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Association of Human Neuroglobin with Cystatin C, a Cysteine Proteinase Inhibitor†

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ABSTRACT: Neuroglobin (Ngb) is a newly discovered globin that is expressed in vertebrate brain. It has been reported that Ngb levels increase in neurons in response to oxygen deprivation, and that Ngb protects neurons from hypoxia. However, the mechanism of this neuroprotection remains unclear. In the present study, we identified human cystatin C, a cysteine proteinase inhibitor, as an Ngb-binding protein by using a yeast two-hybrid system. Surface plasmon resonance experiments verified that Ngb binds to cystatin C dimers, not to the monomers. Because both intracellular cystatin C and the amyloidogenic variant of cystatin C form dimers, Ngb may modulate the intracellular transport (or secretion) of cystatin C to protect against neuronal death under conditions of oxidative stress and/or it may have a role in the development of neurodegenerative diseases.

Globins are iron porphyrin complex (heme)-containing proteins that bind reversibly to oxygen and, as such, play an important role in respiratory function. Neuroglobin (Ngb)[1] is a recently discovered globin of vertebrate brain that has a high affinity for oxygen (*1*−*3*). Ngb can reversibly bind oxygen (*1*, *4*, *5*). The iron atom in the heme prosthetic group of Ngb exists in either the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) redox state. In the absence of exogenous ligands, both the ferric and the ferrous forms of Ngb are hexacoordinated with endogenous protein ligands, distal and proximal histidine (*4*). Oxygen (O_2) or carbon monoxide (CO) can displace the distal histidine of ferrous Ngb to produce ferrous oxygen-bound Ngb (ferrous-O_2 Ngb) or ferrous carbon monoxide-bound Ngb (ferrous-CO Ngb) (*4*).

Ngb is widely expressed in the cerebral cortex, hippocampus (CA1, CA2, CA3, and CA4, especially in the pyramidal layer), thalamus, hypothalamus, and cerebellum (*1*, *3*, *6*, *7*). Recently, it has been suggested that Ngb is involved in the neuronal response to hypoxia and ischemia (*8*, *9*). Ngb expression was reported to increase in response to neuronal hypoxia in vitro and to focal cerebral ischemia in vivo (*8*, *9*). Neuronal survival following hypoxia was reduced by inhibiting Ngb expression with an antisense

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[1] Abbreviations: Ngb, neuroglobin; ferrous-O_2 Ngb, ferrous oxygen-bound Ngb; ferrous-CO Ngb, ferrous carbon monoxide-bound Ngb; G protein, guanine nucleotide-binding protein; Gα, α subunit of heterotrimeric G protein; SPR, surface plasmon resonance; BD, DNA-binding domain; AD, activation domain; SD, synthetic dropout medium; GST, glutathione S-transferase; RU, resonance units.

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oligodeoxynucleotide and was enhanced by Ngb overexpression, supporting the notion that Ngb protects neurons from hypoxic−ischemic insults (*8*). Moreover, Ngb was found to protect the brain from experimentally induced stroke in vivo (*9*).

The mechanism by which Ngb affords neuroprotection under conditions of oxidative stress such as ischemia and reperfusion remains unclear. Recently, we found that ferric Ngb, which is generated spontaneously as a result of rapid autoxidation, binds exclusively to the GDP-bound form of the Gα and behaves as a guanine nucleotide dissociation inhibitor, inhibiting the rate of exchange of GDP for GTP (*10*). The interaction of GDP-bound Gα with ferric Ngb will liberate G$\beta\gamma$, leading to protection against neuronal death (*11*).

The objective of this study was to investigate other mechanisms of Ngb function under oxidative stress. We searched for proteins that could interact with human Ngb by using a yeast two-hybrid system. Here, we report the identification of cystatin C as another Ngb-interacting protein. Surface plasmon resonance (SPR) measurements were used to investigate further the interaction of Ngb and cystatin C.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen. The yeast two-hybrid screen was carried out using a GAL4-based yeast two-hybrid system (MATCHMAKER Two-Hybrid system 3; Clontech, Palo Alto, CA). The screening and assays were performed as recommended by the manufacturer (Clontech). Human Ngb cDNA was amplified by PCR using a human universal Quick-clone cDNA (Clontech). The PCR fragment digested with *Nde*I and *Bam*HI was inserted into the pGBKT7 vector (Clontech) to generate a construct of human full-length 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 transformed into yeast strain AH109 (Mata). The transformants were plated on dropout medium lacking tryptophan (SD/-Trp) because the pGBKT7 vector has a selectable *TRP1* marker. The Pretransformed Human Brain Matchmaker cDNA library, in which a human brain cDNA library has been cloned into a pACT2 vector with a selectable *LEU2* marker for expression as fusions with the GAL4 activation domain (AD) (a.a. 768−881 of GAL4), and which has been pretransformed into yeast strain Y187 (Matα), was purchased from Clontech.

The two transformant cultures were mated to each other and initially plated on dropout medium lacking leucine and tryptophan (SD/-Leu/-Trp) to select for both the bait and prey plasmids. In this two-hybrid system, the GAL4 BD binds to the GAL upstream activating sequence and, if the fusion proteins interact, the AD will be brought into proximity with promoters of four reporter genes (*HIS3*, *ADE2*, *MEL1*, and *lacZ*), thereby activating transcription and permitting growth on selection media (His^-, Ade^-) and the expression of α-galactosidase (*MEL1* product) and β-galactosidase (*lacZ* product). The cotransformants were then plated on dropout medium lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp) to select for colonies that expressed interacting proteins. Liquid cultures were assayed for α- and β-galactosidase to verify and to quantify the two-hybrid interactions. The pACT2 plasmids encoding the library clones were isolated and sequenced using an ABI 3100 Genetic analyzer (Applied Biosystems, Foster city, CA), and homology searches against database sequences were performed using the BLAST algorithm.

In addition, bait and prey plasmid pairs were cotransformed into yeast strain AH109 (Clontech). The transformants were initially plated on dropout medium lacking leucine and tryptophan (SD/-Leu/-Trp) to select for both the bait and prey plasmids. Positive interactions were confirmed by cell growth on histidine- and adenine-depleted yeast synthetic medium and by α- and β-galactosidase assays.

Western Blot Analysis. Transformants were selected on the appropriate SD medium and extracts of soluble proteins were prepared. Protein samples were resolved by electrophoresis on 15% polyacrylamide/SDS gels. Proteins were electroblotted onto Sequi-Blot PVDF membranes (Bio-Rad, Hercules, CA) for 1 h. The membranes were incubated at 4 °C for 1 h with a primary antibody (anti-GAL4 AD monoclonal antibody (Clontech)) in phosphate-buffered saline. After being washed three times at 4 °C with PBS containing 0.05% Tween20, the membranes were incubated with HRP-linked $F(ab')_2$ fragment of sheep anti-mouse Ig (Amersham Biosciences, Buckinghamshire, England) at 4 °C for 1 h. The membrane was again washed three times with the buffer at 4 °C, and the proteins were visualized by ECL Western blotting detection reagents (Amersham Biosciences).

α-*Galactosidase Assays.* The supernatant from the cell culture medium was incubated with *p*-nitrophenyl-α-D-galactoside (PNP-α-Gal) (Wako chemicals, Osaka, Japan). The optical density at 410 nm of *p*-nitrophenol released from the substrate PNP-α-Gal was normalized by the cell density of yeast measured photometrically at 600 nm and was expressed in units of α-galactosidase. One unit of α-galactosidase was defined as the amount of enzyme that hydrolyzed 1 μmol of PNP-α-Gal to *p*-nitrophenol and D-galactose in 1 min at 30 °C in acetate buffer, pH 4.5.

β-*Galactosidase Assays.* The harvested cells were suspended in Z buffer (60 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$) and disrupted by freeze−thawing. An aliquot of this extract was used for the β-galactosidase assays using *o*-nitrophenyl-β-galactoside (ONPG) (Wako chemicals) as the substrate. The optical density at 420 nm of *o*-nitrophenol released from the substrate ONPG was normalized by the cell density of yeast measured photometrically at 600 nm and was expressed in units of β-galactosidase. One unit of β-galactosidase was defined as the amount that hydrolyzed 1 μmol of ONPG to *o*-nitrophenol and D-galactose per min per cell.

Preparation of Protein Samples. Glutathione *S*-transferase (GST) and a fusion protein of GST and human Ngb were prepared as follows. Human Ngb cDNA was cloned into the pGEX-4T-1 vector (Amersham Biosciences) to produce the fusion protein GST-Ngb, and the resulting vector was sequenced with an ABI 3100 Genetic Analyzer (Applied Biosystems). Overexpression of GST-Ngb or GST alone (as a control) was induced in *E. coli* strain BL21 (DE3) (Novagen, Madison, WI) by treatment with isopropyl β-D-thiogalactopyranoside for 4 h. GST-ferric Ngb or GST was purified by using glutathione sepharose 4B beads (Amersham Biosciences) as recommended by the manufacturer. GST-ferrous-CO Ngb was generated by adding sodium dithionite and CO gas to the GST-ferric Ngb followed by gel filtration.

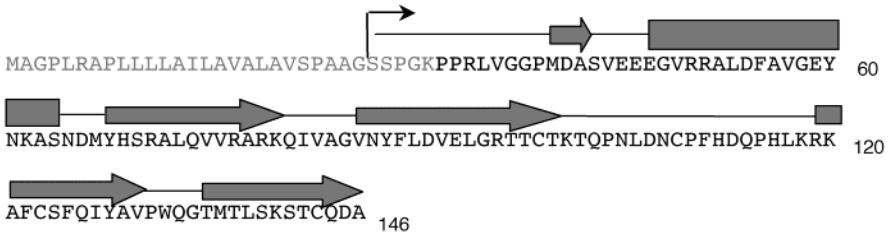

FIGURE 1: An Ngb-interacting protein identified by the yeast two-hybrid system. To search for Ngb-binding proteins, human full-length Ngb was used as a bait to screen a human brain cDNA library. The coding region of the isolated cystatin C cDNA clone obtained in the yeast two-hybrid screen is indicated in bold. The positions of an α-helix and five *β*-sheets, based on the crystal structure of cystatin C (*35*), are shown schematically above the amino acid sequence as a box and arrows, respectively. Numbers on the right correspond to the last amino acid on each line. Human mature cystatin C (a.a. 27−146) is generated from the cystatin C precursor (a.a. 1−146) by the removal of a signal peptide for secretion (a.a. 1−26).

Human cystatin C derived from human urine was purchased from Calbiochem (San Diego, CA). Dimerized cystatin C was produced by incubation at 68 °C for 2 h.

Surface Plasmon Resonance (SPR) Experiments. SPR measurements were performed on a BIAcore X Instrument (Biacore, Uppsala, Sweden). GST, GST-ferric Ngb, or GST-ferrous-CO Ngb was immobilized on the surface of a CM5 sensor chip by using an amine coupling kit (Biacore) according to the manufacturer's instructions. In brief, the carboxymethylated dextran in the CM5 sensor chip was activated by mixing equal volumes of 400 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide in water and 100 mM hydrochloride/*N*-hydroxysuccinimide in water, and injecting the mixture into the instrument at 10 *μ*L/min for 7 min. A solution of 10 *μ*g/mL of GST, GST-ferric Ngb, or GST-ferrous-CO Ngb dissolved in 10 mM acetate buffer (pH 4.5) was then injected over the activated surface of the sensor chip for 7 min at a flow-rate of 10 *μ*L/min. Unreacted sites on the sensor chip were masked by injecting 1 M ethanolamine (pH 8.5) for 7 min. After the immobilization process, nonspecifically bound protein was removed by washing with running buffer (10 mM Hepes, 50 mM NaCl, 0.005% Tween20, pH 7.4) until the value of the resonance units (RU) became nearly constant.

All binding experiments were performed at 25 °C at a flow rate of 5 *μ*L/min. Human cystatin C monomers or dimers in the running buffer were injected for 60 s. Running buffer alone was then applied over the next 60 s. The BIAcore response is expressed in relative RU, i.e., the difference in response between flow cell with immobilized protein and the control flow channel. 1000 RU corresponds to 1 ng/mm^2 of bound ligand. Experimental curves (sensorgrams) were analyzed by means of the BIAevaluation 3.1 software package using the model A + B ⇔ AB to estimate the association and dissociation rate constants k_a and k_d.

RESULTS

Screening of Ngb-Binding Proteins by a Yeast Two-Hybrid System. To identify proteins that were expressed in the brain and that could physically interact with Ngb, we performed a yeast two-hybrid screen using pGBKT7-human Ngb as the bait and a human brain cDNA library cloned into pACT2 as the prey. Out of 2 × 10^6 transformants, we obtained 43 colonies that had positive phenotypes for expressing the selection markers (*HIS3*, *ADE2*, and *MEL1*). Further screening of the library identified several yeast clones that yielded activity in the α- and *β*-galactosidase assays. The pACT2 plasmids containing the cDNA sequence of the putative Ngb interaction partners were recovered from the yeast cells. DNA sequencing of the isolated plasmids, coupled with homology searching against sequence databases, showed that one plasmid contained a partial sequence of cystatin C cDNA, ranging from the codon of a.a. 32 to the stop codon at aa 146 (Figure 1). A database search revealed that the partial sequence shared 100% identity with the cDNA of human cystatin C. The partial sequence is almost the same size as the mature form of cystatin C, in which the N-terminal signal peptide for secretion (a.a. 1−26) has been deleted.

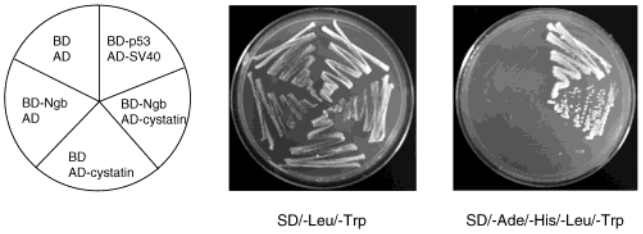

FIGURE 2: Growth of transformed yeast on selective plates. Yeast strain AH109 was cotransformed with pGBKT7-X and pACT2-Y incorporating the indicated constructs (left). Plasmid pGBKT7-X encodes a fusion of the GAL4 BD (a.a. 1−147) and a protein X, whereas plasmid pACT2 (or pGADT7)-Y encodes a fusion of the GAL4 AD (a.a. 768−881) and a protein Y. Plasmids pGBKT7-53 and pACT2-SV40 large T antigen were transformed as a positive control because p53 protein is known to interact with SV40 large T-antigen. Transformed AH109 cells containing both plasmids were streaked out on plates lacking leucine and tryptophan (SD/-Leu/-Trp) or plates lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp).

Human Ngb Interacts with Cystatin C. To confirm further the interaction of Ngb with cystatin C, yeast strain AH109 was cotransformed with a bait plasmid (either pGBKT7-Ngb or pGBKT7) and a prey plasmid (either pCAT2-cystatin C or pACT2 (pGADT7)). Double transformants were first plated on dropout medium lacking leucine and tryptophan (SD/-Leu/-Trp) to select for both the bait and prey plasmids. The cotransformants were then plated on dropout medium lacking adenine, histidine, leucine and tryptophan (SD/-Ade/-His/-Leu/-Trp). As shown in Figure 2, yeast cells transformed with both pGBKT7-Ngb and pCAT2-cystatin C grew on the SD/-Ade/-His/-Leu/-Trp plate, as did a positive control (p53,SV40). By contrast, other double transformants did not grow on these plates (Figure 2).

Liquid cultures were assayed for α- and *β*-galactosidase to verify and to quantify the two-hybrid interactions. The α- or *β*-galactosidase assays showed that, in comparison with other double transformants, yeast cells transformed with both

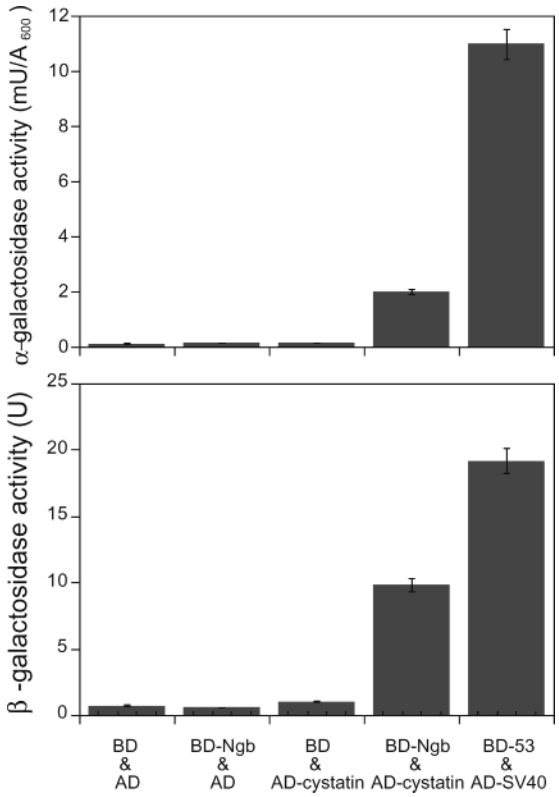

FIGURE 3: α- and *β*-Galactosidase activities of transformed yeast. The experimental conditions were described under Materials and Methods. The values shown are the averages of three independent experiments.

pGBKT7-Ngb and pCAT2-cystatin C had significant galactosidase activity, as did the positive control (Figure 3).

In addition, to check the expression of GAL4 AD-cystatin C, we analyzed transformants by Western blot by using the anti-GAL4 AD monoclonal antibodies and confirmed that the pACT2-cystatin C expression construct yielded a fusion protein of the expected molecular size (~35 kDa) (Figure 4). Taken together, these data indicate that human Ngb interacts with cystatin C.

SPR Detection of Human Ngb Binding to Cystatin C Dimer. To investigate the interaction of Ngb and cystatin C in vitro, we carried out SPR experiments. SPR is a powerful tool for real time measurements of direct protein−protein interactions that does not require labeling of the proteins. For these SPR experiments, we used GST-Ngb. The UV−visible spectra of GST-Ngb indicated that GST-Ngb forms a ferric or ferrous ligand-bound state as does wild-type Ngb (data not shown). At first, we covalently coupled GST-ferric Ngb or GST to a sensor chip and characterized the interaction between Ngb and cystatin C. We found that cystatin C monomers do not bind to the GST-ferric Ngb as well as GST (Figure 5A,B). Next, we investigated the potential interaction of ferrous-O_2 Ngb with cystatin C. Because ferrous-O_2 Ngb is unstable and is rapidly converted into ferric Ngb through autoxidation (*4*), stable ferrous-CO Ngb was used for the SPR experiments. As shown in Figure 5C, the binding affinity of cystatin C monomers to the immobilized GST-ferrous-CO Ngb was significantly low.

As it has been reported that intracellular cystatin C forms a dimer (*12*), and dimerized cystatin C can be generated by incubation at 68 °C for 2 h in vitro (*13*), we next investigated

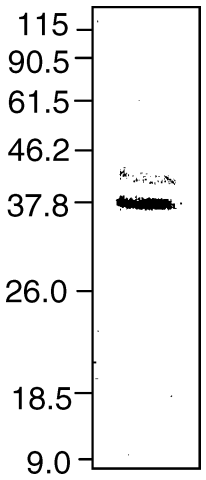

FIGURE 4: Expression of GAL4-AD-cystatin C fusion protein in transformed yeast. For the Western blot analyses, protein samples were resolved by electrophoresis on a 15% polyacrylamide/SDS gel and then electroblotted onto a PVDF membrane. The blot was probed with an anti-GAL4 AD monoclonal antibody, followed by an HRP-linked F(ab′)$_2$ fragment of sheep anti-mouse Ig. Proteins were visualized by ECL Western blotting detection reagents. Molecular size markers (in kilodaltons) are given on the left.

whether any binding occurs between Ngb and dimeric cystatin C. As shown in Figure 5D, cystatin C dimers did not bind to a control sensor chip covalently coupled to GST alone. A representative sensorgram in Figure 5E shows that the resonance response reflecting ferric Ngb-dimeric cystatin C occurred in an analyte concentration-dependent manner. In the association phase (0 ~ 60 s) the intensity of SPR increased, indicating that cystatin C dimers bound to ferric Ngb specifically, whereas in the dissociation phase (60 ~ 120 s) the intensity of SPR decreased, indicating that cystatin C dimers dissociated from the immobilized GST-ferric Ngb. Similarly, Figure 5F showed that cystatin C dimers bound to ferrous-CO Ngb in an analyte concentration-dependent manner. Binding parameters for the interaction of cystatin C dimers with ferric and ferrous-CO Ngb were as follows: association rate constants, $k_a = 3.1 \times 10^3$ and 8.5×10^3 M^{-1} s^{-1}; dissociation rate constants, $k_d = 7.4 \times 10^{-3}$ and 4.7×10^{-2} s^{-1}; and equilibrium dissociation constants, $K_d = k_d/k_a = 2.4$ and 5.5 μM, respectively. No significant resonance signals were obtained from sensor chip surfaces that did not have attached ligands, indicating an absence of nonspecific interactions between the sensor chip surfaces and the analytes (data not shown).

DISCUSSION

In the present work, we identified cystatin C, a cysteine proteinase inhibitor, as a Ngb-binding protein by using a yeast two-hybrid system. The SPR experiments confirmed that human Ngb binds to dimeric, but not monomeric, cystatin C. Recently, we demonstrated that ferric Ngb binds to Gα (GTPase) (*10*). Another link between a proteinase inhibitor and a GTPase has been reported (*14*): products of the *ras* oncogene (Ras), which encode small GTPases that regulate cell growth, proliferation, and differentiation, share partial sequence homology with the cysteine proteinase inhibitor family designated the "cystatin superfamily" and function as inhibitors of cathepsins B and L (*14*). Some proteinases inactivate growth-related proteins such as growth

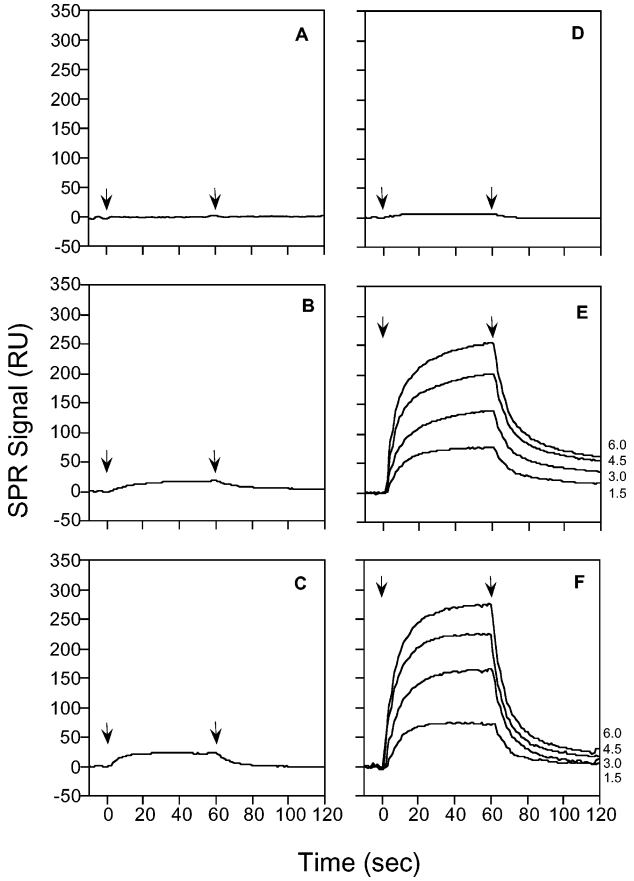

FIGURE 5: SPR analyses of cystatin C binding to Ngb. (A−F) Binding affinities of cystatin C monomers and/or dimers to the immobilized GST (A and D), GST-ferric Ngb (B and E) or GST ferrous-CO Ngb (C and F). GST, GST-ferric Ngb or GST-ferrous-CO Ngb was immobilized on a CM5 sensor chip, with an immobilization level of 9850, 9450, or 10250 RU, respectively. Cystatin C samples incubated at 4 °C (A, B, and C) or 68 °C (D, E, and F) for 2 h were used as analytes. Dimerized cystatin C was produced by incubating cystatin C at 68 °C for 2 h. The on and off processes for ligand binding were recorded on a BIAcoreX. The arrows at 0 and 60 s indicate the start of the injection of ligand (association phase) and the start of the injection of buffer alone (dissociation phase), respectively. In panels A−D, the concentration of cystatin C was 6.0 μM. In panels E and F, the concentrations of cystatin C used were 1.5, 3.0, 4.5, and 6.0 μM.

factor receptors by causing their degradation, whereas some generate cytokines and/or regulators of angiogenesis by causing protein cleavage (*15*−*18*). In fact, Ras can suppress the degradation of epidermal growth factor receptors and thereby affect cell growth (*19*). These results imply that the regulation of G protein-mediated signaling pathways and the inhibition of cysteine proteinase activity may be crucial for cell survival under conditions of oxidative stress.

Possible Roles of a Ngb and Cystatin C Dimer Complex. We have shown that cystatin C interacts with human Ngb. Although cystatin C is known to be a potent endogenous inhibitor of cysteine proteinases such as cathepsin B, H, or L, its precise role in the brain remains unclear. Cystatin C is widely expressed in almost all tissues and is secreted into various biological fluids. Intracellular cystatin C is located in lysosomes (*12*, *20*). It has been reported that the production of cystatin C is upregulated in the central nervous system after ischemia and that CA1 pyramidal neurons of rat hippocampus show delayed expression of cystatin C in transient ischemia of the forebrain (*21*). Moreover, cystatin C has been reported to be involved in oxidative stress-induced apoptosis in cultured neurons (*22*). Also the cystatin B, another member of the cystatin superfamily, plays a role in preventing apoptosis in cerebellar neurons as has been shown in cystatin B-deficient mice (*23*). Therefore, cystatin C may be involved in mechanisms of apoptosis stimulated by oxidative stress in the brain.

Previous studies have demonstrated that cathepsin inhibitors save postischemic CA1 neurons from delayed death and rescue hippocampal slices from cell death induced by oxygen and/or glucose deprivation (*24*, *25*), suggesting that cathepsins participate in the degeneration of neurons after the ischemic insult. Because cystatin C is also a inhibitor of cathepsins, it may be important in preventing neuronal death after severe ischemic damage to the brain through the regulation of cysteine proteinase activities. Since it has been reported that oxidative stress rapidly initiates cathepsins B and L translocation from lysosomes to the cytosol due to the lysosomal membrane injury or rupture (*26*, *27*), cystatin C may be also released from lysosome into the cytoplasm where Ngb exists during oxidative stress conditions.

Our present results are the first to demonstrate an interaction between Ngb and cystatin C. Because it has been reported that cystatins form dimers during intracellular trafficking or under intracellular reducing conditions (*12*, *28*), it can be inferred that Ngb bound to the intracellular (dimeric) form of cystatin C in the yeast two-hybrid system. It should be noted that Ngb does not bind to cystatin C monomers. Because the dimer formation is known to be accompanied by a complete loss of the protein's activity as a cysteine proteinase inhibitor (*12*), these data suggest that Ngb does not modulate cysteine proteinase activity directly. Taken together, these findings suggest that under conditions of oxidative stress Ngb, by interacting with cystatin C dimers, may modulate intracellular transport or processing through the secretory pathway of cystatin C to protect against neuronal death. Moreover, cystatin C has been shown recently to be important for the proliferation and expansion of neural stem cells: cystatin C is an essential extracellular autocrine/paracrine cofactor required for the proliferation of fibroblast growth factor 2 (FGF2)-responsive neuronal stem cells in vitro and in vivo, and it may therefore have a crucial role in neuronal survival as well as in neurogenesis (*29*). Further studies will be necessary to clarify the involvement of Ngb in regulating neuronal death and/or proliferation. Studies are now in progress to elucidate the physiological significance of the Ngb-cystatin C interaction in the brain.

Relationship to Neurodegenerative Diseases. Cystatin C is associated with chronic neurodegenerative processes. The accumulation of a variant of cystatin C with L68Q substitution is known to be the cause of cerebral amyloid formation in patients with "hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I)", also known as "hereditary cystatin C amyloid angiopathy (HCCAA)" (*30*). This disease results in paralysis, the development of dementia due to multiple strokes, and generally death from cerebral hemorrhage before the age of 40 years. In the brains of patients affected with Alzheimer disease (AD), the neuronal concentration of cystatin C protein is increased and its association with amyloid-β has been established (*20*, *31*). In addition, the amyloidogenic L68Q cystatin C variant has

been shown to form dimers more easily than wild-type cystatin C (*32*).

Ngb was reported to undergo more rapid autoxidation than other globins, resulting in the production of reactive oxygen species (*4*). It has been shown that oxidative stress leads to neurodegeneration by promoting the cross-linking and aggregation of amyloidogenic molecules. For example, β-amyloid (Aβ), which has been implicated in Alzheimer's disease, was reported to be cross-linked by H_2O_2 and hemoglobin or hemin (*33*), resulting in the production of insoluble and aggregating molecules that lead to amyloid formation. Furthermore, it has been reported that iron-catalyzed oxidative reactions mediated by cytochrome *c*/H_2O_2 promotes the aggregation of α-synuclein by causing its oxidative cross-linking via the formation of *o*,*o*′-dityrosine (*34*), which could potentially lead to the development of Parkinson's disease and related disorders. Thus, reactive oxygen species produced by Ngb binding to cystatin C dimers might promote the cross-linking of amyloidogenic molecules because cystatin C is known to associate with the Aβ (*31*). Taken together, these results suggest that Ngb may be linked to the development of neurodegenerative disorders such as Alzheimer disease.

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