC truncation, were generated by splicing, using the overlap extension PCR method with the expression plasmid containing the subcloned ORF of TR1 (pcDNA3.1-TR1-FLAG) as a template. In brief, the segments to be joined (fragments A and B) were generated in separate primary