Zebrafish Neuroglobin Is a Cell-Membrane-Penetrating Globin[†]

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Received February 18, 2008; Revised Manuscript Received March 26, 2008

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 oxidative stress conditions, such as ischemia and reperfusion. We previously demonstrated that 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 $G\alpha_i$. Recently, we used a protein delivery reagent, Chariot, and demonstrated that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity. In the present study, we found that chimeric ZHHH Ngb, in which module M1 of human Ngb is replaced by that of zebrafish Ngb, protects PC12 cells against oxidative stress-induced cell death even in the absence of Chariot. Using fluorescein isothiocyanate (FITC)-labeled Ngb proteins, we demonstrated that both zebrafish and chimeric ZHHH Ngb can penetrate cell membranes in the absence of Chariot, suggesting that module M1 of zebrafish Ngb can translocate into cells. This is the first report of a native cell-membrane-penetrating globin.

Neuroglobin (Ngb)[1] is a heme protein, recently discovered in the mammalian brain, which can reversibly bind oxygen (O_2) (*1*−*3*). Mammalian Ngb is widely expressed in the cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum, and retina (*1, 4*−*8*). It was recently suggested that mammalian Ngb might be involved in the neuronal response to hypoxia and ischemia (*9*−*13*). Expression of mammalian Ngb was reported to increase in response to neuronal hypoxia *in vitro* and to focal cerebral ischemia *in vivo* (*9, 10*). 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 (*9, 11, 13*). Mammalian Ngb was reported to protect the brain from experimentally induced stroke *in vivo* (*10, 12*).

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 $G\alpha_i$ (*14*). In contrast, we showed that under normoxia, ferrous ligand-bound Ngb did not have a GDI activity (*14*). These findings led us to propose that human Ngb may be a novel oxidative stress-responsive sensor for signal transduction in the brain (*14, 15*). Recently, we demonstrated that human Ngb competes with $\beta\gamma$ 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 $G\beta\gamma$ (*16*). The enhancement of $G\beta\gamma$ signaling may promote cell survival by the activation of phosphotidylinositol 3-kinase (*17*). Moreover, we used a protein delivery reagent, Chariot (*18*), to investigate whether the GDI activity of human Ngb plays an important role in its neuroprotective activity under oxidative stress conditions and demonstrated that the GDI activity of human Ngb is tightly correlated with its neuroprotective activity (*19*).

Although Ngb was originally identified in mammalian species, it is also present in nonmammalian vertebrates, including the zebrafish, *Danio rerio* (*20, 21*). Mammalian and fish Ngb proteins share about 50% amino acid sequence identity. Fish Ngb has similar oxygen-binding kinetics as mammalian Ngb (*21*). The genes of human and zebrafish Ngb are made of 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 (*20*−*23*). Previously, we demonstrated that zebrafish ferric Ngb did not exhibit GDI activity and that a chimeric ZHHH Ngb, in which module M1 of human Ngb is replaced by that of zebrafish, forms almost the same structure as human Ngb and acts as a GDI for $G\alpha_i$ in a manner similar to human Ngb (*22*). Moreover, we showed that protein transduction of chimeric ZHHH but not zebrafish Ngb with Chariot rescued PC12 cell death caused by hypoxia/reoxygenation (*19*).

In the present study, we investigated, in the absence of Chariot, the protective effect of several Ngb proteins against cell death of PC12 cells after hypoxia/reoxygenation. We

[†] This work was supported in part by Grant-in-Aid 19570121 for Scientific Research (C) (to K.W.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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[1] Abbreviations: Ngb, neuroglobin; G protein, guanine nucleotide-binding protein; GDI, guanine nucleotide dissociation inhibitor; PBS, phosphate-buffered saline; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; TBE, trypan blue exclusion; FITC, fluorescein isothiocyanate.

10.1021/bi800286m CCC: $40.75
Published on Web 04/17/2008

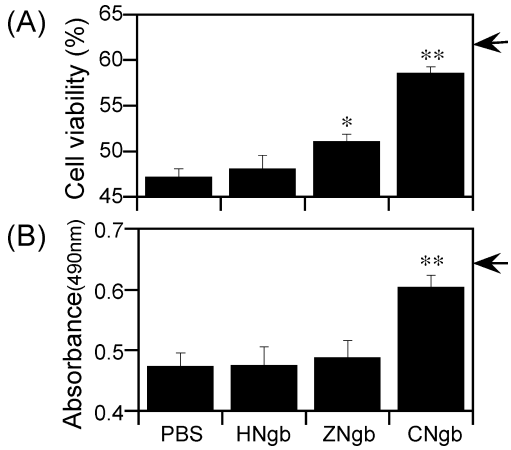

FIGURE 1: Protective effects of human Ngb (HNgb), zebrafish Ngb (ZNgb), and chimeric Ngb (CNgb), in the absence of Chariot, on PC12 cell death induced by hypoxia/reoxygenation. Human Ngb (HNgb), zebrafish Ngb (ZNgb), or chimeric Ngb (CNgb) was applied to PC12 cells without Chariot. Cell viabilities were measured by TBE (A) and MTS (B) assays. All data are expressed as means ± standard error of means (SEM) from three independent experiments, each performed in tripricate. (∗) $p < 0.05$ and (∗∗) $p < 0.0005$, compared to PBS without Chariot by one-way analysis of variation (ANOVA). Each value of chimeric Ngb with Chariot is shown by an arrow at the right side (*19*).

discovered that the chimeric ZHHH Ngb protects PC12 cells against oxidative stress-induced cell death even in the absence of Chariot. We prepared fluorescein isothiocyanate (FITC)-labeled Ngb proteins and investigated their translocation into cells, demonstrating that both zebrafish and chimeric ZHHH Ngb can penetrate the cell membrane even in the absence of Chariot.

EXPERIMENTAL PROCEDURES

Preparation of Proteins. Plasmids for human Ngb, zebrafish Ngb, and chimeric ZHHH Ngb, in which module M1 of human Ngb was replaced by that of zebrafish Ngb, were prepared as described previously (*14, 22*). Overexpression of each Ngb was induced in *Escherichia coli* strain BL 21 (DE 3) following treatment with isopropyl-β-D-thiogalactopyranoside, and each Ngb protein was purified as described previously (*14, 16, 22, 24*−*26*). In brief, the soluble cell extracts were loaded onto DEAE sepharose anion-exchange columns equilibrated with 20 mM Tris-HCl (pH 8.0). Ngb proteins were eluted from the columns with buffer containing 75 mM NaCl and further purified by passage through Sephacryl S-200 HR gel-filtration columns. Purified Ngb was dialyzed overnight against phosphate-buffered saline (PBS). Endotoxin was removed from the protein solutions by phase separation using Triton X-114 (Sigma-Aldrich, St. Louis, MO) (*27, 28*). Trace amounts of Triton X-114 were removed by passage through Sephadex G25 gel (GE Healthcare Bio-Sciences, Piscataway, NJ) equilibrated with PBS.

Cell Culture. A rat pheochromocytoma PC12 cell line (RCB0009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). PC12 cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% (v/v) fetal bovine serum (FBS), 10% (v/v) heat-inactivated horse serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (all from Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed twice weekly, and the cultures were split 1:8 once every week.

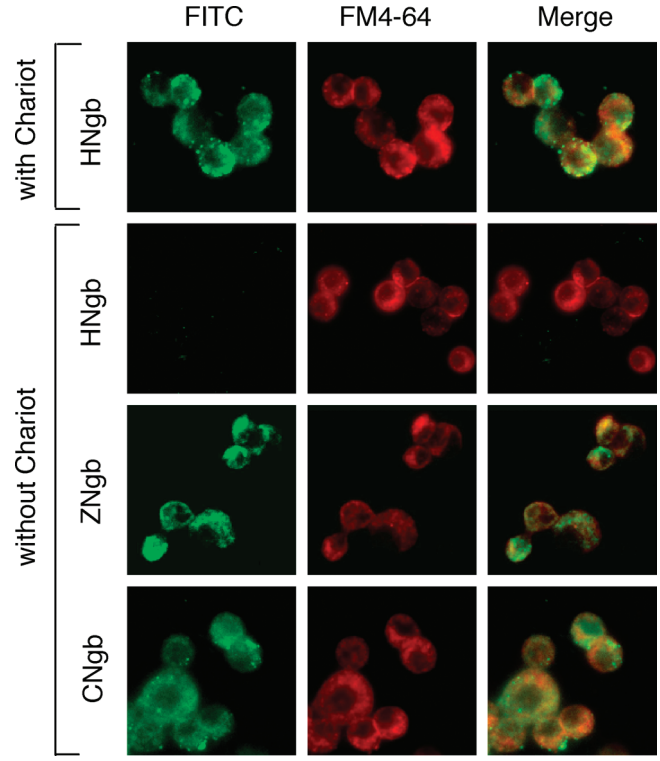

FIGURE 2: Transduction of FITC-labeled human, zebrafish, or chimeric Ngb into PC12 cells. FITC-labeled (green) human (HNgb), zebrafish (ZNgb), or chimeric (CNgb) Ngb was applied to PC12 cells with or without Chariot and in the presence of FM4-64 (red), a fluorescent marker of endocytosis. The cells were then incubated for another 6 h under hypoxia (1% O_2), fixed in 4% (w/v) paraformaldehyde for 30 min, and observed with fluorescence microscopy.

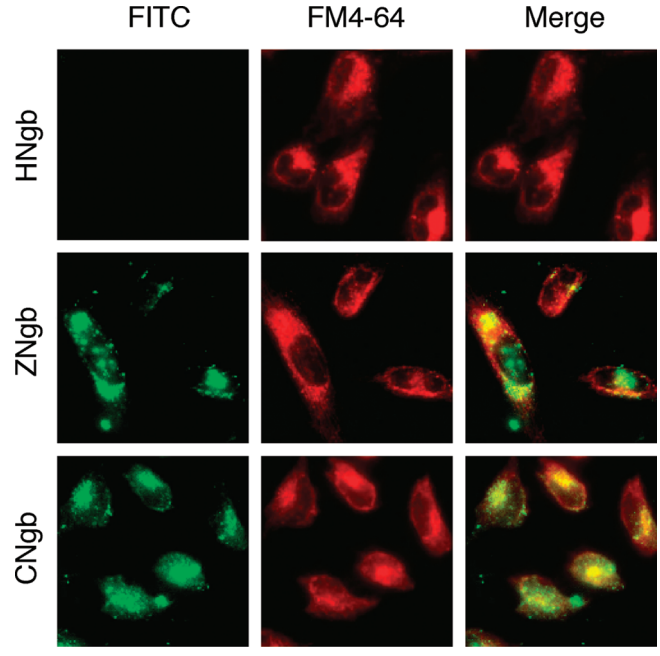

FIGURE 3: Transduction of FITC-labeled human, zebrafish, or chimeric Ngb into HeLa cells. FITC-labeled (green) human (HNgb), zebrafish (ZNgb), or chimeric (CNgb) Ngb was applied to HeLa cells, seeded on glass, without Chariot and in the presence of FM4-64 (red). The cells were then incubated for another 6 h under normoxia. The living, unfixed cells were directly observed with fluorescence microscopy.

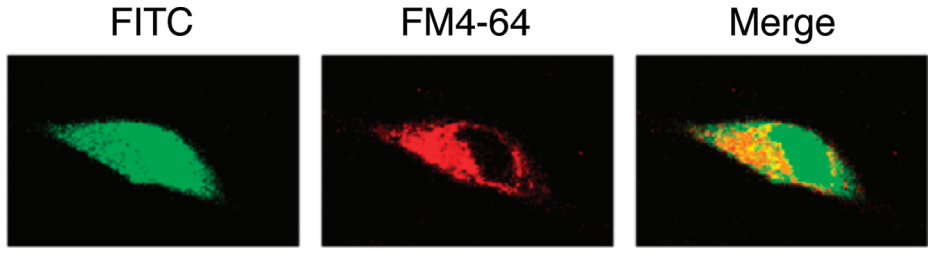

FIGURE 4: Confocal images of FITC-labeled zebrafish Ngb in HeLa cells. FITC-labeled (green) zebrafish Ngb was applied to HeLa cells without Chariot and in the presence of FM4-64 (red). The cells were then incubated for another 24 h under normoxia. The living, unfixed cells were directly observed with confocal laser scanning fluorescence microscopy.

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Zebrafish    1 MEKLSEKDKGLIRDSWESLGKNKVPHGIVLFTR 33
Trout        1 MEKLTEKEKELIRVSWESLGKDKVPHGVIMFSR 33
Pufferfish   1 MEKLSSKDKELIRGSWDSLGKNKVPHGVILFSR 33

Rat          1 MER---LESELIRQSWRAVSRSPLEHGTVLFSR 30
Mouse        1 MER---PESELIRQSWRVVSRSPLEHGTVLFAR 30
Monkey       1 MER---PEPELIRQSWRAVSRSPLEHGTVLFAR 30
Human        1 MER---PEPELIRQSWRAVSRSPLEHGTVLFAR 30
                     A         B
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FIGURE 5: Sequence alignment among module M1 of fish and mammalian Ngb proteins. Multiple sequence alignment was performed using Clustal W with manual adjustments. Arg (R) and Lys (K) residues conserved among fish Ngb proteins are highlighted in yellow. Proline (P) residues in mammalian sequences are highlighted in red. The positions of α helices (A and B) of human Ngb (PDB code 1OJ6) are shown. 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.

HeLa cells (RCB0007) were also obtained from the RIKEN Cell Bank. HeLa cells were maintained in culture in DMEM containing 4.5 g/L glucose, 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (all from Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed twice weekly, and the cultures were split 1:8 once every week.

Hypoxia/Reoxygenation. We modified the method previously reported (*29*−*31*) to examine PC12 cell death caused by hypoxia/reoxygenation. In brief, the cells were plated on poly-D-lysine-coated 96-well tissue culture plates at a density of 1.0×10^5 cells/mL in DMEM containing 2.0 g/L glucose, 2% (v/v) FBS, and 2 mM glutamine for 24 h. Each Ngb was transduced with or without Chariot (Active Motif, Carlsbad, CA) as described below. Hypoxia was induced in a multigas incubator (Astec, Fukuoka, Japan; set to 1% O_2, with 5% CO_2 and 94% N_2) at 37 °C for 24 h. After the hypoxia, the culture medium was replaced with fresh DMEM containing 2.0 g/L glucose, 2% (v/v) FBS, and 2 mM glutamine, and the cells were incubated at 37 °C for 24 h under normoxia (95% air/5% CO_2).

Protein Transduction with Chariot. Protein transduction was performed using Chariot according to the instructions of the manufacturer. Each purified Ngb protein (3 μg per well) was incubated in the absence or presence of diluted Chariot for 30 min at room temperature. Then, the mixture was added to cells that had been washed in DMEM without serum. Fresh DMEM without serum was added, and the cells were incubated at 37 °C for 1 h; FBS was then added to a final concentration of 2%. The cells were incubated at 37 °C for another 2 h to allow for Ngb internalization.

Cell-Viability Assays. Cell viability was measured by trypan blue exclusion (TBE) assays. Trypan blue was added to the cultured cells, and percentages of blue-stained cells were calculated after counting at least 1000 cells via phase-contrast microscopy. Cell viability was also measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Reagent (Promega, Madison, WI), containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)]. The cultured cells were incubated with the MTS reagent at 37 °C for 4 h in a humidified, 5% CO_2 atmosphere. Then, the amount of colored formazan dye formed was quantified by measuring the absorbance at 490 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (BioRad, Hercules, CA).

FITC Labeling of Ngb Proteins. Ngb was conjugated to fluorescein isothiocyanate (FITC; Dojindo, Kumamoto, Japan) according to the instructions of the manufacturer of the Fluoreporter FITC protein-labeling kit (Molecular Probes, Eugene, OR). FITC-labeled Ngb was purified using G25 gel chromatography to eliminate free FITC. The concentrations of Ngb protein and FITC dye in the purified FITC-labeled Ngb were calculated on the basis of their absorbance at 413 and 494 nm, respectively. The molar ratio of dye/protein in the purified FITC-labeled Ngb was determined to be 0.9−1.3 FITC dye molecules/Ngb protein.

Observation of Protein Translocation into Cells with Fluorescence Microscopy. PC12 and HeLa cells were seeded at 2×10^4 cells/mL in 35 mm tissue culture dishes (Corning, Corning, NY) and 35 mm glass-bottomed dishes (Matsunami Glass, Osaka, Japan), respectively. When cells were 60−70% confluent, FITC-labeled Ngb was added with or without Chariot in the presence of 1 μM FM4-64 (Molecular Probes, Eugene, OR), a general fluorescent marker of endocytosis. The cells were incubated under hypoxia (1% O_2) or normoxia at 37 °C for 6 or 24 h. PC12 cells were washed with cold PBS twice, fixed in 4% (w/v) paraformaldehyde for 30 min, and analyzed by fluorescence microscopy (Olympus IX71, Tokyo, Japan). HeLa cells were washed with cold PBS twice, and the living, unfixed cells were directly observed with fluorescence microscopy. Fluorescent images were also collected by confocal laser scanning microscopy (Olympus, FV1000) in a sequential scanning mode.

RESULTS AND DISCUSSION

Chimeric ZHHH Ngb Protects PC12 Cells against Oxidative Stress-Induced Cell Death in the Absence of Chariot. Recently, we used the protein delivery reagent, Chariot, to investigate whether the GDI activity of human Ngb plays an important role in its neuroprotective activity under oxidative stress conditions and demonstrated that the GDI activity of human Ngb is tightly correlated with its neuro-

protective activity (*19*). We showed that, in the presence of Chariot, protein transduction of human or chimeric ZHHH but not zebrafish Ngb resulted in a significant increase in cell viability (*19*). In the present study, we investigated the neuroprotective activities of human, zebrafish, and chimeric ZHHH Ngb in the absence of Chariot. Parts A and B of Figure 1 clearly show that the chimeric ZHHH Ngb protected PC12 cells against cell death caused by hypoxia/reoxygenation even in the absence of Chariot. In contrast, the viabilities of cells incubated with human or zebrafish Ngb without Chariot were not significantly different from the control condition (PBS; Parts A and B of Figure 1). These results suggest that chimeric ZHHH Ngb may efficiently cross biological membranes.

Translocation of Zebrafish and Chimeric ZHHH Ngb into Cells. We first tried to confirm that human Ngb protein was delivered into PC12 cells by Chariot. Because FITC-labeled Ngb proteins easily attached, nonspecifically, to poly-D-lysine-coated dishes, dishes without poly-D-lysine were used for this experiment. As shown in Figure 2, human Ngb was successfully delivered into PC12 cells in the presence of Chariot. Part of the FITC-labeled Ngb fluorescence signal appeared punctate and co-localized with FM4-64, a general fluorescent marker of endocytosis, within cells (Figure 2), suggesting partial localization of FITC-labeled Ngb proteins in endosomes prior to their release into the cytosol. In control experiments without Chariot, human Ngb was not translocated into PC12 cells (Figure 2). Next, we evaluated the ability of zebrafish and chimeric ZHHH Ngb proteins to be translocated into cells in the absence of Chariot. We demonstrated that FITC-labeled chimeric ZHHH and zebrafish Ngb penetrated the cell membrane of PC12 cells in the absence of Chariot (Figure 2). When these results are taken together, they suggest that module M1 of zebrafish Ngb has the ability to translocate into cells. The efficiency of chimeric ZHHH or zebrafish Ngb protein transduction did not depend upon hypoxic or normoxic conditions (data not shown). Moreover, we found that both zebrafish and chimeric Ngb but not human Ngb can be translocated also into HeLa cells in the absence of Chariot (Figure 3). The transduction efficiencies of chimeric and zebrafish Ngb in HeLa cells were dependent upon their incubation time (data not shown). Confocal images of living, unfixed HeLa cells confirmed the intracellular presence of zebrafish Ngb (Figure 4).

In the present study, we showed that chimeric ZHHH and zebrafish Ngb proteins are cell-membrane-penetrating globins. Furthermore, our results suggest that module M1 of zebrafish Ngb is essential for protein transduction of Ngb into cells, because both the zebrafish and chimeric Ngb proteins share the M1 module of zebrafish Ngb. It has been reported that the BETA2/NeuroD protein and the human immunodeficiency virus type 1 (HIV-1) TAT (transactivator of transcription) protein can permeate several cells because of arginine (Arg)- and lysine (Lys)-rich protein transduction domain sequences in their structures (*32*−*34*). The module M1 sequence of zebrafish Ngb shares several conserved basic Arg and Lys residues with other fish Ngb proteins (Figure 5). As shown in Figure 5, the positions of some Lys residues in zebrafish Ngb are occupied by proline (Pro) residues in human Ngb, implying that Ngb might have undergone mutations of Lys to Pro during its evolutionary process from fish to human, thus inhibiting its ability to efficiently transverse cell membranes. Further studies are in progress using site-directed mutagenesis in zebrafish Ngb M1 module to investigate the mechanism of chimeric ZHHH and zebrafish Ngb protein transduction.

Wittenberg and Wittenberg observed an extracellular heme-protein in the choroid blood from perfused retina of two basal teleost fish species (bowfin and bluefish) (*35*). The hemochrome absorption spectra of these proteins were similar to those of fish Ngb. This heme protein may correspond to Ngb, implying that fish Ngb may be secreted. Further research is necessary to investigate the physiological properties of fish Ngb.

Molecular Design of a Novel Cell-Membrane-Penetrating, Neuroprotective Agent. In the present study, we demonstrated that chimeric ZHHH Ngb is a cell-membrane-penetrating, neuroprotective globin. Previously, it was reported that Ngb proteins were fused to the basic Arg-rich protein transduction domain of the HIV-1 TAT protein, which possesses the ability to traverse biological membranes efficiently (*36*−*38*). The TAT-fused Ngb proteins entered cells (*36*−*38*), but the results of their neuroprotective properties are conflicting: Zhou et al. reported that a TAT-fused rat Ngb protected against apoptosis induced by hypoxia (*38*), whereas Peroni et al. demonstrated that treatment with a TAT-fused human Ngb failed to protect against oxygen and glucose deprivation (*37*). Fusion of human Ngb to the TAT sequence might block the binding site of human Ngb with $G\alpha_i$ and/or induce changes in the human Ngb−$G\alpha_i$ interaction because of the existence of many residues with positive charges in the TAT sequence. From our present results on chimeric ZHHH Ngb, as well as our previous results on several module-substituted proteins, we conclude that module substitutions will be useful for designing and producing novel functional proteins (*22, 26, 39*−*43*).

REFERENCES

1. Burmester, T., Weich, B., Reinhardt, S., and Hankeln, T. (2000) A vertebrate globin expressed in the brain. *Nature 407*, 520–523.
2. Dewilde, S., Kiger, L., Burmester, T., Hankeln, T., Baudin-Creuza, V., Aerts, T., Marden, M. C., Caubergs, R., and Moens, L. (2001) Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. *J. Biol. Chem. 276*, 38949–38955.
3. Watts, R. A., III, and Hargrove, M. S. (2001) Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *J. Biol. Chem. 276*, 30106–30110.
4. Mammen, P. P. A., Shelton, J. M., Goetsch, S. C., Williams, S. C., Richardson, J. A., Garry, M. G., and Garry, D. J. (2002) Neuroglobin, a novel member of the globin family, is expressed in focal regions of the brain. *J. Histochem. Cytochem. 50*, 1591–1598.
5. Reuss, S., Saaler-Reinhardt, S., Weich, B., Wystub, S., Reuss, M. H., Burmester, T., and Hankeln, T. (2002) Expression analysis of neuroglobin mRNA in rodent tissues. *Neuroscience 115*, 645–656.
6. Zhang, C., Wang, C., Deng, M., Li, L., Wang, H., Fan, M., Xu, W., Meng, F., Qian, L., and He, F. (2002) Full-length cDNA cloning of human neuroglobin and tissue expression of rat neuroglobin. *Biochem. Biophys. Res. Commun. 290*, 1411–1419.
7. Schmidt, M., Gieβl, A., Laufs, T., Hankeln, T., Wolfrum, U., and Burmester, T. (2003) How does the eye breathe? Evidence for neuroglobin-mediated oxygen supply in the mammalian retina. *J. Biol. Chem. 278*, 1932–1935.
8. Wystub, S., Laufs, T., Schmidt, M., Burmester, T., Maas, U., Saaler-Reinhardt, S., Hankeln, T., and Reuss, S. (2003) Localization of neuroglobin protein in the mouse brain. *Neurosci. Lett. 346*, 114–116.
9. Sun, Y., Jin, K., Mao, X. O., Zhu, Y., and Greenberg, D. A. (2001) Neuroglobin is up-regulated by and protects neurons from hypoxic−ischemic injury. *Proc. Natl. Acad. Sci. U.S.A. 98*, 15306–15311.

10. Sun, Y., Jin, K., Peel, A., Mao, X. O., Xie, L., and Greenberg, D. A. (2003) Neuroglobin protects the brain from experimental stroke *in vivo*. *Proc. Natl. Acad. Sci. USA 100*, 3497–3500.
11. Fordel, E., Thijs, L., Martinet, W., Lenjou, M., Laufs, T., Bockstaele, D. V., Moens, L., and Dewilde, S. (2006) Neuroglobin and cytoglobin overexpression protects human SH-SY5Y neuroblastoma cells against oxidative stress-induced cell death. *Neurosci. Lett. 410*, 146–151.
12. Khan, A. A., Wang, Y., Sun, Y., Mao, X. O., Xie, L., Miles, E., Graboski, J., Chen, S., Ellerby, L. M., Jin, K., and Greenberg, D. A. (2006) Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. *Proc. Natl. Acad. Sci. U.S.A. 103*, 17944–17948.
13. Li, R. C., Pouranfar, F., Lee, S. K., Morris, M. W., Wang, Y., and Gozal, D. (2007) Neuroglobin protects PC12 cells against 102-amyloid-induced cell injury. Neurobiol. Aging, in press.
14. Wakasugi, K., Nakano, T., and Morishima, I. (2003) Oxidized human neuroglobin as a heterotrimeric Gα protein guanine nucleotide dissociation inhibitor. *J. Biol. Chem. 278*, 36505–36512.
15. Wakasugi, K., Kitatsuji, C., and Morishima, I. (2005) Possible neuroprotective mechanism of human neuroglobin. *Ann. N.Y. Acad. Sci. 1053*, 220–230.
16. Kitatsuji, C., Kurogochi, M., Nishimura, S., Ishimori, K., and Wakasugi, K. (2007) Molecular basis of guanine nucleotide dissociation inhibitor activity of human neuroglobin by chemical cross-linking and mass spectrometry. *J. Mol. Biol. 368*, 150–160.
17. Schwindinger, W. F., and Robishaw, J. D. (2001) Heterotrimeric G-protein $\beta\gamma$-dimers in growth and differentiation. *Oncogene 20*, 1653–1660.
18. Morris, M. C., Depollier, J., Mery, J., Heitz, F., and Divita, G. (2001) A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat. Biotechnol. 19*, 1173–1176.
19. Watanabe, S., and Wakasugi, K. (2008) Neuroprotective function of human neuroglobin is correlated with its guanine nucleotide dissociation inhibitor activity. Biochem. Biophys. Res. Commun. *369*, 695–700.
20. Awenius, C., Hankeln, T., and Burmester, T. (2001) Neuroglobins from the zebrafish *Danio rerio* and the pufferfish *Tetraodon nigroviridis*. *Biochem. Biophys. Res. Commun. 287*, 418–421.
21. Fuchs, C., Heib, V., Kiger, L., Haberkamp, M., Roesner, A., Schmidt, M., Hamdane, D., Marden, M. C., Hankeln, T., and Burmester, T. (2004) Zebrafish reveals different and conserved features of vertebrate neuroglobin gene structure, expression pattern, and ligand binding. *J. Biol. Chem. 279*, 24116–24122.
22. Wakasugi, K., and Morishima, I. (2005) Identification of residues in human neuroglobin crucial for guanine nucleotide dissociation inhibitor activity. *Biochemistry 44*, 2943–2948.
23. Go, M. (1981) Correlation of DNA exonic regions with protein structural units in haemoglobin. *Nature 291*, 90–92.
24. Wakasugi, K., Nakano, T., Kitatsuji, C., and Morishima, I. (2004) Human neuroglobin interacts with flotillin-1, a lipid raft microdomain-associated protein. *Biochem. Biophys. Res. Commun. 318*, 453–460.
25. Wakasugi, K., Nakano, T., and Morishima, I. (2004) Association of human neuroglobin with cystatin C, a cysteine proteinase inhibitor. *Biochemistry 43*, 5119–5125.
26. Wakasugi, K., and Morishima, I. (2005) Preparation and characterization of a chimeric zebrafish−human neuroglobin engineered by module substitution. *Biochem. Biophys. Res. Commun. 330*, 591–597.
27. Aida, Y., and Pabst, M. J. (1990) Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J. Immunol. Methods 132*, 191–195.
28. Liu, S., Tobias, R., McClure, S., Styba, G., Shi, Q., and Jackowski, G. (1997) Removal of endotoxin from recombinant protein preparations. *Clin. Biochem. 30*, 455–463.
29. Yoshimura, S.-I., Banno, Y., Nakashima, S., Takenaka, K., Sakai, H., Nishimura, Y., Sakai, N., Shimizu, S., Eguchi, Y., Tsujimoto, Y., and Nozawa, Y. (1998) Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. *J. Biol. Chem. 273*, 6921–6927.
30. Yamada, J., Yoshimura, S., Yamakawa, H., Sawada, M., Nakagawa, M., Hara, S., Kaku, Y., Iwama, T., Naganawa, T., Banno, Y., Nakashima, S., and Sakai, N. (2003) Cell permeable ROS scavengers, Tiron and Tempol, rescue PC12 cell death caused by pyrogallol or hypoxia/reoxygenation. *Neurosci. Res. 45*, 1–8.
31. Koo, B.-S., Lee, W.-C., Chung, K.-H., Ko, J.-H., and Kim, C.-H. (2004) A water extract of *Curcuma longa* L. (Zingiberaceae) rescues PC12 cell death caused by pyrogallol or hypoxia/reoxygenation and attenuates hydrogen peroxide induced injury in PC12 cells. *Life Sci. 75*, 2363–2375.
32. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) In vivo protein transduction: Delivery of a biologically active protein into the mouse. *Science 285*, 1569–1572.
33. Futaki, S. (2005) Membrane-permeable arginine-rich peptides and the translocation mechanisms. *Adv. Drug Delivery Rev. 57*, 547–558.
34. Noguchi, H., Bonner-Weir, S., Wei, F.-Y., Matsushita, M., and Matsumoto, S. (2005) BETA2/NeuroD protein can be transduced into cells due to an arginine- and lysine-rich sequence. *Diabetes 54*, 2859–2866.
35. Wittenberg, J. B., and Wittenberg, B. A. (1975) A hemoprotein implicated in oxygen transport into the eye of fish. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol. 51*, 425–429.
36. Mendoza, V., Klein, D., Ichii, H., Ribeiro, M. M., Ricordi, C., Hankeln, T., Burmester, T., and Pastori, R. L. (2005) Protection of islets in culture by delivery of oxygen binding neuroglobin via protein transduction. *Transplant Proc. 37*, 237–240.
37. Peroni, D., Negro, A., Bähr, M., and Dietz, G. P. H. (2007) Intracellular delivery of neuroglobin using HIV-1 TAT protein transduction domain fails to protect against oxygen and glucose deprivation. *Neurosci. Lett. 421*, 110–114.
38. Zhou, G.-Y., Zhou, S.-N., Lou, Z.-Y., Zhu, C.-S., Zheng, X.-P., and Hu, X.-Q. (2008) Translocation and neuroprotective properties of TAT PTD Ngb fusion protein in primary cultured cortical neurons. *Biotechnol. Appl. Biochem. 49*, 25–33.
39. Wakasugi, K., Ishimori, K., Imai, K., Wada, Y., and Morishima, I. (1994) "Module" substitution in hemoglobin subunits: Preparation and characterization of a "chimera $\beta\alpha$-subunit". *J. Biol. Chem. 269*, 18750–18756.
40. Inaba, K., Wakasugi, K., Ishimori, K., Konno, T., Kataoka, M., and Morishima, I. (1997) Structural and functional roles of modules in hemoglobin: Substitution of module M4 in hemoglobin subunits. *J. Biol. Chem. 272*, 30054–30060.
41. Wakasugi, K., Ishimori, K., and Morishima, I. (1997) "Module"-substituted globins: Artificial exon shuffling among myoglobin, hemoglobin α- and β-subunits. *Biophys. Chem. 68*, 265–273.
42. Wakasugi, K., Quinn, C. L., Tao, N., and Schimmel, P. (1998) Genetic code in evolution: Switching species-specific aminoacylation with a peptide transplant. *EMBO J. 17*, 297–305.
43. Wakasugi, K., Nakano, T., and Morishima, I. (2005) Oxidative stress-responsive intracellular regulation specific for the angiostatic form of human tryptophanyl-tRNA synthetase. *Biochemistry 44*, 225–232.

BI800286M