DeLIGHTful Proteins: Engineering NIRW Light Activated Guanylate Cyclase

Alhena Islam[1,2] and Mark Gomelsky[2]

[1]College of Arts and Sciences (COL 2024), University of Pennsylvania; [2]Department of Molecular Biology, University of Wyoming

Introduction

Controlling Cells with Light

- Cell-based therapies have great treatment potential
  - Light as control tool
    - ❖ Non-invasive
    - ❖ Spatial precision
    - ❖ Temporal control
- **Near Infra-Red Window (NIRW) (660-880 nm) light**
  - Deep penetration (Fig. 1)
  - No harmful effects
- **Optogenetics:** Use of engineered photoactivated proteins to regulate biological processes (1)

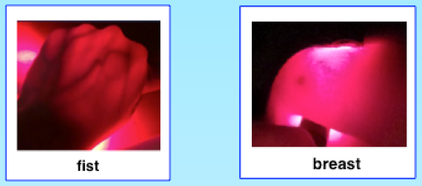

Fig. 1. Light penetration through human tissues.

Bacteriophytochrome Optogenetic Systems

- **Bacteriophytochrome (BphP)** responds to NIRW light (Fig. 2)
  - Biliverdin IX α (BV IX α) absorb light

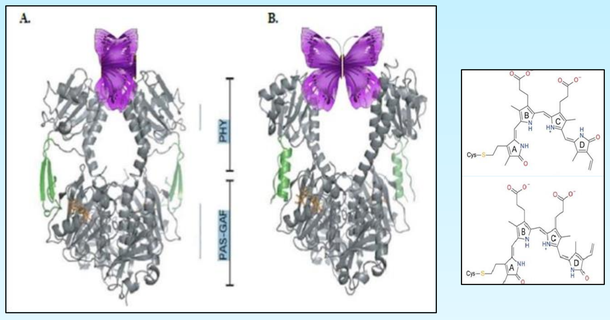

Fig. 2. Left: Inactive ground state showing an "out of phase" conformation (A); Active signaling state showing an "in phase" arrangement of output domains of a BphP (B). Right: D ring of BV IX α flipsover.

- **Guanylate cyclase (GC)**
  - Produce **cyclic guanosine monophosphate (cGMP)** (2)
    - ❖ Regulate fewer biological pathways
      - ○ Offer targeted treatment options
- **Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel (HCN)** regulated by cGMP (Fig. 3) (3)
  - Control neuronal and pacemaker cells

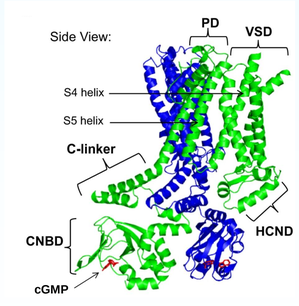

Fig. 3. Structure of HCN channel depicting cGMP binding to CNBD.

- **NIRW light activated GC will enable specific control of physiological functions related to cGMP**

Cyclases PaaC7 and PagC7

- PaaC7 and PagC7
  - Weak light activated AC/GC (4)
  - High dynamic range
- Coiled-coil region between BphP and AC/GC
  - More rigid conformation

Design of NIRW Light Activated GC Constructs

Strategy 1: Changing the Preference

- Observed conserved amino acids (AA) (Fig. 4)

Fig. 4. Amino acids' sequence of IlaM5-AC, various AC, and GC (alignment).

- Introduced E and R or K mutations to IlaM5-AC
  - Produced **IlaM5 ER_{23}** and **IlaM5 EK_{37}**

Strategy 2: Changing the Linker

- Linker: AA sequence between BphP and human GC
- Changed the linker length
  - Produced F0-F7 (Fig. 5)

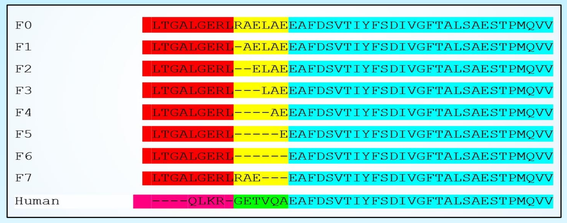

Fig. 5. Fusion 0-7 designs; Red sequence is end of BphP, yellow sequence is linker sequence, and blue sequence is beginning of GC.

- NNK mutagenesis in linker
  - Produced **NNK 67 and NNK 85**
  - Mutation of A to R of second to last amino acid

Testing of Constructs

- Blue/White LacZ assay
  - Blue colonies if cGMP present
  - Plates exposed to NIRW light or covered with foil
- Reporter strains A388 and A390
  - A388 contain transcriptional factor crp (Fig. 6) (5)
  - A390 contain transcriptional factor crp_G

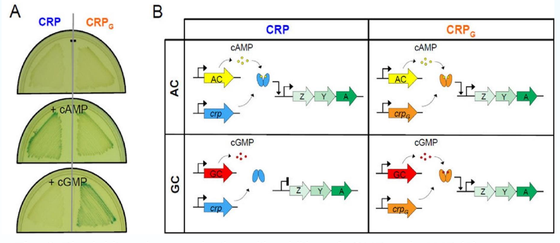

Fig. 6. Blue/white colony production with addition of cGMP and cAMP (A); Comparison of cAMP and cGMP binding to crp and crp_G and *lacz* activation (B) (5).

Initial Results

- IlaM5 ER_{23} and IlaM5 EK_{37} not light activated
- F0, F2, F3, NNK 67, and NNK 85 potentially light activated
- Heterogenous results warranting further studies

Objective

- Verify photoactivity of past potential NIRW light activated GC constructs
- Control HCN channel activity through membrane localization of weaker NIRW light activated GC

Methods

Photoactivity Verification of GC Constructs

- F0, F2, F3, NNK 67, and NNK 85 chosen for verification
- Constructs transformed into A388 and A390
- Plated transformants
- Resuspended six colonies of each construct
  - Replica plated colonies
- Blue/White LacZ assay was performed

Photoactivity Verification of IlaM5 BphO

- Transformed IlaM5 BphO into BIK strain
- Same procedures were followed to plate and test as in photoactivation verification of GC

Designing Membrane Localized GC Construct

- pAAV IlaM5 used as vector backbone (VB)
  - Plasmid cut with EcoRI and NrUI
- PCR amplified BphP from pAAV IlaM5
- Assembled PaaC7, BphP, and VB with Gibson Assembly

Results and Discussion

Photoactivity Verification of GC Constructs

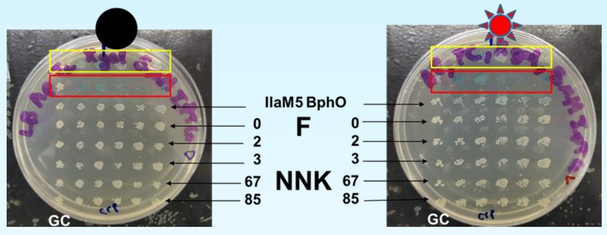

Fig. 7. Blue/White screening of designed GC constructs in A388 at 5 μM IPTG; Positive control in red box (constitutively active AC) and negative control in yellow box (empty pET vector); Left, dark condition; Right, light condition.

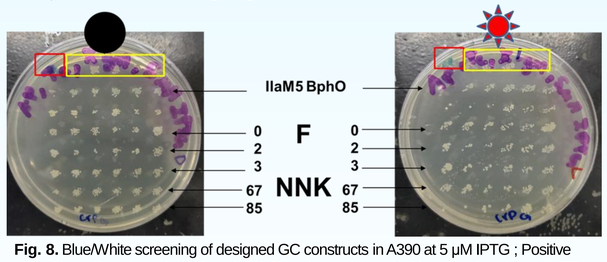

Fig. 8. Blue/White screening of designed GC constructs in A390 at 5 μM IPTG ; Positive control in red box (constitutively active GC) and negative control in yellow box (empty pET vector); Left, dark condition; Right, light condition.

GC constructs in A388

- F0, F2, F3, NNK 67, and NNK 85 produced only white colonies (Fig. 7)
  - Expected white colonies
  - cGMP not bind to crp
- IlaM5 BphO produced blue colonies in light and dark (Fig. 7)
  - No significant difference between light and dark
  - Expected greater difference

GC constructs in A390

- F0, F2, F3, and NNK 85 produced only white colonies (Fig. 8)
  - Indicated minimal cGMP production
  - Not light-dependent
- NNK 67 and 85 produced few blue colonies in light and dark (Fig. 8)
  - Some cGMP production and not light-dependent

Photoactivity Verification of IlaM5 BphO

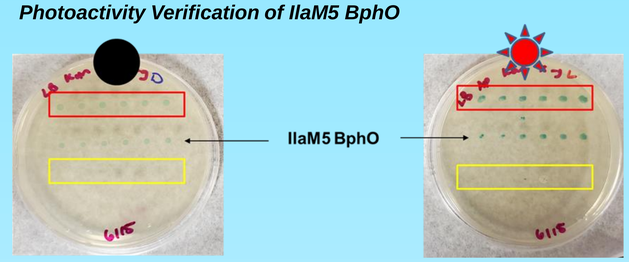

Fig. 9. Blue/White screening of IlaM5 BphO in BIK strain; Positive control in red box (IlaM5-AC) and negative control in yellow box (empty pET vector); Left, dark condition; Right, light condition.

- IlaM5 BphO produced cAMP in light-dependent manner (Fig. 9)
  - Minimal blue in dark, robust blue in light

pAAV PaaC7 Construct

- Three-piece Gibson assembly was successful (Fig. 10)

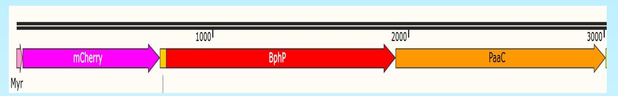

Fig. 10. Scheme of PaaC7 construct

Conclusion and Future Directions

Conclusion

- Past GC constructs were not light-dependent
- IlaM5 BphO not as light-dependent in A388

Future Directions

- Verify PaaC7 light-dependent activity
- Convert PaaC7 to PagC7
- Clone in myristoylation (Myr) tag
- Test light-dependent HCN channel activity
- PagC7 membrane localization enable greater HCN channel control

Acknowledgments

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