Cytoglobin Is a Respiratory Protein in Connective Tissue and Neurons, Which Is Up-regulated by Hypoxia*§

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Cytoglobin is a recently discovered vertebrate globin distantly related to myoglobin, and its function is unknown. Here we present the first detailed analysis of the distribution and expression of cytoglobin. Northern and Western blotting experiments show the presence of cytoglobin mRNA and protein in a broad range of tissues. Quantitative PCR demonstrates an up-regulation of cytoglobin mRNA levels in rat heart and liver under hypoxic conditions (22 and 44 h of 9% oxygen). Immunofluorescence studies with three antibodies directed against different epitopes of the protein consistently show cytoglobin in connective tissue fibroblasts as well as in hepatic stellate cells. Cytoglobin is also present in chondroblasts and osteoblasts and shows a decreased level of expression upon differentiation to chondrocytes and osteocytes. Cytoglobin is located in the cytoplasm of these cell types. Evidence against an exclusively nuclear localization of cytoglobin, as recently proposed, is also provided by transfection assays with green fluorescent protein fusion constructs, which demonstrates the absence of an active nuclear import. The differential expression of cytoglobin argues against a general respiratory function of this molecule, but rather indicates a connective tissue-specific function. We hypothesize that cytoglobin may be involved in collagen synthesis. Cytoglobin expression was also observed in some neuronal subpopulations of the central and the peripheral nervous systems. Surprisingly, cytoglobin is localized in both the cytoplasm and nucleus of neurons, indicating a possible additional role of this protein in neuronal tissues.

Globins are small heme-containing proteins that have the ability to bind O2 and other gaseous ligands (1). Most globins efficiently capture and deliver O2, thus sustaining the aerobic metabolism of the respiratory chain (2–6). Some globins may also act as enzymes utilizing molecular O2 in oxidation reactions (7, 8). Vertebrates harbor four distinct globin types that differ in tissue distribution and function. The heterotetrameric hemoglobin facilitates the transport of O2 in the red blood cells of the circulatory system (1). The myocytes of the cardiac and striated muscles contain myoglobin, a monomeric protein that enhances O2 diffusion to the mitochondria of this metabolically highly active tissue and acts as a temporal O2 store (4–6). Myoglobin may also act as an NO dioxygenase (8). A respiratory function has also been attributed to neuroglobin (Ngb), a recently described vertebrate monomeric globin (9) that is expressed in neurons of the central and peripheral nervous systems (9–11). Ngb is also present in endocrine tissues (10), and a particularly high Ngb concentration was observed in the mammalian retina (12). Most recently, cytoglobin (Cygb) has been identified as the fourth member of the vertebrate globin family. Cygb has been identified in the expressed sequence tag (EST) databases from mouse, man, and zebrafish and found to be expressed in a broad range of mammalian tissues (13–14). In an independent study, Kawada et al. (15) had characterized the rat homologue of Cygb as a heme protein that shows enhanced expression in the stellate cells of the fibrotic liver, thus dubbing it “stellate cell activation-associated protein” or STAP (15, 16). Cygb binds O2 reversibly via the Fe2+ ion of the heme group (14–18). Similar to some plant hemoglobins, Ngb and some other globins (19), recombinant Cygb displays a hexacoordinated heme structure of the Fe2+ in the deoxy form (14, 17, 18). Mammalian Cygb has the highest degree of sequence conservation observed among the vertebrate globins, with mouse and human Cygb differing in only 4.7% of the amino acids (13). Phylogenetic analyses suggest that Cygb and myoglobin form a common clade, which is distinct from neuroglobin and hemoglobin and originated from a globin common ancestor more than 450 million years ago (13, 20). Mammalian Cygb is longer than most globins and covers 190 amino acids instead of the typical 140–150 amino acids. This is due to two ~20 amino acid extensions at both the N and the C terminus. Nevertheless, the globin fold consisting of eight α-helices, and important globin-specific residues involved in O2 binding are conserved in the Cygb protein (20, 21). In addition to the globin-typical introns at positions B12.2 (i.e. between codon positions 2 and 3 of the 19th amino acid of the B-helix) and G7.0, the mammalian Cygb...
organs were immediately removed, postfixed for 1 h in the same fixative, and stored at 4 °C in phosphate-buffered 30% sucrose. Bones were decalcified in 12.5% EDTA (Mallinckrodt, Hennef, Germany) in distilled water. Perfusion-fixation was carried out at a constant rate of 10 ml/min with 200–300 ml of ice-cold 4% paraformaldehyde, 1.37% L-lysine, 0.21% sodium periodate in PBS (23). The recombinant protein (cytg) was isolated and loaded on the gel. Antibody dilutions were performed according to the manufacturer's instructions, and stored at 4 °C for several days at room temperature with 0.1% NaN₃. Animal handling and experiments were conducted according to a protocol that was approved by the county government office (Bezirksregierung Rheinhesen-Pfalz, Az 177-07/961-30). The mice were anesthetized with tribromoethanol (0.3 g/kg body weight) and were transcardially perfused with 100 ml of phosphate-buffered saline (PBS) at room temperature, with 15,000 IU of heparin/liter added. Perfusion-fixation was carried out at a constant rate of 10 ml/min with 200–300 ml of ice-cold 4% paraformaldehyde, 1.37% L-lysine, 0.21% sodium periodate in PBS (23). The right atrium was opened to enable venous outflow. Selected tissues and organs were immediately removed, postfixed for 1 h in the same fixative, and stored at 4 °C in phosphate-buffered 30% sucrose. Bones were decalcified in 12.5% EDTA (Mallinckrodt, Hennef, Germany) in distilled water (pH 7.2–7.4) for several days at room temperature with gentle agitation.

White rats (Rattus norvegicus) with a weight of ~150 g were kept in individual cages under controlled temperature conditions of 22–24 °C. Hypoxia was achieved by incubating the rats for 22 or 44 h in a sealed chamber (70/70/50 cm), which was constantly flushed at 3.5 liters/min with a premixed gas containing 9% oxygen. The rats were killed by intramuscular injection of Imalgene 100 (100 mg/kg of body weight; Rhone-Merieux, France). Heart and liver tissues were immediately taken and frozen in liquid nitrogen.

**Antibody Preparation**—Polyclonal antibodies were raised in rabbits against synthetic peptides that had been designed according to conserved regions of human and mouse Cygb (Fig. 1A). The antibodies are directed against the amino acid positions 2–16 of the N terminus (Cygb1: H_N-EKVPGEMEIERE-RS-CONH₂), 66–80 (Cygb2: H_N-MEDPLEMPQPKRK-CONH₂), or 92–106 (Cygb3: H_N-VENLHPDPKVSVL-CONH₂) of the middle region, or 175–190 of the C terminus (Cygb4: H_N-PNATTPATLPSSGP-CONH₂). Each of the antibodies was purified from the serum using the appropriate synthetic peptide coupled to a SulfoLink column (Pierce), following the manufacturer's instructions, and stored at 4 °C in 50 mM Tris, 100 mM glycine, pH 7.4, supplemented with 0.1% NaN₃.

**ELISA (enzyme-linked immunosorbent assay)—**ELISA plates were coated by 2 h of incubation with 50 ng of Cygb in PBS. The plates were blocked for 1 h with 1% bovine serum albumin in PBS and subsequently washed three times with PBST (PBS supplemented with 0.05% Tween 20). Antibody incubation was carried out overnight at 4 °C. The plates were washed three times with PBST, and incubated for 90 min at 37 °C with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Dianova), diluted 1:10,000 in PBS. After additional washings, the bound antibody was quantified by staining in 1 mg/ml o-phenylenediamine dissolved in phosphate-citrate buffer, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol. After heat denaturation at 95 °C for 5 min, the protein samples (50 µg of total protein) were loaded to a 14% SDS-polyacrylamide gel. Antibody detection was carried out on protein samples transferred to nitrocellulose for 2 h at 0.8 mA/cm². Nonspecific binding sites were blocked by incubation for 2 h with 2% nonfat dry milk in TBST (10 mM Tris, pH 7.4, 140 mM NaCl, 0.3% Tween-20). The membranes were washed four times for 10 min in TBST and incubated with the goat anti-rabbit antibody coupled with alkaline phosphatase (Dianova) (1:10,000 in TBST). The filters were washed in TBST as above, and detection was carried out with nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

**Immunohistochemistry**—Cryosections (14-µm thick) of perfusion-
fixed organs were placed on glass slides coated with gelatin or poly-

t-lysine. Non-specific binding sides were blocked at room temperature for 1 h with 1% bovine serum albumin in PBS. The sections were incubated with anti-Cygb antibodies (1:500 to 1:1000 in PBS) in the blocking solution overnight at 4°C. The sections were washed 3× 8 min in PBS and incubated for 90 min at room temperature in the dark with the secondary antibody (goat anti-rabbit IgG coupled to Cy3, Dianova), diluted 1:1000 in blocking solution. The sections were washed as described above and embedded in Elvanol polyvinyl alcohol (Mowiol, Cal-

biochem). In some experiments, the Hoechst dye 33342 (Hoechst, War-

rington, PA) was added to the Elvanol to stain the nuclei. The sections were analyzed using an Olympus BX51 research microscope equipped with a digital camera. Images were combined using the Adobe Photo-

shop 7.0 program, which was also used to adjust image contrast and

brightness and to add labels.

Northern Blotting—A commercial Northern blot (Biocat, Heidelberg,

Germany) containing normalized amounts of human mRNA was hy-

bridized under standard conditions with a human Cygb cDNA probe, labeled radioactively by random-priming (Roche Applied Science, Mannheim, Germany). Final washing conditions were 65°C at 0.1× standard saline citrate (SSC). The blot filter was exposed for 24 h using an intensifying screen and Kodak XROMat film.

Transfection of Cygb-GFP Fusion Constructs—For construction of fusions between Cygb and the green fluorescent protein (GFP) gene, the complete human Cygb coding region was the first PCR-amplified using primer pairs with restriction enzyme recognition sites attached to their 5′-ends (XhoI and KpnI for the N-terminal Cygb-GFP constructs and XhoI and Xbal for the C-terminal GFP-Cygb constructs). The PCR

amplificates were then cut with the appropriate enzymes (New England Biolabs, Frankfurt/M, Germany) and directionally ligated into double-

restricted pEGFP-N1 and pEGFP-C1 vectors (Clontech, Heidelberg, Germany), which contain the gene for enhanced green fluorescent pro-

tein. We constructed four different gene fusions: CyGEGFP-N1 and CyGB-EGFP-C1, in which the gene sequences are directly adjacent to each other, and two constructs containing an additional 6-amino acid spacer (AGC ACC TGG AGC TGC ACC) inserted between the two genes (CyGB-EGFP-N1 spacer and CyGB-EGFP-C1 spacer) to possibly fa-
cilitate an easier folding of the smaller globin protein part. All CyGB-

EGFP fusion vectors were transformed into Escherichia coli RRI1 AM15 and verified by DNA sequencing. Plasmid DNA purified using the E.Z.N.A. Plasmid Miniprep Kit I (Peqlab, Erlangen, Germany) was of sufficient quality for successful transfection.

Vero cells (ATCC CCL81) and Hela cells (ATCC CCL2) were rou-
tinely cultured in MEM supplemented with 10% fetal bovine serum, 100 IU of penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO2 incubator. The day prior to transfection cells were plated into 35-mm glass-bottomed dishes (MatTek Corp.) at a density of 200,000. On the day of transfection, 1 μg of each DNA was used with 3 μl of FuGENESI (Roche Applied Science, Mannheim, Germany) to transfect the cells according to the manufacturer’s instructions. GFP transfected the nucleosomes were detected and analyzed at the Advanced Light Microscopy Facility at EMBL, Heidelberg. Cells were imaged 18 h after transfection in carbanate-free culture medium equilibrated with 10 mM HEPES pH 7.4 (24) on a Leica DMIRBE microscope with a ×63 NA 1.4PL Apo objective. Confocal images were taken using an UltraView real-time confocal system (PerkinElmer Life Sciences) consisting of a Nikon Eclipse TE200 microscope equipped with a 1.3 NA PlanFluar ×100 objective. Images were processed using NIH ImageJ software (Bethesda, MD).

RNA Isolation and Real-time RT-PCR—Total RNA from rat was isolated using the “tri-reagent” kit (Molecular Research Center, Cincinnati, OH), and concentrations were measured using an Amersham Biosciences GeneQuant Photometer. The 22-h hypoxic and the normoxic

tissues were analyzed by Northern blotting. Only antibodies

directed against different synthetic Cygb peptides (Fig. 1A) were raised in rabbits. After purification by affinity chromato-

graphy, the antibodies (αCygb1-4) showed strong positive re-

action with recombinant Cygb in Western blotting and in

ELISA (see Supplemental Data, Fig. S1). The reaction was inhibited by preadsorption of the antibodies with a 100-fold molar excess of purified recombinant Cygb, demonstrating a specific interaction with the antigen. Three of the antibodies (αCygb1-3) gave essentially identical results in immunofluo-

rescence experiments, while no specific immunostaining was

observed with the fourth antibody (αCygb4) (see Supplemental Data Fig. S2). The antibodies were further tested on various tissues by Western blotting. Only antibodies αCygb2 and αCygb3 showed the expected bands of 21 kDa in various tis-

sues, which is about the same size as the recombinant protein (Fig. 1B). The antibodies are directed against peptides from the globin D-helix, and the EF-region, respectively (Fig. 1A). The additional bands observed in the Western blot at various positions in some tissues represent nonspecific cross-reactions and do not disappear by preadsorption of the antibody with recom-

binant Cygb (see Supplemental Data Fig. S3). By contrast, the staining of the Cygb band is specific and was blocked by pread-

sorption. Antibodies αCygb1 and αCygb4, directed against the N or C terminus, respectively, showed only weak reactions in Western blot experiments.

RESULTS

Antibodies Against Cygb—Four polyclonal antibodies that are directed against different synthetic Cygb peptides (Fig. 1A) were raised in rabbits. After purification by affinity chromato-

graphy, the antibodies (αCygb1-4) showed strong positive reaction with recombinant Cygb in Western blotting and in

ELISA (see Supplemental Data, Fig. S1). The reaction was inhibited by preadsorption of the antibodies with a 100-fold molar excess of purified recombinant Cygb, demonstrating a specific interaction with the antigen. Three of the antibodies (αCygb1-3) gave essentially identical results in immunofluo-

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sorption. Antibodies αCygb1 and αCygb4, directed against the N or C terminus, respectively, showed only weak reactions in Western blot experiments.
Detection of Cygb mRNA and Protein in Mammalian Tissues—The presence of Cygb mRNA in a broad range of tissues was confirmed by Northern blotting (Fig. 2). Using a radioactively labeled cDNA probe, we detected Cygb at different levels in all organs tested. The Cygb mRNA forms a single band of about 2 kb, which agrees well with the size predicted from EST data (accession numbers BC018822 and NM_134268).

Examination of various organs with antibodies aCygb1–3 showed Cygb protein to be present in distinct cell populations (Fig. 3). In the liver, strong staining was observed in the small hepatic stellate cells (HSCs), which are scattered between the large hepatocytes (Fig. 3, A and C). In addition, fibroblasts forming the connective tissue in the vicinity of blood vessels are decorated with the antibodies. Immunostaining was inhibited after preadsorption of the antibodies with the recombinant antigen (Fig. 3B). No reaction was observed in hepatocytes, endothelial, as well as sinusoidal and red blood cells. Cygb was found in the cytoplasm of the HSCs, while there was no detectable labeling of the nuclei (Fig. 3C). The fibroblasts were decorated with the Cygb antibodies in heart, muscle, colon, kidney, and tendon (Fig. 3, D–G). Particularly strong immunofluorescence was observed in the connective tissue of the Lamina propria mucosae that supports the gut epithelium of the colon (Fig. 3E). In the cartilage of the tracheal wall, about 10% of the chondral cells were found to be stained, which we suspect to be the active chondroblasts, while in the mature chondrocytes no Cygb was detected (Fig. 3F). In the tendon, the matrix fibroblasts were strongly labeled, while less intense staining was present in fibroblasts of the adjacent muscle (Fig. 3F). Cytoglobin expression was also observed in skin fibroblasts (data not shown). The osteoblasts and the osteocytes of the bones were both stained with the antibodies, with the osteocytes showing a weak labeling (Fig. 3J). As in the HSCs (Fig. 3C), anti-Cygb labeling in the fibroblasts, chondroblasts, osteoblasts, and osteocytes was apparently restricted to and uniformly distributed within the cytoplasm, and was also present in cytoplasmic extensions (Fig. 3, D, E, and G–J). No staining of the extracellular matrix or the nuclei of these cells was detected with any of the antibodies. Most other cell types of these organs, such as

Detection of Cygb protein in mouse tissues. Indirect anti-Cygb immunofluorescence was carried out with aCygb antibodies on cryosections from liver (A–C), heart (D), gut (E and F), kidney (G), trachea (H), tendon (I), and bone (J). In some sections, the nuclei were stained with Hoechst dye 33342 (green staining; C, D, F). Bright immunofluorescence (red staining) is visible in HSC and the liver fibroblasts (FB) that surround the portal vein, whereas the hepatocytes (HC) are not labeled (A). No immunoreaction was detected in the liver upon preabsorption of the antibody with recombinant Cygb (B). Higher magnification of the liver section shows staining of the cytoplasm of stellate cells (HSC) (C). In the heart, significant cytoplasmic staining was only found in the cardiac fibroblasts (FB), but not in the myocytes (D). The inset on the right-hand side shows a merged figure of the heart tissue counterstained with Hoechst dye 33342. Staining of the colon shows cytoglobin in fibroblasts of the connective tissue in the Lamina propria mucosae (E). Higher magnification of the myenteric plexus in the colon shows cytoplasmic localization of Cygb in the fibroblasts, and cytoplasmic and nuclear staining in the neurons (N) (F). In the kidney, fibroblasts are stained, but not endothelial or other cells (G). Staining of the wall of the trachea show bright immunolabeling of the chondroblasts in the cartilage (CB) (H). In a cross section of the wrist (I), staining of fibroblasts was observed in both muscle (M) and tendon (T). In the bone (J), osteoblasts (OB) show strong and osteocytes (OC) show weak anti-Cygb immunofluorescence. Size bars are given at the lower right-hand side.
the endothelial cells in the kidney and the colon (Fig. 3, E and G), the goblet cells of the colon mucosa, and the muscle cells in the heart (Fig. 3D) or the skeletal muscle (Fig. 3I) did not show any signal that was considered to be above background.

Furthermore, we found the neurons of the myenteric plexus in the colon to express Cygb (Fig. 3F). However, in these cells Cygb immunolabeling was found in both the cytoplasm (including the nerve processes) and the nuclei. The specificity of this anti-Cygb staining is corroborated by the observation that in the adjacent fibroblasts anti-Cygb immunostaining is exclusively present in the cytoplasm. Cygb was also found in the central nervous system. Cygb-immunoreactive neurons were scattered throughout several regions of the brain. The immunofluorescent signals were strong and clearly distinguishable from the background (Fig. 4). For example in the cerebral cortex, stained neurons were found in all laminae but their number amounted to less than ten percent of the total of neurons. A similar situation was found in the caudate putamen where only a subpopulation of neurons exhibited immunofluorescence. As seen in higher magnifications (Fig. 4, B and C), the signal was present in the cell soma (cytoplasm and nucleus) and in cell processes. Antibodies aCygb1 to 3 gave identical staining results in the brain, whereas aCygb4 did not show any signal.

Analysis of Cygb Intracellular Localization by GFP Fusions—The intracellular localization of Cygb was further studied by constructing gene fusions between Cygb and GFP, and transfecting these constructs into cell lines for analyzing the distribution of the fluorescent signal generated upon expression of the fusion genes. N-terminal (Cygb-GFP) as well as C-terminal (GFP-Cygb) fusions were made, and fusions with and without a 6-amino acid spacer between Cygb and GFP were transfected, with all constructs giving the same result. Upon transfection into Hela and Vero cells, GFP signal was distributed equally between the cytoplasm and nucleus (Fig. 5, which argues against a specific nuclear import of Cygb. It is conceivable, however, that the nuclear GFP signal is due to passive diffusion of the fusion protein into the nucleus. The fusion protein size (21 kDa for Cygb plus 27 kDa for GFP) is in the range of the 40–50-kDa cutoff for passive nuclear entry.

Cygb mRNA Expression under Hypoxic Conditions—The expression level of Cygb in heart and liver was tested by comparing rats, which had been kept in normoxia or exposed to hypoxia (9% oxygen for 22 or 44 h). Quantitative real-time RT-PCR shows that in rat heart and liver Cygb mRNA is up-regulated upon exposure to hypoxia (9% oxygen for 22 or 44 h). Quantitative real-time RT-PCR experiments revealed an up-regulation of Cygb mRNA in both organs under hypoxic conditions. In rat heart, Cygb mRNA was up-regulated by a factor of 1.8 ± 0.3 after 22 h and by 2.5 ± 0.9 times after 44 h (Fig. 6). We also observed a 2.2 ± 0.8 and a 2.3 ± 0.5 fold increased expression in rat liver after 22 or 44 h, respectively. The up-regulation of Cygb under hypoxia is significant at the p < 0.01 level. Overall, the mRNA expression was much stronger in heart than in liver (not shown), confirming the Northern blot data.

DISCUSSION

Although globins are certainly among the best studied proteins in terms of function, structure, genetics, and evolution, Cygb was discovered only recently as the fourth member of the vertebrate globin superfamily (13–15). Several functions for Cygb have been proposed (20), but its actual role in animal metabolism is still unknown (13–15, 22).

Localization of Cygb within Tissues and Cells—Cygb was initially observed to be expressed in the HSCs of rat and human liver (15, 16). Here we show that Cygb is also present in cells that form the connective and supportive tissues, represented by the fibroblasts, chondrocytes and bone cells. These cell types have a common ontogenetic origin, as chondrocytes as well as osteocytes differentiate from fibroblasts (25). The exact origin of the HSCs is still a matter of debate (26), although it is generally assumed that HSCs and fibroblasts are related derivatives of the mesoderm (27). Additional Cygb expression was found in distinct neural cell populations of the central and peripheral nervous systems. Taken together, these data fit well with the apparently ubiquitous presence of Cygb in a broad range of tissues and organs (13, 14, 16).

By applying three antibodies that were independently raised against different Cygb peptides, as well as by using various methods for tissue fixation and immunodetection, we consis-

FIG. 5. Transfection of Cygb-GFP fusion constructs. A, a control plasmid, only containing GFP without a Cygb fusion partner. B, GFP signal distribution in Hela cells after transfection and expression of an N-terminal Cygb-GFP fusion construct. C, GFP signal distribution in Vero cells. The fluorescence in Hela and Vero cells is distributed almost equally over the cytoplasm and the nucleus, and indistinguishable from the controls. This argues against a specific nuclear import mediated by Cygb.

FIG. 6. Hypoxia regulation of Cygb mRNA. Quantitative real-time RT-PCR shows that in rat heart and liver Cygb mRNA is up-regulated upon exposure to hypoxic conditions.
tently identified the Cygb protein to be localized in the cytoplasm of only distinct cell populations from the non-neuronal tissues. In each case, the specificity of the antibodies and thus the reliability of our results were confirmed by preadsorption experiments, which never showed any immunostaining.

In a recent study Geuens et al. (22) put forward the idea that Cygb is an exclusively nuclear protein, which is present in virtually all cells from a broad range of tissues. Thus both cellular and subcellular distribution of Cygb was proposed to be distinct from our results, e.g. these authors suggested Cygb to be localized in the nuclei of hepatocytes, while we observed Cygb in the liver only in the cytoplasm of HSCs and fibroblasts. However, our data are in agreement with independent immunohistochemical studies on rat and human liver that identified Cygb in the cytoplasm of HSCs (15, 16). Moreover, biochemical experiments on isolated hepatocytes (15) provided convincing evidence against the presence of Cygb in this cell type, neither cytoplasmic nor nuclear. The same biochemical study found Cygb in isolated HSCs, which agrees with our data. Additional evidence against an exclusive nuclear localization of Cygb was provided by the experiments that employed Cygb-GFP fusion constructs, which did not show any active transport of the fusion protein into the nucleus. Calculations using Reinhardt’s method (28) predicted Cygb to be a cytoplasmic protein (94.1% reliability). This score is in the same range as that for myoglobin, a protein of undisputed cytoplasmic localization. Given the consistency of our results and their agreement with independent data sets (15, 16), we conclude that Cygb is essentially a cytoplasmic protein in fibroblasts and their derivatives. Thus we cannot confirm the results of Geuens et al. (22), which we consider to be most likely the result of a nonspecific cross-reaction of their polyclonal anti-Cygb antibody with a nuclear protein.

In striking contrast to the situation in the fibroblast-related cells, however, Cygb was found in both the cytoplasm and nuclei of some neurons in the peripheral and central nervous systems. The reliability of this result is confirmed by the fact that all three antibodies (αCygb1–3) used for immunohistochemistry showed identical staining patterns, which could be blocked by preadsorption with the antigen. The differential localization of Cygb in neurons versus fibroblasts and related cells is surprising and suggests the existence of a neuron-specific factor that enables the active translocation or passive diffusion of Cygb from the cytoplasm into the nucleus. This might be explained either by a particular role of Cygb in neurons (see below), and/or by the fact that specific physiological conditions are required to initialize the translocation into or the retention within the nucleus.

**Implications for Cygb Function—**Any evaluation of Cygb function must take the cellular and subcellular distribution of the protein into account. Cygb is found only in distinct cell populations that are, in contrast to the myoglobin- and Ngb-expressing cells (5, 6, 9–12), not generally associated with particular high metabolic rates and thus oxygen consumption. Therefore, a general role of Cygb in facilitating O2 diffusion to the respiratory chain of the mitochondria, as was assumed recently (13, 15, 17), seems unlikely. For similar reasons a role of Cygb as terminal oxidase that supports respiration upon temporary anaerobic conditions may be excluded. Our results are also difficult to reconcile with a function of Cygb in oxygen sensing (22), because there is no obvious explanation why such an O2-sensor should be restricted to the fibroblast-derived cell populations and some neuronal subpopulations. Kawada et al. (15) suggested that Cygb may protect the HSCs from reactive oxygen species (ROS), which are known to be generated e.g. by hepatocytes during inflammation (29). Nevertheless, to the best of our knowledge there is no evidence that Cygb-containing cells are particularly sensitive to ROS.

On the other hand, it is tempting to assume that the particular role of Cygb in fibroblasts, chondroblasts, osteoblasts, and HSCs is linked to collagen synthesis. This process requires O2 and takes place in the cell types that express Cygb. It is conceivable that Cygb supplies molecular oxygen to the prolyl-4-hydroxylase, an iron-containing dioxygenase that employs O2 for the hydroxylation of proline residues in the procollagen molecule (30, 31). The hydroxyproline residue is essential in stabilizing triple helical collagen chains. Although the amount of oxygen consumed by the collagen hydroxylation process is unknown, the degree of collagen hydroxylation in fact depends on the available oxygen (32). The idea of a collagen-linked function of Cygb agrees with the fact that active, collagen-secreting chondroblasts and osteoblasts show a high level of Cygb expression, whereas the inactive, mature chondrocytes and osteocytes that do not synthesize collagen have considerably reduced levels of Cygb. This hypothesis is also in line with the observation that Cygb synthesis is strongly enhanced in activated HSCs of the fibrotic liver (15). Activation of HSCs also occurs during viral infection, wound healing etc (33) and is accompanied by a considerable increase in collagen synthesis (34, 35). It should be noted that the production of collagen mRNA and protein is strongly increased under hypoxia in vitro and in vivo (35–39). As we have shown here, the expression of Cygb is significantly enhanced under hypoxia as well. If our hypothesis is correct, the higher level of Cygb at low oxygen conditions accommodates the increased oxygen demand of the hypoxia-induced collagen synthesis. It remains to be established whether there is a direct interaction of Cygb with the prolyl-4-hydroxylase, or, which seems more likely in analogy to the function of myoglobin, that Cygb facilitates the diffusive transport of O2 to the enzyme.

Nevertheless, other functions of Cygb are still conceivable (20) and compatible with the data, e.g. Cygb may protect the prolyl-4-hydroxylase from noxious ROS or may decompose ROS that might be generated by this enzyme. Cygb may also be involved in a ROS-mediated signaling pathway that activates processes specific for the fibroblast-related cells, e.g. the synthesis of collagen (34, 40).

Cygb is also found in some neurons of brain and the peripheral nervous system, but there is no evidence that collagen is synthesized in these cells. In contrast to the fibroblast-related cell types, the Cygb-containing neurons showed staining of both the cytoplasm and the nuclei. Thus, a distinct function of Cygb in neuronal cells may be assumed. On the one hand, Cygb may also provide O2 to enzymatic reactions. For example, the NO synthases (NOS) require O2 for the production of NO from L-arginine (41). On the other hand, Cygb may be involved in a ROS (NO)-signaling pathway in neurons. Given the phylogenetically ancient position of Cygb among the vertebrate globins (13), future studies will not only elucidate the function of this recently discovered heme protein, but may also provide insights into the evolutionary changes that eventually led to respiratory myoglobins and hemoglobins.

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**Note Added in Proof—**Recently, Nakatani et al. (Nakatani, K., Okuyama, H., Shimahara, Y., Saeki, S., Kim, D. H., Nakajima, Y., Seki, S., Kawada, N., and Yoshizato, K. (2004) Lab. Invest. 84, 91–101) also reported the presence of Cygb in the fibroblast cell lineage.
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