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Functional characterization of fish neuroglobin: Zebrafish neuroglobin is highly expressed in amacrine cells after optic nerve injury and can translocate into ZF4 cells ☆

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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. Mammalian Ngb is involved in neuroprotection under conditions of oxidative stress, such as ischemia and reperfusion. We previously found that zebrafish Ngb can penetrate the mammalian cell membrane. In the present study, we investigated the functional characteristics of fish Ngb by using the zebrafish cell line ZF4 and zebrafish retina. We found that zebrafish Ngb translocates into ZF4 cells, but cannot protect ZF4 cells against cell death induced by hydrogen peroxide. Furthermore, we demonstrated that a chimeric ZHHH Ngb protein, in which module M1 of human Ngb is replaced by that of zebrafish, is a cell-membrane-penetrating protein that can protect ZF4 cells against hydrogen peroxide exposure. Moreover, we investigated the localization of Ngb mRNA and protein in zebrafish retina and found that Ngb mRNA is expressed in amacrine cells in the inner nuclear layer and is significantly increased in amacrine cells 3 days after optic nerve injury. Immunohistochemical studies clarified that Ngb protein levels were increased in both amacrine cells and presynaptic regions in the inner plexiform layer after nerve injury. Taken together, we hypothesize that fish Ngb, whose expression is upregulated in amacrine cells after optic nerve injury, might be released from amacrine cells, translocate into neighboring ganglion cells, and function in the early stage of optic nerve regeneration. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Globins are iron porphyrin complex (heme)-containing globular proteins that bind reversibly to oxygen (O_2) and, as such, play an important role in respiratory function. Mammalian neuroglobin (Ngb) is widely expressed in the brain and retina [1,2]. Neuronal survival after oxidative stress can be reduced by inhibiting the expression of mammalian Ngb with an antisense oligodeoxynucleotide and enhanced by overexpressing mammalian Ngb, supporting the notion that mammalian Ngb protects neurons from hypoxic–ischemic insults [3–7]. Mammalian Ngb has been reported to protect the brain from experimentally induced stroke *in vivo* [8,9]. Moreover, overexpression of mammalian Ngb in the hearts of transgenic mice reduced ischemic injury to myocardial cells [9], suggesting that mammalian Ngb can protect non-neuronal cells as well as neuronal cells against oxidative stress-induced cell death.

Hypotheses concerning the neuroprotective mechanism of human Ngb have been reported [10–14]. Initially, Ngb was suggested to be an O_2 storage protein like myoglobin (Mb) [1]. However, the low concentration of Ngb in brain tissues except for the retina perhaps argues against a role for Ngb in storing and carrying significant amounts of O_2. As an alternative mechanism, it has been reported that Ngb may act as an intracellular scavenger of reactive oxygen species (ROS) and/or nitric oxide [15–19]. In contrast to Mb, the reaction of ferric Ngb with hydrogen peroxide does not generate the very reactive

Abbreviations: Ngb, neuroglobin; Mb, myoglobin; ROS, reactive oxygen species; G protein, guanine nucleotide-binding protein; GDI, guanine nucleotide dissociation inhibitor; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DIG, digoxigenin; Stx, syntaxin; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; HNgb, human neuroglobin; ZNgb, zebrafish neuroglobin; CNgb, chimeric ZHHH neuroglobin; NAC, N-acetylcysteine

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cytotoxic ferryl (Fe^{4+}) species [18]. This property may be beneficial under conditions of oxidative stress.

To investigate other function of human Ngb under conditions of oxidative stress, we previously performed yeast two-hybrid screening using human Ngb as a bait and identified flotillin-1, a lipid raft microdomain-associated protein, as a binding partner of human Ngb [20]. We demonstrated that human Ngb is present in lipid rafts only during oxidative stress and that lipid rafts are crucial for neuroprotection by Ngb [7]. We found that human ferric Ngb, which is generated under oxidative stress conditions, binds exclusively to the GDP-bound form of the α-subunit of heterotrimeric G protein ($G\alpha_{i/o}$), which is present in lipid rafts, and acts as its guanine nucleotide dissociation inhibitor (GDI) by inhibiting the rate of exchange of GDP for GTP on $G\alpha_{i/o}$ [7,21–24]. Moreover, we demonstrated that human Ngb inhibits the decrease in cAMP concentration that occurs under oxidative stress by functioning as a GDI for $G\alpha_{i/o}$, leading to protection against cell death [7].

Although Ngb was originally identified in mammalian species, it is also present in non-mammalian vertebrates [25,26]. Mammalian and fish Ngb proteins share about 50% amino acid sequence identity (Fig. 1). Fish Ngb proteins are also hexacoordinated globins with similar oxygen-binding kinetics [26]. We found that zebrafish Ngb does not exhibit GDI activity [23]. In order to clarify residues of human Ngb that are crucial for its GDI activity, we prepared human Ngb mutants with a focus on residues differing between human and zebrafish Ngb proteins and on residues with positive or negative charges exposed on the protein surface, and demonstrated that Glu53, Arg97, Glu118, and Glu151 of human Ngb are crucial for the GDI activity of human Ngb (Fig. 1) [23]. Moreover, by using wild-type Ngb and several Ngb mutants, we demonstrated that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity [4,7].

The genes of human and zebrafish Ngb comprise four exons interrupted by three introns, and exons 1, 2, 3, and 4 encode compact protein structural 'modules', termed M1, M2, M3, and M4, respectively (Fig. 1) [22,23,25–27]. We previously showed that a chimeric ZHHH Ngb protein, in which the module M1 of human Ngb is replaced by that of zebrafish, acts as a GDI for $G\alpha_{i/o}$ in a manner similar to human Ngb [23]. We demonstrated that chimeric ZHHH Ngb rescues PC12 cells from death caused by hypoxia/reoxygenation, as does human

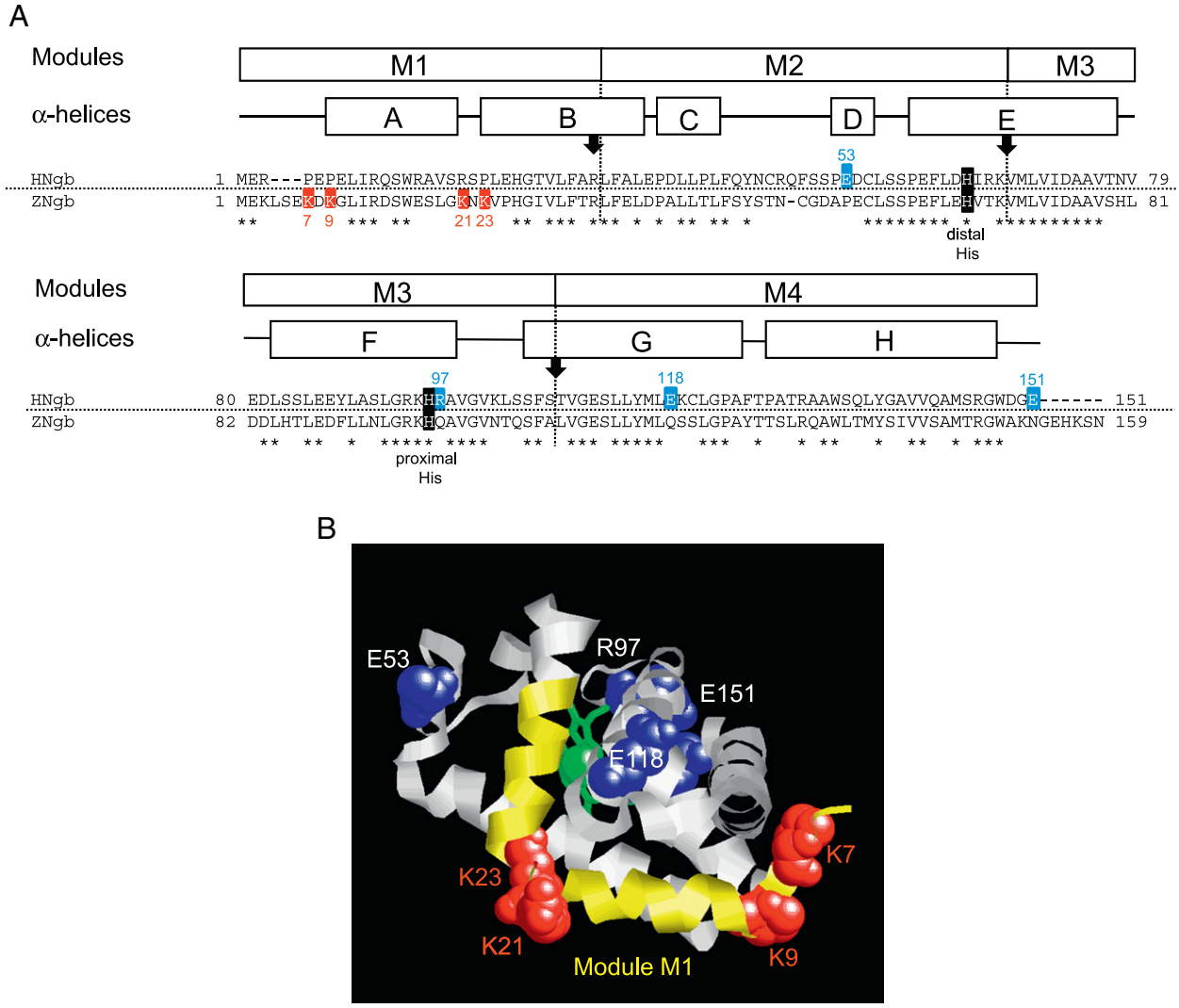

Fig. 1. Structure of Ngb. (A) Sequence alignment of human Ngb (HNgb) and zebrafish Ngb (ZNgb). Multiple sequence alignment was performed by Clustal W with manual adjustments. The positions of modules M1–M4, α-helices A–H (PDB ID: 1OJ6), and the proximal (His96) and distal (His64) histidine residues of HNgb are shown. Residues crucial for the GDI activity of HNgb and the cell-membrane-penetrating activity of ZNgb are marked in blue and red, respectively. Consensus amino acids are indicated by an asterisk. Numbers on the left and right of the sequences correspond to those at the beginning and end of the sequences, respectively. Gaps in the sequences are indicated by dashes. Intron positions in HNgb and ZNgb are indicated by arrows. (B) Tertiary structure of HNgb (PDB ID: 1OJ6). Residues in HNgb crucial for its GDI activity are indicated in blue. Locations of Lys residues critical for protein transduction in ZNgb are highlighted in red. The sequence alignment showed that amino acids K7, K9, K21, and K23 of ZNgb correspond to P4, P6, R18, and P20 in HNgb, respectively; thus, the residues 4, 6, 18, and 20 of the HNgb are represented by red space-filling balls, and residues K7, K9, K21, and K23 are indicated directly. Module M1 is highlighted in yellow. Heme is indicated in green.