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Review

Species-specific functional evolution of neuroglobin

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ABSTRACT

Neuroglobin (Ngb) is a recently discovered vertebrate heme protein that is expressed in the brain and can reversibly bind oxygen. Human Ngb is involved in neuroprotection under oxidative stress conditions such as ischemia and reperfusion. We previously demonstrated that, on the one hand, human ferric Ngb binds to the α-subunit of heterotrimeric G proteins ($G\alpha_i$) and acts as a guanine nucleotide dissociation inhibitor (GDI) for $G\alpha_i$. On the other hand, zebrafish Ngb does not exhibit GDI activity. By using wild-type and Ngb mutants, we demonstrated that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity. The crucial residues for both GDI and neuroprotective activity, corresponding to Glu53, Arg97, Glu118, and Glu151 of human Ngb, are conserved among boreotheria of mammalia. Recently, we found that zebrafish, but not human, Ngb can translocate into cells and clarified that module M1 of zebrafish Ngb is important for protein transduction. By performing site-directed mutagenesis, we showed that Lys7, Lys9, Lys21, and Lys23 of zebrafish Ngb are crucial for protein transduction activity. Because these residues are conserved among fishes, but not among mammals, birds, reptilians, or amphibians, the ability to penetrate cell membranes may be a unique characteristic of fish Ngb proteins. Moreover, we clarified that zebrafish Ngb interacts with negatively charged cell-surface glycosaminoglycan. Taken together, these results suggest that the function of Ngb proteins has been changing dynamically throughout the evolution of life.

Contents

1. Introduction

Neuroglobin (Ngb) is a heme protein, recently discovered in the mammalian brain, which can reversibly bind oxygen (Burmester et al., 2000; Dewilde et al., 2001; Trent et al., 2001). Mammalian Ngb is widely expressed in the cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum, and retina (Burmester et al., 2000; Mammen et al., 2002; Reuss et al., 2002; Zhang et al., 2002; Schmidt et al., 2003; Wystub et al., 2003). It has been recently suggested that mammalian Ngb might be involved in the neuronal response to hypoxia and ischemia (Sun et al., 2001; 2003; Fordel et al., 2006; Khan et al., 2006; Li et al., 2008). For example, mammalian Ngb has been reported to protect neurons from hypoxic–ischemic insults (Sun et al., 2001; 2003). In addition, expression of mammalian Ngb has been found to increase in response to neuronal hypoxia *in vitro* and to focal cerebral ischemia *in vivo* (Sun et al., 2001; 2003). Furthermore, neuronal survival following hypoxia or oxidative stress conditions can be reduced by inhibiting Ngb expression with an antisense oligodeoxynucleotide and enhanced by Ngb overexpression, supporting the notion that mammalian Ngb protects neurons from hypoxic–ischemic insults (Sun et al., 2001; Fordel et al., 2006; Li et al., 2008). Lastly, mammalian Ngb has been reported to protect the brain from

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experimentally induced stroke *in vivo* (Sun et al., 2003; Khan et al., 2006; Li et al., 2010).

Although Ngb was originally identified in mammalian species, it is also present in non-mammalian vertebrates, including zebrafish (Awenius et al., 2001; Fuchs et al., 2004). Mammalian and fish Ngb proteins share about 50% amino acid sequence identity (Fig. 1). Fish Ngb has oxygen-binding kinetics similar to those of mammalian Ngb (Fuchs et al., 2004). The iron atom in the heme prosthetic group of vertebrate Ngb proteins normally exists in either the ferrous (Fe^{2+}) or ferric (Fe^{3+}) redox state. Both the ferric and ferrous forms of Ngb are hexacoordinated to their endogenous protein ligands, namely the proximal and distal histidine (His) residues, and oxygen can displace the distal His residue of ferrous Ngb to produce ferrous oxygen-bound Ngb (Dewilde et al., 2001; Fuchs et al., 2004).

In this review, we summarize our current findings on novel functions of the human and zebrafish Ngb proteins.

2. Human Ngb as a guanine nucleotide dissociation inhibitor (GDI) for $G\alpha_i$ under oxidative stress conditions

We previously found that human ferric Ngb binds exclusively to the GDP-bound form of the α-subunit of heterotrimeric G protein ($G\alpha_i$) and acts as a guanine nucleotide dissociation inhibitor (GDI) by inhibiting the rate of exchange of GDP for GTP on $G\alpha_i$ (Wakasugi et al., 2003). Under normoxia, by contrast, ferrous ligand-bound Ngb did not have GDI activity (Wakasugi et al., 2003). These findings led us to propose that human Ngb may be a novel oxidative stress-responsive sensor for signal transduction in the brain (Wakasugi et al., 2003; 2005). Recently, we demonstrated that human Ngb competes with the βγ-subunits of heterotrimeric G protein ($G\beta\gamma$) for binding to $G\alpha_i$, suggesting that the interaction of GDP-bound $G\alpha_i$ with ferric Ngb liberates $G\beta\gamma$ (Kitatsuji et al., 2007). The enhancement in $G\beta\gamma$ signaling may promote cell survival by the activation of phosphotidylinositol 3-kinase (Schwindinger and Robishaw, 2001).

We have shown that zebrafish Ngb does not exhibit GDI activity (Wakasugi and Morishima, 2005) and cannot rescue cell death (Watanabe and Wakasugi, 2008a; 2008b). Previously, we examined key differences between human and zebrafish Ngb sequences by creating human mutant proteins with a particular focus on exposed residues with positive or negative charges. We found that human R47A, K102N, K119N, and D149A Ngb mutants had the same GDI activity as human wild-type Ngb, whereas E53Q, R97Q, E118Q, and E151N Ngb mutants did not, indicating that Glu53, Arg97, Glu118, and Glu151 of human Ngb are crucial for the GDI activity of human Ngb (Wakasugi and Morishima, 2005; Wakasugi et al., 2005). To gain further insight into the relationship between GDI activity and the neuroprotective function of human Ngb, we investigated the neuroprotective activity of human Ngb mutants. On the one hand, human R47A, K102N, K119N, and D149A Ngb mutants, which retained GDI activity, protected PC12 cells against cell death caused by hypoxia/reoxygenation as did human wild-type Ngb (Watanabe and Wakasugi, 2008a). On the other hand, human E53Q, R97Q, E118Q, and E151N Ngb mutants, which did not function as GDI proteins, did not rescue cell death under oxidative stress conditions (Watanabe and Wakasugi, 2008a). These results clearly show that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity.

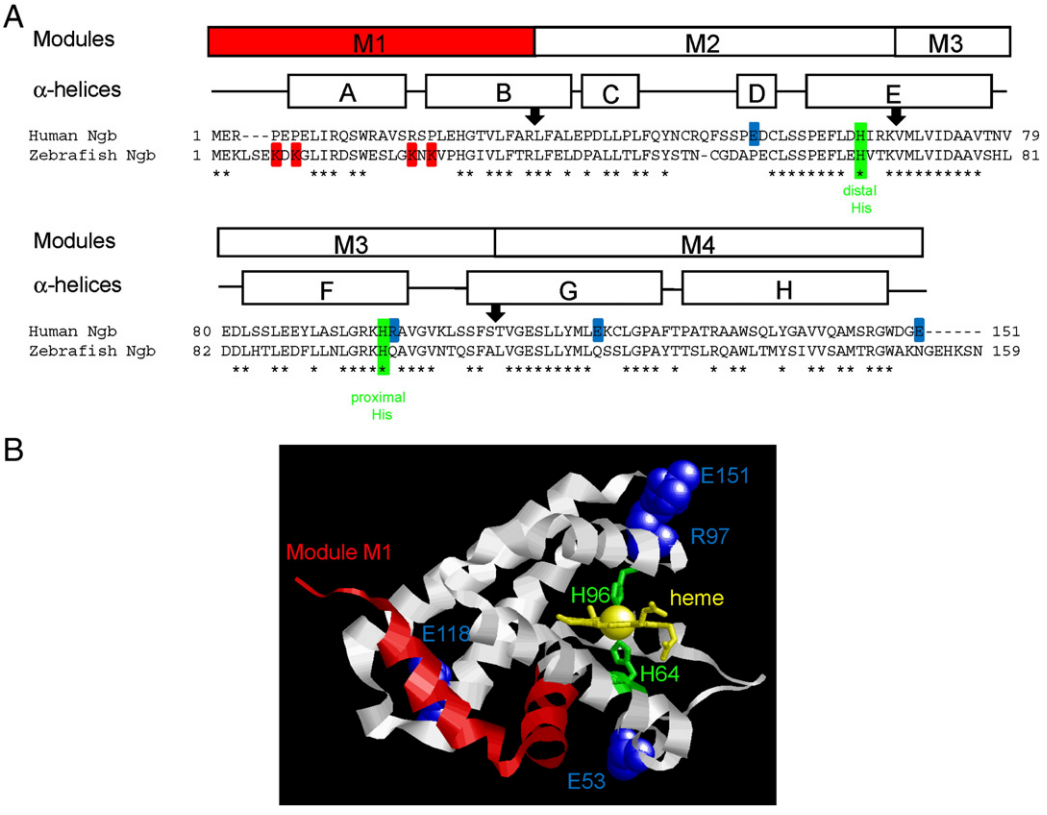

Fig. 1. Structure of Ngb. (A) Sequence alignment of human and zebrafish Ngb proteins. Multiple sequence alignment was performed by Clustal W with manual adjustments. The positions of α-helices A–H (Protein Data Bank Code: 1OJ6), modules M1–M4, and the proximal (His96) and distal histidine (His64) residues of human Ngb are shown. Consensus amino acids are indicated by an asterisk. Numbers on the left and right of the sequences correspond to those at the beginning and the end of the sequences, respectively. Gaps in the sequences are indicated by dashes. Intron positions in human and zebrafish Ngb at B12.2 (i.e. between codon positions 2 and 3 of the 12th amino acid of the globin helix B), E11.0, and G7.0 are indicated by arrows. The residues crucial for the GDI activity of human Ngb and the cell-membrane-penetrating activity of zebrafish Ngb are marked in blue and red, respectively. (B) Tertiary structure of human Ngb (Protein Data Bank code: 1OJ6). Residues in human Ngb crucial for its GDI activity (E53, R97, E118, and E151) are indicated in blue. Module M1 is highlighted in red. The proximal and distal His residues are indicated in green.