Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Neuroscience Letters 374 (2005) 207–211

Neuroscience Letters

www.elsevier.com/locate/neulet

Divergent distribution of cytoglobin and neuroglobin in the murine eye

Marc Schmidt[a], Tilmann Laufs[b], Stefan Reuss[c], Thomas Hankeln[b], Thorsten Burmester[a,*]

[a] *Institute of Zoology, Molecular Animal Physiology, University of Mainz, Müllerweg 6, D-55099 Mainz, Germany*
[b] *Institute of Molecular Genetics, Johannes Gutenberg, University of Mainz, Müllerweg 6, D-55099 Mainz, Germany*
[c] *Department of Anatomy, Johannes Gutenberg, University of Mainz, Müllerweg 6, D-55099 Mainz, Germany*

Received 5 September 2004; received in revised form 7 October 2004; accepted 21 October 2004

Abstract

Neuroglobin (Ngb) and cytoglobin (Cygb) are two vertebrate globins with yet poorly defined functions. Previous studies had demonstrated a high expression level of neuroglobin in the mammalian retina, being in line with a respiratory function. Here we show that in the mouse eye, cytoglobin is localised in fibroblasts of the ciliary processes and the choroidea. In the neuronal retina, cytoglobin is expressed in a subset of neurons of the ganglion cell and inner nuclear layers. Cytoglobin is also present in the inner plexiform layer, but absent from the pigment cells. Neuroglobin is localised in photoreceptor inner segments, the plexiform layers and the ganglion cell layer. The divergent distribution of neuroglobin and cytoglobin in the mammalian retina suggests distinct functions of these proteins in the vertebrate nervous system. While neuroglobin seems to be associated with oxygen consumption, a respiratory function of cytoglobin is unlikely.
© 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: Cytoglobin; Fibroblast; Neuroglobin; Neuron; Oxygen-binding protein; Reactive oxygen species; Retina

Recent studies led to the discovery of neuroglobin (Ngb) and cytoglobin (Cygb) as novel members of the superfamily of vertebrate globins [2,4,10,14,19]. These two different globin types show divergent evolutionary origins and tissue distributions, which are also different from the well-known hemoglobin and myoglobin. Ngb is a predominantly neuronal protein [4,15,21], which is highly expressed in the mammalian retina [17]. Ngb displays a distinct distribution in the retina, and is mainly localized in the ganglion cell layer, the plexiform layers, as well as the ellipsoid region of the inner segments. This pattern has been associated with the distribution of mitochondria and considered as support for the hypothesis of a respiratory function of Ngb [3]. Cytoglobin has been identified in human, mouse, rat and zebrafish tissues [2]. The typical globin fold consisting of eight alpha-helices and other important globin-specific residues involved in O_2-binding are conserved in Cygb [5,9]. While Cygb has been named according to its expression in many different types of mammalian tissues [1,2,19], recent studies have shown that this globin is mainly present in fibroblast cell lineage, which also includes the osteoblasts, chondroblasts and hepatic stellate cells [11,16]. The function of Cygb is still uncertain. Most globins are involved in O_2 delivery and storage, thus sustaining the aerobic metabolism in the mitochondria [20]. While initially considered a possible respiratory protein [2,19], Kawada et al. [10] suggested Cygb to act as peroxidase involved in the detoxification of reactive oxygen species (ROS) in the liver. Geuens et al. [7] speculated that Cygb may act as an oxygen sensor molecule located in the nucleus of the cell and Nakatani et al. [11] suggested a function in proliferation of vitamin A storing cells. Based on its predominant expression in the fibroblast cell-lineage and other data, we recently hypothesised some function of Cygb in the collagen metabolism [16]. Additional Cygb expression was observed in distinct populations of neurons in the mouse brain, as well as in some ganglia of the peripheral nervous system. For further understanding of Cygb function, we have investigated the distribution of this protein in the mouse retina.

Polyclonal antibodies against Ngb and Cygb were prepared as described before [16,21]. Briefly, an anti-Ngb antibody was raised in rabbits against a synthetic Ngb-peptide

* Corresponding author. Tel.: +49 6131 3924477; fax: +49 6131 3924652.
E-mail address: burmeste@uni-mainz.de (T. Burmester).

0304-3940/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2004.10.071

(H_2N-CLSSPEFLDHIRKVML-$CONH_2$). Four anti-Cygb antibodies were raised against different peptides, of which three antibodies (αCygb1: H_2N-EKVPGEMEIERRERS-$CONH_2$; αCygb2: H_2N-MEDPLEMERSPQLRK-$CONH_2$; αCygb3: H_2N-VVENLHDPDKVSSVL-$CONH_2$) worked in immunostaining [16]. Perfusion-fixation of adult Balb/C mice was carried out at a constant rate of 10 ml/min with 150–200 ml of ice-cold 4% paraformaldehyde, 1.37% L-lysine, 0.21% sodium-periodate in PBS. Animal handling and experiments were conducted according to an approved protocol (Bezirksregierung Rheinhessen-Pfalz, Az 177-07/961-30). The eyes were immediately removed, postfixed for 1 h in the same fixative and stored at 4 °C in 30% sucrose/PBS no longer than a week. 14 μm cryosections of the perfusion-fixed retina were placed on glass slides and non-specific binding sides were blocked at room temperature for 1 h with 1% BSA in PBS. The sections were incubated with anti-Cygb (dilution 1:100) or anti-Ngb antibodies (1:200) overnight at 4 °C. The sections were washed 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 1:200. The sections were washed and embedded in Elvanol (Mowiol, Calbiochem). The Hoechst dye 33342 (Hoechst, Warrington, 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 and labelled with the Adobe Photoshop 7.0 program. For Western blotting, proteins were extracted from brain and retina by homogenizing the tissues in PBS (7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 145 mM NaCl) with 0.1% SDS. Tissue extracts (about 100 μg protein per lane) were diluted with sample buffer (65 mM Tris–HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol) and denatured at 95 °C for 5 min. Recombinant Cygb [9] was used as positive control. After gel electrophoresis on a 14% SDS-polyacrylamide gel, the proteins were transferred on nitrocellulose filters. Non-specific binding sites were blocked by incubation for 2 h with 2% BSA in TBST (10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 0.3% Tween-20). The membranes were incubated with anti-Cygb antibodies, diluted 1:1,000 in 1% BSA/TBST. Then the membranes were washed four times 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.

In Western blotting experiments α-Cygb antibodies showed specific staining of a band with a molecular mass of 21 kDa in protein extracts from both mouse brain and retina (Fig. 1). The reaction was inhibited by pre-absorption of the antibodies with an excess of purified recombinant Cygb (not shown). Cryosections of the total mouse eye were obtained from material that had been fixed by perfusion with 4% paraformaldehyde. The sections were examined with three independently derived anti-Cygb antibodies [16]. The quality and specificity of these antibodies had been demonstrated

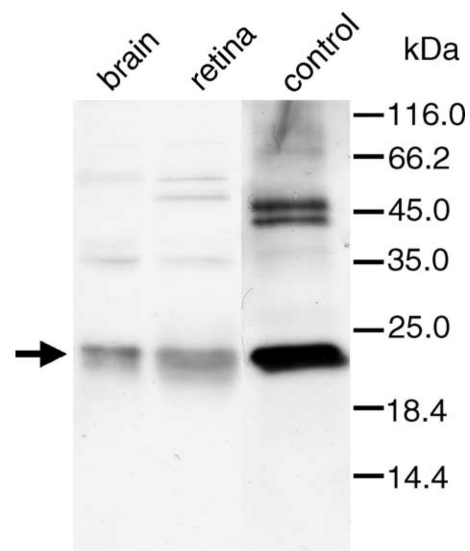

Fig. 1. Western blot analysis of Cygb protein in mouse brain and retina. Cygb protein with a molecular mass of 21 kDa (arrow) was detected in protein extracts from brain and retina. Recombinant Cygb was loaded on the right lane as control; the band at 45 kDa may represent a stable dimer.

in the previous study by competition and pre-adsorption experiments, pre-adsorption controls on retinal section show no specific staining. All three antibodies showed identical staining results. Expression of Cygb was observed in non-neuronal cells of the eye (Fig. 2A–C). The stained cells most likely represent stromal fibroblasts of the ciliary processes that support the vitreous body. Weak anti-Cygb staining in the iris was also present in cells we suspect to be the fibroblasts. Strong Cygb staining was observed in the ganglion cell layer and the inner nuclear layer of the neuronal retina, and the fibroblasts of the choroidea (Fig. 2D–F). Co-staining with Hoechst dye, which labels the nuclei of the cells, showed that in both the ganglion cell layer and in the inner nuclear layer, only a fraction of neurons were labelled. Anti-Cygb immunoreactivity was also found in the inner plexiform layer and in some regions of the outer plexiform layer. No Cygb was present in the outer nuclear layer, and in the inner and outer segments of the photoreceptor cells. Closer examination of the neuronal retina showed that the most prominent anti-Cygb staining was present in the nuclei of the neurons from the ganglion cell and inner nuclear layers (Fig. 2J–L). However, only a fraction of the nuclei was found strongly stained with the anti-Cygb antibodies, while most nuclei displayed only weak or no detectable anti-Cygb labelling. In addition to the nuclei, the perinuclear regions and the nuclei-free inner plexiform layer show anti-Cygb staining. Again, this result was confirmed with independently derived anti-Cygb antisera. Immunostaining of the retina with a specific anti-Ngb antibody was carried out as described [17,21]. Cryosections from the same animal that had been used for the Cygb-staining experiments were employed. The results confirmed previous data [17] and showed bright anti-Ngb immunofluorescence in the ganglion cell layer, the inner and outer plexiform lay-

ers and the inner segments (Fig. 2M–O). Minor perinuclear Ngb-staining was found in the nuclear layers.

Cygb was initially observed to be expressed in the hepatic stellate cells of rat and human liver [10], while later studies have shown that Cygb is also present in the cytoplasm of cells that form the connective and supportive tissues, represented by the fibroblasts, chondroblasts and bone cells [11,16]. The basic expression pattern of Cygb in non-neuronal cells is confirmed by the present study of the mouse eye, which shows Cygb in fibroblasts of structures such as the choroidea and

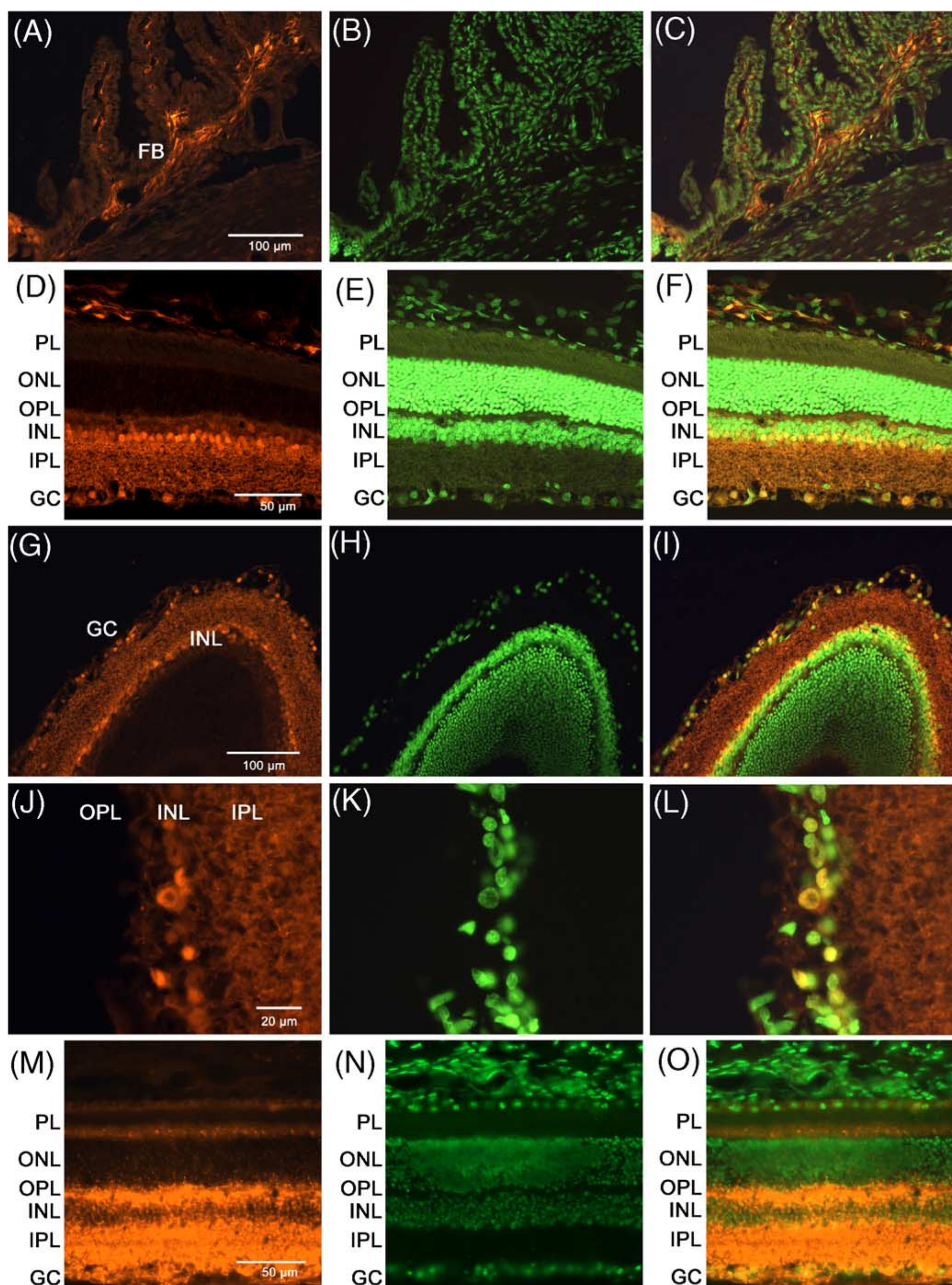

Fig. 2. Immunohistochemical localization of Cygb and Ngb protein in mouse eye. Indirect anti-Cygb immunofluorescence was carried out with αCygb (A–L) and αNgb (M–O) antibodies (red staining). The nuclei were counter-stained with Hoechst dye 33342 (green staining; B, E, H, K), merged pictures are shown in C, F, I and L. Experiments were carried out with antibodies αCygb2 (A–F; J–L) and αCygb3 (G–I). Strong anti-Cygb immunofluorescence is visible in the fibroblasts (FB) of the ciliary body (A) and in the choroidea (D). Neuronal cell bodies were stained in the ganglion cell layer (GC) and in the inner nuclear layer (INL) (D, G). No Cygb was present in the outer nuclear layer (ONL), outer plexiform layer (OPL) and photoreceptor layer (PL). Anti-Cygb staining was also present in the inner plexiform layer (IPL) (D, G). At higher magnification, Cygb staining was detected in the nuclei of ganglion cell and inner nuclear layer neurons (J, L). Ngb is located in the inner segments of the photoreceptor cells (PL), the outer and inner plexiform layers (OPL, NPL) and the ganglion cell layer (GC) (M, O).

ciliary processes (Fig. 2). Additional Cygb expression had been found in distinct neural cell populations [16]. However, in contrast to the fibroblast-related cells, which show an exclusive presence of Cygb in the cytoplasm, in neurons Cygb was found in both the cytoplasm and nuclei.

The mammalian retina is a highly oxygen-consuming tissue that is divided into morphological and functionally distinct layers [22]. In contrast to Ngb, which shows an about 50–100-fold higher concentration in the retina than in brain [17], Cygb is expressed at similar levels in these two tissues. Ngb is present in the inner segments, plexiform layers and the ganglion cells, while Cygb is only localized in the ganglion cell layer and inner nuclear layer. The divergent distribution of Ngb and Cygb in the retina strongly suggests different functions of these two members of the vertebrate globin family. Recent studies favour an important role of Ngb in oxygen homeostasis of neurons [3,17,18]. This hypothesis is in line with the high expression and the distribution of Ngb in the retina, which also suggests an association of Ngb with mitochondria and thus oxygen consumption [17,22]. Ngb may carry out a myoglobin-like role [20] in facilitating oxygen diffusion from the cellular surface to the mitochondria [3]. Other proposed Ngb functions [3,14], such as a role in detoxification of harmful ROS, may not be formally excluded, but considering the fact that in the retina most ROS are induced by light in the outer segments [12,13] where Ngb is absent, this hypothesis may be considered unlikely.

Neither expression levels nor intra-retinal distribution provide any evidence that Cygb – although being most closely related to myoglobin [2] – is involved in cellular oxygen supply. Interestingly, Cygb is expressed in some but not all neurons of the ganglion cell layer and inner nuclear layer that we suspect to be amacrine cells. It is noteworthy that – in contrast to the fibroblast cell lineage – in neurons from retina and the central and peripheral nervous systems [16], Cygb protein is localized in both the cytoplasm and the nucleus. Presently, the function of Cygb in the neuronal nuclei is difficult to interpret. For similar reasons as for Ngb it is unlikely that Cygb is involved in the general detoxification of ROS. However, it is conceivable that in the neurons Cygb may act as an oxygen (or NO) sensor that commutes between the cytoplasm and the nucleus, where it carries out a regulatory function. This may be analogous to the globin-coupled oxygen sensors of some bacteria [6]. On the other hand, Cygb may also provide oxygen to enzymatic reactions such as NO synthesis, which require O_2 for the production of NO from L-arginine. In this context it is noteworthy that NO synthases have also been found in the nucleus [8]. Future studies are warranted to elucidate the exact role of Cygb in both connective tissues and neurons.

Acknowledgements

We thank Anke Bentmann for her help with the immunostaining experiments, Uwe Wolfrum for discussion and comments, and Jürgen Markl for excellent working facilities. This work was supported by the Deutsche Forschungsgemeinschaft (Bu956/5 and Ha2103/3) and the European Union (QLG3-2002-01548).

References

[1] K. Asahina, N. Kawada, D.B. Kristensen, K. Nakatani, S. Seki, M. Shiokawa, C. Tateno, M. Obara, K. Yoshizato, Characterization of human stellate cell activation-associated protein and its expression in human liver, Biochim. Biophys. Acta. 1577 (2002) 471–475.

[2] T. Burmester, B. Ebner, B. Weich, T. Hankeln, Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues, Mol. Biol. Evol. 19 (2002) 416–421.

[3] T. Burmester, T. Hankeln, Neuroglobin: a respiratory protein of the nervous system, News Physiol. Sci. 19 (2004) 110–113.

[4] T. Burmester, B. Weich, S. Reinhardt, T. Hankeln, A vertebrate globin expressed in the brain, Nature 407 (2000) 520–523.

[5] D. de Sanctis, S. Dewilde, A. Pesce, L. Moens, P. Ascenzi, T. Hankeln, T. Burmester, M. Bolognesi, Crystal structure of cytoglobin: the fourth globin type discovered in man displays heme hexa-coordination, J. Mol. Biol. 336 (2004) 917–927.

[6] T.A.K. Freitas, S. Hou, M. Alama, The diversity of globin-coupled sensors, FEBS Lett. 552 (2003) 99–104.

[7] E. Geuens, I. Brouns, D. Flamez, S. Dewilde, J.P. Timmermans, L. Moens, A globin in the nucleus!, J. Biol. Chem. 278 (2003) 30417–30420.

[8] A. Giordano, C. Tonello, A. Bulbarelli, V. Cozzi, S. Cinti, M.O. Carruba, E. Nisoli, Evidence for a functional nitric oxide synthase system in brown adipocyte nucleus, FEBS Lett. 514 (2002) 135–140.

[9] D. Hamdane, L. Kiger, S. Dewilde, B.N. Green, A. Pesce, J. Uzan, T. Burmester, T. Hankeln, M. Bolognesi, L. Moens, M.C. Marden, The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin, J. Biol. Chem. 278 (2003) 51713–51721.

[10] N. Kawada, D.B. Kristensen, K. Asahina, K. Nakatani, Y. Minamiyama, S. Seki, K. Yoshizato, Characterization of a stellate cell activation-associated protein (STAP) with peroxidase activity found in rat hepatic stellate cells, J. Biol. Chem. 276 (2001) 25318–25323.

[11] K. Nakatani, H. Okuyama, Y. Shimahara, S. Saeki, D.H. Kim, Y. Nakajima, S. Seki, N. Kawada, K. Yoshizato, Cytoglobin/STAP, its unique localization in splanchnic fibroblast-like cells and function in organ fibrogenesis, Lab. Invest. 84 (2004) 91–101.

[12] D.T. Organisciak, H.M. Wang, A. Xie, D.S. Reeves, L.A. Donoso, Intense-light mediated changes in rat rod outer segment lipids and proteins, Prog. Clin. Biol. Res. 314 (1989) 493–512.

[13] J.S. Penn, M.I. Naash, R.E. Anderson, Effect of light history on retinal antioxidants and light damage susceptibility in the rat, Exp. Eye Res. 44 (1987) 779–788.

[14] A. Pesce, M. Bolognesi, A. Bocedi, P. Ascenzi, S. Dewilde, L. Moens, T. Hankeln, T. Burmester, Neuroglobin and cytoglobin. Fresh blood for the vertebrate globin family, EMBO Rep. 12 (2002) 1146–1151.

[15] S. Reuss, S. Saaler-Reinhardt, B. Weich, S. Wystub, M.H. Reuss, T. Burmester, T. Hankeln, Expression analysis of neuroglobin mRNA in rodent tissues, Neuroscience 115 (2002) 645–656.

[16] M. Schmidt, F. Gerlach, A. Avivi, T. Laufs, S. Wystub, J.C. Simpson, E. Nevo, S. Saaler-Reinhardt, S. Reuss, T. Hankeln, T. Burmester, Cytoglobin is a respiratory protein expressed in connective tissue and neurons that is up-regulated by hypoxia, J. Biol. Chem. 279 (2004) 8063–8069.

[17] M. Schmidt, A. Giessl, T. Laufs, T. Hankeln, U. Wolfrum, T. Burmester, How does the eye breathe? Evidence for neuroglobin

mediated oxygen supply in the mammalian retina, J. Biol. Chem. 278 (2003) 1932–1935.

[18] Y. Sun, K. Jin, X.O. Mao, Y. Zhu, D.A. Greenberg, Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 15306–15311.

[19] J.T. Trent III, M.S. Hargrove, A ubiquitously expressed human hexacoordinate hemoglobin, J. Biol. Chem. 277 (2002) 19538–19545.

[20] J.B. Wittenberg, B.A. Wittenberg, Myoglobin function reassessed, J. Exp. Biol. 206 (2003) 2011–2020.

[21] S. Wystub, T. Laufs, M. Schmidt, T. Burmester, U. Maas, S. Saaler-Reinhardt, T. Hankeln, S. Reuss, Localization of neuroglobin protein in the mouse brain, Neurosci. Lett. 346 (2003) 114–116.

[22] D.Y. Yu, S.J. Cringle, Oxygen distribution and consumption within the retina in vascularised and avascular retinas and in animal models of retinal disease, Prog. Retinal Eye Res. 20 (2001) 175–208.