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PKA MODULATES IRON TRAFFICKING IN THE STRIATUM VIA SMALL GTPASE, RHES

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Abstract—Ras homolog enriched in striatum (Rhes), is a highly conserved small guanosine-5′-triphosphate (GTP) binding protein belonging to the Ras superfamily. Rhes is involved in the dopamine receptor-mediated signaling and behavior though adenylyl cyclase. The striatum-specific GTPase share a close homology with Dexras1, which regulates iron trafficking in the neurons when activated though the post-translational modification called s-nitrosylation by nitric oxide (NO). We report that Rhes physiologically interacted with Peripheral benzodiazepine receptor-associated protein7 and participated in iron uptake via divalent metal transporter 1 similar to Dexras1. Interestingly, Rhes is not S-nitrosylated by NO-treatment, however phosphorylated by protein kinase A at the site of serine-239. Two Rhes mutants – the phosphomimetic form (serine 239 to aspartic acid) and constitutively active form (alanine 173 to valine) – displayed an increase in iron uptake compared to the wild-type Rhes. These findings suggest that Rhes may play a crucial role in striatal iron homeostasis. © **2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: Rhes, iron uptake, striatum, phosphorylation, G protein, PKA.

INTRODUCTION

Iron is an essential metal ion critical for basic cellular processes, such as mitochondrial ATP generation and DNA replication (Hentze and Kuhn, 1996; McCord, 1998; Todorich et al., 2009). Iron deficiency studies show that iron plays an important role in normal division of cells, including neuronal precursor cells, astrocytes and oligodendrocytes (Moos and Morgan, 1998; Ke and Qian, 2007). In addition, iron is required for several neuronal specific functions, such as dopaminergic neurotransmitter synthesis and myelination (Moos and Morgan, 1998; Rouault and Cooperman, 2006; Todorich et al., 2009). Due to its unique chemical nature, however, iron can have deleterious effects by generating reactive oxygen species through Fenton's reaction when in excess (McCord, 1998). The precise mechanism and participating molecules are not clearly understood, but iron accumulation and its pathological implications have been reported in numerous neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease and Parkinson's disease (PD) (Berg and Hochstrasser, 2006; Barnham and Bush, 2008; Todorich et al., 2009; Khalil et al., 2011; Nunez et al., 2012; Schneider and Bhatia, 2012).

A novel ras homolog enriched in striatum (Rhes), is a guanosine-5′-triphosphate (GTP)-binding protein. Like all G proteins, Rhes contains the conserved domain including the GTP binding domain, a C-terminal prenylation site, and the magnesium binding domain. The physiological role of Rhes is not fully understood, however, it has been reported to involve in PI3K activation (Vargiu et al., 2004; Todorich et al., 2009) and regulatory actions on AKT (Protein Kinase B) pathway (Bang et al., 2012; Harrison et al., 2013) at a cellular level and modulation of dopamine receptor-mediated behavior with Rhes mutant mice (Harrison and Lahoste, 2006; Errico et al., 2008; Quintero et al., 2008). Recently, it has been shown that the interaction of Rhes with a huntingtin protein gives rise to selective vulnerability to striatal pathology in Huntington's disease (Subramaniam and Snyder, 2011).

Rhes amino acids share 67% identity in the open reading frames with Dexras 1, a brain-enriched member of the Ras family of small G proteins (Falk et al., 1999; Vargiu et al., 2004). Moreover, these two have a common feature of an extended C-terminal tail which differentiates them from the conventional Ras family members (Falk et al., 1999; Graham et al., 2001). Dexras1 has been shown to activate G protein signaling via selectively binding to Gαi2, increasing GTPγS binding to Gi and Go and activating extracellular signal-regulated kinases 1 and 2 (Erk 1 and 2) (Cismowski et al., 2000; Graham and Prossnitz, 2002). Particularly, we discovered that Dexras1 participates in iron uptake through *N*-methyl-D-aspartate (NMDA) receptor-mediated

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Abbreviations: λ PPase, λ phosphatase; A173V, alanine-173 valine; DMT1, divalent metal transporter 1; EDTA, ethylenediaminetetraacetic acid; G31V, glycine-31 valine; GEF, guanidine exchange factor; GTP, guanosine-5′-triphosphate; GST, glutathione S-transferase; HRP, horseradish peroxidase; IRE, iron-responsive element; nNOS, neuronal nitric oxide synthase, NO, nitric oxide; NTBI, non-transferrin-bound iron; PAP7, peripheral benzodiazepine receptor-associated protein7; PBS, phosphate-buffered saline; PKA, protein kinase A; Rhes, ras homolog enriched in striatum; S239A, serine-239 alanine; S239D, serine-239 aspartic acid; Tf, transferrin; TfR, transferrin receptor.

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signaling. Specifically, stimulation of NMDA receptors activates neuronal nitric oxide synthase (nNOS), leading to *S*-nitrosylation and activation of Dexras1 which, via peripheral benzodiazepine receptor-associated protein7 (PAP7) and divalent metal transporter 1 (DMT1), physiologically induces iron uptake (Cheah et al., 2006). More recently, we found that Dexras1 is required for NMDA-elicited neuronal toxicity via NO and iron influx (Chen et al., 2013).

Since Rhes is highly expressed in the striatum where the level of iron is the highest and shares a close homology with Dexras1 which controls neuronal iron trafficking (Falk et al., 1999; Cheah et al., 2006), we wondered whether Rhes is involved in the neuronal iron uptake in the striatum. We found that wild-type Rhes interacts with PAP7, a scaffolding protein between Dexras1 and DMT1, as an iron transporter and an active form of Rhes enhances iron uptake compared to a native form. Our in vitro phosphorylation assay revealed that protein kinase A (PKA) specifically phosphorylates at the residue of 239 in Rhes. Surprisingly, the phosphomimetic mutant of serine-239 to aspartic acid (S239D) induced an increase of iron uptake while the phosphodead mutant of serine-239 to alanine (S239A) did not. These observations indicate that PKA-mediated phosphorylation of Rhes activates Rhes GTPase and regulates the intracellualar iron influx.

EXPERIMENTAL PROCEDURES

Cells and generation of mutant constructs

HEK 293T cells were maintained in DMEM with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin–streptomycin at 37 °C with 5% CO_2 atmosphere in a humidified incubator. Wild-type Rhes was cloned into pCMV-Myc (Clontech, CA, USA) and subsequently S293A and S293D mutants were created with the QuickChange (Agilent Technologies, CA, USA) method according to the manufacturer's instruction.

Iron uptake assay

Non-transferrin-bound iron (NTBI) uptake assays were performed as previously described (Cheah et al., 2006). In brief, HEK293T cells were transfected with Rhes-Myc or mutants using Polyfect reagent (Qiagen, CA, USA). After 48 h, the cells were washed with phosphate-buffered saline (PBS) then resuspended into iron uptake buffer (25 mM Tris, 25 mM MES, 140 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.8 mM CaCl2 [pH 5.5]) and transferred to glass test tubes. Ascorbic acid was added to 1 mM $FeSO_4$ at a 44:1 ratio. $^{55}FeCl_3$ (PerkinElmer Life Science, CA, USA) was added to the iron/ascorbic acid mixture, which was then added to the cells in iron uptake buffer to a final concentration of 20 μM. Cells were incubated at 37 °C with shaking for 15 min. The cells were washed twice with cold PBS plus 0.5 mM EDTA and harvested. An aliquot of resuspended cells was taken for protein assay using the Bio-Rad Protein Assay Reagent; the protein concentrations of individual samples were used to quantitate ^{55}Fe incorporation (cpm/μg protein). Samples were normalized to control. Statistical comparisons of iron uptake were performed by student's *t*-test. All NTBI (non-transferrin bound iron) uptake experiments were repeated at least three times, each sample in triplicate.

Glutathione S-transferase (GST) pull-down assay

GST or GST-tagged PAP7 constructs were cotransfected with Rhes-Myc constructs into HEK293T cells using PolyFect (Qiagen, CA, USA), with a transfection efficiency of greater than 90%. Cells were lysed 48 h after transfection in buffer A (100 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 15% glycerol, 1 mM PMSF, 25 mg/ml antipain, 50 mg/ml leupeptin, 50 mg/ml aprotinin, 25 mg/ml chymostatin, and 25 mg/ml pepstatin). Lysates were precleared with pansorbin cells (Calbiochem, Germany), then 1 mg of total protein was incubated with glutathione–sepharose beads overnight at 4 °C. Beads were washed with wash buffer (50 mM Tris [pH 7.4], 500 mM NaCl, 10 mM b-glycerophosphate) twice, then once with buffer A. Beads were quenched in sample buffer (100 mM Tris [pH 6.8], 10% glycerol, 250 mM b-mercaptoethanol, 2% sodium dodecyl sulfate, and bromophenol blue). Total protein (50 mg) was loaded as input. Rhes-Myc binding was examined using an anti-myc antibody (Roche, AZ, USA) followed by incubation with anti-mouse secondary conjugated to horseradish peroxidase (HRP) (Jackson Immunoresearch); blots were then stripped and probed with an anti-GST antibody conjugated to HRP to detect PAP7. Chemiluminescence (Fisher Scientific, MA, USA) was used to detect bands on the Western blot.

In vitro phosphorylation

Immunoprecipitation and in vitro kinase assay were performed as previously described (Faul et al., 2007). Cells transfected with Rhes-Myc or Myc were lysed in buffer A, and then centrifuged at 12,000*g* for 10 min at 4 °C. After preclearing with 125 μl of Protein A beads prepared as a 20% (v/v) suspension for 1 h at 4 °C, supernatants were incubated with anti-Myc antibody and the immunocomplexes were precipitated by the addition of Protein A bead suspensions. The immunoprecipitates were collected by centrifugation and washed twice with buffer A and twice with PBS. The kinase assay was performed by incubating cell lysates in phosphatase buffer containing 20 mM $MgCl_2$ with or without λ phosphatase (λ PPase) for 3 h at 30 °C. After thoroughly washing with PBS three times, protein-bound beads were incubated with kinase buffer containing 1 mM MgATP, λ PPase inhibitors, and trace mount of [γ-32P] ATP for 30 min at 30 °C. When indicated, protein kinases, such as PKA, protein kinase C (PKC), and casein kinase 2 (CK2) were added to the reaction mixture. The kinase reaction was stopped by adding 4× SDS loading buffer and the samples were subjected to SDS–PAGE. Protein transferred blot was exposed to X-ray film for autoradiography. The blot was incubated with blocking buffer of 10% skimmed milk then proved with anti-Myc antibody for detection of input signal. To