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IDENTIFICATION OF NEUROGLOBIN-INTERACTING PROTEINS USING YEAST TWO-HYBRID SCREENING

Z. YU,[a]* N. LIU,[a] Y. WANG,[b] X. LI[b] AND X. WANG[a]*

[a]*Neuroprotection Research Laboratory, Departments of Neurology and Radiology, Massachusetts General Hospital, and Program in Neuroscience, Harvard Medical School, Charlestown, MA, USA*

[b]*School of Pharmaceutical Sciences, Wenzhou Medical College, Wenzhou, Zhejiang, China*

Abstract—Neuroglobin (Ngb) is a globin protein that is highly and specifically expressed in brain neurons. A large volume of evidence has proven that Ngb is a neuroprotective molecule against hypoxic/ischemic brain injury and other related neurological disorder; however, the underlying mechanisms remain poorly understood. Aiming to provide more clues in understanding the molecular mechanisms of Ngb's neuroprotection, we performed yeast two-hybrid screening to search for proteins that interact with Ngb. From a mouse brain cDNA library, we found totally 36 proteins that potentially interact with Ngb, and 10 of them were each identified in multiple positive clones. The shared sequences within these multiple clones are more likely to be Ngb-interacting domains. In primary cultured mouse cortical neurons, immunoprecipitation was performed to confirm the interactions of selected proteins with Ngb. The discovered Ngb-interacting proteins in this study include those involved in energy metabolism, mitochondria function, and signaling pathways for cell survival and proliferation. Our findings provide molecular targets for investigating protein interaction-based biological functions and neuroprotective mechanisms of Ngb.

Key words: neuroglobin, yeast two-hybrid screening, molecular interaction.

Neuroglobin (Ngb) is a globin family member identified in 2000 (Burmester et al., 2000) that is predominantly expressed in neurons of the neural systems including retina, and some endocrine tissues (Reuss et al., 2002; Wystub et al., 2003; Fordel et al., 2004; Brunori and Vallone, 2006). As a globin protein, Ngb binds with high affinity to various gaseous ligands such as O_2, CO, and NO (Dewilde et al., 2001). During the past decade, accumulating evidences have demonstrated that Ngb is protective for neurons against hypoxic/ischemic insults (Khan et al., 2006; Greenberg et al., 2008; Burmester and Hankeln, 2009; Yu et al., 2009a). Enhanced Ngb gene expression inversely correlates with the severity of histological and functional deficits after ischemic stroke (Sun et al., 2001, 2003; Hundahl et al., 2006; Peroni et al., 2007; Wang et al., 2008). Moreover, Ngb has been speculated to have translational importance with broad impact on neurological disorders. For example, Ngb over-expression has been found to be protective against beta-amyloid-induced neurotoxicity in mouse (Khan et al., 2007).

As an oxygen-binding protein, Ngb was originally thought to function in O_2 storage and transportation. However, due to its high O_2 binding rate and low O_2 dissociation rate, plus its low protein level ($\sim$1 μM) in the brain (Brunori and Vallone, 2006), Ngb is more likely to function in O_2 sensing rather than O_2 storage and transportation (Kriegl et al., 2002; Fago et al., 2004). Further study suggested Ngb may serve as a hypoxia sensor and initiate signal transduction in neuronal cells (Wakasugi and Morishima, 2005). It was reported that ferric human Ngb exerts guanine-nucleotide dissociation inhibitor (GDI) activity by preventing the Gα subunit from binding to the G$\beta\gamma$ complex and thus activates downstream signal transduction pathway, which is protective against oxidative stress (Schwindinger and Robishaw, 2001; Wakasugi et al., 2003).

Ngb was also proposed to modulate nitric oxide (NO) homeostasis since the oxygenated derivative of Ngb, Ngb-O_2, reacts with NO rapidly to produce NO_3^- and met-Ngb (Brunori et al., 2005). This pathway disposes of NO, which may in turn protect cellular respiration jeopardized by the inhibitory effect of NO on cytochrome c oxidase activity (Moncada and Erusalimsky, 2002; Brunori et al., 2004).

Another important physiological implication of Ngb is its effect in maintaining mitochondrial function in brain under hypoxic/ischemic condition, and this may be related to Ngb's role in reactive oxygen species (ROS) scavenging (Herold et al., 2004; Rayner et al., 2006; Fordel et al., 2007). At the subcellular level, Ngb is associated with mitochondria and linked to the oxidative metabolism (Burmester et al., 2007). Our laboratory has demonstrated that Ngb over-expression improved mitochondrial function and reduced oxidative stress in primary cultured neurons after hypoxia (Liu et al., 2009). Ngb over-expression also protected PC12 cells against beta-amyloid toxicity and attenuates beta-amyloid-induced mitochondrial dysfunction (Li et al., 2008).

Importantly, although the above hypotheses are inspiring in explaining Ngb's neuroprotection mechanisms, most of them are based on indirect or correlative experimental data. Thus better understanding of the molecular mechanism of Ngb's biological function and neuroprotective roles would have fundamental and translational significance,

*Corresponding author. Tel: +1-617-724-9503 or +1-617-724-9513; fax: +1-617-726-7830.
E-mail address: zyu@partners.org (Z. Yu) or wangxi@helix.mgh.harvard.edu (X. Wang).

Abbreviations: co-IP, co-immunoprecipitation; ETF, electron-transferring flavoprotein; mPTP, mitochondria permeability transition pore; Ngb, neuroglobin; OGD, oxygen-glucose deprivation; ROS, reactive oxygen species.

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which may eventually improve the development of Ngb-targeted therapeutics against stroke and other neurological disorders. We strongly believe that the molecular interaction between Ngb and other proteins is an important basis on above regards. As the first step, in this study we aimed to identify the Ngb-interacting proteins using yeast two-hybrid screening system and further validate their bindings in primary cultured mouse cortical neurons.

EXPERIMENTAL PROCEDURES

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using a GAL4-based yeast two-hybrid system (MATCHMAKER Two-Hybrid System 3; Clontech, Palo Alto, CA, USA); screening and assays were performed following the manufacturer's instruction (Clontech). Mouse Ngb cDNA was amplified by PCR; the PCR fragment was then digested with *Nde*I and *Bam*HI, and inserted into the pGBKT7 vector (Clontech) to generate a construct of mouse Ngb cDNA fused in-frame to the GAL4 DNA-binding domain (BD) (amino acids [a.a.] 1–147 of GAL4) as the bait. The vector was then transformed into yeast strain Y187 and the transformants were plated on dropout medium lacking tryptophan (SD/−Trp) because the pGBKT7 vector had a selectable *TRP1* marker. The pre-transformed Mouse Brain Matchmaker cDNA Library in pGADT7 vector was purchased from Clontech.

For yeast two-hybrid screening, the mating reaction between Y187 transformed with pGBKT7-Ngb construct and AH109 pre-transformed with mouse brain library (in pGADT7) was performed and selected on Quadruple Drop Out (stringent selection) medium. Positive clones were further tested on medium containing X-alpha-Gal, which tests alpha-galactosidase activated by positive Ngb-target protein interaction. The pGADT7 plasmids encoding the library clones were isolated and sequenced using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA), and homology searches against database sequences were performed using the BLAST algorithm on NCBI (National Center for Biotechnology Information).

Primary cortical neuronal culture

All animal experiments were performed following protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Primary neuronal culture was prepared from the cortex of embryonic day 15 mouse. In brief, the cortical neurons were suspended in neuron-defined culture medium and plated onto poly-D-lysine-coated 35-mm dishes (6×10^5 cells per dish). Neural basal medium supplemented with 2% B27, 0.3-mM L-glutamine, and 1% penicillin–streptomycin was used. Half of the medium was replaced every 3 days. Protein extraction was carried out at day 8 of neuronal culture.

Western blot

Western blot was performed as previously described (Yu et al., 2009b). Ngb protein levels were examined by Western blot using mouse anti-Ngb antibody (Santa Cruz). Selected Ngb-interacting proteins were detected with their respective antibodies after immuno-precipitation with anti-Ngb antibody.

Co-immunoprecipitation (co-IP)

Proteins were extracted from primary cultured mouse cortical neurons, and immuno-precipitation was performed using 2-μg polyclonal antibodies against mouse Ngb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After 3-h incubation, protein G sepharose was added and incubated overnight at 4 °C, and then centrifuged for 1 min at 12 000×*g*. The precipitates were rinsed with immuno-precipitation buffer (0.5% NP-40, Tris–Cl pH 8.0, 0.15-M NaCl) four times to remove non-specific binding molecules. IgG was used as negative control for precipitation. The co-immunoprecipitates were analyzed by Western blot. Antibodies against Atp1b1, Cyc1, Ubc, Dvl1, Etfa, Gabarap1, and VDAC antibody were used to detect these proteins.

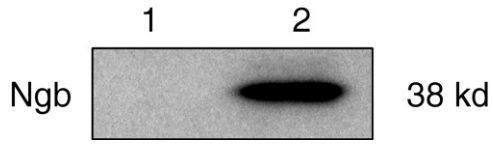

Fig. 1. Expression of Ngb-Gal4 BD fusion protein in yeast. Yeast strain Y187 was transformed with pGBKT7-Ngb and transformants were selected on appropriate SD medium. Soluble protein extracts were prepared and protein samples were subjected to SDS-PAGE. Ngb expression was detected using anti-Ngb antibody. Lane 1: control Y187 transformed with pGBKT7 vector. Lane 2: Y187 transformed with pGBKT7-Ngb.

RESULTS

Expression of Ngb protein in yeast strain Y187 transformed with pGBKT7-Ngb vector

To conduct yeast two-hybrid screening, we first cloned mouse Ngb cDNA into pGBKT7 vector. The resulted pGBKT7-Ngb vector was transformed into yeast strain Y187, and Ngb expression was confirmed by Western blot (Fig. 1).

Genes found from yeast two-hybrid screening with multiple positive clones

After mating reaction between pGBKT7-Ngb-transformed Y187 and AH109-containing mouse cDNA library, examples of clone selection were shown in Fig. 2. It shows nine clones containing Atp1b1 sequence and five clones containing Cyc1 sequence are positive for both Drop Out medium and X-alpha-Gal selection (showing blue color), and they are considered potential Ngb-interacting proteins. However, for Eef1a1, it can grow in Quadruple Drop Out medium, but negative in X-alpha-Gal assay, so is not considered an Ngb-interacting protein.

Totally 36 proteins were found to interact with Ngb. Among them, 10 proteins were each found in multiple positive clones (Table 1), and 26 were each found in one positive clone (Table 2). For proteins found in multiple clones, the gene inserts correspond to different parts of the same gene, most of them have common sequences and may designate the potential Ngb-interacting domains. These proteins with positive clone numbers from high to low were Na^+/K^+ ATPase beta 1 Subunit (Atp1b1), cytochrome c-1 (Cyc1), ubiquitin C (Ubc), disheveled-dsh homolog 1 (Dvl1), synaptotagmin I (Syt1), Na^+/K^+ ATPase beta 3 polypeptide (Atp1b3), electron-transferring flavoprotein alpha subunit (Etfa), GABA(A) receptor-associated protein like 1 (Gabarapl1), microtubule-associated protein 1A (Mtap1a), and voltage-dependent anion channel 1