A GLOBIN IN THE NUCLEUS!

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Abbreviations: Hb: hemoglobin; Mb: myoglobin; Ngb: neuroglobin; mNGB: mouse neuroglobin; Cygb: cytoglobin; mCygb: mouse cytoglobin; hCYGB: human cytoglobin; STAP: stellate cell activation-associated protein; PBS: phosphate
buffered salin; FITC: Fluorescein Isothiocyanate; H+L: heavy and light chains; GFAP: Glial fibrillary acidic protein; DAPI: 4’-6-Diamidino-2-phenylindole; sGC: soluble guanylyl cyclase; EcDos: *E. coli* direct oxygen sensor; PAS: sensory domain named after the eukaryotic proteins Period, Arnt and Simple-minded; NPAS2: neuronal PAS protein 2; CRP: cAMP receptor protein.
Abstract

Cytoglobin and neuroglobin are recently discovered members of the globin family. *In situ* hybridization localized neuroglobin mainly in brain and retina, while cytoglobin was expressed ubiquitously in all analysed tissues. In the present study, polyclonal antibodies were raised against both proteins and the distribution of them was studied by immunocytochemistry at tissue and subcellular level. Cytoglobin immunoreactivity was uniformly distributed and found in all tissues studied. At the subcellular level, cytoglobin immunoreactivity was exclusively detected in the cell nucleus. In contrast, neuroglobin immunoreactivity was detected in specific brain regions with varying intensities and in the islet of Langerhans in the pancreas. The immunoreactivity was restricted to the cytoplasm of neurons and endocrine beta cells.

The nuclear localization of cytoglobin opens new perspectives for possible function(s) of globin-folded proteins as transcriptional regulators.
Introduction

The widespread occurrence of hemoglobins (Hbs) in virtually all kingdoms indicates that the gene for Hb is very ancient, and that Hbs may serve functions other than simple O2 carriers (1-4). The actual function of the proteins of this superfamily is mainly associated with O2 transport/storage. However, the reported involvement of bacterial (5), invertebrate (6) and vertebrate Hbs/myoglobins (Mbs) (7;8) in the detoxification of NO, might illustrate a more primitive function of these molecules (3). Involvement in other functions as O2 scavenger (4), O2 sensor (9), O2 consuming enzymes (10) or shadow pigments (11) have been suggested and may illustrate the coincidental use of a stable protein fold during evolution.

Recently, two new members of the vertebrate globin family, namely neuroglobin (Ngb) and cytoglobin (Cygb), have been discovered (12-16). Both are monomeric (151 and 190 amino acid residues, respectively), intracellular proteins, displaying all determinants of the globin fold. Sequence analyses reveal low sequence identity with vertebrate Hb and Mb (20-25%), as well as a very ancient origin, i.e., much older than Mb (16). The heme-iron atom of Ngb and Cygb is hexacoordinated, showing a His-Fe-His binding scheme, both histidines being the proximal and the distal histidine, respectively (13;15;17). Ngb has a high recombination rate ($k_{on}$) and a slow dissociation rate ($k_{off}$), indicating a high intrinsic affinity for the ligands (O2/CO). Before binding, the external ligands must compete with the internal 6th ligand, resulting in an observed O2 affinity of the recombinant proteins similar to that of Mb (1 torr at 37°C).

The function of Ngb and Cygb is a matter of debate. In response to hypoxia, Ngb is
upregulated in vivo and in vitro, and protects the neurons against hypoxic damage (18). High concentrations (~100 µM) of Ngb are observed in the retina and its subcellular distribution correlates with the localization of the mitochondria (19). Both observations suggest a role in intracellular O₂ supply.

A protein, identical to Cygb and described in liver tissue as stellate cell activation-associated protein (STAP), was found to have a peroxidase activity in vitro and to be upregulated upon the development of hepatic inflammation and fibrosis (20;21). Other potential functions of Cygb have not yet been documented.

The present study aimed at elucidating the immunocytochemical localization of Ngb and Cygb in different mice tissues and the localization of Cygb in the cell nucleus.
Experimental procedures

Expression cloning and purification of recombinant Ngb and Cygb

Mouse Ngb (mNgb) expression and purification were done as described previously (17). Expression and purification of human cytoglobin (hCYGB) was done as follows. The expression plasmid containing hCYGB in pET3a (13) was transformed into *E. coli* strain BL21(DE3)pLysS. Except for the omission of 5-aminolevulinic acid hydrochloride, cells were grown and harvested as described for mNgb (17). Cells were suspended in 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluorid and 5 mM dithiotreitol. After suspension, 1/10 volume 10% triton X-100, 10% deoxycholic acid, 500 mM Tris-HCl pH 7.5, 20 mM EDTA was added and the cells were exposed to three freeze-thaw cycles and sonication until complete lysis. Inclusion bodies were isolated by centrifugation at 3,300 g for 10 min and the pellet was suspended in and washed three times with 1% triton X-100, 1 mM EDTA and 50 mM Tris-HCl pH 7.5. Inclusion bodies were solubilized in 6 M guanidinium hydrochloride, 50 mM Tris-HCl pH 7.5 and 1% 2-mercapto-ethanol during 1 hr at 0°C. After elimination of the insoluble material by centrifugation (10 min 10,000 g), hCYGB was reconstructed by adding a 1.4 M excess of hemin and dialyzed against 5 mM Tris-HCl pH 8.5 at 4°C. The reconstructed hCYGB was further purified as described for mNgb.

Immunohistochemistry

Polyclonal antibodies against purified recombinant mNgb and hCYGB were raised in rabbits and monitored by ELISA using standard protocols. The anti-mNgb and anti-hCYGB antibodies were purified from sera by ammonium sulfate precipitation and affinity chromatography using the corresponding immobilized antigen coupled to Cyanogen

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Bromide-activated Sepharose™ 4b (Amersham Pharmacia) as matrix (22).

Adult Swiss mice (n=6) were used for immunohistochemistry. All procedures were approved by the local ethics committee of the University of Antwerp and conformed to European Community regulations. After lethal injection with Nembutal (sodium pentobarbital) mice were transcardially perfused with physiological saline, followed by Zamboni’s fixative (30 ml for 10 minutes). Tissues (brain, liver, heart, striated muscle, lung, kidney and small bowel) were removed and post-fixed in Zamboni’s fixative for 30 min at room temperature, after which they were treated for improving the immunocytochemical conditions according to Llewellyn-Smith et al. (23). After being stored overnight in 20% sucrose in phosphate buffered saline (PBS) at 4°C, tissues were mounted in Tissue Tek (Sakura Finettek, Europe). Twenty-µm-thick cryostat sections were thaw-mounted on poly-L-lysine-coated microscope slides and dried for 2 h at 37°C. All primary and secondary antisera were diluted in PBS containing 10% normal goat serum (DAKO X 0907, Carpinteria, CA) 0.1% bovine serum albumin, 0.05% thimerosal and 0.01% NaN₃ (PBS). All incubations were carried out at room temperature. After preincubation with PBS containing 1% Triton X-100, sections were incubated overnight with rabbit polyclonal antibodies against mNgb (diluted 1:2000) or hCYGB (diluted 1:1000), followed by incubation with Fluororescein Isothiocyanate (FITC)-conjugated Fab fragments of goat anti-rabbit IgG heavy and light chains (H+L) (diluted 1:200; 1 h; Jackson ImmunoResearch 111-097-003, West Grove, PA). Double labeling studies for these globins and specific markers were performed to determine the nature of mNgb- or hCYGB-immunoreactive cells. Since all antibodies had been raised in rabbits, the double labeling procedure initially described by Negoescu et al. (24) and modified by Brouns et al. (25) had to be applied. Briefly, an incubation with primary
antibodies raised against mNgb or hCYGB was performed overnight, after which the sections were incubated with FITC-conjugated Fab fragments of goat anti-rabbit IgG (H+L) (diluted 1:200) for 6 h, followed by a 2 h incubation with unlabeled Fab fragments of goat anti-rabbit IgG (Jackson ImmunoResearch 111-007-003) to block all possible remaining binding sites of the first primary antibody. The sections were then incubated overnight with the second primary antibody, which was directed against glial fibrillary acidic protein (anti-GFAP, rabbit polyclonal, 1:200, DAKO Z334), calretinin (rabbit polyclonal, 1:5,000, Swant 7696, Bellinzona, Switzerland) or calbindin (rabbit polyclonal, 1:2,000, Swant CB-38). These antibodies were detected with a Cy3-labeled goat anti-rabbit IgG (H+L) (GAR-Cy3; diluted 1:4,000, 1h, Jackson ImmunoResearch 111-165-144). Negative controls were performed by substitution of non-immune sera for the primary or secondary antisera and antibody-specificity was checked by preabsorption of the mNgb and hCYGB antisera with the antigen. To this end, antibodies were diluted 1:1000 in PBS and incubated overnight at room temperature with 1 to 10 µg protein per 100 µl.

For the detection of Ngb and Cygb in mouse pancreas, the tissue was fixed in buffered 4% paraformaldehyde for 4 h and embedded in paraffin. Sections of 4 µm were immunostained with anti-mNgb (1:1000) or anti-hCYGB (1:1000) following the same procedures as described above. To determine the immunoreactive endocrine cells co-localizing with Ngb expression, the following markers were used: insulin (guinea pig polyclonal, 1:10,000, DAKO A564), somatostatin (rat monoclonal, 1:100, Biogenesis 8330-0009, Poole, UK) and glucagon (rabbit polyclonal, 1:200, Affiniti GA 1181, Exeter, UK). The antibodies were detected with Cy3-labeled donkey anti guinea pig IgG (H+L) (diluted 1:500, Jackson ImmunoResearch 706-165-148), a Cy3-labeled donkey anti rat IgG (H+L) (diluted 1:500, Jackson ImmunoResearch 712-165-153) or GAR-Cy3 respectively.
Sections were cross-explored using fluorescence (Olympus BX50 or Zeiss Axiophot) microscopy. Fluorescence micrographs of DAPI-stained tissues were made with on the Olympus Microscope with a Sony 9100 VID CAM, and processed with AnalySIS software. To obtain detailed images of labeled neurons, a confocal laser scanning microscope (Zeiss LSM 410) equipped with image reconstruction facilities (Imaris 2.7 software; Bitplane AG, Zürich, Switzerland; Silicon Graphics Indigo 2 workstation) was used. Excitation of the FITC-fluorophore and the Cy3-fluorophore was achieved with an argon laser (488 nm) and a Helium/Neon laser (543nm), respectively.

**Western blotting**

mCygb was isolated from a nuclear extract of mouse liver. The nuclei were isolated according to the method of Blobbel and Potter as cited by Tata (26) and mCygb was isolated by affinity chromatography on a matrix-immobilized anti-hCYGB antibody column. Extracts of the nuclear and the cytosolic fraction were analyzed by SDS-PAGE. For immunodetection the anti-hCYGB antibody (1:1000) was used.
Results and Discussion

ELISA and Western blotting confirmed the specificity of the antibodies against mNgb and hCYGB, which did not cross-react or produce any reaction against recombinant Mb and Hb, and no cross-reaction was seen between mNGB and hCYGB (additional Fig*).

Immunoreactivity of mCygb was detected in all studied tissues (Fig. 1). Within the brain, mCygb immunoreactivity was uniformly distributed over the different brain regions. No co-localization of mCygb- and GFAP-immunoreactivity was observed but some mCygb-immunoreactivity positive cells also displayed immunoreactivity for calbindin, a calcium-binding protein known to be present in neurons within the central nervous system (Fig. 1), and calretinin, corroborating that, mCygb immunoreactivity is limited to neurons within the brain. In all mCygb-positive cells the immunoreactivity was specifically confined to the cell nucleus, as evidenced by combined detection of mCygb-immunoreactivity and 4’-6-Diamidino-2-phenylindole (DAPI), a specific nuclear staining (Fig. 2).

The nuclear localization of mCygb was independently confirmed by isolating mCygb from a nuclear liver extract by affinity chromatography on a matrix-immobilized anti-hCYGB antibody column, followed by Western blot analysis (Fig. 3).

In contrast, mNgb was expressed in distinct tissues: no immunoreactivity was detected in liver, heart, striated muscle, lung, small bowel or kidney. In pancreas, mNgb immunoreactivity was restricted to the islets of Langerhans. It was visible in the brain, as such confirming the results of Mammen et al. (27) and Reuss et al. (28). Within the brain, mNgb immunoreactivity was found focally having different staining intensities. The most intense mNgb immunoreactivity was found in the medial vestibular nucleus (Fig. 4) and the

* supplemental data
paraolivary nucleus, less intense reactivity was seen in the thalamic and subthalamic regions and in the cortex, whereas none was detected in the hippocampus and corpus callosum. These results mainly confirm previous *in situ* hybridization data (27;28). However, in contrast to Reuss et al. (28) who claimed a more uniform distribution of mNgb throughout the distinct brain regions, we found a more focal distribution pattern of mNgb. This difference could be the result of the used techniques: with *in situ* hybridization the distribution of the mRNA was studied, whereas the present study detected the distribution of the protein. Combined detection of mNgb and GFAP, calretinin and calbindin revealed the absence of GFAP and calretinin immunoreactivity from mNgb-immunopositive cells. These mNgb-immunoreactivity cells clearly resembled a neuronal morphology. Single staining (Fig. 4) as well as double labeling with an antibody raised against calbindin revealed the cytosolic localization of mNgb both at the level of the perikaryon and neurites.

The observation that mNgb and mCygb occur in neurons of the same regions suggests that both molecules might be expressed in the same cells. This hypothesis was substantiated by the presence of both mNgb- and mCygb-immunoreactivity in cultured HN33 cells (data not shown) and by PCR experiments on neuronal cells *in vitro* (1).

In spite of their different embryological origin, pancreatic β-cells and neuronal cells share the expression of similar proteins such as glutamic acid decarboxylase, tyrosine hydroxylase and neurofilament proteins. The expression of the same genes in two different cell types could be explained either by the presence of specific transcriptional activators (Islet-1, Pax-6, Beta-2...) or by the absence of specific transcriptional repressors (example NRSF= neuron-restrictive silencing factor). mNgb was shown to be expressed in the endocrine pancreatic islets of Langerhans. This was confirmed by double labeling

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1 Fordel, E., Geuens, E., Dewilde, S., Rottier, P., Carmeliet, P., Grooten, J., Moens, L. (2003) *in preparation*
experiments with specific markers for the different types of islet cells: insulin, which is only present in β-cells; glucagon, an α-cell specific protein and somatostatin, a δ-cell marker (29). No specific co-localization was observed with glucagon or somatostatin, mNgb-immunoreactivity was present in the β-cells in the center of the islet (fig. 5). However, the immunoreactivity intensities of mNgb in the β-cells was quite variable, especially when comparing with that of insulin. Nevertheless, mNGB-immunoreactivity was observed in each islet of the studied sections.

The presence of a globin in the nucleus of animal cells is totally unexpected. A precedent has been described in plants, i.e. in cultured alfalfa cells, where a hypoxia-inducible non-symbiotic Hb was localized in the nucleus by immuno-electron microscopy methods (30).

How globin-like molecules enter the nucleus is unclear, since no known nuclear transport signal could be detected in Cygb or the non-symbiotic alfalfa Hb. However, given the small size and mass of these molecules (44 x 44 x 25 Å and Mr ~17,000-21,000), simple diffusion cannot be excluded (31;32).

The function of a globin - a heme-protein - in the nucleus is unclear, but a role other than supplying O2 to the energy producing machinery of the cell is obvious. Heme-based sensors represent an important class of signal-transducing proteins, reacting on the binding of diatomic molecules, such as NO, O2 and CO (33;34). Among this group are soluble guanylyl cyclase (sGC; (35)), the transcription regulator CooA (36), the direct O2 sensors [E.coli direct oxygen sensor (EcDos) and FixL] (37;38) and the neuronal PAS (sensory domain named after the eukaryotic proteins Period, Arnt and Simple-minded) protein 2 [NPAS2;
With the exception of FixL, which is a high-spin and pentacoordinated protein, the heme-iron atom occurring in these proteins is always in the hexacoordinated low-spin state, displaying cytochrome-like spectra.

The overall structure of these molecules is variable. They may exist as a single molecule containing a heme-based sensor domain and an effector domain with enzymatic activity. This is the case for FixL and EcDos, where a PAS-folded heme-based sensor domain is covalently linked to a kinase and a phosphodiesterase domain, respectively (38). CooA, a homodimer, consists of a heme-containing cAMP receptor protein (CRP) linked to a small DNA-binding domain with a typical helix-turn-helix motif (42). In contrast, NPAS2 is part of a non-covalent tetrameric complex, containing two NPAS2 subunits and two BMAL1 subunits, the latter allowing specific DNA binding (39;43).

All these molecules are, upon activation, involved in different signal transduction pathways: FixL will control the expression of N₂ fixation genes in Rhizobia; EcDos will regulate the aerobic respiration in E. coli; CooA, a bacterial CO sensor, will regulate the transcription of two operons encoding a CO oxidizing-system and NPAS2 is a gas-responsive transcription factor involved in the regulation of the circadian rhythm (38;39).

In bacterial flavohemoglobins, globin-folded domains occur linked to a FAD/NADP binding dehydrogenase domain and are used in the detoxification of NO as a dioxygenase (44). In contrast, the hemoglobin of Vitreoscilla binds non-covalently to a dehydrogenase containing a flavin domain to form its functional complex (45-47).

Cygb is a hexacoordinated, low-spin hemoprotein that reversibly binds O₂ and CO and also allows reaction with NO (13;15). The nuclear localization of Cygb suggests a
function in gene regulation upon activation by a di-atomic ligand. A direct interaction of Cygb with DNA is unlikely. However, this hemoprotein may bind to an unknown partner with DNA-binding characteristics as observed in NPAS2 (39). Interaction of a globin-folded molecule with a binding partner has been illustrated by the Vitreoscilla Hb, which interacts with a reductase domain and with subunit I of cytochrome bo (ubiquinol oxidase) of E. coli and Pseudomonas aeruginosa (43-47). Subunit I contains the binuclear center for the reduction of O₂ to water. As such, Vitreoscilla Hb delivers O₂ directly to the terminal oxidase.

The nuclear localization of Cygb suggests a possible interaction with other protein(s) potentially involved in the regulation of gene transcription as a reaction to a stimulus. This finding might trigger the search for yet another function of a globin-folded domain.
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Figure legends

Fig 1: Single confocal optical sections of mCygb immunoreactivity in different mouse tissues.

A: Double staining of mCygb (green FITC fluorescence) with calbindin (CB, red Cy 3 fluorescence) in the mouse brain. Calbindin D28k immunoreactive neurons (neuronal cytoplasmatic marker) show mCygb immunoreactivity in the nucleus (n) of the cells. The nucleolus (asterisk), however, is not stained.

B: mCygb immunostaining (green) in the nuclei of hepatocytes in mice.

C. mCygb immunostaining (green) in the nuclei of renal glomerular cells and cells of proximal and distal tubuli in mice.

D. mCygb immunostaining (green) in the nuclei of cells of the pancreas in mice.

Note that in B, C and D the very faint background staining seen in the red channel was used to visualize the contours of the cells. c: cytoplasm; n: nucleus; *: nucleolus

Fig. 2: Fluorescence micrograph of mouse liver stained with mCygb (green FITC fluorescence) and DAPI, a marker for cell nuclei.

A. Immunocytochemical staining for mCygb

B. DAPI staining of the same position as in (A) conforming the presence of mCygb in nuclei of hepatocytes.

c: cytoplasm; n: nucleus

Fig. 3.: Western blot analysis of mouse liver subcellular fractions.

Detection was done with anti-hCYGB antibodies. In lanes 2 and 3 similar protein concentrations (10 µg) of an affinity-purified nuclear extract (2) and a cytosolic extract (3)
were analyzed, whereas in lane 1 recombinant mCygb is used as a positive control.

Fig. 4: Maximum value projections of confocal optical sections of mNgb-containing cells in mouse brain.

mNgb-containing neurons in the medial ventricular nuclei of the mouse brain.

Immunoreactivity is present in the cytoplasm of the neuronal cell body and in the neuronal extensions (asterisks). The nucleus remains unstained. c: cytoplasm; n: nucleus, *: neuronal extension

Fig. 5: Single confocal optical sections of mNgb (green, FITC fluorescence) immunoreactivity in mouse pancreas.

Double staining of mNgb with glucagon (A) and insulin (B) (both red, Cy-3 fluorescence) showing the mNgb immunoreactivity in cells of the islets of Langerhans.
Supplemental data

Supplemental figure: Reaction of anti-mNgb and anti-hCYGB antibodies with ELISA

Reaction of the anti-mNgb (white) and anti-hCYGB (gray) antibody (diluted 1:500) against different coatings (4.5 µg/well).

The anti-hCYGB antibody gives a strong reaction against recombinant hCYGB and against a nuclear extract of liver. The anti-NGB antibody gives a specific reaction against recombinant mNGB and against a brain extract. Both antibodies give no reaction against Hb and Mb and also no cross-reaction against each other.
A globin in the nucleus
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