

p19 detected in the rat retina and pineal gland is a guanylyl cyclase-activating protein (GCAP)[★]*

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The Ca^{2+} -dependent activation of retina-specific guanylyl cyclase (retGC) is mediated by guanylyl cyclase-activating proteins (GCAPs). Here we report for the first time detection of a 19 kDa protein (p19) with GCAP properties in extracts of rat retina and pineal gland. Both extracts stimulate synthesis of cGMP in rod outer segment (ROS) membranes at low (30 nM) but not at high (1 μM) concentrations of Ca^{2+} . At low Ca^{2+} , immunoaffinity purified p19 activates guanylyl cyclase(s) in bovine ROS and rat retinal membranes. Moreover, p19 is recognized by antibodies against bovine GCAP1 and, similarly to other GCAPs, exhibits a Ca^{2+} -dependent electrophoretic mobility shift.

Molecules of cyclic GMP (cGMP) interact directly with cationic channels located in the plasma membrane of the outer segments of retinal photoreceptor cells and keep the chan-

nels open. Light triggers a cascade of biochemical events which lead to the hydrolysis of cGMP by photoreceptor-specific phosphodiesterase (PDE6) and closure of the channels

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Abbreviations: GC, guanylyl cyclase; GCAP, guanylyl cyclase-activating protein; IBMX, 3-isobutyl-1-methylxanthine; NCS, neuronal calcium sensors; retGC, retina-specific guanylyl cyclase; ROS, rod outer segments.

(reviewed by Gorczyca 1999; Palczewski *et al.*, 2000). In consequence, the cytoplasmic concentration of Ca^{2+} ($[\text{Ca}^{2+}]_{\text{free}}$), which is high (about 600 nM) in the darkness, drops to a low level (< 50 nM). The lowered concentration of calcium ions is the signal for resynthesis of cGMP by guanylyl cyclase (GC), beginning the recovery of the photoreceptor cell to the dark state. The photoreceptor-specific GC, named also retinal GC (retGC), is negatively regulated by Ca^{2+} (Lolley & Racz, 1982; Koch & Stryer, 1988). The enzyme is active below 100 nM ($[\text{Ca}^{2+}]_{\text{low}}$) and inactive above 500 nM ($[\text{Ca}^{2+}]_{\text{high}}$) concentrations of Ca^{2+} . This Ca^{2+} -dependent activation of retGC is mediated by Ca^{2+} -binding proteins named guanylyl cyclase-activating proteins (GCAPs) and contributes to the recovery of the dark state by photoreceptor cells (Dizhoor *et al.*, 1994; Gorczyca *et al.*, 1994). Three different GCAPs (GCAP1, GCAP2 and GCAP3) have been identified in the vertebrate retina to date (Palczewski *et al.*, 1994; Dizhoor *et al.*, 1995; Gorczyca *et al.*, 1995; Frins *et al.*, 1996; Haeseleer *et al.*, 1999). These highly homologous proteins share structural properties with a larger family of proteins called Neuronal Calcium Sensors (NCS) (Burgoyne & Weiss 2001).

Although the retina is the primary site where GCAPs are expressed, GCAP1 was also detected in the bovine pineal gland (Venkataraman *et al.*, 2000) and expression of genes encoding GCAP1 and GCAP2 was reported for the chicken pineal gland (Semple-Rowland *et al.*, 1999). Together with the findings that also retGC is present in pineal glands of both species these observations indicate that the synthesis of cGMP in the pineal gland may be regulated in a Ca^{2+} -dependent manner but the physiological significance of this mechanism is unclear.

The aim of our study was to elucidate whether the 19 kDa protein (p19) we had detected in extracts of rat retina and pineal gland using antibodies specific to bovine GCAP1 belongs to the GCAP subfamily. In

this report we present data indicating that p19 is most likely a rat ortholog of bovine GCAP1.

MATERIALS AND METHODS

Chemicals. Acrylamide, bis (*N,N'*-methylene-bis-acrylamide), ammonium persulfate, SDS, TEMED, and Bromophenol Blue were purchased from BioRad Laboratories (Hercules, CA, U.S.A.); GTP, Tris, 2-mercaptoethanol, glycerol, glycine, EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), benzamidine, leupeptin, IBMX, dithiothreitol (DTT), MgCl_2 , and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Neutral alumina (ICN alumina) was purchased from ICN Biomedicals GmbH (Eschwege, Germany), while CNBr-activated Sepharose 4B from Amersham Pharmacia Biotech AB (Uppsala, Sweden). $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ was purchased from PerkinElmer, Life Sciences (Boston, MA, U.S.A.). Alkaline phosphatase conjugated anti-rabbit IgG and BCIP/NBT color development substrate were purchased from Promega (Madison, WI, U.S.A.).

Antibodies. Monoclonal antibody mAbG2 was generated in BALB/c mice against truncated (GCAP180) bovine GCAP1 (Gorczyca *et al.*, 1995); rabbit polyclonal antibodies pAb23 and pAb850 were raised against recombinant bovine GCAP1 or GCAP2 expressed in *E. coli* (Gorczyca, 2000).

Animals. Wistar rats 2–3 months old weighing about 180–250 g came from the Institute of Medicine of Work in Łódź. The animals were treated in accordance with procedures approved by the local Ethics Committee For Animal Experimentation in Łódź.

Preparation of rat retinal and pineal gland extracts. Rat retinas and pineal glands were isolated as described by Zawilska *et al.* (1997). Briefly, the animals were killed in the light phase of a 24 h lighting cycle (preceded by 0.5 h adaptation to darkness) by decapita-

tion and their pineal glands and retinas were quickly isolated under dim red light (intensity about 4 luxes). The obtained organs were then homogenized in water containing inhibitors of proteases (leupeptin, PMSF, benzamidine) using an Ultra Turrax homogenizer. The homogenates were clarified by centrifugations at $30\,000 \times g$. The resulting supernatants (the extracts) were collected and used directly in retGC activity assays or in purification procedures as a source of GCAPs. The particulate fraction obtained after the first centrifugation, termed crude retinal (or pineal gland) membranes, was used in GC assays as a source of particulate GCs activity.

Preparation of ROS membranes. Fresh bovine eye balls obtained from a local slaughterhouse were adapted to the darkness and all further procedures were carried out under dim red light at 4°C . The retinas were dissected within 2–4 h after the death of the animals and rod outer segments (ROS) were isolated using discontinuous sucrose gradient according to McDowell (1993). The obtained ROS pellet was then suspended in 50 mM Hepes, pH 7.8, 10 mM NaCl, homogenized and centrifuged at $30\,000 \times g$ for 10 min at 4°C . The supernatant was discarded and the pellet was washed again under the same conditions. Finally, the pellet was resuspended in the same buffer. The obtained suspension, termed crude ROS membranes or washed ROS (wROS), was used in the retGC activity test.

Purification of p19. The 19 kDa protein was purified from the rat retinal extract by immunoaffinity chromatography according to the modified procedure previously described by Gorczyca (2000) for purification of bovine GCAPs. Briefly, a C10/10 column (Amersham Pharmacia Biotech) containing 2 ml of Sepharose 4B coupled with pAb850 (pAb850-Sepharose) against bovine GCAP2 was followed by an identical column containing 2 ml of mAbG2-Sepharose against bovine GCAP1. The extract obtained from 300 rat retinas in a volume of 20 ml was loaded at a flow rate of

2.0 ml/h onto the first column and the unbound material was directly loaded onto the second column connected with the first one in tandem. The columns were then washed with 25 mM Tris/HCl, pH 7.5, 50 mM NaCl, disconnected, and washed with 25 mM Tris/HCl, pH 7.5, 0.2 M NaCl to remove nonspecifically adsorbed proteins. Bound proteins were eluted with 0.1 M glycine, pH 2.5. Fractions (1 ml) were collected, immediately neutralized with 1.5 M Tris/HCl, pH 8.8, and analyzed for stimulatory effect on GC activity. In some experiments only the mAbG2-Sepharose column was used for p19 purification.

GC activity assay. GC activity was determined in crude membranes (ROS or retinal), prepared as described above and supplemented either with the extracts (retinal or pineal) or with purified p19. The assay used is a modification of the retGC assay described previously (Gorczyca *et al.*, 1995; Gorczyca, 2000). Briefly, 64 μl of the final reaction mixture contained 50 mM Hepes, pH 7.8, 60 mM KCl, 10 mM NaCl, 10 mM MgCl_2 , 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.4 mM EGTA, CaCl_2 in amounts calculated using the computer program "Chelator 1.0" (Schoenmakers *et al.*, 1992) to obtain the desired concentration of $[\text{Ca}^{2+}]_{\text{free}}$, 1 mM $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (specific activity 30 000–50 000 d.p.m./nmole), and crude membranes containing 100 μg of protein, as determined by the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.). The reaction, initiated by adding the membranes, was terminated after 10 min incubation at 30°C by adding 0.4 M HCl. All the above steps were performed at dim red light. The sample was neutralized with 0.2 mM Tris/HCl, pH 7.4 and the product ($[\text{P}^{32}]\text{cGMP}$) was separated from the substrate ($[\alpha\text{-}^{32}\text{P}]\text{GTP}$) using neutral alumina gel. Finally, the radioactivity of the product was measured using a liquid scintillation counter.

SDS/PAGE and Western blotting. Both extracts (retinal and pineal gland), each containing 30 μg of protein as determined by the BCA Protein Assay, were resolved under re-

ducing conditions in SDS/PAGE using 12.5% acrylamide gels and electrotransferred onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) or Immobilon™ P membranes (Millipore, Bedford, MA, U.S.A.). The membranes were then blocked with 2% (w/v) casein and incubated for 1.5 h with anti-GCAP1 antibodies. After washing with 50 mM Tris, pH 7.5, 150 mM NaCl and 0.05% Tween 20 (TBST) and subsequently three times with 50 mM Tris, pH 7.5, 150 mM NaCl (TBS), the membranes were incubated with anti-rabbit IgG goat antibodies conjugated to alkaline phosphatase and visualized using a NBT/BCIP-based substrate.

RESULTS AND DISCUSSION

Antibodies against GCAP1 recognize a 19 kDa protein in extracts of rat retina and pineal gland

Polyclonal antibody 23 (pAb23), specific to bovine GCAP1, recognizes in rat retinal extract and in rat pineal gland extract a protein with apparent molecular mass of 19 kDa

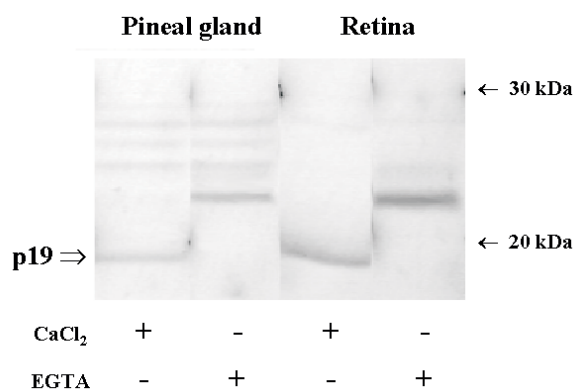


Figure 1. Western blot analysis of rat pineal gland and retinal extracts.

Proteins from each extract (30 μ g), obtained as described in Materials and Methods, were subjected to SDS/PAGE in the presence of either 1 mM CaCl₂ or 1 mM EGTA, electrotransferred onto nitrocellulose membrane and immunostained with antibody (pAb23) against bovine GCAP1. Thin arrows indicate positions of molecular mass standards.

(Fig. 1). In the extract of pineal gland some additional bands of higher molecular mass are weakly immunostained. However, only the 19 kDa protein displays a characteristic Ca²⁺/EGTA-dependent electrophoretic mobility shift and in the presence of EGTA it migrates as a 23 kDa band. Since such a property is typical for GCAPs (Gorczyca *et al.*, 1995; Frins *et al.*, 1996), we assume that all other bands are due to unspecific staining. The intensity of p19 staining is weaker in the pineal extract than in the retinal extract indicating that this protein is expressed in the pineal gland at a lower level than in the retina. Our results also support the findings of Cuenca *et al.* (1998), who using another antibody against bovine GCAP1 have shown by means of immunohistochemical techniques the presence of GCAP1 in the rat retina. Hence, p19 is most likely a rat ortholog of bovine GCAP1.

Retinal and pineal extracts activate retGC in crude ROS membranes

If p19 detected in both rat extracts was a GCAP, it should activate in a Ca²⁺-dependent manner retGC in the ROS membranes. To check this we used the assay of guanylyl cyclase activity. The extracts obtained from the rat retina and from the rat pineal gland markedly (six- and almost three-fold, respectively) stimulate at [Ca²⁺]_{low} cGMP synthesis in crude membranes of bovine rod outer segments (Fig. 2). In some experiments bovine retinal extract was used as a positive control. Under the same experimental conditions it stimulates the GC activity more than 7-fold (not shown). Neither of the rat extracts stimulate the GC activity at [Ca²⁺]_{high} indicating that they indeed activate the retGC and thus display GCAP-like properties. Moreover, the lower potency of the pineal than the retinal extract to activate retGC at [Ca²⁺]_{low} corresponds to the above observed (Fig. 1) weaker expression of p19 in the pineal gland in comparison with the retina. Since GCAP1 amino acid sequences reveal very high interspecies

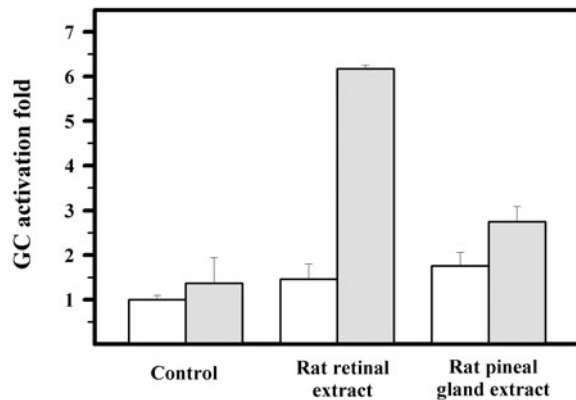


Figure 2. Stimulation of retGC activity by rat retinal and pineal extracts.

The retGC activity was measured in bovine wROS (see Materials and Methods) at 30 nM (filled bars, [Ca²⁺]_{low}) and 1 μ M (open bars, [Ca²⁺]_{high}) concentrations of free Ca²⁺ either in the absence (control) or in the presence of extract (100 μ g of protein). The basal activity of retGC, measured at [Ca²⁺]_{high} in the absence of extracts, was taken as 1.0.

homology (Gorczyca & Sokal, 2002), in the next step we attempted to isolate p19 from the rat retinal extract by means of immunoaffinity chromatography using antibodies against bovine GCAP1.

Immunoaffinity purified p19 is recognized by anti-GCAP1 antibodies and activates retGC in crude ROS and retinal membranes

Specific fractions eluted from the mAbG2-Sepharose column were concentrated, subjected to SDS/PAGE, electrotransferred onto ImmobilonTM P and either stained using Coomassie Brilliant Blue (CBB) or immunostained with pAb23. In the presence of CaCl₂ the very weak band corresponding to the molecular mass of 19 kDa is stained by CBB. There is also strong staining of an unknown protein of about 27 kDa. Although the same bands are immunostained by the pAb23 antibody, the intensity of staining is similar for both. Moreover, in the presence of EGTA only p19 exhibits the shift characteristic for GCAPs and migrates as a 22–23 kDa protein (Fig. 3). These results show that p19, present

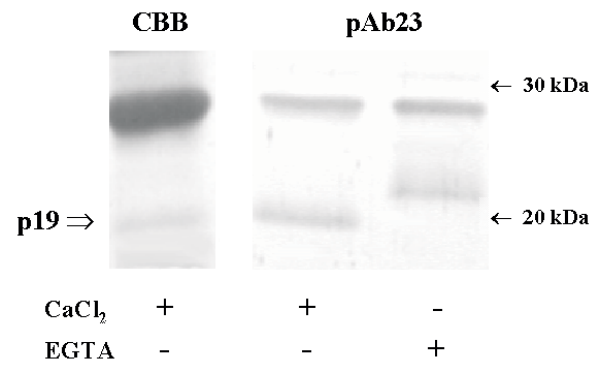


Figure 3. Detection of immunoaffinity purified p19.

Immunoaffinity purified p19 was concentrated, subjected to SDS/PAGE, electrotransferred onto ImmobilonTM P membrane and then either stained with Coomassie Brilliant Blue R-250 (CBB) or immunostained using pAb23. For CBB staining 40 μ l of concentrated fraction was loaded onto the gel, while for immunostaining only 10 μ l was applied. Other details are described in Fig. 1.

in the fractions obtained after immunoaffinity chromatography, displays GCAP1-like properties. The yield of the purification is very low as one can purify only about 9–10 μ g of p19 from 300 rat retinas.

To check whether the analyzed fractions contain a protein with retGC stimulating activity, they were tested in the GC assay using wROS and crude rat retinal membranes (Fig. 4). Bovine GCAP1 (0.2 μ g of protein), purified from bovine retinal extract in the same way as p19 but without the concentration step, was used as a positive control. It stimulates retGC in wROS about 3-fold. In the presence of p19, GC activity is elevated at [Ca²⁺]_{low} by a factor of 1.8 in bovine wROS and by a factor of 1.4 in crude rat retinal membranes. At the same time there is no stimulation of GC activity by p19 at [Ca²⁺]_{high} in both membrane fractions. These results provide further evidence that the analyzed fractions contain GCAP. The lower stimulatory potency of p19 in comparison with bovine GCAP1 may be a result of p19 inactivation during isolation. Interestingly, the fractions obtained after immunoaffinity chromatography of rat retinal extract on

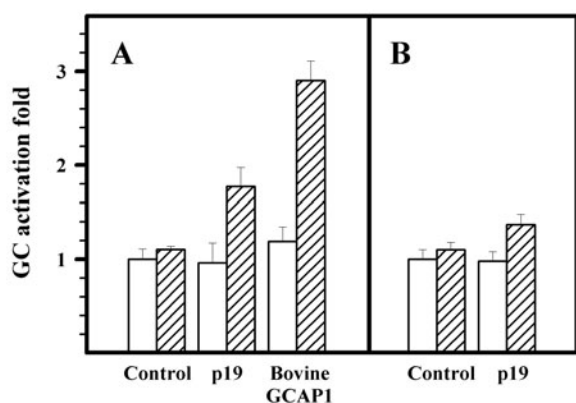


Figure 4. Stimulation of retGC activity by p19.

The retGC activity was measured at $[Ca^{2+}]_{low}$ (filled bars) or $[Ca^{2+}]_{high}$ (open bars) in bovine wROS (A) or in crude rat retinal membranes (B) either in the absence (control) or in the presence of immunoaffinity purified p19 (0.2 μ g of protein). Immunoaffinity purified bovine GCAP1 (0.2 μ g of protein) was used as a positive control. All other details are described in Fig. 2.

pAb850-Sepharose do not contain any GCAP-like activity, although the pAb850 antibody was able to stain a band corresponding to molecular mass of about 21 kDa in both the retinal and pineal rat extracts (not shown). On the basis of all the above results one can assume that p19 is a rat ortholog of bovine GCAP1 but further studies are required to prove it definitively.

In the retinal photoreceptor cells the role of GCAP1 as a key element in the phototransduction is well established (recently reviewed by Gorczyca & Sokal, 2002). Although the presence of GCAP1 was also proved in the chicken (Semple-Rowland *et al.*, 1999) and bovine (Venkataraman *et al.*, 2000) pineal glands, its physiological role there is still elusive. It is only hypothesized that since pinealocytes contain most of the retinal proteins characteristic for phototransduction and the pineal bodies of lower vertebrates and newborn mammals directly respond to light (Falcon, 1999; Meissl, 1997; Tosini *et al.*, 2000), this Ca^{2+} -dependent regulation of retGC in the gland might be a part of a phototransduction process. In mammals, during their

maturation, the direct light sensitivity of the pineal gland disappears and it is suggested that the elements of the phototransduction machinery are either no longer expressed or are adopted by other signaling systems. GCAP1 and retGC are still present in bovine pineal glands isolated from mature animals and it is postulated that the Ca^{2+} -dependent regulation of cGMP synthesis in the pineal gland is linked to α -adrenergic signaling through regulation of $[Ca^{2+}]_{free}$ by the $\alpha_{2D/A}$ -adrenergic receptor (Venkataraman *et al.*, 1998; 2000).

In summary, our results show that p19, recognized in the rat retinal and pineal extracts by an antibody specific to bovine GCAP1, belongs to the family of guanylyl cyclase-activating proteins (GCAPs). They also indicate that p19 is most likely a rat ortholog of bovine GCAP1 thus supporting observations of others, extending them to new species, and suggesting that Ca^{2+} /GCAP1/retGC signaling in pineal gland is well conserved in evolution. What is the physiological role of this signaling pathway in the pineal gland remains an open question.

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