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Research Article

BIOCHEMICAL CHARACTERIZATION OF PHOTOACTIVATED ADENYLYL CYCLASE FROM *NAEGLERIA GRUBERI*

Meenakshi Tanwar[1], Manuela Stierl[2], Sindhu Kandoth Veetil[1], Alfons Penzkofer[3] Peter Hegemann[2], and Suneel Kateriya[1]*

[1]*Department of Biochemistry, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110021, India*

[2]*Institut für Biologie/Experimentelle Biophysik, Humboldt Universität zu Berlin, Invalidenstrasse 42, D-10115 Berlin, Germany,*

[3]*Fakultät für Physik, Universität Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany*

Abstract: Blue light sensors using FAD (BLUF) domains are flavin based blue light photoreceptors. The BLUF domains are often fused with various effector domains. BLUF domain coupled with adenylyl cyclase domain is known as photoactivated adenylyl cyclase (PAC). *Naegleria gruberi* genome database analysis revealed the presence of four PACs. Each of the photoactivated adenylyl cyclases from *Naegleria gruberi* (NgPACs) is composed of a BLUF domain and an adenylyl cyclase domain. Light regulated enzymatic activity of recombinant NgPAC1 protein was assayed in dark and after blue light irradiation by measuring the cAMP level. Experimental results showed that the NgPAC1 protein exhibits light regulated cyclase activity. In this report, we have also demonstrated that the recombinant NgPAC1 exits as an oligomer in solution.

Keywords: Photoactivated adenylyl cyclase; *Naegleria gruberi*; light regulated cyclase activity; BLUF; NgPAC; optozymes.

Introduction

Cyclic nucleotide cAMP is a ubiquitous secondary messenger in all organisms. cAMP plays a key role in controlling biological processes in eukaryotes and prokaryotes. The synthesis of cAMP from ATP is catalyzed by adenylate cyclase (AC). Adenylate cyclase (AC) works as a central relay station which receives and amplifies primary signals and in turn, cAMP activates target proteins, and finally result in cellular response to primary stimulus (Linder, 2006). In lower organisms, adenylyl cyclase (AC) consists of multidomain architecture where cyclase homology domain (CHD) is fused with various sensor domains like histidine kinase, GAF domain (AC cyaB1 from *Anabaena sp*. PCC 7120) and BLUF domain (PAC from *Euglena gracilis).* In *Euglena gracilis,* the BLUF domain (sensor of blue light using FAD) is coupled with adenylyl cyclase and is known as photoactivated adenylyl cyclase (PAC). In *Euglena gracilis,* photoactivated adenylyl cyclase (euPAC) is responsible for photobehavioral activity (Iseki *et al.*, 2002). euPAC α (112 kDa) and β (94 kDa) are of large size and consist of two BLUF and two cyclase domains. A smaller photoactivated adenylyl cyclase with single BLUF and cyclase domains has been reported in *Beggiatoa* (bPAC) (Stierl *et al.*, 2011; Ryu *et al.*, 2010). These PACs (euPAC and bPAC) show increase in cyclase activity upon blue light illumination. These PACs have been used as optogenetic tools to manipulate the cAMP level within the cell (Schroder-Lang *et al.*, 2007; Stierl *et al.*, 2011). Sequences similar to PAC have also

Corresponding Author: **Suneel Kateriya**
E-mail: skateriya@south.du.ac.in

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been found in other organisms like *Naegleria gruberi, Naegleria australiensis, Oscillatoria acuminate* and *Pseudanabaena biceps*.

Naegleria gruberi is a free-living amoeboflagellate protozoan. *Naegleria* genome is crucial for studies of cell biology, evolution, environmental and medical microbiology. Recently, *Naegleria gruberi* genome has been sequenced (Fritz-Laylin *et al.*, 2010). The genome database search revealed the presence of photoactivated adenylyl cyclases (NgPAC). Four PACs are present in *Naegleria gruberi*. Photoactivated adenylyl cyclases are also reported in *Naegleria australiensis* (Yasukawa *et al.*, 2013). In the present report, we have identified four photoactivated adenylyl cyclase (PACs) sequences from *Naegleria gruberi*. Absorption and fluorescence characteristics of NgPAC1 from *Naegleria gruberi* have been studied earlier (Penzkofer *et al.*, 2011), which shows the presence of functional BLUF domain in NgPAC1. The biochemical characteristics and light regulated cyclase activity of NgPAC1 are presented here in detail. Relative low molecular mass (40 kDa), appreciable solubility and light regulated cyclase activity make NgPACs potential new tools for non-invasive light dependent control of cAMP level in cells and useful for neuroscience applications.

Materials and Methods

Genome mining for identification of putative new photoactivated adenylyl cyclases from Naegleria gruberi (NgPAC)

BLAST search was performed using photoactivated adenylyl cyclase from *Euglena gracilis* (euPAC) as a template to search PACs from *Naegleria gruberi*. The NgPAC sequences were fetched from the JGI genome database (*http://genome.jgi.doe.gov/Naegr1*). The retrieved sequences were analyzed for the presence of BLUF and cyclase domains using CDART program (*http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi*) (Geer *et al.*, 2002). The secondary structure prediction of the NgPAC1 protein was performed using Psipred program (*http://bioinf.cs.ucl.ac.uk/psipred/*) (McGuffin *et al.*, 2000). For homology analysis, the NgPAC sequences were aligned to bPAC using CLUSTALW multiple alignment tool (*http://www.ebi.ac.uk/Tools/clustalw2/index.html*) (Larkin *et al.*, 2007).

Purification of recombinant photoactivated adenylyl cyclase

The NgPAC1 gene was synthesized (codon optimized for *E. coli* expression) from Gene Art, Germany. The gene was cloned into pASK 43 vector using BamHI and XhoI restriction enzymes and was further confirmed by automated DNA sequencing. The NgPAC1 pASK construct was transformed into *E. coli* expression strain (Bl-21DE3λ). Transformed cells were inoculated into 5 mL of Luria-Bertani broth supplied with 100 µg/ml ampicillin. Overnight grown culture was inoculated into 500 mL of Terrific Broth medium (TBM). The culture was shaken at 200 rpm at 37 ^{o}C to an optical density of 0.6 at 600 nm. They were then supplemented with anhydrotetracyclin to a final concentration of 200 µM and were grown at 16 ^{o}C for 48 hrs. Harvested cells were resuspended in 1X PBS, pH 7.4 and lysed by sonication on ice. Lysed cells were centrifuged at 29,700 x g for 50 min at 4 ^{o}C to obtain soluble fractions. NgPAC1 recombinant protein was purified from soluble fraction in the presence of safe light (red light or in darkness) by immobilized metal affinity chromatography (IMAC) using Co^{2+} metal ion resins (Clontech Laboratories Inc. USA) according to supplier's instructions.

Size-exclusion chromatography of NgPAC1 protein for quaternary structure investigation

Size exclusion chromatography was carried out with an AKTA explorer chromatography system (GE Healthcare, USA) on HiLoad 16/60 Superdex 200 (1.0 X 30 cm) prep grade column at flow rate of 1ml/min with 1X PBS (pH 7.4) at 4 ^{o}C. Oligomeric state of the NgPAC1 protein was determined by comparing with standard molecular weight marker (Bio-Rad, USA), separated under isocratic conditions. Peaks of the chromatogram were fit to a Gaussian curve with the IGOR Pro program (Wave Metrics Inc., USA).

Glutaraldehyde crosslinking of the recombinant NgPAC1 protein

Crosslinking of NgPAC1 protein was carried out using freshly prepared 2.5% (v/v) glutaraldehyde

(Sigma). Purified NgPAC1 protein was incubated with 5 µl of 2.5% glutaraldehyde at 37 °C. The reaction was stopped after 5 min, by the addition of 1 M Tris-Cl (pH 8). The cross linked protein samples were resolved on 10% SDS-PAGE.

Light regulated cyclase assay with recombinant NgPAC1 protein

cAMP level was detected by a competitive immunoassay using an ELISA kit (ENZO Biomol, Germany) according to manufacturer's instructions. Activity assays were performed at room temperature. The cyclase activity of the purified recombinant protein NgPAC1 was assessed in the assay buffer (50 mM Tris-Cl pH 7.5, 1 mM $MgCl_2$, 300 mM KCl) containing 10 µg of purified protein. The reaction was started by the addition of 0.1mM ATP. Enzymatic assays of the protein were executed under two conditions, in darkness and upon irradiation with blue light for the duration of the assay using blue LED (460 nm, 25 µW/cm^2/nm, 20 nm full spectral bandwidths). At different time intervals, the reactions were stopped by addition of 0.1 M HCl.

Results and Discussion

Photoactivated adenylyl cyclases identified from Naegleria gruberi

Genome mining of *Naegleria gruberi* yielded four photoactivated adenylyl cyclases (PACs). These PACs were named as NgPAC1, NgPAC2, NgPAC3 and NgPAC4. Conserved domain analysis of all NgPACs suggested the presence of BLUF domain and cyclase domain. NgPAC1, NgPAC2 and NgPAC3 consisted of one BLUF and cyclase domains (Figure 1A i), while NgPAC4 consisted of two BLUF and two cyclase domains in the sequence (Figure 1A ii). Secondary structure analysis suggested that the BLUF domain is composed of five β strands and two α helices. The conserved flavin binding residues of the canonical BLUF domain were also present in the BLUF domain of NgPACs (Figure1B, C). All the conserved catalytic residues of adenylyl cyclase domain were present in all the NgPACs. The metal binding residue Asp, essential adenine binding residue Lys or Thr and transition state stabilizing residue Asn and Arg were also found to be conserved in NgPACs (Figure 1C). These relevant characteristics indicate that NgPACs would be working as light-gated cyclases upon functional expression.

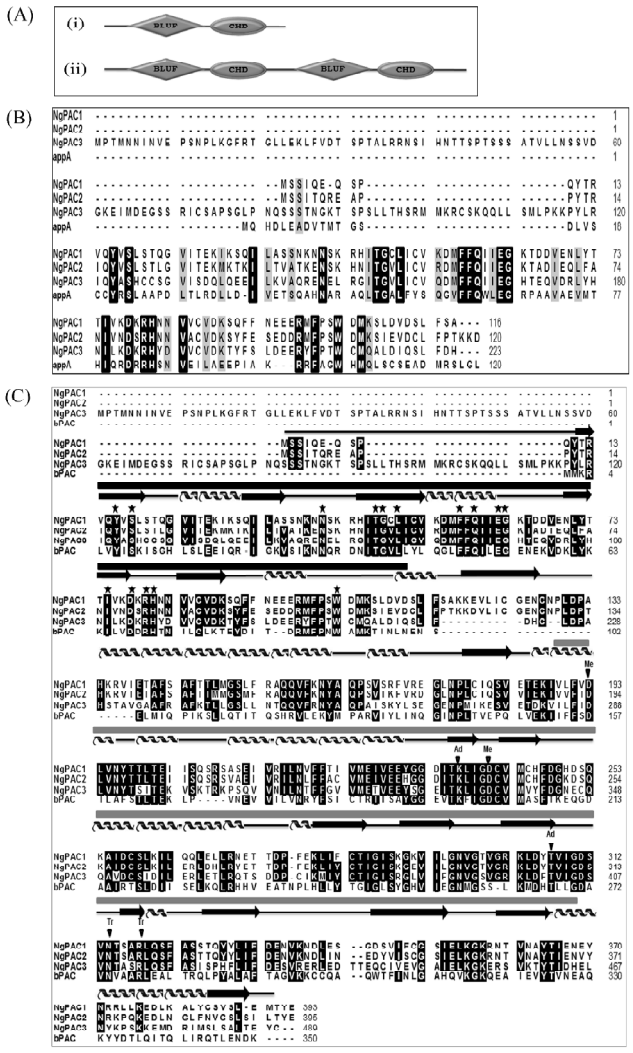

Figure 1: **Domain architecture and homology analysis of the photoactivated adenylyl cyclases from *Naegleria gruberi*.**

(A) i) Domain architecture of NgPAC1, NgPAC2 and NgPAC3. ii). Domain architecture of NgPAC4. (B) Sequence alignment of BLUF domain of NgPAC1 (XP-002674370), NgPAC2 (XP-002672444.1), NgPAC3 (XP-002644371)) with AppA Bluf domain. Highly conserved residues for functional BLUF domain are displayed in white against black background. (C) Sequences of photoactivated adenylyl cyclases from *Naegleria gruberi* NgPAC1, NgPAC2, NgPAC3 and bPAC (from *Beggiatoa*) were aligned to each other. Identical amino acid residues are displayed in white against black background. Flavin binding pocket residues are shown with star. Catalytic conserved residues of cyclase domain - metal binding residue Asps (Me), essential adenine binding residue Lys or Thr and transition state stabilizing residue Asn and Arg are displayed with black arrows heads. The protein consists of both α-helical and β-sheets coils and same are represented with helical cartoon and arrow, respectively. BLUF domain and cyclase domain region are indicated by black and grey bar, respectively.

Recombinant NgPAC1 exists as oligomer in solution

NgPAC1 was overexpressed in *E. coli* with 6x histidine tag fused to N-terminus. Recombinant NgPAC1 was efficiently expressed in soluble form and purification of the expressed protein was monitored at each step by SDS-PAGE and vizualized by coomassie staining (Figure 2). A major band of 40 kDa was observed on the gel which was equivalent to its calculated molecular mass. The affinity purified NgPAC1 protein was analyzed by size exclusion chromatography. In the gel filtration chromatography, major fraction of the protein eluted as oligomer (>670 kDa), while smaller fraction of the protein eluted as monomer (40 kDa; Figure 3A). Oligomeric nature of NgPAC1 protein was further confirmed by glutaraldehyde chemical cross linking which revealed that the protein exists in higher oligomeric states (Figure 3B). Cyclases are known to form dimers for regulation of cyclase activity and active sites are located at the dimer interface (Linder *et al.*, 2006). Earlier report also suggested that BLUF proteins exist as dimers (Anderson *et al.*, 2005), pentamers and higher oligomers (Kita *et al.*, 2005). For functional activity and regulation between BLUF and cyclase domains NgPAC1 may exist as higher oligomer. It can be concluded that the higher oligomeric forms of the protein may be required for functional activity.

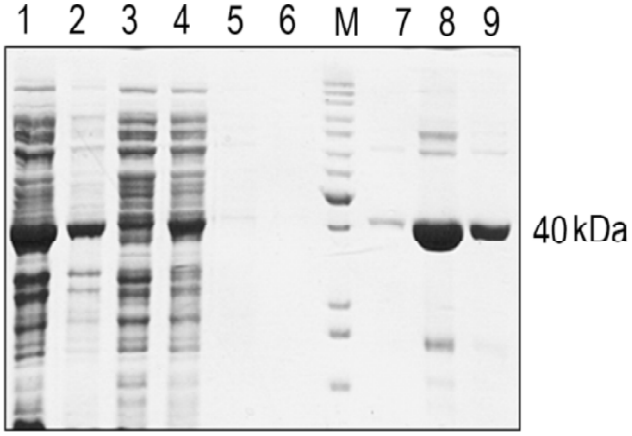

Figure 2: **SDS-PAGE analysis of the recombinant NgPAC1.**

Purification profile of affinity purified recombinant NgPAC1 protein. Lane 1: Total cell lysate (TCL), Lane 2: Soluble fraction (SF), Lane 3: Insoluble fraction (ISF), Lane 4: Flow through (FT), Lane 5 and 6: washing, Lane 7: Eluent fraction 1(E1), Lane 8: E2, Lane 9: E3. M stands for marker.

Light regulated activity of recombinant NgPAC1

To assign light regulated cyclase activity of NgPAC1, cAMP level was measured with recombinant purified protein in the dark and after illumination with blue light. Upon blue light illumination, there was significant increase in NgPAC1 cyclase activity in comparison to the cyclase activity in the dark (Figure 4A). Like bPAC, NgPAC1 showed light regulated cyclase activity. However, cyclase activity of bPAC is higher than that of NgPAC1 activity (Figure 4B). In comparison to bPAC, NgPAC1 showed a higher basal cyclase activity in dark.

For further application of NgPAC1 as optogenetic tool to manipulate the cAMP level in cell, there is a need to engineer NgPAC1 to reduce its basal cyclase activity. There is also a requirement of PACs with different kinetic

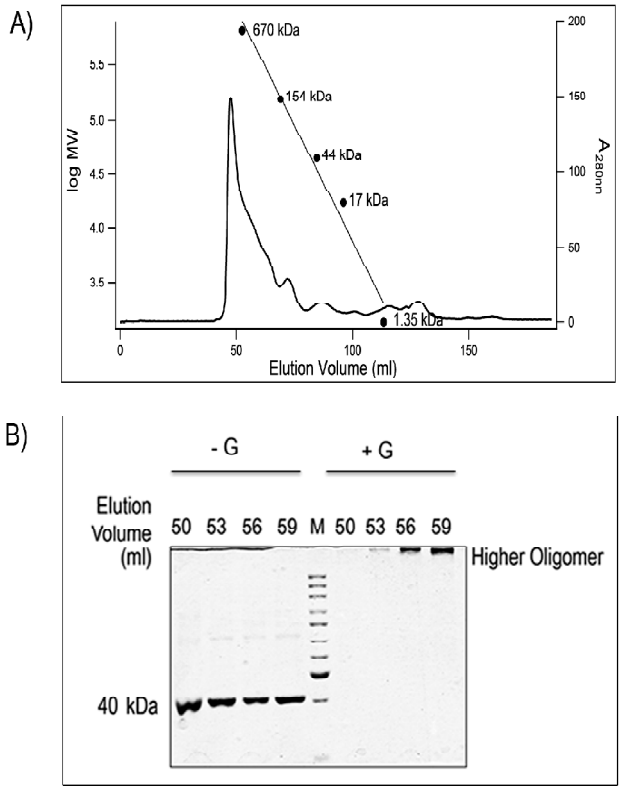

Figure 3: **Oligomeric characterization of NgPAC1.**

(A) SEC chromatogram showing oligomeric states of the NgPAC1. Straight line represented the calibration of standard molecular weight marker comprising thyroglobulin (670 kDa), α-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35kDa). (B) SDS-PAGE profile of NgPAC1 glutaraldehyde crosslinked samples showing higher oligomers with glutaraldehyde (G). M stands for marker.

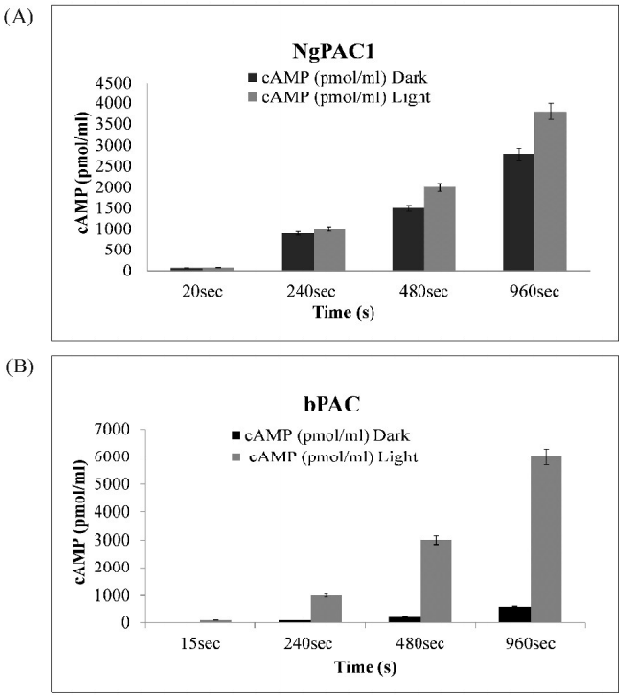

Figure 4: **Light regulated enzymatic cyclase activity.**

(A) Light regulated adenylyl cyclase activity of NgPAC1 in dark and upon continuous illumination with blue light. (B) Adenylyl cyclase activity of bPAC in dark and upon continuous illumination with blue light. Solid bar in grey color and bar with black lining indicate the cAMP level in dark and after illumination with blue light respectively.

activities which would enable the manipulation of distinct cells and tissues with different temporal level expression.

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