MOLECULAR CHARACTERIZATION AND EXPRESSION OF $G_{q/11}$ PROTEIN IN FISHES

Thesis

Presented to the Graduate Council of Texas State University-San Marcos in Partial Fulfillment of the Requirements for the Degree Master of SCIENCE

by

Varsha Radhakrishnan, B.F.Sc.

San Marcos, Texas
May 2007
MOLECULAR CHARACTERIZATION AND EXPRESSION
OF $G_{\alpha11}$ PROTEIN IN FISHES

Committee Members Approved:

__________________________
Dana M. García, Chair

__________________________
Joseph R. Koke

__________________________
Dittmar Hahn

Approved:

__________________________
Nihal Dharmasiri

J. Michael Willoughby
Dean of the Graduate College
ACKNOWLEDGEMENTS

First of all, I would like to thank my mentor Dr. Dana García for her patience, encouragement and overall support through the ups and downs of my research project. Dr. García and her family always treated me like an extended family member, which made it easy for me to talk to her about anything and everything. She is truly a remarkable lady and has contributed a lot to both my personal and professional life.

I offer my sincere thanks to Dr. Joseph Koke who helped me with confocal and electron microscopy and also with my few initial attempts in starting a primary culture of bluegill RPE. I appreciate and thank Dr. Dittmar Hahn for his support and advice whenever I needed them and also for allowing me full access to his lab. Thanks are also due to Dr. Nihal Dharmasiri for his general advice, critical comments and help with a few molecular techniques.

My special thanks to Dr. Michael Forstner who made me realize my “real potential” and helped me with phylogenetic methods and to Dr. Chris Nice who let me borrow materials from his lab whenever I needed them. I also thank Mr. Prasad Phatarpekar, Dr. Simon Durdan and Mr. Chad Copeland, my former lab mates, for their patience in listening and answering my endless questions. My thanks to Mr. James Neece and Ms. Margaux Salas for introducing me to basic molecular techniques when I first joined the lab. Among our current lab members, I offer my special thanks to Ms. Liz Crittenden and Mr. Adam Johnson for helping me with sectioning and providing me with
fish tissues whenever I needed it. I would also like to thank undergraduate students in our lab who have helped me with my project at one time or the other, especially Mr. CJ Schubert and Ms. Hannah Riezenman. I am immensely grateful to members from other labs, Mr. Zach Gompert, Ms. Allana Welsh and Ms. Katie Saul, for their help and guidance with some techniques during the course of my research.

Last but not the least, I would like to thank and dedicate this thesis to my family for encouraging and supporting me to pursue my ambition. Without their continued support and prayers, I could not have successfully completed this project.

This work was supported by National Science Foundation grants (IOB-0615762, IOB-0634704, IOB 05-33445 and IOB-0235523) and a Texas State Research Enhancement Award awarded to Dr. Dana García.

This manuscript was submitted on January 27, 2007.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
</tbody>
</table>

## CHAPTER

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>II.</td>
<td>MATERIALS AND METHODS</td>
</tr>
<tr>
<td>III.</td>
<td>RESULTS</td>
</tr>
<tr>
<td>IV.</td>
<td>DISCUSSION</td>
</tr>
</tbody>
</table>

REFERENCES                                                                 | 68  |
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ga-protein classification, expression and their effectors in mammalian tissues</td>
<td>9</td>
</tr>
<tr>
<td>2. Table showing oligonucleotide primers used for the isolation of Ga(_{11}) and Ga(_q) fragment from bluegill</td>
<td>16</td>
</tr>
<tr>
<td>3. List of scientific name, common name, code name, and accession numbers of DNA and amino acid sequences used in alignment and phylogenetic analyses</td>
<td>20</td>
</tr>
<tr>
<td>4. Table showing percent identity and percent similarity of bluegill Ga(_{11})-like fragment and Ga(<em>q) fragment with Ga(</em>{11}) fragments of other vertebrates at the amino acid level using BL2SEQ program</td>
<td>29</td>
</tr>
<tr>
<td>5. Table showing percent identity and percent similarity of bluegill putative Ga(<em>q) and Ga(</em>{11}) fragments with Ga(_q) fragments of other vertebrates at the amino acid level using BL2SEQ program</td>
<td>35</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pigment granule movements in fish retinal pigment epithelium</td>
<td>3</td>
</tr>
<tr>
<td>2. Model of $G_{\alpha q/11}$ protein activation</td>
<td>8</td>
</tr>
<tr>
<td>3. Position of PCR fragments for the $G_{\alpha 11}$-like fragment obtained using primers listed in Table 2</td>
<td>25</td>
</tr>
<tr>
<td>4. A comparison of the gene structure of mammalian $G_{\alpha 11}$ with the $G_{\alpha 11}$-like gene fragment retrieved from bluegill cDNA</td>
<td>26</td>
</tr>
<tr>
<td>5. Nucleotide sequence of bluegill $G_{\alpha 11}$-like fragment and the deduced amino acid residues</td>
<td>27</td>
</tr>
<tr>
<td>6. Alignment of deduced amino acid sequences of bluegill $G_{\alpha 11}$-like fragment with published vertebrate $G_{\alpha 11}$ sequences using CLUSTAL W</td>
<td>28</td>
</tr>
<tr>
<td>7. Position of PCR fragments for bluegill $G_{\alpha q}$ obtained using primers listed in Table 2</td>
<td>31</td>
</tr>
<tr>
<td>8. Nucleotide sequence of the bluegill $G_{\alpha q}$ fragment and the deduced amino acid residues</td>
<td>32</td>
</tr>
<tr>
<td>9. A comparison of the gene structure of mammalian $G_{\alpha q}$ with the $G_{\alpha q}$ gene fragment retrieved from bluegill cDNA</td>
<td>33</td>
</tr>
<tr>
<td>10. Alignment of deduced amino acid sequence of bluegill $G_{\alpha q}$ fragment with published vertebrate $G_{\alpha q}$ sequences using CLUSTAL W</td>
<td>34</td>
</tr>
<tr>
<td>11. Consensus parsimony tree for nucleotide alignment obtained by heuristic search of the dataset</td>
<td>37</td>
</tr>
<tr>
<td>12. Neighbor joining tree for DNA alignment with Tamura-Nei model as distance correction obtained by heuristic search with five thousand random stepwise replications</td>
<td>38</td>
</tr>
<tr>
<td>13. The consensus maximum likelihood tree topology using GTR+I+G model of evolution</td>
<td>39</td>
</tr>
</tbody>
</table>
14. Tree showing Bayesian analysis of posterior probability using the GTR+I+G model of evolution........................................................................................................................................40
15. Consensus parsimony tree for protein alignment obtained using heuristic search of the dataset ........................................................................................................................................41
16. RT-PCR results showing expression of Gaq and Ga11-like (Gα10) mRNA in bluegill (a) and zebrafish (b)..................................................................................................................42
17. Immunolocalization of Gaq/11 in bluegill choroid (a) and RPE/photoreceptors (b)....44
18. Immunolocalization of Gaq/11 in bluegill retina...........................................................45
19. Immunolocalization of Gaq/11 in zebrafish RPE/photoreceptors ............................46
20. Immunolabeling of Gaq/11 in 7 dpf zebrafish eyes.......................................................48
21. Immunolabeling of Gaq/11 in 14 dpf zebrafish eyes.......................................................49
22. Immunolabeling of Gaq/11 in 21 dpf zebrafish eyes.......................................................50
23. Immunolabeling of Gaq/11 in 28 dpf zebrafish eyes.......................................................51
24. Immunolabeling of Gaq/11 in adult zebrafish eyes.......................................................52
ABSTRACT

MOLECULAR CHARACTERIZATION AND EXPRESSION
OF G_q/11 PROTEIN IN FISHES

by

Varsha Radhakrishnan, B.F.Sc.

Texas State University-San Marcos
May 2007

SUPERVISING PROFESSOR: DANA M. GARCÍA

Teleost fishes and lower vertebrates adjust to changing light conditions by diurnal movements of melanin pigment granules in their retinal pigment epithelium (RPE). RPE is not directly activated by light and several observations suggest that some extracellular signal from the retina may trigger the cascade of events leading to the granular movement in RPE. Findings by González et al. (2004) and Phatarpekar et al. (2005) suggest that pigment granule movements in fish RPE may be induced via G-protein coupled muscarinic receptors. Based on these findings, I hypothesize that bluegill (Lepomis macrochirus) RPE expresses at least one member of the G_q/11 family that mediates
the signal from the activated muscarinic receptor to the effector. To test this hypothesis, I characterized cDNAs encoding two G-proteins from the Gq/11 family. The subtype identity of these novel cDNAs and the encoded G-protein fragments from bluegill were examined using phylogenetic analyses. Their expression was examined in adult bluegill and zebrafish eyes using RT-PCR and immuno-histochemistry. Immunolabeling was also used to study the ontogeny of Gαq/11 expression in zebrafish eyes. My results indicate that bluegill express both bona fide Gαq and a second member of the Gαq/11 family, but that only Gαq is expressed in eye. These findings imply that Gαq could play a specific role in pigment movement in fish eyes. It also lays the groundwork for the future elucidation of the function of these genes in carbachol-induced pigment granule dispersion.
CHAPTER I

INTRODUCTION

Light adaptation is the ability of the visual system to adjust its sensitivity and performance to the ambient level of illumination. In a normal day-night cycle, the illumination on earth’s surface varies by more than ten orders of magnitude, making light adaptation highly important for the survival of an organism. In vertebrate eyes, photoadaptation occurs at several stages within the retinal network (Purpura et al., 1990; Schmitt and Dowling, 1999). For example, in mammals and higher vertebrates, the amount of light reaching the retina is controlled by their adjustable pupil. In addition, within the photoreceptors, many mechanisms and several stages for adaptation have been reported (see Hood and Birch, 1993). For instance, nocturnal organisms like cats, mice and rats have a rod-dominant retina while some organisms like the lizards and ground squirrels have a cone-dominant retina (Farber et al., 1981). In lower vertebrates like teleost fishes and amphibians that have a fixed pupil, light adaptation is achieved by retinomotor movement, a collective term used to describe movements of the rod and cone photoreceptors in the retina and the movement of melanin pigment granules in the retinal pigment epithelium (Douglas, 1982; Burnside and Nagle, 1983). Retinal pigment epithelium (RPE) is a monolayer of tissue located between the neural retina and choroid that functions in the physiological support of the retina (see Zinn and Marmor, 1979).
In teleost fishes, during light, the melanin pigment granules migrate from the cell body of the RPE into the apical projections that interdigitate with the rod photoreceptors (Figure 1a), shielding them from photobleaching (Douglas, 1982; Burnside and Nagle, 1983; King-Smith et al., 1997; García, 1998). Likewise, during light, the cones remain contracted and closer to incoming light than rods (Cavallaro and Burnside, 1988). In dark, the position of rod and cone photoreceptors are reversed, and the melanin pigment granules aggregate at the base of the RPE (Figure 1b) allowing the photoreceptors to maximize light capture (Burnside and Nagle, 1983; Cavallaro and Burnside, 1988; Iuvone et al., 2005).

Photoreceptors (rods and cones) respond directly to light and some endogenous circadian cues (McCormack and Burnside, 1991; Burnside, 2001). For example, serotonin (Zaunreiter et al., 1998b), melatonin (Zaunreiter et al., 1998a), and dopamine (Dearry and Burnside, 1989) have been linked to the retinal circadian clock in fishes. But RPE do not respond directly to light. In other words, RPE isolated from retina does not disperse pigment in response to light, nor does RPE with dispersed pigments aggregate pigment granules in the dark (Garcia and Burnside, 1994). Rather, pigment dispersion in RPE is presumed to be dependent on neurotransmitters and perhaps other small molecules diffusing from the neural retina (García and Burnside, 1994).
Figure 1. Pigment granule movements in fish retinal pigment epithelium. Cartoon depicting a cell of retinal pigment epithelium (a) during light, showing fully dispersed pigment granules with the rods (red) elongated and cone (green) contracted and (b) during dark when the pigment granules are aggregated, with the rods (red) contracted and cone (green) elongated between the apical processes.
Using pharmacological studies, Dearry and Burnside (1988) first reported that dopamine, acting via D2 receptors, may regulate retinomotor movements in green sunfish (*Lepomis cyanellus*) RPE, specifically by causing light-adaptive responses. Their studies also showed that dopamine induces light-adaptive cone contraction in isolated dark cultured retina and that when D2 receptors were blocked, cone contraction was also inhibited (Dearry and Burnside, 1986). Furthermore, dopaminergic cells were identified in the inner plexiform layer of retina by Mangel and Dowling (1987).

Later, it was shown that retinomotor movements persisted in fish whose dopaminergic cells had been ablated by treatment with 6-hydroxydopamine (Douglas *et al.*, 1992; Ball *et al.*, 1993). This suggested that neurochemicals in addition to dopamine may be involved in light-induced pigment granule dispersion. The evidence that the cholinergic agent, carbachol, can induce pigment granule dispersion (García, 1998) was the first study that raised the possibility of acetylcholine acting in addition to dopamine in regulating retinomotor movements. The idea of cholinergic signaling in light adaptation is relatively new and much less well derived. Studies have shown that flicker stimulation increases acetylcholine release and that the amount of release is proportional to flicker frequency (Vivas and Drujan, 1980). Furthermore, cholinergic cells have been identified in the amacrine cells of the retina (Arenzana *et al.*, 2005). Thus, after numerous studies on retinomotor movements in the last two decades, a possibly new model of signal transduction has been suggested for light adaptation by González *et al.* (2004) and Phatarpekar *et al.* (2005). Their results suggest that light adaptive pigment granule dispersion in fish RPE may be elicited via specific G-protein coupled receptors called muscarinic receptors.
Muscarinic receptors are cholinergic, heptahelical receptors that are of five subtypes in mammals (M1-M5) (Caulfield and Birdsall, 1998), though only M2, M3 and M5 receptor subtypes have been described in fishes. Zebrafish has been shown to express both M2 and M5 muscarinic receptors (Hsieh and Liao, 2002) while only M3 subtype has been described in catfish melanophores (Hayashi and Fujii, 1994). Using molecular and pharmacological studies, Phatarpekar et al. (2005) characterized M2 and M5 receptor subtypes from bluegill cDNA and studied their expression in bluegill RPE and other tissues. Their results show that M5 but not M2 receptor subtype is expressed in bluegill RPE and hence is the more likely mediator of carbachol-induced pigment granule dispersion.

The model of signal transduction suggested by Phatarpekar et al. (2005) involves the activation of the M5 receptor by carbachol which triggers a member of the G\(_{q/11}\) protein family involved in phosphoinositide hydrolysis, that further activates a cascade of events leading to pigment granule dispersion in RPE. Though this model is well supported by several studies, it is still in need of further testing to clarify the specific G\(\alpha\)\(_{q/11}\)-protein, its effector and further downstream events. Pharmacological agents like GPAnt-2a (Yajima et al., 1997) and YM-254890 (Takasaki et al., 2004) have been reported to be highly selective in blocking G\(\alpha_{q/11}\) proteins. While these drugs are powerful tools to study G\(\alpha_{q/11}\)-mediated signaling, they have been characterized predominantly for mammals and may not be highly selective for fish. Furthermore, no pharmacological agents that are capable of selectively activating or blocking individual members of G\(\alpha_{q/11}\) family have been identified thus far or made commercially available. So molecular characterization of G\(\alpha_{q/11}\) subtypes and studies of their expression in fish
RPE might help resolve the step following muscarinic receptor activation in the model suggested by Phatarpekar et al. (2005).

Findings by Phatarpekar et al. (2005) led me to hypothesize that \( \text{G} \alpha_q \) and/or \( \text{G} \alpha_{11} \) may be expressed in bluegill RPE and may have a specific role in the signaling pathway that leads to pigment granule dispersion. The focus of my thesis research was to molecularly characterize \( \text{G} \alpha_q \) and \( \text{G} \alpha_{11} \) in bluegill sunfish (\textit{Lepomis macrochirus}) and to study their expression in fish eyes using RT-PCR and immuno-histochemistry.

G-proteins are guanine nucleotide-binding, heterotrimeric, membrane-associated proteins that function as molecular transducers, relaying signals from cell-surface receptors to downstream effectors. They are composed of \( \alpha \) (39-52 kDa), \( \beta \) (35-36 kDa) and \( \gamma \) (8-10 kDa) subunits (Birnbaumer, 1990; Simon et al., 1991). They have a conserved structure and share a common molecular switch mechanism mediated by the binding and hydrolysis of GTP (Bourne et al., 1990). In resting state, the \( \alpha \)-subunit is bound to GDP and all three subunits remain associated as a trimer (Figure 2a). Activation of the receptor by an agonist induces a shift in receptor conformation, triggering the G-protein activation by replacement of GDP by GTP in the \( \alpha \)-subunit and dissociation of the \( \alpha \)-subunit from the \( \beta \gamma \) dimer (Figure 2b). Both the \( \alpha \)-subunit and the \( \beta \gamma \) dimer can then interact independently with specific effectors (Gilman, 1987; Birnbaumer, 1990). The hydrolysis of GTP to GDP causes the reassociation of the \( \alpha \)-, \( \beta \)- and \( \gamma \)-subunits leading to G-protein inactivation. As of 2003, 28\( \alpha \), 5\( \beta \) and 12\( \gamma \) subunits have been identified and sequenced (Cabrera-Vera et al., 2003). Based on their \( \alpha \) subunit sequence homology and function, \( \text{G} \alpha \)-proteins are broadly divided into \( \text{G} \alpha_s \), \( \text{G} \alpha_i \), \( \text{G} \alpha_{q/11} \) and \( \text{G} \alpha_{12/13} \) (Simon et al.,
The G-proteins under each family, their function and expression in various mammalian tissues are given in Table 1.

The $\alpha_{q/11}$ class of G-proteins have been the focus of numerous studies not only due to their profound effects on the pathology of chronic inflammations overall (Das et al., 2000), but also due to their role in heart disease (Webster and Bishopric, 2003), Alzheimer’s disease (Kelly et al., 2005), asthma (Gosens et al., 2006), autism (Allgeier et al., 1994), and many others. The four members of this family, namely $\alpha_q$, $\alpha_{11}$, $\alpha_{14}$ and $\alpha_{15/16}$, are pertussis toxin-insensitive and are involved in phosphoinositide hydrolysis.
Figure 2. Model of $\text{G}_q/11$ protein activation. Diagram showing (a) $\text{G}_q/11$ in its resting state with all subunits associated as a trimer and (b) $\text{G}_q/11$ activation by replacement of GDP with GTP and dissociation from $\beta\gamma$ dimer in response to ligand binding receptor. The activated GTP-associated $\text{G}_q/11$ activates phospholipase C-$\beta$ (PLC $\beta$) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).
Table 1. Gα-protein classification, expression and their effectors in mammalian tissues. This table was adapted from a review by Wettschureck and Offermanns (2005).

<table>
<thead>
<tr>
<th>G-protein family</th>
<th>Members</th>
<th>Expression</th>
<th>Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gαs</td>
<td>Gαs</td>
<td>Ubiquitous</td>
<td>▲AC (all types)</td>
</tr>
<tr>
<td></td>
<td>Gαolf</td>
<td>Olfactory epithelium, brain</td>
<td>▲AC</td>
</tr>
<tr>
<td></td>
<td>GαsXL</td>
<td>Neuroendocrine</td>
<td>▲AC</td>
</tr>
<tr>
<td>Gαi</td>
<td>Gαi1</td>
<td>Widely distributed</td>
<td>▼AC(I, III, V, VI, VIII, IX)</td>
</tr>
<tr>
<td></td>
<td>Gαi2</td>
<td>Ubiquitous</td>
<td>▼AC</td>
</tr>
<tr>
<td></td>
<td>Gαi3</td>
<td>Widely distributed</td>
<td>▼AC</td>
</tr>
<tr>
<td></td>
<td>1Gαo1</td>
<td>Neuronal, neuroendocrine</td>
<td>▼VDCC</td>
</tr>
<tr>
<td></td>
<td>2Gαo2</td>
<td>Neuronal, neuroendocrine</td>
<td>▼VDCC</td>
</tr>
<tr>
<td></td>
<td>3Gαo3</td>
<td>Brain</td>
<td>Unclear</td>
</tr>
<tr>
<td></td>
<td>Gαz</td>
<td>Neuronal, platelets</td>
<td>▲AC(V,VI), Rap1GAP</td>
</tr>
<tr>
<td></td>
<td>Gαgust(3)</td>
<td>Taste cells, brush cells</td>
<td>▲PDE</td>
</tr>
<tr>
<td></td>
<td>Gαs1</td>
<td>Rods, taste cells</td>
<td>▲PDE 6 (γ-subunit)</td>
</tr>
<tr>
<td></td>
<td>Gαs2</td>
<td>Cone photoreceptors</td>
<td>▲PDE 6 (γ-subunit)</td>
</tr>
<tr>
<td>Gαq/11</td>
<td>Gαq</td>
<td>Ubiquitous</td>
<td>▲PLC β 1-4</td>
</tr>
<tr>
<td></td>
<td>Gα11</td>
<td>Almost ubiquitous</td>
<td>▲PLC β 1-4</td>
</tr>
<tr>
<td></td>
<td>Gα14</td>
<td>Lung, kidney, testes, spleen</td>
<td>▲PLC β 1-4</td>
</tr>
<tr>
<td></td>
<td>4,5Gα15/16</td>
<td>Hematopoietic cells, keratinocytes</td>
<td>▲PLC β 1-4</td>
</tr>
<tr>
<td>Gα12/13</td>
<td>Gα12</td>
<td>Ubiquitous</td>
<td>PDZ-RhoGEF/LARG, Btk, Gapl, cadherin</td>
</tr>
<tr>
<td></td>
<td>Gα13</td>
<td>Ubiquitous</td>
<td>P115RhoGEF, PDZ-RhoGEF/LARG, Radixin</td>
</tr>
</tbody>
</table>

1,2,3 - Nürnberg et al., 1994; 4 - Taylor et al., 1991; 5 - Wu et al., 1992.
▲, activates; ▼, inhibits; AC, adenylyl cyclase; PDE, phosphodiesterase; PLC, phospholipase C; VDCC, voltage-dependent Ca2+ channel; RhoGEF, Rho guanine nucleotide exchange factor; Btk, Bruton’s tyrosine kinase.

All four members activate phospholipase C-β isomers that stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), which yields inositol 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG), both of which can act as intracellular second messengers (Wu et al., 1992; Broadley and Kelly, 2001). IP3 stimulates the
release of calcium by binding to receptors located on the endoplasmic reticulum. DAG and an increase in intracellular calcium levels activate protein kinase C (Broadley and Kelly, 2001). Calcium has many other effectors as well.

The sequence homology and expression of the members of $\text{Ga}_q/11$ family vary. While $\text{Ga}_q$ and $\text{Ga}_{11}$ share 88% homology in mammals and are expressed ubiquitously (Strathmann and Simon, 1990; Milligan and Mitchell, 1993), $\text{Ga}_{14}$ is 81% identical to $\text{Ga}_q$ and its expression is restricted to kidney, spleen, lung and testes in mammals (Wilkie et al., 1992). $\text{Ga}_{15}$ and its human counterpart $\text{Ga}_{16}$ are less than 60% homologous to $\text{Ga}_q$ and are restricted to hematopoietic tissue (Amatruda et al., 1991; Wilkie et al., 1992) and keratinocytes in the skin (Rock et al., 1997) in mammals. Despite being members of the $\text{Ga}_{q/11}$ family, $\text{Ga}_{14}$ and $\text{Ga}_{15/16}$ are not likely to be involved in light adaptive pigment granule movements for two reasons.

Firstly, both members have restricted expression in specific tissues like spleen, kidney, testes, lung and hematopoietic cells, respectively. Moreover, there has not been any report about the expression of these subtypes in vertebrate eyes. Secondly, despite their promiscuity in receptor coupling, neither $\text{Ga}_{14}$ nor $\text{Ga}_{16}$ has been shown to couple to muscarinic receptors. While $\text{Ga}_{14}$ has been reported to couple to histamine receptors (Kuhn et al., 1996) and $\alpha_1$-adrenergic and interleukin-8 receptors in transfected COS-7 cells (Wu et al., 1992), $\text{Ga}_{15/16}$ has been shown to be coupled to chemokine receptors for interleukin 8, C5a, and fMLP (Offermanns and Simon, 1995), and histamine receptors (Kuhn et al., 1996). But both $\text{Ga}_q$ and $\text{Ga}_{11}$ couple indiscriminately to a wide range of receptors including muscarinic, histamine, parathyroid hormone, thyrotropin-releasing hormone, gastrin-releasing peptide, vasopressin, gonadotrophin releasing hormone,
angiotensin II, bradykinin and $\alpha_1$-adrenergic receptors (Shapira et al., 1998), and both members are ubiquitously expressed in mammalian tissues.

These observations raise the possibility that $G_{\alpha_{11}}$ and/or $G_{\alpha_q}$ may be involved in transmitting the signal from the muscarinic receptor to the effector. In addition, both members have played a role in light adaptation in many vertebrate and invertebrate models. For example, using immuolabelling and in situ hybridization techniques, some studies (Peng et al., 1997; Kasahara et al., 2002) showed that $G_{\alpha_{11}}$ is expressed in chick photoreceptors and pinealocytes, and that its activation may play a role in light adaptive phase shifting in pinealocytes. Peng et al. (1997) showed $G_{\alpha_{11}}$ immunoreactivity in the rod photoreceptors and $G_{\alpha_q}$ immunoreactivity in the amacrine layer of bovine retina.

Among invertebrates, $G_{\alpha_q}$ expression has been documented in Drosophila (Strathmann and Simon, 1990), crayfish (Terakita et al., 1996) and in squid photoreceptors (Suzuki et al., 1995). Other studies have also shown the involvement of $G_{\alpha_o}$-mediated signaling in invertebrate vision (Terakita et al., 1996).

In spite of the numerous studies on the signaling pathway of retinomotor movements in fish, little is known about the G-protein subtypes expressed in fish eyes or their role in light adaptation. To my knowledge, there is only one study (Sarwar et al., 1996) on G-protein subtypes expressed in fish. Sarwar et al. (1996) aligned $G_{\alpha}$ subunits from Japanese puffer fish (Takifugu rubripes) and compared their similarities with those of mammalian G-proteins. Though their study showed that Takifugu sequences were homologous to their mammalian orthologs, 90% of the amino acid sequences used in their study were partial (representing 30% of the expected sequence), and no phylogenetic inferences about relationships among G-protein subtypes were evaluated.
Similarly, though Ga proteins are expressed throughout the prenatal development of vertebrates, only a few studies have addressed the role of Ga-protein in vertebrate development (Wettschureck and Offermanns, 2005). Most insights into their role in development were from studies on mutant mice lacking specific Ga-subtypes. It was shown that null mutations of the genes coding for individual Ga-subtypes proved to be lethal to embryos (Offermanns et al., 1998), thus indicating their pivotal role in development. Another report by Shah and Hausman (1996) suggest the involvement of Ga_{q/11} in retinal development in chick.

In this thesis, I report my results from examining the G-protein subtypes expressed in bluegill using a PCR approach, laying the groundwork for future elucidation of their role in carbachol-induced pigment granule dispersion. The subtype identity of one novel G-protein cDNA fragment (Ga_{q}) from bluegill was confirmed using several phylogenetic methods. Analysis of the second G-protein cDNA fragment failed to yield a definitive identity based on the current nomenclature, raising the possibility that a new subclass should be designated (e.g. G_{q1} and G_{q2} or G_{10}). The expression of these genes was examined in bluegill and zebrafish eyes using RT-PCR and immuno-histochemistry. Immunolabelling was also used to study the ontogeny of G-protein expression in zebrafish eyes. My results indicate that of the two G-proteins uncovered, only Ga_{q} is expressed in retina and RPE. These findings suggest that Ga_{q} could be involved in the signal transduction pathway linking muscarinic receptor activation to pigment granule dispersion.
CHAPTER II

MATERIALS AND METHODS

Fish maintenance

Bluegill (*Lepomis macrochirus*) and zebrafish (*Danio rerio*) were kept in aerated 55 gallon and 10 gallon aquaria, respectively, on a 12 hour light/12 hour dark cycle room for at least two weeks prior to use. The protocols for animal use were approved by the Texas State Animal Care and Use Committee (Protocol # 069744F82). Prior to killing, the fish were anesthetized using tricaine methysulfonate (Western Chemical Inc., Scottsdale, AZ).

Isolation of total RNA and generation of cDNA

Total RNA was isolated from 10 mg samples of bluegill brain, heart, retina and RPE using an RNAqueous-4PCR kit (Ambion, Austin, TX), following the manufacturer’s instructions. For zebrafish, the RPE and retina were too difficult to separate without contamination from other tissues and hence total RNA from whole eyes was extracted. Briefly, the tissue was isolated and homogenized in RNase-free tubes provided in the kit using guanidinium thiocyanate and passage through silica-based buffer. Total RNA (8 μl) was then used to generate cDNA, using the RETROscript RT-PCR kit (Ambion, Austin, TX), following the manufacturer’s instructions. The two-step
protocol involved denaturing of total RNA and oligo (dT) primers at 82°C for 3 min before adding remaining RT solutions, including RT buffer and reverse transcriptase.

**PCR amplification of cDNA**

The cDNA transcripts were amplified in a 50μl polymerase chain reaction. The primers for the reaction were originally designed based on the alignment of vertebrate Ga11 and Gaq sequences. The complete coding sequences of Ga11 and Gaq were downloaded in FASTA format (Pearson, 1990) from GenBank (Bilofsky and Burks, 1988) database. As no fish Ga11 and Gaq sequences were available for alignment, chick Ga11 and mouse Gaq were used as query sequences to search the *Takifugu rubripes* genome available at [http://www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html](http://www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). The putative *Takifugu* sequences were then aligned with other vertebrate G-protein sequences using CLUSTAL W program (Thompson *et al.*, 1994). Highly homologous regions near the 5' and 3' ends of the alignment were selected for designing primers. The forward and reverse primers designed were about 18-23 bps long and their compatibility, G+C percent and self-complementarity were checked using an oligonucleotide properties calculator available online at [http://www.basic.northwestern.edu/biotools/OligoCalc.html](http://www.basic.northwestern.edu/biotools/OligoCalc.html). The list of primers used for amplifying putative Ga11 and Gaq from bluegill brain cDNA are given in Table 2. The primers were purchased from BioSynthesis Inc, Lewisville, TX. The primers were reconstituted in TE buffer (comprising 10 mM Tris pH 7.5 and 1mM EDTA) and were diluted further in PCR-grade water before use.
PCR was carried out by adding 1 μl cDNA, 3 μl MgCl (25 mM), 10 μl 5X GoTaq flexi buffer, 2 μl dNTPs (10 mM each), 0.5 μl GoTaq Flexi DNA polymerase (5 μ/μl) (Promega Inc, WI), 1 μl each of the primers and 31.5 μl nuclease-free water and subjecting the mixture to the following conditions: one cycle of initial denaturation at 94°C for 1 min 4 sec, 40 cycles each of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min 30 sec, and a cycle of final extension at 72°C for 5 min. The PCR products and 1kb GoTaq DNA ladder (Promega Inc, WI) were loaded onto a 1% agarose gel inundated with TAE buffer (containing 0.4 M Tris, 0.01 M EDTA and 0.2 M acetic acid, pH 8.5) and were subjected to electrophoresis at 120V for 1hr. To verify the presence and the size of the product, the gel was stained in ethidium bromide for 5 min and observed under ultraviolet light after destaining in distilled water. A photograph of the gel was obtained using Nikon COOLPIX 990 digital camera with a UV filter, and the picture was processed with Adobe Photoshop Version 5.5 software (Adobe Systems Inc., San Jose, CA). PCR products of approximately 1000 bp were purified using High Pure PCR Product Purification Kit (Roche Diagnostics Corp, IN) following the instruction manual, and sequenced using automated cycle sequencing method at Retrogen Inc, CA. The sequences obtained were analyzed and edited individually using the software Sequencher 4.2 (Gene Codes Inc., Ann Arbor, MI).
Table 2. Table showing oligonucleotide primers used for the isolation of $G_{\alpha_{11}}$ and $G_{\alpha_q}$ fragment from bluegill. The length of the primers, their percent GC content and melting temperatures are also provided.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Primer length</th>
<th>G+C content (%)</th>
<th>Melting temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G11F1</td>
<td>ATG ACT CTG GAG TCC ATG ATG G</td>
<td>22</td>
<td>50</td>
<td>66</td>
</tr>
<tr>
<td>G11F2</td>
<td>GAA TCA GAC AAC GAG AAC CGC A</td>
<td>22</td>
<td>50</td>
<td>66</td>
</tr>
<tr>
<td>G11F3</td>
<td>GTA CTC AGA CGA GGA TAA GAG</td>
<td>21</td>
<td>47.6</td>
<td>62</td>
</tr>
<tr>
<td>G11R1</td>
<td>TTG AGG TTG AGG ATG GTG TC</td>
<td>20</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>G11R2</td>
<td>GCT GTA GGA TGG TGT CTT TG</td>
<td>20</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>G11R3</td>
<td>GGC GGT CAT AGG CCT CTT GG</td>
<td>20</td>
<td>65</td>
<td>58</td>
</tr>
<tr>
<td>GqF</td>
<td>AGC GAG GAG GCC AAG GAA GC</td>
<td>20</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>GqR</td>
<td>TCC TCC ATT CGG TTC TCA TTG TCT</td>
<td>24</td>
<td>45.8</td>
<td>70</td>
</tr>
<tr>
<td>$\beta$-actin F</td>
<td>CTC CAT CAT GAA GTG CGA CGT</td>
<td>21</td>
<td>52</td>
<td>54</td>
</tr>
<tr>
<td>$\beta$-actin R</td>
<td>CAG ACG GAG TAT TTG CGC TCA</td>
<td>21</td>
<td>52</td>
<td>54</td>
</tr>
</tbody>
</table>

The identity of the sequenced genes were initially evaluated by comparing the obtained sequence with known nucleotide sequences in GenBank (Bilofsky and Burks, 1988) using the search tool BLASTn (nucleotide-nucleotide BLAST). This tool compares the query sequence against all available nucleotide sequences, and the percentage similarity between the top hits (best matches) and the query sequence was obtained. The nucleotide sequence was then translated into the corresponding amino acid sequence using the ‘Translate tool’ available at the proteomics website [http://ca.expasy.org/tools/dna.html](http://ca.expasy.org/tools/dna.html). The deduced amino acid sequence was used to search the available protein sequences in GenBank (Bilofsky and Burks, 1988) using BLASTp (protein-protein BLAST) tool. The percent similarity and percent identity of bluegill amino acid sequences with other vertebrate $G_{\alpha_q}$ and $G_{\alpha_{11}}$ sequences were also calculated.

Once the identity of the genes were tentatively confirmed using different search tools, the putative bluegill G-protein fragments were again aligned with their vertebrate orthologs and more outer primers (Table 2) were designed on the 5' and 3' ends to get the ends of the gene. All primers once designed, were checked for their compatibility using the oligonucleotide calculator and were purchased from BioSynthesis Inc, Lewisville, TX. The PCR products were sequenced each time and the final sequence fragments were edited and incorporated into the initial contiguous sequence using Sequencher 4.2 (Gene Codes Inc. Ann Arbor, MI) to form a contiguous sequence.

Phylogenetic analyses

In order determine the subtype identity of the sequenced fragments, complete coding sequences of forty-four G-protein α subunits were downloaded in FASTA format (Pearson, 1990) from GenBank (Bilofsky and Burks, 1988) (Table 3) and were aligned using Sequencher 4.2 (Gene Codes Inc., Ann Arbor, MI, USA). Different alignments were tried by first aligning Gα sequences of each subtype, followed by aligning each subtype with other subtypes. The criterion for optimal alignment was the perfect alignment of "landmarks" or highly conserved motifs in all G-protein sequences. The DNA sequences were translated into proteins in MacClade 4.05 OS X (Maddison and Maddison, 2001) using universal genetic code and for every gap in the protein sequence, a corresponding triplet gap was introduced manually in the aligned nucleotide sequence. The protein and DNA alignment files were executed in PAUP* (Swofford, 1998), and the
G-protein sequences from mold (*Mucor circinelloides*) and fungi (*Ustilago maydis*) were used as outgroups in all analyses. Before reconstructing phylogenies using different methods, the g1 statistic was used to estimate the phylogenetic signal for the dataset by generating a frequency distribution of thousand random trees.

Tree topologies were obtained under parsimony criterion using heuristic search with five thousand random replications using stepwise additions for both the DNA and protein alignments. The third position codons were downweighted for the parsimony analysis as the nucleotides at the third codon positions evolve faster than those at the first and second codon positions. Bootstrap statistical support for individual clades was obtained by five thousand random replications with one stepwise addition using heuristic search. A neighbor joining tree was generated using distance as the optimality criterion. Tamura-Nei model, which assumes unequal base frequencies and three substitution rates, was used for distance correction and the rates for variable sites were assumed to be equal.

Modeltest 3.7 (Posada and Crandall, 1998) was used to select the model of evolution for Bayesian and maximum likelihood analysis using DNA alignment. The General time reversible + I (invariant sites) + G (gamma distribution) model was chosen using the Akiike criterion for having the highest log likelihood. Maximum likelihood was estimated in PAUP* using the parameter estimates obtained from Modeltest. Fifty bootstrap replications using one heuristic random stepwise addition was used to determine statistical support values. The Bayesian analysis with GTR+I+G as evolutionary model, was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). *Ustilago maydis*, a fungus, Gpa1 was set as the outgroup for the analysis. An initial Markov Chain Monte Carlo search was set to run ten thousand generations with a sample
frequency of one hundred. The runs were repeated with increasing number of generations until the sum of the log likelihoods of trees converged to a stable value. Based on the number of generations taken to stabilize the log likelihood value, a final run was set, with the number of generations set to a million with a sample frequency of hundred. The burnin, which represents the number of trees that would be ignored while the consensus tree is created, was set to twenty five percent of the number of trees sampled (2500). The tree file produced in MrBayes was opened in PAUP*, and a consensus tree was generated.
Table 3. List of scientific name, common name, code name, and accession numbers of DNA and amino acid sequences used in alignment and phylogenetic analyses. NA represents sequences that are unpublished and lack accession numbers. * represents sequences of putative partial bluegill G-protein fragments used in the current study (LmGi was characterized by Sandra Becerra).

<table>
<thead>
<tr>
<th>Code used in tree</th>
<th>Scientific name</th>
<th>Common name</th>
<th>Ga Subtype</th>
<th>Accession # for nucleotide</th>
<th>Accession # for amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>RnG11</td>
<td>Rattus norvegicus</td>
<td>House rat</td>
<td>G11</td>
<td>AF239674</td>
<td>AAF81690</td>
</tr>
<tr>
<td>CgG11</td>
<td>Cricetulus griseus</td>
<td>Hamster</td>
<td>G11</td>
<td>DQ 202706</td>
<td>ABA77548</td>
</tr>
<tr>
<td>HsG11</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>G11</td>
<td>AF493900</td>
<td>AAM12614</td>
</tr>
<tr>
<td>XiG11</td>
<td>Xenopus laevis</td>
<td>African clawed frog</td>
<td>G11</td>
<td>BC 042284</td>
<td>AAH42284</td>
</tr>
<tr>
<td>LmG11*</td>
<td>Lepomis macrochirus</td>
<td>Bluegill sunfish</td>
<td>G11</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>DrG11</td>
<td>Danio rerio</td>
<td>Zebrafish</td>
<td>G11</td>
<td>XM 686074</td>
<td>XP 691166</td>
</tr>
<tr>
<td>CgGq</td>
<td>Cricetulus griseus</td>
<td>Hamster</td>
<td>Gq</td>
<td>DQ202705</td>
<td>ABA77547</td>
</tr>
<tr>
<td>HsGq</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>Gq</td>
<td>AF493896</td>
<td>AAM12610</td>
</tr>
<tr>
<td>MmGq</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Gq</td>
<td>M 55412</td>
<td>AAA63306</td>
</tr>
<tr>
<td>RnGq</td>
<td>Rattus norvegicus</td>
<td>House rat</td>
<td>Gq</td>
<td>AF234260</td>
<td>AAF59930</td>
</tr>
<tr>
<td>LmGq*</td>
<td>Lepomis macrochirus</td>
<td>Bluegill sunfish</td>
<td>Gq</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HsG14</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>G14</td>
<td>BC027886</td>
<td>AAH27886</td>
</tr>
<tr>
<td>BtG14</td>
<td>Bos taurus</td>
<td>Cow</td>
<td>G14</td>
<td>NM 17323</td>
<td>NP 776746</td>
</tr>
<tr>
<td>MmG14</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>G14</td>
<td>NM 008137</td>
<td>NP 032163</td>
</tr>
<tr>
<td>MmG15</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>G15</td>
<td>M80632</td>
<td>AAA37713</td>
</tr>
<tr>
<td>MmGs</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Gs</td>
<td>BC062654</td>
<td>AAH62654</td>
</tr>
<tr>
<td>RnGs</td>
<td>Rattus norvegicus</td>
<td>House rat</td>
<td>Gs</td>
<td>M12673</td>
<td>AAA41261</td>
</tr>
<tr>
<td>CgGs</td>
<td>Cricetulus griseus</td>
<td>Hamster</td>
<td>Gs</td>
<td>DQ202704</td>
<td>ABA77546</td>
</tr>
<tr>
<td>BtGs</td>
<td>Bos taurus</td>
<td>Cow</td>
<td>Gs</td>
<td>NM 181021</td>
<td>NP 851364</td>
</tr>
<tr>
<td>HsG13</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>G13</td>
<td>NM 006572</td>
<td>NP 006563</td>
</tr>
<tr>
<td>HsG12</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>G12</td>
<td>NM 007353</td>
<td>NP 031379</td>
</tr>
<tr>
<td>MmG12</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>G12</td>
<td>NM 010302</td>
<td>NP 034432</td>
</tr>
<tr>
<td>TrG12</td>
<td>Takifugu rubripes</td>
<td>Fugu</td>
<td>G12</td>
<td>L79907</td>
<td>AAL77634</td>
</tr>
<tr>
<td>HsGt1</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>Gt1</td>
<td>BC095505</td>
<td>AAH95505</td>
</tr>
<tr>
<td>MmGt1</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Gt1</td>
<td>NM 008140</td>
<td>NP 032166</td>
</tr>
<tr>
<td>DrGt1</td>
<td>Danio rerio</td>
<td>Zebrafish</td>
<td>Gt1</td>
<td>BC059464</td>
<td>AAH59464</td>
</tr>
<tr>
<td>XiGt1</td>
<td>Xenopus laevis</td>
<td>African clawed frog</td>
<td>Gt1</td>
<td>BC111509</td>
<td>AAA11510</td>
</tr>
<tr>
<td>MmGt2</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Gt2</td>
<td>NM 008141</td>
<td>NP 032167</td>
</tr>
<tr>
<td>TrGt2</td>
<td>Takifugu rubripes</td>
<td>Fugu</td>
<td>Gt2</td>
<td>L79898</td>
<td>AAL77635</td>
</tr>
<tr>
<td>RnGt2</td>
<td>Rattus norvegicus</td>
<td>House rat</td>
<td>Gt2</td>
<td>NP 112297</td>
<td>NP 112297</td>
</tr>
<tr>
<td>MmGi1</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Gi1</td>
<td>NM 010305</td>
<td>NP 034435</td>
</tr>
<tr>
<td>HsGi1</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>Gi1</td>
<td>BC026326</td>
<td>AAH26326</td>
</tr>
<tr>
<td>GgG1</td>
<td>Gallus gallus</td>
<td>Chick</td>
<td>Gi1</td>
<td>NM 205403</td>
<td>NP 990734</td>
</tr>
</tbody>
</table>
Table 3-Continued. List of scientific name, common name, code name, and accession numbers of DNA and amino acid sequences used in alignment and phylogenetic analyses. NA represents sequences that are unpublished and lack accession numbers. * represents sequences of putative partial bluegill G- protein fragments used in the current study (LmGi was characterized by Sandra Becerra).

<table>
<thead>
<tr>
<th>Code used in tree</th>
<th>Scientific name</th>
<th>Common name</th>
<th>Gα Subtype</th>
<th>Accession # for nucleotide</th>
<th>Accession # for amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MmGi3</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Gi3</td>
<td>NM 010306</td>
<td>NP 034436</td>
</tr>
<tr>
<td>LmGi*</td>
<td>Lepomis macrochirus</td>
<td>Bluegill sunfish</td>
<td>Gi</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MmGo</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Go</td>
<td>NP 034438</td>
<td>NP 034438</td>
</tr>
<tr>
<td>RnGo</td>
<td>Rattus norvegicus</td>
<td>House Rat</td>
<td>Go</td>
<td>NM 017327</td>
<td>NP 059023</td>
</tr>
<tr>
<td>HsGo</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>Go</td>
<td>NM 138736</td>
<td>NP 620073</td>
</tr>
<tr>
<td>MmGz</td>
<td>Mus musculus</td>
<td>Mouse</td>
<td>Gz</td>
<td>NM 010311</td>
<td>NP 034441</td>
</tr>
<tr>
<td>RnGz</td>
<td>Rattus norvegicus</td>
<td>House Rat</td>
<td>Gz</td>
<td>NM 013189</td>
<td>NP 037321</td>
</tr>
<tr>
<td>HsGz</td>
<td>Homo sapiens</td>
<td>Man</td>
<td>Gz</td>
<td>NM 002073</td>
<td>NP 002064</td>
</tr>
<tr>
<td>UmGpa1</td>
<td>Ustilago maydis</td>
<td>Fungus (smut)</td>
<td>Gpa1</td>
<td>UMU85775</td>
<td>AAC49724</td>
</tr>
<tr>
<td>McGpa1</td>
<td>Mucor circinelloides</td>
<td>Fungus (mold)</td>
<td>Gpa1</td>
<td>DQ286549</td>
<td>ABB85289</td>
</tr>
</tbody>
</table>

Immunolocalization of Gαq/11 in fishes

The expression of Gα11 and/or Gαq in bluegill and zebrafish eyes was studied to infer whether these G-proteins could play a role in light adaptive granular movements in the RPE. Towards that end, bluegill sunfish and zebrafish were killed by double pithing according to the IACUC protocols, and their eyes were excised and fixed in 4% paraformaldehyde in PBS (comprising 137 mM NaCl, 27mM KCl, 43 mM Na2HPO4.7H2O and 14 mM KH2PO4, pH 8) overnight at 4°C. For zebrafish 7 days post fertilization (dpf), 14 dpf and 21 dpf, the whole fish was fixed while for 28 dpf and adults, whole eyes were excised and fixed in 4% PFA in PBS. The tissue was washed three times in PBS and then transferred into vials containing 30% sucrose in PBS at 4°C overnight for cryoprotection. The samples were then washed and embedded in the embedding medium (Tissue-tek® O.C.T. compound, Sakura Finetek USA, Inc., product code # 4583) and the Tissue-tek containing samples were trimmed to 1mm cubes using razor blade. The tissue containing cubes were sectioned using a cryotome and the sections (10
micrometer thickness) were collected on coverslips coated with 0.5% gelatin. The sections were dried at 4°C overnight and were washed three times with phosphate buffered saline containing 0.1% Tween-20 (PBST).

Sections were incubated in 20% non-fat powdered milk in PBS for 2 hours at room temperature to block non-specific binding. The coverslips were washed three times in PBST and were incubated in rabbit anti-mouse Gq/11 polyclonal antibody (Chemicon, USA) (1:100) 2 hours at room temperature. The ability of the antibody to detect the Gq/11 protein in fish was tested first using the dot blot technique. Negative controls were incubated in PBS. Coverslips were washed three times with PBST and were incubated with Cy3-labeled anti-rabbit IgG (Invitrogen Inc, USA) for two hours at room temperature (1:200). The coverslip was washed again in PBS and mounted on glass slides in a medium consisting of 90% glycerol in water containing 1 mg/ml p-phenylenediamine. Images were acquired with a confocal microscope using an Olympus IX-70 fitted with a Bio-Rad MRC 1024 confocal scan-head via the Keller port and with Olympus BH2-RFCA epifluorescence microscope fitted with Olympus C-35AD-4 camera.
CHAPTER III

RESULTS

Molecular characterization of a \( \alpha_{q/11} \) gene fragment from bluegill brain cDNA

In order to molecularly characterize members of the \( \alpha_{q/11} \) gene family, primers were first designed to amplify \( \alpha_{11} \). Using the primer set G11F1 and G11R1 shown in Table 2, an approximately 500 bp DNA fragment was amplified from bluegill brain cDNA. The sequenced products from both primers formed a single, bidirectional, contiguous sequence, and the second primer set G11F2 and G11R2 (see Table 2) was designed based on this sequence to obtain the non-overlapping 3' region. A third primer set, G11F3 and G11R3 (see Table 2), was also designed to obtain the non-overlapping 5' region. The fragments obtained using the three primer sets and their positions relative to the contiguous sequence are shown in Figure 3. All three sequence fragments when joined generated an ~ 893 bp contiguous sequence. Using BLAST, this sequence was compared with G-protein sequences from other vertebrates, and it showed highest identity (up to 85 %) with \( \alpha_{11} \) sequences. The \( \alpha_{11} \)-like fragment retrieved from bluegill was compared with \( \alpha_{11} \) in mammals (see Figure 4) and the results showed that out of the seven exons, the retrieved bluegill gene fragment was missing only exon 1 and a portion of exon 2, exons 3-7 being fully sequenced. The 893 bp long sequence was translated in
six reading frames, and a single open reading frame encoding 296 amino acids was encoded in frame 2 (Figure 3).

The deduced amino acid sequence when compared with other vertebrate G-protein sequences also showed highest homology (up to 99%) with Gα11 sequences. Both the nucleotide and the deduced amino acid sequences of the bluegill Gα11-like fragment are shown in Figure 5. An alignment of the deduced amino acid sequence of the bluegill Gα11-like fragment with known vertebrate sequences is shown in Figure 6. The percentage identity and similarity between this sequence and vertebrate Gα11 sequences is shown in Table 4. The highest percent identity and similarity was observed with a zebrafish (97% and 99%, respectively) Gα11-like sequence. Since Gα11 is known to be highly similar to Gαq in mammals, the percent similarity of the putative bluegill Gα11 fragment with Gαq sequences from other vertebrates was also evaluated (see Table 5). In fact, based on the comparison of fish and mammalian Gα11 and Gαq “signature” residues, I find that of the 21 residues inferred for bluegill Gα11-like fragment (highlighted in green in Figure 6), 9 residues are Gαq-like.
Figure 3. Position of PCR fragments for the $\alpha_{11}$-like fragment obtained using primers listed in Table 2. The position of primers relative to the sequence and their direction are also denoted in the figure. The horizontal black lines represent the gene fragments generated from the primers given in Table 2. The names of the primers are provided above or below the primer.
Figure 4. A comparison of the gene structure of mammalian Ga₁₁ with the Ga₁₁-like gene fragment retrieved from bluegill cDNA. The exons E1-E7 are shown as red boxes and are interrupted by 6 introns (represented as black lines). The numbers above the red boxes indicate the expected length of each exon in mammalian Ga₁₁ sequences. The lines in green represent the length of the exons retrieved in the current study from bluegill cDNA (~893 bp) and the dotted pink lines represent the part of the gene still uncharacterized from bluegill (~193 bp). This diagram is not to scale and is based on the output from Ensembl comparison of the bluegill Ga₁₁-like gene fragment with mammalian Ga₁₁ sequences.
Figure 5. Nucleotide sequence of bluegill Gα11-like fragment and the deduced amino acid residues. The nucleotide sequence is 893 bp long (in black), and there are 296 deduced amino acid residues (in blue).
Figure 6. Alignment of deduced amino acid sequences of bluegill Gα11-like fragment with published vertebrate Gα11 sequences using CLUSTAL W. The G1-G5 boxes (GTP-binding domains) are highlighted in grey and switches I-III are highlighted in blue. The amino acid residues highlighted in green represents the residues that differ between Gα11 and Gαq, while those highlighted in yellow represent the residues critical for binding with phospholipase C-β. The residues constituting the α1-α5 helices and β1-β5 sheets identified from the crystalline structure of Gα are underlined in black. The stars at the bottom of the alignment indicate homology, and the double dots represent amino acids that are conserved but not identical among all organisms. The accession numbers of the sequences used in the alignment are provided in Table 4.
Table 4. Table showing percent identity and percent similarity of bluegill Gα₁₁-like fragment and Gα_q fragment with Gα₁₁ fragments of other vertebrates at the amino acid level using BL2SEQ program. The numbers in black represent percent identity and numbers in parentheses represent percent similarity. * indicates a predicted sequence.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Scientific name</th>
<th>Accession number</th>
<th>% identity to bluegill Gα₁₁</th>
<th>% identity to bluegill Gα_q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Gα₁₁</td>
<td>Homo sapiens</td>
<td>AAM12614</td>
<td>88 (95)</td>
<td>86 (93)</td>
</tr>
<tr>
<td>Mouse Gα₁₁</td>
<td>Mus musculus</td>
<td>NP_034431</td>
<td>85 (95)</td>
<td>84 (93)</td>
</tr>
<tr>
<td>Rat Gα₁₁</td>
<td>Rattus norvegicus</td>
<td>AAF81690</td>
<td>85 (95)</td>
<td>84 (93)</td>
</tr>
<tr>
<td>Hamster Gα₁₁</td>
<td>Cricetulus griseus</td>
<td>ABA77548</td>
<td>86 (95)</td>
<td>85 (93)</td>
</tr>
<tr>
<td>Chick Gα₁₁</td>
<td>Gallus gallus</td>
<td>NP_989565</td>
<td>88 (95)</td>
<td>84 (94)</td>
</tr>
<tr>
<td>Zebrafish Gα₁₁*</td>
<td>Danio rerio</td>
<td>AHH85433</td>
<td>97 (99)</td>
<td>85 (94)</td>
</tr>
</tbody>
</table>

**Molecular characterization of Gα_q gene fragment from bluegill brain cDNA**

The primers for Gα_q listed in Table 2 were used to amplify ~741 bp DNA fragment from bluegill brain cDNA. The products from the primer set GqF and GqR generated a single, bidirectional, contiguous sequence (~692 bp). The sequence fragments obtained using the primer set and their positions relative to the contiguous sequence are shown in Figure 7. This sequence was compared with G-protein sequences from other vertebrates, and it showed highest homology with Gα_q sequences (up to 85%). The sequence was translated in six reading frames and a single open reading frame encoding 230 amino acids was encoded in frame 3 (see Figure 7). The deduced amino acid sequence when compared with other vertebrate G-protein sequences also showed highest homology with Gα_q sequences (up to 98%). Both the nucleotide and the deduced amino acid sequences of the putative bluegill Gα_q are shown in Figure 8. A comparison of the gene structure of Gα_q (in mammals) and the Gα_q fragment retrieved from bluegill cDNA is given in Figure 9, and the results showed that out of the seven exons in the expected mammalian sequences, the retrieved bluegill Gα_q gene fragment was missing...
exon 6 and 7 and the first forty-four residues of exon 1, the rest being fully sequenced.

An alignment of the deduced amino acid sequence of bluegill Gαq with other vertebrate Gαq sequences is shown in Figure 10. Upon comparing bluegill and mammalian Gα11 and Gαq “signature” residues, it was observed that of the 21 residues inferred for bluegill Gαq (highlighted in green in Figure 10), only two residues appear to be Gα11-like. The percentage identity and similarity between this sequence and other vertebrate Gαq sequences is shown in Table 5.
Figure 7. Position of PCR fragments for bluegill $\alpha_q$ obtained using primers listed in Table 2. The position of primers relative to the sequence and their direction are also denoted in the figure. The horizontal black arrows represent the gene fragments generated from the primers given in Table 2, and the primer names are provided above the respective primers.
gcaaggaagccaggcggatcaacgagatcgaagagcagctccgccgggaacaagagggac
K E A R R I N D E I E R Q L R R D K R D
gcacgccgggaactgaaactgtgtctttcgcacccgggaagtgtgaagagacacattc
A R R E L K L L L L L G T G E S G K S T F
tcaaaacagatgaggattatccatgcaacccgctactccgatgaggaacaagaggttttc
I K Q M R I I H G T G Y S D E D K R G F
accaaaactgtgctctacagacatctttcagccagcagccatgtcagccagccatatggag
T K L V Y Q N I F T A M Q A M I R A M E
acgctcaagatcccttacaaatatgagcacaacaaagggcaatgccaacattgtgagagag
T L K I P Y K Y E H N K G N A N I V R E
gtggacqtagaaaggtctccatgtcagagatccctttattgtagatcaatcaagaagctta
V D V E K V S M F E N P Y V D A I K S L
tggaatgaccagcagatcaggagtctgtatgtctgagagagggaggtatgaccatctctcagac
W N D P G I Q E C Y D R R E Y Q L S D
tctaccaaatattacctgaaacgcgtttggagcagcagccagcagccgtacttccctcact
S T K Y Y L N A L D R I A E P A Y L P T
caccaagtgtgttgagggttagagctccccaccaccggcatcatgcaataccctgttgcac
Q Q D V L R V R V P T T G I M Q Y P F D
cgcaagtgtgctcatattcaggagtggtggtgtacagqggagtggagggaggtgagggagggag
L Q S V I F R M V D V G G Q R S R R K
tggatccactgttttgagaacgcagcagctcactccattatgtctctgtgtagcgcctacgcaggtat
W I H C F E N V T S I M F L V A L S E Y
gaccaagtgttggagatccagacaatgaga
D Q V L V E S D N E

Figure 8. Nucleotide sequence of the bluegill Gαq fragment and the deduced amino acid residues. The nucleotide sequence is 692 bp long, and there are 230 deduced amino acid residues.
Figure 9. A comparison of the gene structure of mammalian $G_\alpha_q$ with the $G_\alpha_q$ gene fragment retrieved from bluegill cDNA. The exons E1-E7 are shown as red boxes and are interrupted by 6 introns (represented as black lines). The numbers above the red boxes indicate the length of each exon (in base pairs) in mammalian $G_\alpha_q$ sequences. The lines in yellow represent the length of the exons retrieved in the current study from bluegill cDNA (~692 bp) and the dotted pink lines represent the part of the gene still uncharacterized from bluegill (~44 bp on the 5' end of the sequence and ~344 bp on the 3' end of the sequence). This diagram is not to scale and is based on the output from Ensembl comparison of the bluegill $G_\alpha_{11}$-like gene fragment with mammalian $G_\alpha_{11}$ sequences.
Figure 10. Alignment of deduced amino acid sequence of bluegill Gαq fragment with published vertebrate Gαq sequences using CLUSTAL W. The G1-G5 boxes (GTP binding domains) are highlighted in grey and switches I-III highlighted in blue. The amino acid residues highlighted in green represents the residues that are different between Gα11 and Gαq while those highlighted in yellow represents the residues that are critical for binding with phospholipase C-β. The residues constituting the α1-α5 helices and β1-β5 sheets of the crystalline structure are underlined in black. The stars at the bottom of the alignment represent homology, and the double dots represent amino acids conserved but not identical among organisms. The accession numbers of the sequences used in the alignment are provided in Table 5.
Table 5. Table showing percent identity and percent similarity of bluegill putative Ga_q and Ga_{11} fragments with Ga_q fragments of other vertebrates at the amino acid level using BL2SEQ program. The numbers represent percent identity, and numbers in parentheses represent percent similarity.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Scientific name</th>
<th>Accession number</th>
<th>% identity to bluegill Ga_q</th>
<th>% identity to bluegill Ga_{11} -like fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Ga_q</td>
<td><em>Homo sapiens</em></td>
<td>AAM12610</td>
<td>94 (98)</td>
<td>89 (96)</td>
</tr>
<tr>
<td>Mouse Ga_q</td>
<td><em>Mus musculus</em></td>
<td>AAA63306</td>
<td>94 (98)</td>
<td>89 (96)</td>
</tr>
<tr>
<td>Rat Ga_q</td>
<td><em>Rattus norvegicus</em></td>
<td>AA59930</td>
<td>93 (98)</td>
<td>88 (96)</td>
</tr>
<tr>
<td>Chick Ga_q</td>
<td><em>Gallus gallus</em></td>
<td>NP_001026598</td>
<td>93 (97)</td>
<td>89 (96)</td>
</tr>
</tbody>
</table>

Phylogenetic analyses

The phylogenetic signal for the dataset was estimated to be -0.576, indicating a strong signal. In all tree topologies obtained using protein and nucleotide alignments employing different phylogenetic methods, mostly similar grouping of G-protein subtypes were observed although the resolution and bootstrap statistical support for some taxa varied among the methods employed. In all tree topologies, the Ga-subtypes grouped to form a single in-group. Within the in-group, they formed two monophyletic units. One unit consisted of Ga_{11}, Ga_q, Ga_{14}, Ga_{15}, Ga_{12}, Ga_{13} and Ga_s, and the other group consisted of Ga_{11}, Ga_{12}, Ga_{i1}, Ga_{i2}, Ga_{i3}, Ga_z and Ga_o subtypes. In all trees obtained using parsimony, neighbor joining, maximum likelihood and Bayesian analysis of the nucleotide alignment (Figures 9-12, respectively), the functional differences among and within Ga subtypes were clearly reflected. In general, all four members of the G_q/11 family that activate phospholipase C were grouped together and were paraphyletic with Ga_{12/13} and Ga_s families, both of which were paraphyletic to one another. The Ga_i group that inhibits adenylyl cyclase were grouped together in all tree topologies. The bluegill
Ga₉/₁₁ sequence retrieved using primers designed based on mammalian Ga₁₁ sequences formed a sister taxon with zebrafish Ga₁₁ sequence and was paraphyletic with mammalian and amphibian Ga₁₁ sequences. Though the mammalian Ga₁₁ sequences grouped to form a single monophyletic unit, the lower vertebrate Ga₁₁ sequences were grouped with the Ga₉ sequences.

The putative bluegill Ga₉ formed a sister group with all mammalian orthologs of Ga₉, and all taxa grouped to form a single monophyletic unit. High bootstrap support (100%) was observed in the grouping of the bluegill Ga₁₁-like fragment with the zebrafish Ga₁₁-like gene; the support for bluegill Ga₉ was slightly lower. The parsimony tree obtained using the protein alignment (Figure 15) showed mostly similar grouping to that of the topology obtained using nucleotide alignment. One major difference observed in the protein-based tree topology was the unresolved Ga₉ group. In this topology, three distinct monophyletic in-groups (instead of two as in other trees) were formed with Ga₉ and Ga₁₂/₁₃ families grouped together, the Ga₉ family and the Gaᵢ family, all of which form a polychotomous group. High bootstrap support (>70%) was observed for both bluegill sequences in the protein alignment. The putative bluegill Gaᵢ (identified and characterized by Sandra Becerra) was grouped with other Gaᵢ members and was closer to Gaᵢ₁ and Gaᵢ₃ rather than Gaᵢ₂. High bootstrap support (>90%) was observed for this grouping in both the nucleotide-based and protein-based tree topologies.
Figure 11. Consensus parsimony tree for nucleotide alignment obtained by heuristic search of the dataset. The numbers indicate bootstrap statistical support values obtained using five thousand random replications of the dataset. Red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for various taxa and their scientific names are given in Table 3.
Figure 12. Neighbor joining tree for DNA alignment with Tamura-Nei model as distance correction obtained by heuristic search with five thousand random stepwise replications. The red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for taxa and their scientific names are given in Table 3.
Figure 13. The consensus maximum likelihood tree topology using GTR+I+G model of evolution. The numbers represent bootstrap statistical support values obtained using fifty random replications of the dataset using heuristic search. The red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for taxa and their scientific names are given in Table 3.
Figure 14. Tree showing Bayesian analysis of posterior probability using the GTR+I+G model of evolution. The numbers represent the posterior probability values and Gα sequences are classified based on their function into four families and are color coded. Red lines represent Gαq family, blue represents Gα12/13, green represents Gαs and Gαi is denoted by black lines. The codes for taxa and their scientific names are given in Table 3.
Figure 15. Consensus parsimony tree for protein alignment obtained using heuristic search of the dataset. The numbers indicate bootstrap statistical support values obtained using five thousand random replications of the dataset. Red lines indicate unpublished putative partial G-protein sequences from bluegill sunfish. The codes for various taxa and their scientific names are given in Table 3.
RT-PCR analysis

To assess if $G_\alpha_q$ and $G_\alpha_{11}$-like mRNA are expressed in fish, RT-PCR was performed on total RNA extracted from bluegill brain, heart, retina and RPE and from zebrafish brain, heart and whole eyes using the primers shown in Table 2. Figure 16 shows that while $G_\alpha_{11}$-like gene ($G_\alpha_{10}$) is expressed in fish brain, $G_\alpha_q$ is expressed in all tissues tested. $\beta$-actin, expressed ubiquitously in all tissues, served as positive controls for the cDNAs. When the $G_\alpha_q$ primers and primers designed to amplify bluegill $G_\alpha_{11}$ were used to perform RT-PCR on total RNA isolated from zebrafish, only $G_\alpha_q$ and $\beta$-actin primers were successful in amplifying the respective genes. $G_\alpha_{10}$ primers designed for bluegill failed to work on zebrafish total RNA.

![Figure 16](image)

**Figure 16.** RT-PCR results showing expression of $G_\alpha_q$ and $G_\alpha_{11}$-like ($G_\alpha_{10}$) mRNA in bluegill (a) and zebrafish (b). $\beta$-actin expression was used as a positive control. The primers used for $G_\alpha_q$, $G_\alpha_{11}$ and $\beta$-actin are given in Table 2.

Immunolocalization of $G_\alpha_{q/11}$ in fish eyes

The RT-PCR results showed that $G_\alpha_q$ but not $G_\alpha_{11}$-like mRNA was detected in bluegill retina and RPE, suggesting the possibility that $G_q$-protein, but not the $G_{11}$-like protein, might be expressed in retina and RPE. Other $G_{q/11}$ proteins not disclosed in this
study could not be ruled out. To study the $\text{G}_{\alpha_{q/11}}$ protein expression in these tissues, immunolabeling using anti-$\text{G}_{\alpha_{q/11}}$ antibody was carried out. The results showed $\text{G}_{\alpha_{q/11}}$ immunoreactivity in bluegill choroid, RPE and photoreceptors (Figure 17). The labeling observed in the RPE in Figure 17 could be $\text{G}_{\alpha_{q/11}}$ labeling in the rod photoreceptors that are closely interdigitated with the apical processes of RPE. Considerable labeling was also observed in the retina that was absent in the control (Figure 18). Similar $\text{G}_{\alpha_{q/11}}$ labeling was observed in zebrafish eyes (Figure 19).
Figure 17. Immunolocalization of \( \text{Ga}_{\alpha/11} \) in bluegill choroid (a) and RPE/photoreceptors (b). The red arrow points to labeling, which appears as specks absent in the control (c), in spite of the high autofluorescence observed.
Figure 18. Immunolocalization of $\text{G}_\alpha_q/11$ in bluegill retina. The white arrow points to the $\text{G}_\alpha_q$ localization (appearing as red specks) in the retina (a) and (c). F-actin staining (green color) using Alexa Fluor 488- conjugated phalloidin (a and c) and nuclear staining using TOPRO (a) act as positive controls. Controls (b and d) lacking phalloidin and TOPRO treatment show no $\text{G}_\alpha_q$ labeling as indicated by the absence of red specks. All images were taken at 60X magnification.
Figure 19. Immunolocalization of $G_{\alpha_{q/11}}$ in zebrafish RPE/photoreceptors. The red arrows in (a) and (b) show labeling of (appearing as small white specks) in the apical processes of RPE and/or photoreceptors as they are interdigitated with one another (c) represents the control.
To study whether $\Gamma_{\alpha_{q/11}}$ is expressed in fish eyes during development, immunolabeling was carried out for 7 dpf, 14 dpf, 21 dpf, 28 dpf and adult zebrafish eyes. At 7dpf, labeling for $\Gamma_{\alpha_{q/11}}$ was observed in eyes though it was difficult to differentiate the specific sites of labeling (Figure 20A and 20B). The control sections did not show the labeling (Figure 20C and 20D). At the metamorphic stage (14dpf), labeling was visible in the retina (Figure 21A) that was absent in the control (Figure 21B). At 21 dpf, $\Gamma_{\alpha_{q/11}}$ immunoreactivity was observed distinctly at in the inner retinal layers (Figure 22A, 22B and 22C) and the controls (22D, 22E and 22F) lacked labeling. At 28dpf or the juvenile stage, retina and choroid showed labeling (Figure 23A, 23B and 23C), while the controls lacked labeling (23E, 23F and 23E). In the retina, distinct labeling was observed in the photoreceptor outer segments, the outer and inner nuclear layers and the ganglion cell layer (Figure 23C). At the adult stage (>90dpf), labeling was shown by retina, choroid and photoreceptors and RPE (Figures 24A, 24B and 24C). The labeling at the RPE could not be differentiated from the photoreceptor labeling as the apical processes of RPE are closely interdigitated with rod photoreceptors.
**Figure 20. Immunolabeling of Ga\_q/11 in 7 dpf zebrafish eyes.** Labeling of Ga\_q/11 protein in larval (7 dpf) zebrafish eyes. The treatment sections were obtained using (A) 20X and (B) 60X objectives. The white arrow points to labeling seen as red specks. The control sections taken using the (C) 20X, (D) 60X objectives lack labeling as indicated by the absence of the specks. The scale bars in (A) and (B) represents 100\(\mu\)m and 50\(\mu\)m, respectively.
Figure 21. Immunolabeling of $\Gamma_{aq/11}$ in 14 dpf zebrafish eyes. Labeling of $\Gamma_{aq/11}$ protein in 14 dpf zebrafish eyes. The treatment and control sections were obtained using a 60X objective. The white arrow points to labeling seen as red specks, which is absent in control. The scale bar in (A) represents 50µm.
Figure 22. Immunolabeling of $\text{G}a_{q/11}$ in 21 dpf zebrafish eyes. Labeling of $\text{G}a_{q/11}$ protein in 21 dpf zebrafish eyes. The treatment sections were obtained using (A) 10X, (B) 20X and (C) 60X objectives. The white arrow points to labeling seen as red specks. The control sections obtained using the (D) 10X, (E) 20X and (F) 60X objectives lack labeling as indicated by the absence of the specks. The scale bars in A, B and C represent 150µm, 100µm and 50µm, respectively.
Figure 23. Immunolabeling of $\text{Ga}_q/11$ in 28 dpf zebrafish eyes. Labeling of Gaq/11 protein in juvenile (28 dpf) zebrafish eyes. The treatment sections were obtained using the (A) 10X, (B) 20X and (C) 60X objectives. The white arrow points labeling seen as red specks. The control sections were obtained using (D) 10X, (E) 20X and (F) 60X objectives and lack labeling as indicated by the absence of the specks. The scale bars in A, B and C represent 150μm, 100μm and 50μm, respectively.
Figure 24. Immunolabeling of $G_{\alpha q/11}$ in adult zebrafish eyes. Labeling of $G_{\alpha q/11}$ protein in adult zebrafish eyes. The treatment sections are (A) at 10X, (B) at 20X and (C) at 60X. The white arrow points labeling seen as red specks. The control sections (D) obtained using 10X, (E) at 20X and (F) at 60X objectives lack labeling as indicated by the absence of the specks. The scale bars in A, B and C represent 150µm, 100µm and 50µm, respectively.
CHAPTER IV

DISCUSSION

The objective of this study was to use a molecular approach to characterize the $G_\alpha_q$ and $G_\alpha_{11}$ subtypes of G-proteins from bluegill sunfish and to study their expression in fish eyes using reverse transcription polymerase chain reaction and immunohistochemistry. My results indicate that bluegill brain expresses *bona fide* $G_\alpha_q$ which is also expressed in retina and RPE. These findings suggest that $G_\alpha_q$ could be involved in the signal transduction pathway linking muscarinic receptor activation to pigment granule dispersion. Lastly, the expression of $G_\alpha_{q/11}$ was observed in all developmental stages tested in zebrafish, implying that these G-protein subtypes may be involved in the embryonic development of fish.

**Molecular characterization of $G_\alpha_q$ and $G_\alpha_{11}$ from bluegill brain cDNA**

I have successfully amplified 692 bp and 893 bp of bluegill $G_\alpha_q$ and $G_\alpha_{11}$-like gene fragments from brain cDNA, representing ~70% and ~84%, respectively, of the expected sequence, based on comparison with other known vertebrate $G_\alpha_q$ and $G_\alpha_{11}$ sequences. BLAST searches at the nucleotide and amino acid levels showed that both sequences are similar to their respective vertebrate homologs. They also showed more than 80% similarity to each other. Both sequences showed the critical amino acid
residues and specific domains required for effector activation, receptor coupling and GTPase activity. Phylogenetic analyses confirmed the assignment of the G-protein fragment from bluegill described herein to the \( \text{Ga}_q \) subtype. However, the identity of the fragment retrieved using primers intended to amplify \( \text{Ga}_{11} \) could not be assigned to any of the known \( \text{G}_q/11 \) groups with certainty using phylogenetic methods. This gene fragment does not appear to be orthologous to mammalian \( \text{Ga}_{11} \) sequences, but appears to be more closely related to \( \text{Ga}_q \) and \( \text{Ga}_{14} \). Therefore, it is likely that this \( \text{Ga}_q \)-like fragment retrieved from bluegill may be unique in its own way, and I propose that this fragment be referred to as \( \text{Ga}_{10} \). Furthermore, it grouped with zebrafish \( \text{Ga}_{11} \); therefore, I propose that the zebrafish gene also be redesignated as \( \text{Ga}_{10} \). At this juncture, it is perhaps important to add that it is not clear if bluegill or lower vertebrates express \( \text{Ga}_{11} \), as the primers that were meant to amplify this gene, amplified a different variant (\( \text{Ga}_{10} \)), and search of the zebrafish and fugu genomic databases failed to yield any other full length \( \text{G}_q/11 \) sequences. Sarwar et al. (1996) characterized 14 of the 16 known mammalian homologs of \( \text{Ga} \) multigene family in fugu using a combined approach of low stringency screens of genomic cosmid libraries and PCR amplification using degenerate primers. Though they identified a \( \text{Ga}_q \) and \( \text{Ga}_{11} \) in fugu, the fragments contained only exons 5, 6 and 7, representing only about 30% of the total anticipated sequence. Thus the study reported here is the first one to characterize \( \text{Ga}_{q/11} \)-subtypes in fish using sequences representing more than 70% of the coding sequence. Furthermore, because Sarwar et al. (1996) failed to include phylogenetic analyses for the assignment of their G-protein subtypes from fugu, our assignment of \( \text{Ga}_q \) from bluegill is more reliable.
Critical amino acids

Amino acids conserved across all Ga subtypes

The alignment of amino acid sequences of Ga subtypes showed that they consist of a GTPase domain and an alpha helical domain. The GTPase domain, common to all GTPases, consists of five alpha helices surrounded by six β strands (Orun, 2006) (underlined areas in Figures 6 and 10). The alpha helical domain is the most divergent of all Ga protein families and is involved in burying the GTP within the core of the protein (Cabrera-Vera et al., 2003). The GTPase domain has five distinct motifs involved in GTP binding and hydrolysis (Cabrera-Vera et al., 2003) which are marked in gray boxes in Figures 5 and 8. These five motifs are conserved across all Ga protein subtypes and may be divided as G1 or P, G2 or E, G3, G4 and G5 (Sandhya and Vemuri, 1997). The G1 motif in mammals show a consensus sequence GXXXXGK(S/T) at positions 45-53 and are known to interact with GTP and GDP. This region is found in most proteins that bind purine nucleoside triphosphates, like protein kinases and ATPases (Walker et al., 1982). The bluegill putative Gaq fragment characterized herein shows a G1 motif consisting of amino acid residues GTGESGKS, exactly identical in both sequence and position to the G1 motif found in mammals. However, in the bluegill putative Ga10 sequence, the residues corresponding to this motif are not known yet as they are encoded 5' to the sequence obtained herein.

The E or G2 motif has a consensus sequence D-(X)n-T. The conserved threonine residue in this motif has been reported to be responsible for Mg²⁺-binding (Paduch et al., 2001), and is located at position 85 in the sequences aligned here. The G2 motif was found in both bluegill Ga10 and Gaq sequences (Figures 6 and 10, respectively), and both
sequences had the necessary threonine residue for binding with Mg$^{2+}$. In bluegill Ga$_{10}$, T$^{76}$ and K$^{77}$ in the G2 motif were replaced by I$^{76}$ and R$^{77}$, respectively, but bluegill Ga$_q$ did not show any such replacement. But as all other residues of the G2 motif in bluegill Ga$_{10}$ appears to be identical to the G2 motif in mammalian Ga$_{11}$ sequences, the substitution of the two amino acids may be functionally neutral.

The G3 motif in mammals has a consensus sequence DXXG, and this conserved region has also been shown to contribute to GTPase activity (Sandhya and Vemuri, 1997). The G3 motif in both bluegill sequences have the amino acids DVGG at position 205-209, as in mammals (Figures 6 and 10, respectively). The consensus sequence NKXD of the G4 motif interacts with the guanine ring in mammals and stabilizes the guanine nucleotide-binding site (Sprang, 1997). Though this motif was completely conserved in bluegill Ga$_{10}$, this region is not yet characterized in bluegill Ga$_q$ as this motif is encoded 3' to the sequence obtained herein. The G5 motif consists of the consensus sequence (T/G/C) (C/S)A and acts as a recognition site for the guanine base (Sprang, 1997). This region was conserved in bluegill Ga$_{10}$, but the corresponding region has not been characterized in bluegill Ga$_q$ yet.

**Amino acids that distinguish Ga$_q/11$ from other Ga subtypes**

The Ga$_q$ and Ga$_{11}$ subtypes form a distinct class of G-proteins as they show less than 50% amino acid sequence identity with other Ga subtypes. Ga$_q$ and Ga$_{11}$, when compared with other Ga subtypes, exhibit several noteworthy differences, though no unique amino acid replacements were noted (Yokoyama and Starmer, 1992). N-terminal sequences of Ga$_q$ and Ga$_{11}$ in mammals were reported to differ distinctly from other G-
protein N-terminals due to the presence of methionine residues preceding the methionine that was predicted by homology to be the first codon in Ga_q and Ga_{11} (Strathmann and Simon, 1990). In other words, Ga_q and Ga_{11} have an additional N-terminal sequence not seen in other G-protein subtypes. The six amino acids found in both sequences (MTLESI) are reported to be highly conserved among organisms suggesting functional significance (Strathmann and Simon, 1990).

In bluegill Ga_q and Ga_{10}, the N-terminal regions have not been sequenced yet and could not be compared to other vertebrate sequences. It should perhaps be noted that this region (MTLESI) is also missing in invertebrate Ga_q sequences. The N-termini of some Ga subtypes are subjected to N-myristoylation on a glycine at the second position (Gly-1) (Buss et al., 1987). But Strathmann and Simon (1990) showed that Ga_q and Ga_{11} differ from other G-protein subtypes by lacking myristoylation, as their glycine at that position is replaced by alanine. In bluegill sequences, this replacement could not be confirmed as the residues at this region are not yet sequenced. The residues at position 46-50 represent the highly conserved “GAGES-box” motif (GTGES) that has been implicated in GTP-binding in all G-protein α subunits (Strathmann and Simon, 1990). Mutations at this region have been shown to affect the GTPase activity of G-proteins (Masters et al., 1989). This region was conserved at the same position in bluegill Ga_q (see Figure 10), but this motif could not be confirmed in bluegill Ga_{10} as it has not been fully sequenced yet. The cysteine residue (position 356) at the C-terminal region of most mammalian G-proteins can be ADP-ribosylated by pertussis toxin, thereby inactivating the G-protein (Strahmann and Simon, 1990). Bluegill Ga_{10}, like other mammalian Ga_{10} sequences, lack cysteine at position 356. The C^{356} at this position is replaced by Y^{356}. Bluegill Ga_q
sequence did not include the complete C-terminus and hence this replacement could not be observed (Figure 10). Other G-protein subtypes like $\alpha_{s}$, $\alpha_{olf}$ and $\alpha_{z}$ also lack cysteine at this position (Lochrie and Simon, 1988), indicating that these proteins are insensitive to modification induced by pertussis toxin.

*Amino acids that differentiate $\alpha_{q}$ and $\alpha_{11}$*

The deduced amino acid sequence of $\alpha_{11}$ in mammals are reported to be 88% identical to that of $\alpha_{q}$ and almost all the differences between them are concentrated at the N-terminal half of the protein (Strathmann and Simon, 1990). Out of the 42 amino acid differences among the two subtypes in mouse, 38 are located in the N-terminal region (1-200), and only 4 differences are located in the other half of the protein (201-359). It is also reported that both sequences may be difficult to discriminate using *in vitro* studies as they are about 97% identical at the C-terminal region, the domain that is important for receptor and effector specificity (Strathmann and Simon, 1990). However, Lochrie and Simon (1988) showed that there are strict amino acid sequence differences that identify and differentiate the two subtypes. In bluegill sequences, similar differences in amino acid residues were observed, and the residues that differ between $\alpha_{q}$ and $\alpha_{11}$ in mammalian sequences are highlighted in green in Figures 6 and 10. However, there were several cases in which the $\alpha_{10}$ sequence had $\alpha_{11}$-like residues and others where the residues were more $\alpha_{q}$-like.
Amino acids critical for receptor coupling

The $G\alpha$ subunit has specific sites on the sequence that interact with receptors and the best characterized receptor contact region is located at the COOH-terminus (Hamm, 1998). Studies using chimeric $G\alpha$ subunits suggest that the last five amino acids may contribute to the specificity of receptor G-protein interaction (Hamm, 1998). However, the carboxyl terminal amino acids alone may not be involved in receptor binding. $G\alpha_{oA}$ and $G\alpha_{oB}$, both of which have the same C-terminal amino acids, interact with different receptors (Rens-Domiano and Hamm, 1995). Furthermore, multiple sites in $G\alpha_{16}$ were required for its interaction with C5a receptor (Rens-Domiano and Hamm, 1995). Key residues in receptor coupling have also been identified in the N-terminus and other regions of specific $G\alpha$ subtypes (Cabrera-Vera et al., 2003). The conserved Arg$^{340}$ residue of the DRY motif on G-protein coupled receptors (GPCRs) is reported to be an important factor in the receptor sequence leading to GDP release and $G\alpha$ activation (Oliveira et al., 1993).

Furthermore, Oliveira et al. (1999) suggested that the binding site of Arg$^{340}$ on the GPCR could be either Asp$^{333}$ or Asp$^{337}$ in the $\alpha5$ helix of the G-protein. This possibility suggests that receptor-$G\alpha$ binding specificity may not be controlled by one specific domain, but could be the result from a network of contacts between the two and may differ for each $G\alpha$ subunit and receptor, resulting in a large number of possible combinations of G-protein-receptor interactions. In bluegill $G\alpha_{11}$-like fragment, the C-terminal regions are similar to mammalian $G\alpha_{11}$ sequences but the $G\alpha_q$ fragment retrieved from bluegill is incompletely sequenced in the C-terminus and hence its identity with respect to its receptor-coupling motif could not be confirmed.
Amino acids critical for binding to effector (PLC)

The sites of interaction between Ga subunit and its effectors are still not fully understood. The alpha helical domain has shown to be important for the specificity of the effector-coupling to G-proteins (Rens-Domiano and Hamm, 1995). Berlot and Bourne (1992) have shown that three discrete regions are important for the interaction of Ga with specific effector proteins like adenylyl cyclase or phospholipase C. These regions correspond to the residues 236-240 (region I), 276-285 (region II) and 349-356 (region III) in Ga subtypes. In Ga, at least two such putative regions of effector interaction have been identified (Rens-Domiano and Hamm, 1995).

Helper et al. (1995) demonstrated that Cys⁹ and Cys¹⁰ (with or without palmitoylation) are required for the activation of PLC-β1 in Ga. They also showed that these residues are important for interaction with the receptor to a certain extent and that the amino terminus of Ga conveys a hydrophobic character to the protein distinct from that contributed by palmitate. Arkinstall et al. (1995) used multiple overlapping synthetic peptides to map regions of Ga that react with recombinant PLC-β1. With this approach they identified two distinct regions of Ga (Ser²⁵¹-Gln²⁵⁶ and Ala³⁰⁶-Asp³¹⁹) that inhibited Ga-mediated PLC-activation. Venkatakrishnan and Exton (1995) on the other hand, used transient expression of chimeras and measurement of inositol phosphate production in HEK-293 cells to reveal that Ile²¹⁷-Lys²⁷⁶ contained the PLC activation site. Alanine scanning mutagenesis of this region also helped them narrow down two clusters of amino acid residues (Asp²⁴³, Asn²⁴⁴, Glu²⁴⁵ and Arg²⁵⁶, Thr²⁵⁷) that are specifically required for PLC interaction (highlighted in yellow in Figures 6 and 10). While both bluegill sequences showed the amino acid residues 243-245 needed for PLC activation, the
residues 256-257 could not be confirmed in Gaq as this region is still not sequenced (Figure 10).

Amino acids critical for binding with βγ dimer

The amino terminus of Ga subunit has been shown to be critical for its interaction with the βγ dimer. The specific residues of the amino terminus involved in interaction with Gβγ may vary among and within each Ga family. For example, residues 7-11 on the Gaαo subtype were shown to be critical for interaction with Gβγ (Rens-Domiano and Hamm, 1995), but for Gaαs, the residues at this position were not critical for binding with βγ-subunit. Another possible βγ-binding site on Ga sequence could be the switch regions as GTP binding decreases Gβγ affinity (Rens-Domiano and Hamm, 1995). The exact βγ-binding site on Gaαq/11 has not been identified yet though it has been speculated that the binding site is located in the amino terminus of the sequence (Rens-Domiano and Hamm, 1995).

Phylogeny and evolution of G-proteins

A number of different mechanisms like tandem gene duplication, genome duplication or tetraploidization and reverse transcription (resulting in pseudogene formation) have been implicated in the development of multigene families (Wilkie et al., 1992; Wilkie and Yokoyama, 1992). There is also evidence that suggests that the eukaryotic genome has undergone multiple genome duplication events and that the most recent event occurred approximately three hundred million years ago (Wilkie et al., 1992). The phylogeny of Ga multigene family in mammals have shown that Gaαq and Gaαi
subtypes each form single monophyletic units, suggesting that they are not closely related to one another. Previous studies have also shown that the Ga12/13 family is closely related to the Gaq/11 family and that their common ancestor diverged from the Gas family (Wilkie et al., 1992; Wilkie and Yokoyama, 1994).

The topology of all trees generated in the study reported herein (which includes fish and amphibian G-protein sequences) showed a slightly different grouping of G-protein subtypes. All tree topologies identified the two genes characterized in the current study as belonging to the Gaq/11 family based on the available nomenclature from mammalian sequences. However, one noteworthy difference in the trees reported herein is that the bluegill Ga10 (LmG11), zebrafish predicted Ga11 (DrG11) and frog Ga11 (XlG11) are clustered separately from the mammalian Ga11 sequences, and they appear to be more closely related to Gaq and Ga14 than they are to mammalian Ga11. This result suggests that the lower vertebrate Ga11 sequences are unique in their own way, and I propose they should be renamed (for example, as Ga10, due to their shared ancestry with Gaq and Ga14). Bluegill Gaq formed a sister clade to the mammalian Gaq sequences but as all Gaq sequences were monophyletic, the bluegill Gaq fragment can be considered as bona fide Gaq. The bluegill Gaq (sequenced by Sandra Becerra) may be named as Gaq1/i3 as it is more closely related to Gaq1 and Gaq3 than to Gaq2.

In the phylogenetic analyses conducted in this study, the grouping of mammalian G-protein subtypes appears to be similar to the G-protein phylogenies put forth by Yokoyama and Starmer (1992) and Wilkie et al. (1992). However, they are drastically different from studies by Simon et al. (1991) and Strathmann and Simon (1991). In their studies, they reported that Gaq and Gai are closely related to one another. This difference
in inferences about G-protein phylogeny could be due to the lack of an outgroup in their studies or to their assumption of a constant rate of amino acid replacement in their analysis. Previous research has shown that the nucleotides at the third codon positions evolve faster than those at the first or second codon positions (Felsenstein, 1978) and hence should be given a lower weight during phylogenetic analysis. Similarly, the rate of transition is much higher than the rate of transversion (Collins et al., 1994), and should be down-weighted while conducting parsimony analysis to make an accurate phylogenetic inference. Neither Simon et al. (1991) nor Strathmann and Simon (1991) down-weighted the nucleotides of their dataset at the third codon positions, and both studies failed to down-weight the nucleotide transitions, calling their results into question.

The evolution of Ga multigene family has been explained by a simple model in mammals. Mammalian Ga_{11} and Ga_{15} are closely related as are Ga_{q} and Ga_{14}. Previous mapping studies suggest that a single progenitor gene was tandemly duplicated giving rise to Ga_{q} and Ga_{11} (Wilkie et al., 1992). These genes were duplicated again onto another chromosome, giving rise to four Gq classes. This hypothesis was supported by the fact that on mouse chromosome 10, the genes for Ga_{11} and Ga_{15} were tandemly duplicated in a head-to-tail array (Davignon et al., 1996). Their gene structure showed that the coding sequence of each gene was contained in seven exons, and the two genes together spanned about 43 kb with 6 kb of intergenic region. Moreover, Ga_{q} and Ga_{14} were found to cosegregate on chromosome 19 in mouse (Yokoyama and Starmer, 1992; Davignon et al., 1996). In the tree topologies generated in the current study, mammalian, Ga_{q} and Ga_{14} were closely related as were mammalian Ga_{11} and Ga_{15}. 
Previous findings suggest that \( \Gamma_{\alpha_i}, \Gamma_{\alpha_o}, \) and \( \Gamma_{\alpha_i} \) genes may be descendents of a common tandemly duplicated progenitor (Wilkie et al., 1992; Wilkie and Yokoyama, 1994). In their tree topology, \( \Gamma_{\alpha_z} \) was observed to be the most divergent among \( \Gamma_i \) class of proteins, and it also mapped separately from all other \( \Gamma_{\alpha} \) genes in other studies (Yokoyama and Stamer, 1992; Wilke et al., 1992). The intron organization of mammalian \( \Gamma_{\alpha_z} \) was shown to be unique with an additional intron in the 5' untranslated region (Kaziro et al., 1991). Wilkie et al. (1992) inferred based on its gene structure that \( \Gamma_{\alpha_z} \) resembled an incomplete pseudogene that may have arisen from a reverse transcription event. They also speculated, based on its highly conserved amino acid sequence in rats and humans, that this gene could have been recruited as a functional gene prior to the divergence of rodents and primates (Wilkie et al., 1992). In the study reported herein, \( \Gamma_{\alpha_z} \) formed a sister taxon with \( \Gamma_{\alpha_o} \), and \( \Gamma_{\alpha_t} \) formed a separate, highly divergent group within the \( \Gamma_i \) ingroup.

\textbf{\( \Gamma_{\alpha_q} \) and \( \Gamma_{\alpha_{11}} \) localization in fish tissues}

Though \( \Gamma_{\alpha_q} \) and \( \Gamma_{\alpha_{11}} \) have been reported to be expressed ubiquitously in all tissues (Strathmann and Simon, 1990; Wettschureck and Offermanns, 2005), numerous studies have independently illustrated that despite their high similarity, there are some tissues where only one subtype is expressed. For example, human and dog platelets express only \( \Gamma_{\alpha_q} \) but not \( \Gamma_{\alpha_{11}} \) (Milligan and Mitchell, 1993; Ushikubi et al., 1994). Another study, particularly relevant to the results reported herein, showed that \( \Gamma_{\alpha_{11}} \) is the only G-protein besides transducin (\( \Gamma_{\alpha_i} \)) identified in the rod outer segments of bovine retina (Peng et al., 1997). They also noted that \( \Gamma_{\alpha_q} \) expression was detected in the inner
retinal layers. My results using RT-PCR show that Gαq but not Gα10, mRNA is expressed in bluegill RPE and retina. Immunolocalization suggests Gαq/11 immunoreactivity in the photoreceptors, but the rod photoreceptors are so tightly interdigitated with the RPE that the labeling cannot be distinguished between the apical processes of the RPE and the photoreceptor outer segments using confocal microscopy. Electron microscopy would be more effective in distinguishing the labeling between the RPE and the photoreceptor outer segments.

The expression profile of Gq/11 in chick developing retina showed that this subtype is expressed from early embryonic stage and their levels did not change during later stages of development (Shah and Hausman, 1996). However, in developing retina from rat, Gα distribution (Gαi1, Gαi2 and Gαo) differed, suggesting that each subtype may have a unique function at various stages of development (Oguni et al., 1996). In zebrafish eye development reported here, the Gαq/11 immunoreactivity was observed at the larval (7dpf), metamorphic (14 dpf and 21 dpf), juvenile (28 dpf) and adult stages. In all stages, Gαq/11 expression was detected in the retina, though the differentiation of labeling in the photoreceptor outer segments, outer nuclear layer and inner nuclear nuclear layers were evident only after the juvenile stages. Though the levels of expression at different stages were not determined, it is clear that Gαq does play a role in the proper development of fish eyes.

**Future studies**

My role in our laboratory’s overall research was to elucidate the G-protein subtype that may be involved in carbachol-induced pigment granule dispersion in bluegill
RPE. My results indicate that \( \alpha_q \) is expressed in RPE, and therefore could be the G-protein subtype involved in transducing the signal from the muscarinic receptor to the effector, producing a cascade of events that lead to pigment granule dispersion in fish eyes. However, as the primers designed to retrieve \( \alpha_{11} \) amplified a novel sequence (\( \alpha_{10} \)), the next step should be to check whether fish express \( \alpha_{11} \) like mammals and to check if this gene is expressed in RPE like \( \alpha_q \). Furthermore, studies using techniques like RNA interference (siRNA) that can knock down specific G-protein subtypes in fish RPE will help demonstrate their role in pigment movements. More knockout studies on downstream signaling components like PLC, IP3, DAG, PKC and PDE will also help elucidate whether the model of signal transduction suggested by Phatarpekar \textit{et al.} (2005) is supported.

Another important study that I would recommend for the future is to look into the expression of \( \alpha_q \) and/or \( \alpha_{11} \) in rod outer segments of bluegill. For the last couple of years, many studies have reported the presence of a phosphoinositide pathway in vertebrate photoreceptor (especially rod) outer segments. Despite these findings, the components involved in the pathway were unclear until Peng \textit{et al.} (1997) identified \( \alpha_{11} \) subtype as the only G-protein besides transducin in rod outer segments of bovine retina. They also identified PLC \( \beta 4 \) in rod outer segments. My RT-PCR results in bluegill retina suggest that there is a high possibility of \( \alpha_q \) expression in bluegill rod outer segments. Thus, future studies on fish rods may raise the interesting possibility that they differ from other vertebrates in expressing \( \alpha_q \) but not \( \alpha_{11} \) in their retinal photoreceptors. Another obvious question that comes out of this study then would be whether \( \alpha_q \) and \( \alpha_{11} \) bear any function other than phototransduction in this location.
Lastly, it would be interesting to evaluate the differences in the expression of genes encoding all G-protein subtypes between dark adapted and light adapted fish RPE using microarray analysis. Microarrays may also be used to study expression levels of muscarinic receptors, G-proteins and other signaling components during fish eye development.
REFERENCES


VITA

Varsha Radhakrishnan was born in Chennai, India on August 4, 1980, the daughter of Mr. Kodakkadath Radhakrishnan and Mrs. Yamuna Radhakrishnan. After completing her Bachelor of Fisheries Science at Kerala Agricultural University in India in 2004, she joined Texas State University-San Marcos to pursue a Master’s of Science degree in Biology. While at Texas State, she worked as graduate assistant for Vertebrate Physiology, as laboratory instructional assistant for courses like Cellular Physiology and Functional Biology and as research assistant for Dr. García. As a graduate student at Texas State, she gave oral and poster presentations at international and local scientific meetings. She is the co-author of a paper that was published in 2006 and she will be submitting her thesis work for publication this year. Upon completion of her graduate degree, she will be joining Ph.D. and will be continuing research in a similar field.

Permanent Address: “Shreepadmam”

Behind GHS, Wadakanchery

Thrissur, Kerala, India 680582.

This thesis was typed by Varsha Radhakrishnan.