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CLONING, SEQUENCING, AND EXPRESSION OF TWO BOVINE RETINAL ISOFORMS OF 14-3-3 PROTEIN WHICH COPURIFY WITH A PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C

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By

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ABSTRACT

CLONING, SEQUENCING, AND EXPRESSION OF TWO BOVINE RETINAL ISOFORMS OF 14-3-3 PROTEIN WHICH COPURIFY WITH A PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C

By

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A broad range of organisms and tissues contain members of the highly conserved 14-3-3 family of proteins. These 27-30kDa acidic proteins have been associated with a diversity of functions, the most notable being their ability to bind to phosphoserines of signaling proteins, such as Raf-1, Protein Kinase C, and cdc 25 in a sequence-specific manner. There are seven known mammalian isoforms of 14-3-3 proteins, designated α through η, all of which appear to be highly expressed in the bovine retina. Two of the seven isoforms, α and δ, appear to be phosphorylated forms of the β and ζ isoforms respectively. Richard Pinke has reported that all or some of these bovine retinal isoforms copurify with phosphoinositide-specific phospholipase C (PLC) activity [Pinke et al., 1997], and suggested that one or more of these 14-3-3 isoforms, perhaps as hetero- or homodimers may function as an adaptor, chaperone or regulator of rod outer segment PLCs. Work was initiated to clone and sequence each of the various retinal 14-3-3 isoforms. The bovine brain sequences for the η and ζ isotypes were previously published. Epsilon was recently cloned, sequenced, and expressed by a colleague in the lab using a Yeast Two-Hybrid system. Only the Bovine β and γ 14-3-3 nucleotide sequences remained unknown. Using reverse transcriptase polymerase chain reaction (RT-PCR), the author cloned both the β and γ 14-3-3 open reading frames.

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And most importantly my parents, JoAnne and James Jones, and brothers, Lance and Corey, know that I love you and in you lies my strength.

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ABBREVIATIONS

cDNA	Complementary DNA
DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate
IP_3	Inositol 1,4,5 triphosphate
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PFU	Plaque forming units
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PI-PLC	Phosphatidylinositol hydrolyzing PLC
PKC	Protein kinase C
PLC	Phospholipase C
PNK	Polynucleotide kinase
ROS	Rod Outer Segment
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
sscDNA	Single-stranded cDNA
SM Buffer	Phage suspension medium
SSC	Nucleic acid transfer buffer

β and γ-Specific Bovine Retinal 14-3-3

INTRODUCTION

Vertebrate visual transduction depends upon a complex array of mechanisms each of which is dependent on others for regulation. Although one of the mechanisms (cGMP-mediated-photoexcitation) has been studied extensively by in vitro methodologies [Stryer, 1986], its modulation by other complexes within the cell is still under investigation. Phosphatidylinositol-specific phospholipases C (PI-PLCs) are a class of enzymes involved in various signal transductions. They cleave PIP_2 phosphodiester linkages to yield two potent second messengers-inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). In many cell types IP_3 mobilizes intracellular Ca^{2+} levels. Although Ca^{2+} plays a dominant role in regulating light/dark adaptations and electrical amplification (gain) of the absorbed photon energy of the photoreceptor cell, it is not certain that photoreceptor Ca^{2+} flux is triggered by IP_3. DAG activates protein kinase C (PKC) which is known to phosphorylate rhodopsin and other photoreceptor proteins. The activation of a PLC can ultimately be expected to have a ripple effect on many other cellular processes; but what regulates PLC activity in the photoreceptor rod outer segment (ROS) is unclear. PLCs are frequently activated by transmembrane receptors, either directly or via a G-protein or a kinase [Majerus et al., 1990]. Since both the activators and the substrate are membrane-associated, it follows that PLCs need to bind to a membrane to be functional. However PLC activity has been demonstrated in both particulate and cytosolic

fractions [Rhee et al., 1989]. These findings suggest that PLCs are capable of moving between the cytosol and membrane by some specific mechanism. Furthermore this mechanism may constitute a way of regulating PLC-generated second messengers.

Multiple forms of PI-PLCs were found in bovine ROS both in soluble and particulate fractions [Gehm and McConnell, 1990b]. Although no direct role for PLC in vertebrate phototransduction has been identified to date, there have been persistent reports of light-activated PI-PLC activities in vertebrate photoreceptors [Jelsema, 1989; Das et al., 1986]. We have undertaken the study of function and regulation of the bovine retinal isoforms of PI-PLC. In the process of purifying to apparent homogeneity a 31,33 kDa dimeric protein with phosphatidylinositol hydrolyzing activity; non-PLC sequences belonging to an already known family of proteins were discovered. Edman degradation of trypsin fragments of this dimeric protein yielded 27 peptide sequences coding for different isoforms of 14-3-3, two trypsin sequences but no discernible X,Y-type PLC sequences [Pinke et al., 1997]. Western blots have thus far failed to reveal significant presence of any known eukaryotic PLCs in ROS. Exhaustive computer analysis showed little sequence identity between published PLCs and the different isoforms of 14-3-3 but hydrophobic cluster analysis (HCA) demonstrated a striking structural similarity between 14-3-3 and certain bacterial PLCs. This raised the possibility that 14-3-3s were actual PLCs or that they copurified with an authentic, extremely active PLC bound very tightly to 14-3-3.

14-3-3 is the name given to a family of acidic proteins due to a particular migration pattern on two-dimensional gel electrophoresis. They are highly conserved and are ubiquitous from yeast to mammals. At least seven known mammalian isoforms have

been identified which exist naturally as homo- or heterodimers. Our laboratory has shown that all seven isoforms have been found in the rod photoreceptor cells, most if not all in the ROS. 14-3-3s are sequence-specific phosphoserine-binding proteins which appear to participate in a broad spectrum of biological signaling processes. Binding of most signaling proteins such as Raf-1, cdc 25, Protein Kinase C, and most recently discovered PLC γ, appears to contain a putative motif, RSXSXP for 14-3-3 binding. No physiological function has yet been attributed to these retinal isoforms of 14-3-3 protein, but such a motif in PLC γ suggests that 14-3-3s are involved in PLC-related signaling pathways. Due to the above findings, work was initiated to clone and sequence each of the bovine retinal isoforms of 14-3-3. It is our belief that if the proteins can be recombinantly expressed, it will enable determination of their possible roles with respect to regulation of PLC activity. Two of the seven bovine isoforms were previously published (η and ζ), two isoforms are phosphorylated forms of others (α and δ), still another was cloned, sequenced and expressed by a colleague (ϵ), and this thesis describes the cloning, sequencing and expression of the last two (β and γ).

LITERATURE REVIEW

14-3-3 is the name given to a family of acidic proteins originally isolated as abundant cytosolic, bovine brain proteins [Moore et al., 1967]. Further studies revealed that 14-3-3s are a ubiquitous group of 27-30 kDa proteins which functionally exist in a dimeric form. Their highly conserved motifs are seen in mammalian as well as non-mammalian sequences suggesting an evolutionary history dating back prior to the separation of species. 14-3-3 proteins from yeast, plants, Drosophila and Xenopus as well as several mammals have been cloned and sequenced. To date, seven mammalian brain isoforms of 14-3-3 have been described, named alpha, beta, gamma, delta, epsilon, zeta, and eta after their respective elution positions on reverse-phase high performance liquid chromatography (HPLC) [Ichimura et al.,1988]. Five of these have been sequenced [Aitken et al., 1992] and the α and δ isoforms are phosphorylated forms of the β and ζ isoforms respectively [Aitken et al., 1995]. This phosphorylation is at a consensus motif for proline directed (cyclin-dependent) kinases. The discovery of tau in T-cells [Nielson, 1991] and sigma in epithelial cells [Leffers et al., 1993; Prasad et al., 1992] expanded this list of mammalian isoforms. Although some mammalian isoforms may be tissue or cell specific, all tissues and cells examined to date express a combination of isoforms [Aitken et al., 1992]. In both budding and fission yeast, two isoforms of 14-3-3 have been isolated [vanHeusden et al., 1995] and found to be essential genes; yeast with disrupted 14-3-3

genes are nonviable [Gelperin et al., 1995]. Further studies of 14-3-3 isoform diversity in other organisms remain incomplete, and it is not yet known how many organisms have multiple isoforms.

14-3-3 proteins have been ascribed a diverse range of activities including a broad spectrum of biological signal transduction mechanisms in a variety of cell types. They bind to phosphoserines of signaling proteins such as Raf-1, Protein Kinase C, and cdc 25, in a sequence specific manner. A20, phosphatidylinositol 3-kinase, Bcr-Abl, and platelet glycoprotein GpIb-IX all interact with 14-3-3 proteins, and all are involved in signal transduction. It was recently discovered that Phospholipase C (PLC)-γ also contains this 14-3-3 binding motif, RSXSXP, suggesting that 14-3-3(s) are involved in PLC-related signaling pathways [Muslin et al., 1996]. Pinke et al., (1997) found that some or all of these 14-3-3s are closely associated with Phospholipase C (PLC) activity in the ROS. Cloning experiments were done in order to confirm expression in bovine retina of 14-3-3 genes, and to extend our knowledge of the role of 14-3-3 in retinal cells.

MATERIALS AND METHODS

MATERIALS. All radiolabeled compounds were purchased from New England Nuclear. 14-3-3 antibodies were purchased from Santa Cruz Biotechnology. Taq DNA polymerase was purchased from GIBCO BRL. DNA modifying enzymes were purchased from Boehringer Mannheim. Reagents for media preparation were purchased from Difco. The pCR2.1 vector was purchased from Invitrogen and the pTrc99A expression vector was procured from Pharmacia Biotech. All other chemicals were purchased from Sigma.

RNA ISOLATIONS. RNA was isolated by the method of Chomczynski [1987]. Bovine eyes were obtained from MURCO INC of Plainwell, Michigan. Within 3 min of stunning retinas were removed from eyes and dropped into liquid nitrogen. Frozen retinas were placed in a 15 ml polyproplene tube and homogenized in a solution of 4M guanidinium thiocynate, 25mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol [SolnD]. Sequentially, 0.1 ml of 2M sodium acetate, pH4.0, 1 ml of phenol (water saturated), and 0.2 ml of chloroform:isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigourously for 10 sec and cooled on ice for 15 min. Samples were centrifuged at 10,000g for 20 min at 4°C. The aqueous phase containing the RNA was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at -20°C for at least 1h to precipitate the RNA.

Sedimentation at 10,000g for 20 min was again performed and the resulting RNA pellet was dissolved in 0.3 ml of SolnD, transferred into a 1.5ml eppendorf tube, and precipitated with 1 volume of isopropanol at -20°C for 1h. Following centrifugation in an Eppendorf centrifuge for 10 min at 4°C, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried (15 min) and dissolved in 50μl DEPC-treated ddH_2O. Absorbance spectra, 300nm to 200nm, were taken from samples of the RNA to determine purity. A_{260}/A_{280} ratios were ~1.9. The integrity of the RNA was assessed by formaldehyde-1% agarose gel electrophoresis as described by Fourney et al. (1988).

POLY A^+ RNA ISOLATION. Poly A^+ RNA was isolated from total RNA using Stratagene's Poly (A) Quik mRNA Isolation Kit (Stratagene, catalogue #200348).

cDNA LIBRARY PRODUCTION. A cDNA library was made in Stratagene's Uni-Zap XR vector system (Stratagene, catalogue #200400). cDNA synthesis was initiated using poly A^+ RNA as a template ,an oligo d(T) linker primer, and MMLV-RT for first strand synthesis and DNA polymerase I for second strand synthesis following a protocol by Stratagene. The cDNA termini were blunted and Eco RI adaptors were ligated to the ends. The adaptor ends were treated with a kinase and the cDNA was restriction digested to be ligated into the Uni-Zap XR vector. The vector construct was packaged using Stratagene's gigapack II packaging extracts (Stratagene, catalogue #200402). The cDNA library was amplified, titered and stored at 4°C (For long-term storage, 1ml samples of the library were stored at -70°C).

LIBRARY PLATING. The prepared bovine retinal λZapII cDNA library was diluted with SM buffer to $3x10^5$ pfu/ml. 2 μl aliquots were mixed with 600 μl of an

overnight culture of XL1-Blue MRF′ E. coli grown in LB broth plus 0.2% maltose-10mM $MgSO_4$. The mixture was incubated at 37° for 20 min. Then 7.5 ml aliquots of molten (50°) NZY top agarose were added, mixed, and poured onto dry, 37°, 150mm NZY agar plates. After the top agarose hardened the plates were inverted and incubated at 37° until the plaques were 0.1-0.2 mm in diameter (typically 4-5 h). Then the plates were stored at 4° for at least 2h.

LIBRARY REPLICAS. Nitrocellulose filters (132 mm) were wetted in 5x SSC, blotted on filter paper, and placed for 5 min on the surface of the plates. Filters were removed and placed plaque-side-up on a sheet of 3MM paper saturated with 0.5 N NaOH, 1.5 M NaCl for 5 min. Filters were transferred to a sheet of 3MM paper saturated with 0.5 M Tris-Cl, pH 8.0, 1.5 M NaCl for 5 min, then to a sheet of 3MM paper saturated with 2x SSC for 5 min. Filters were blotted and air dried on 3MM paper , then dried in a vacuum oven at 80° for 2h. Finally the filters were stored at room temperature in a vacumn dessicator.

IMMUNOLOGICAL SCREENING. The prepared Bovine retinal cDNA library was screened with a 14-3-3 commercial antibody using the picoBlue Immunoscreening Kit made by Stratagene (Stratagene, catalogue # 200371). Expression screening of $1x10^6$ plaques with an antibody directed against human 14-3-3β (C-20) led to the identification of 40 positive clones. After four consecutive screenings 12 positive clones were isolated and subjected to restriction digest analysis. Only 4 of these positive clones had the predicted restriction product sizes and were subjected to Sanger dideoxy sequencing.

OLIGONUCLEOTIDE SYNTHESIS AND PURIFICATION. Oligonucleotides designed based on 14-3-3 rat brain β and γ sequences [Watanabe et al., 1993] were

synthesized by the MSSSF, Department of Biochemistry, Michigan State University on an Applied Biosystems 3948 nucleic acid synthesizer and purifier. 60nM quantities of each oligo was synthesized, automatically purified and UV quantified at A_{260}.

INTERNAL PCR PROBE SYNTHESIS. Sense and antisense primer sets were synthesized to two highly conserved regions among all 14-3-3 isotypes using Rat β and γ sequences to specialize the primers for those respective isotypes. These primer sets were utilized in RT-PCR as described below. The 540 bp amplified products were cloned into Invitrogen's pCR2.1 vector as described below. Clones having the correct size restriction inserts were sequenced by the Sanger Dideoxy method using ^{35}S. Those clones which were confirmed to contain the 540 bp 14-3-3 β or γ specific sequences were released from the Invitrogen vector, gel purified and radiolabeled using [α-^{32}P] dGTP and T4 polynucleotide kinase (PNK).

DNA SCREENING. The bovine retinal cDNA library was screened with the radiolabeled double stranded internal PCR probes following the protocol by Sambrook et al., (1989). $1x10^6$ plaques were screened with either the β or γ-specific probes. Positive plaques were plaque purified and re-plated for secondary and tertiary screenings.

RT-PCR. First strand cDNA synthesis using 5μg of Bovine retinal total RNA, an oligo $d(T)_{12-18}$ primer and Superscript II Reverse Transcriptase (GIBCO BRL) was carried out following the GIBCO BRL protocol. PCR was used to generate the fragment of DNA containing the open reading frame only, using the first strand cDNA as a template and oligonucleotide primers which included the start and stop codon regions of the β and γ 14-3-3 genes. Protocols developed by Perkin Elmer Cetus were used with empirically determined adjustments made for particular amplifications. Typical reaction conditions

were 20mM Tris-Cl, pH 8.4, 50mM KCl, 1.5mM $MgCl_2$, 200μM each dNTPs, 100pmole sense and antisense primers, 5 Units/μl Taq DNA Polymerase, 1-200ng sscDNA. These samples were subjected to a 6 min 94°C initial denaturation step, followed by 30 cycles containing a 1 min 94°C denaturation step, a 1 min 55°C primer annealing step, and a 3 min 72°C elongation step before a 10 min final extension @ 72°C.

PCR PRODUCT PURIFICATION. The PCR products were purified using Promega's Wizard PCR Preps DNA Purification Kit (Promega, catalogue #A7170).

DNA SEQUENCING. DNA sequencing was performed using two different methods. The purified PCR-amplified products and the sense and antisense primers used for PCR were submitted to the Sequencing Facility, Plant Biology Building, MSU for automated dye primer sequencing. All other templates were sequenced using Sequenase version 2.0 modified bacteriophage T7 DNA polymerase (USB) and its accompanying reagents and protocols with [^{35}S]-dATPαS and with either the M13 -40 forward primer or sequence-specific oligonucleotide primer. Reaction products were separated on a 60 cm, 7 M UREA, 8% polyacrylamide gel.

COMPUTER ANALYSIS OF SEQUENCES was done on Gateway 2000 PC equipment in our laboratory, using GCG software purchased from the University of Wisconsin.

CLONING PCR PRODUCTS. The TA Cloning Kit (Invitrogen, catalogue #K2000-01) was utilized. The kit came with a precut vector, pCR2.1, which has 3′ single T overhangs. This allowed direct ligation of the PCR products without having to resort to restriction cutting and subsequent cleanup. The PCR products, which inherently have 3′ single A overhangs as catalyzed by Taq DNA Polymerase, were ligated to the vector with

T4 DNA ligase. The recombinant DNAs were used to transform competent INV1αF′ E. coli which were provided with the kit. Transformants were then screened by their ability to catabolize the chromogenic substrate, X-gal (blue/white screening).

SUBCLONING INTO EXPRESSION VECTOR. Directional cloning was used to subclone the PCR products into the pTrc99A expression vector (Pharmacia, catalogue #27-5007-01). The pTrc99A vector and the pCR2.1 cloned PCR products were digested with HindIII and NcoI restriction enzymes. The cut PCR products and pTrc99A vector fragments were gel purified using the Geneclean II Kit (BIO 101, catalogue #1001-400) and the PCR fragment was ligated into the new vector following the protocol from Sambrook et al., (1989).

BACTERIAL EXPRESSION OF 14-3-3 β AND γ ISOFORMS. XL1-Blue cells transformed with the pTrc99A β or γ constructs were grown overnight at 37° in LB media containing 50 μg/ml ampicillin. The overnight cultures were diluted 1:10 with fresh medium containing 50 μg/ml ampicillin. The diluted cultures were grown at 37° to mid-log phase (A600= 0.6-1.0). 1.0 mM IPTG was added to each culture in order to induce expression. The cultures were incubated at 37° for an additional 3-5 hours before the proteins were isolated.

AGAROSE GEL ELECTROPHORESIS. A 1% agarose gel was prepared to provide resolution of the larger molecular weight products [Sambrook et al., 1989]. Gels and chamber buffers contained 1xTAE, 0.5μg/ml ethidium bromide.

SOUTHERN BLOT ANALYSIS. Southern analysis was performed by the methods of Sambrook et al., (1989).

ACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS. Proteins were electrophoresed in denaturing conditions by the method of Sambrook et al., (1989).

WESTERN BLOT ANALYSIS. Western blot analysis was performed as described by Martin et al., (1993). Antibodies specific for the carboxy terminus of the β and γ isoforms were purchased from Santa Cruz Biotechnology.

RESULTS

cDNA Library Screening. Expression screening of a bovine retinal cDNA library utilizing the commercial 14-3-3β (C-20) antibody directed against human 14-3-3 was unsuccessful. Of the four clones isolated after repetitive screenings and restriction digest analysis, none appeared to have 14-3-3 sequence but three clones contained sequence of that of peripherin. It may be that 14-3-3 is in low abundance in the cDNA library as compared to peripherin. Under the guidance of my committee, I refrained from any further immunological screening and tried a new approach.

Two internal dsDNA probes specific for the β and γ genes were made by PCR (see Methods). Plaque lifts of $1x10^6$ pfus were hybridized with each probe and the resulting autorads revealed 25 plaques that hybridized to the 14-3-3 γ probe and 10 plaques that hybridized to the β probe. The plaques were purified and subjected to secondary and tertiary screenings where 5 of the original 25 hybridized again to the γ probe and 3 of 10 to the β probe. Restriction analysis of the positive clones was inconclusive. PCR analysis with the eight positive clones resulted in the correct size product and Southern blots using the same internal PCR probes used to probe the library were positive. No 14-3-3 sequences were obtained from any of the positive clones. The β specific probe isolated an enolase which showed 65% homology to the probe. The γ probe also isolated a non 14-3-3 sequence exhibiting less than 50% identity.

The sequence of the probes had been confirmed and their size of 540 bp was appropriate for screening. This led me to believe that the problem was either with the library or with the screening technique. Before screening again, the complexity of the library and the screening protocol should be examined. Since both screening attempts had failed, another approach was devised patterned after Jones et al., (1995).

RT-PCR. Two sets of PCR primers were synthesized to include the start and stop codon regions of the published rat brain 14-3-3 β and γ sequences (see Fig 1). These were used in RT-PCRs as described in methods. The PCR reactions for both β and γ using these primers and sscDNA as the template yielded a single product of approximately the expected size of the total open reading frames (ORFs), 740 bp (see Fig 2). The PCR products were blotted onto nitrocellulose and probed with β or γ specific internal PCR probes (see Methods). The β and γ 740 bp products each hybridized to the β or γ specific internal probes respectively (see Fig 3). This suggested that if not all at least some of the open reading frame for both the β and γ genes had been amplified.

Direct Sequencing. The 740 bp PCR products were column purified using Promega's Wizard PCR DNA purification System. The samples were directly sequenced using automated dye primer sequencing. In separate reactions the sense and appropriate antisense PCR primers for each product were used as sequencing primers. According to the BLAST sequence search module (NIH), the resulting sequences showed a high homology to those of 14-3-3. Additional primers were synthesized to sequence further in both directions (see Fig 1). The full length ORFs for both the β and γ genes were sequenced (see Fig 4). These sequences exhibited 89% homology with the rat 14-3-3 sequences used to make the primers and >60% identity to the other published bovine

Figure 1. Oligonucleotide Primers

A. The two PCR primer pairs were complementary to 1) the rat brain 14-3-3 β [Watanabe et al., 1993] and 2) the rat brain 14-3-3 γ [Watanabe et al., 1993] sequences. They were constructed similar to those used by Jones et al. [1995]. Each one was 32-33 bases long with a 8 base region at the 5′ end that contained a 6 base restriction site and 2 base "clamp" to aid in subsequent cloning of PCR products.
B. These are the additional primers made to extend the sequence and confirm the complete open reading frame of both the β and γ genes.

Figure 1A Initial PCR Primer Pairs

1) Rat Brain 14-3-3 β:

Sense primer corresponding to bases 1-24

M D K S E L V Q

5′ GGAATTCC ATG GAC AAA AGT GAG CTG GTA CAG 3′

NcoI

Antisense primer corresponding to bases 711-735

* N E G E G A D

5′ ACGCAAGC TTA GTT CTC TCC CTC TCC AGC ATC T 3′

HindIII

2) Rat Brain 14-3-3 γ:

Sense primer corresponding to bases 1- 25

M V D R E Q L V

5′ GGAATTCC ATG GTG GAC CGA GAG CAA CTG GTG C 3′

NcoI

Antisense primer corresponding to bases 720-744

* N N G E G G D

5′ ACGCAAGC TTA GTT GTT GCC TTC GCC GCC GTC G 3′

HindIII

Figure 1B. Secondary Sequencing Primers

Sense, γ-14-3-3 Bases 94-114

5′ GAG CTG AAT GAG CCA CTG TCC 3′

Sense, γ-14-3-3 Bases 603-622

5′ CGA CGA TGC CAT CGC CGA GC 3′

Antisense, γ-14-3-3 Bases 94-114

5′ GGA CAG TGG CTC ATT CAG CTC 3′

Sense,β-14-3-3 Bases 422-443

5′ CTG TGT CGA ACT CCC AGC AGG C 3′

Sense, β-14-3-3 Bases 46-66

5′ GCC GAG CGC TAC GAT GAC ATG 3′

Sense,β-14-3-3 Bases 82-103

5′ GCG GTC ACA GAG CAG GGG CAC G 3′

Antisense,β14-3-3 Bases 82-103

5′ CGT GCC CCT GCT CTG TGA CCG C 3′.

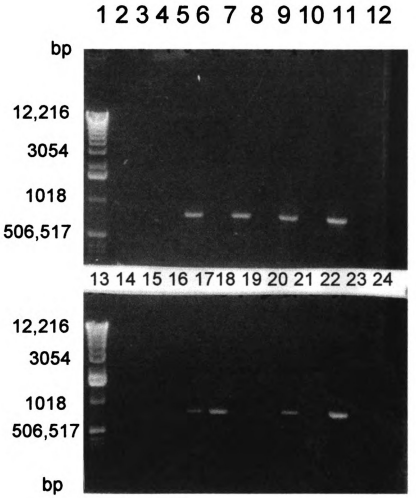

Figure 2. RT-PCR Products

The PCR primers (Figure 1A) were combined in RT-PCRs using, oligo d(T)-primed sscDNA. The products were fractionated on a 1% agarose gel and compared with MW markers (lane 1 and 13, 1 ug 1kb DNA ladder). The PCRs used either the sense and antisense #1 primers (lanes 5, 7, 9, and 11) yielding a predominant band of 735 bp the expected size of the Beta gene, or used the sense and antisense #2 primers (lanes 17, 18, 21, 23) yielding a predominant band of 744 bp the expected size of the Gamma gene. These products were isolated by the Wizard DNA purification Kit (Promega) and subsequently sequenced and cloned.

Southern of Figure 2 lanes 1-12,
Beta-specific PCR products

Southern of Figure 2 lanes 13-24,
Gamma-specific PCR products

Figure 3. Southern Analysis of PCR Products.

The PCR products were fractionated on a 1% agarose gel with ~9 ug DNA/lane. The DNA was transferred to a nitrocellulose membrane by standard blotting techniques. The internal PCR probes, Beta and Gamma, were individually labeled using [∝-32P]dCTP and T7 polynucleotide kinase and hybridized to their perspective membranes containing four lanes of Beta or Gamma-specific DNA. After washing, autorads of the membranes were taken.

Figure 4. Nucleotide and Deduced Amino Acid Sequences of Bovine 14-3-3 β and γ

Figure 4A. Bovine Retinal 14-3-3 β Sequences.

```
1 ATGGACAAAAGTGAGCTGGTACAGAAAGCCAAGCTCGCCGAGCAGGCCGA
    M  D  K  S  E  L  V  Q  K  A  K  L  A  E  Q  A  E
51 GCGCTACGATGACATGGCTGCGGCCATGAAGGCGGTCACAGAGCAGGGGC
     R  Y  D  D  M  A  A  A  M  K  A  V  T  E  Q  G
101 ACGAGCTCTCCAACGAGGAGAGAAACCTGCTGTCCGTCGCCTACAAGAAT
    H  E  L  S  N  E  E  R  N  L  L  S  V  A  Y  K  N
151 GTGGTCGGCGCCCGCCGTTCGTCCTGGCGTGTCATCTCCAGCATCGAACA
     V  V  G  A  R  R  S  S  W  R  V  I  S  S  I  E  Q
201 GAAAACTGAGAGGAACGAGAAGAAGCAGCAGATGGGCAAAGAGTACCGCG
       K  T  E  R  N  E  K  K  Q  Q  M  G  K  E  Y  R
251 AGAAGATCGAGGCCGAGCTGCAGGACATCTGCAATGACGTGCTGCAGCTG
    E  K  I  E  A  E  L  Q  D  I  C  N  D  V  L  Q  L
301 TTGGATAAATACCTTATTCCCAATGCTACACAACCAGAAAGTAAGGTGTT
     L  D  K  Y  L  I  P  N  A  T  Q  P  E  S  K  V  F
351 CTACTTGAAAATGAAAGGCGATTATTTTAGATATCTTTCTGAGGTGGCTT
       Y  L  K  M  K  G  D  Y  F  R  Y  L  S  E  V  A
401 CTGGAGACAATAAACAAACCACTGTGTCGAACTCCCAGCAGGCTTACCAA
     S  G  D  N  K  Q  T  T  V  S  N  S  Q  Q  A  Y  Q
451 GAAGCATTTGAAATTAGTAAGAAAGAAATGCAGCCTACACACCCCATTCG
       E  A  F  E  I  S  K  K  E  M  Q  P  T  H  P  I  R
501 ACTGGGGCTGGCACTTAATTTCTCCGTCTTTTATTATGAGATTCTAAACT
         L  G  L  A  L  N  F  S  V  F  Y  Y  E  I  L  N
551 CTCCTGAAAAGGCTTGCAGCCTGGCAAAAACGGCGTTTGATGAGGCGATT
     S  P  E  K  A  C  S  L  A  K  T  A  F  D  E  A  I
601 GCTGAATTGGACACACTGAATGAAGAGTCTTACAAAGACAGCACCCTGAT
       A  E  L  D  T  L  N  E  E  S  Y  K  D  S  T  L  I
651 TATGCAGCTGCTTAGGGACAATCTCACTCTGTGGACGTCGGAAAACCAGG
         M  Q  L  L  R  D  N  L  T  L  W  T  S  E  N  Q
701 GGGACGAAGGAGATGCTGGAGAGGGAGAGAACTAA
    G  D  E  G  D  A  G  E  G  E  N
```

Figure 4B. Bovine Retinal 14-3-3 γ Sequences

```
  1 ATGGTGGACCGAGAGCAACTGGTGCAGAAAGCCCGGCTGGCGGAGCAGGC
    M  V  D  R  E  Q  L  V  Q  K  A  R  L  A  E  Q  A
 51 GGAGCGCTATGACGATATGGCCGCGGCCATGAAGAACGTGACGGAGCTGA
      E  R  Y  D  D  M  A  A  A  M  K  N  V  T  E  L
101 ATGAGCCACTGTCCAATGAAGAAAGAAACCTTCTCTCTGTCGCCTACAAG
    N  E  P  L  S  N  E  E  R  N  L  L  S  V  A  Y  K
151 AATGTAGTCGGGGCACGCCGTTCTTCCTGGAGGGTTATCAGTAGCATCGA
      N  V  V  G  A  R  R  S  S  W  R  V  I  S  S  I  E
201 GCAGAAGACATCTGCCGACGGTAACGAGAAGAAAATAGAGATGGTCCGTG
      Q  K  T  S  A  D  G  N  E  K  K  I  E  M  V  R
251 CTTACCGTGAAAAAATAGAGAAAGAGTTGGAGGCCGTATGTCAGGATGTG
    A  Y  R  E  K  I  E  K  E  L  E  A  V  C  Q  D  V
301 CTGAGCCTGCTGGATAACTACCTGATCAAGAATTGCAGCGAGACCCAGAT
      L  S  L  L  D  N  Y  L  I  K  N  C  S  E  T  Q  I
351 TGAGAGCAAAGTGTTTTACCTGAAGATGAAAGGGGACTATTACCGCTACC
      E  S  K  V  F  Y  L  K  M  K  G  D  Y  Y  R  Y
401 TGGCCGAAGTCGCCACCGGCGAGAAGAGGGCGACCGTCGTGGAGTCATCT
    L  A  E  V  A  T  G  E  K  R  A  T  V  V  E  S  S
451 GAGAAGGCCTACAGCGAAGCCCACGAAATCAGCAAGGAGCACATGCAGCC
      E  K  A  Y  S  E  A  H  E  I  S  K  E  H  M  Q  P
501 CACTCACCCCATTAGATTAGGCCTGGCCCTTAACTACTCCGTCTTCTACT
      T  H  P  I  R  L  G  L  A  L  N  Y  S  V  F  Y
551 ACGAAATCCAGAACGCCCCGGAACAAGCCTGCCACTTGGCCAAGACCGCC
    Y  E  I  Q  N  A  P  E  Q  A  C  H  L  A  K  T  A
601 TTCGACGATGCCATCGCCGAGCTTGACACCCTCAACGAAGACTCCTACAA
      F  D  D  A  I  A  E  L  D  T  L  N  E  D  S  Y  K
651 GGACTCCACGCTGATCATGCAGCTGCTCCGTGACAACCTCACGCTCTGGA
      D  S  T  L  I  M  Q  L  L  R  D  N  L  T  L  W
701 CGAGCGACCAGCAAGACGACGACGGCGGCGAAGGCAACAACTAA
    T  S  D  Q  Q  D  D  D  G  G  E  G  N  N
```

brain 14-3-3 isotypes (Table 1). A lineup was done to compare the bovine retinal 14-3-3 amino acid sequences (Fig 5). There are distinct regions of high homology among the various isoforms. The 27 peptide sequences isolated from the dimeric protein copurifing with ROS PLC activity as described by Pinke et al., (1997) were superimposed on the lineup as seen by the highlighted sequences.

Cloning PCR Products For Expression. The two 740 bp PCR products were cloned using the TA Cloning Kit (Invitrogen). This system takes advantage of the fact that Taq polymerase catalyzes the addition of a single A to the 3′ end of the newly amplified cDNA yielding a single base overhang. The supplied vector, pCR2.1, comes precut and has a complementary 3′ single T overhang allowing direct ligation of PCR products using T4 ligase. The INV1αF′ E. coli transformed with these constructs theoretically should yield white colonies when grown on LB agar plus 50 μg/ml ampicillin and spread with 1mg/ml X-gal, whereas those transformants that have self-ligated vectors should yield blue colonies denoting α-complementation. Some transformants with recombinant DNA were initially identified by this blue/white screening method. However, additional transformants with recombinant plasmids were discovered in subsequent screenings that still had partially functional β-galactosidases that in the presence of X-gal yielded light blue or white colonies with a blue center.

Streaks of 30 transformants from the β(740) ligation and γ(740) ligation (these included all white, some light blue and blue colonies) were grown on nitrocellulose filters. The filters were each incubated with their respective β- or γ-specific internal PCR probe (see Methods). The β-specific probe hybridized to 9 out of the 30 transformants and the γ-specific probe hybridized to 4 out of 30 transformants.

Table 1. Comparison of 14-3-3 PCR Clones with Published Sequences

Bovine Retina	**β-14-3-3 from Rat Brain**		**γ-14-3-3 from Rat Brain**		**η-14-3-3 from Bovine Brain**		**ζ-14-3-3 from Bovine Brain**	
	cDNA	AA	cDNA	AA	cDNA	AA	cDNA	AA
β	89%	98%	72%	84%	72%	83%	74%	90%
ε	66%	75%	65%	73%	64%	72%	65%	78%
η	71%	82%	84%	91%	100%	100%	69%	83%
γ	70%	83%	89%	99%	80%	91%	68%	83%
ζ	75%	90%	67%	83%	69%	83%	100%	100%

Table 1. Comparisons of 14-3-3 Sequences. The table shows the percent similarity of both the cDNA and the amino acid sequences among the seven identified bovine retinal 14-3-3 isoforms (α and δ excluded because they are phosphorylated forms of β and ζ respectively) with those of published rat and bovine brain 14-3-3 sequences.

Figure 5. 14-3-3 Bovine Retinal Amino Acid Sequences.

This is a lineup of the now known Bovine 14-3-3 amino acid sequences. Those sequences in red represent those determined by our lab, those in black have been previously published, and the blue sequences are the consensus. The highlighted areas represent the 27 peptide sequences obtained by Pinke et al., (1997). Some of the 27 sequences were repetitive; the highlighted areas of the consensus sequence suggests that peptides were sequenced for all of the isoforms in this region.

Figure 5.

```
            1                                                  50
      Beta  .M.DKSELVQ KAKLAEQAER YDDMAAAMKA VTEQGHELSN EERNLLSVAY
   Epsilon  MD.DREDLVY QAKLAEQAER YDEMVESMKK VAGMDVELTV EERNLLSVAY
       Eta  MG.DREQLLQ RARLAEQAER YDDMASAMKA VTELNEPLSN EDRNLLSVAY
     Gamma  MV.DREQLVQ KARLAEQAER YDDMAAAMKN VTELNEPLSN EERNLLSVAY
      Zeta  ..MDKNELVQ KAKLAEQAER YDDMAACMKS VTEQGAELSN EERNLLSVAY
 1433proseq M..Dre.Lvq kAkLAEQAER YDdMaaaMKa Vte..eeLsn EeRNLLSVAY

            51                                                 100
      Beta  KNVVGARRSS WRVISSIEQK TER..NEKKQ QMGKEYREKI EAELQDICND
   Epsilon  KNVIGARRAS WRIISSIEQK EENKGGEDKL KMIREYRQMV ETELKLICCD
       Eta  KNVVGARRSS WRVISSIEQK TMADGNEKKL EKVKAYREKI EKELETVCND
     Gamma  KNVVGARRSS WRVISSIEQK TSADGNEKKI EMVRAYREKI EKELEAVCQD
      Zeta  KNVVGARRSS WRVVSSIEQK TE..GAEKKQ QMAREYREKI ETELRDICND
 1433proseq KNVvGARRsS WRviSSIEQK tea.gnEkK. .mvreYReki E.ELediCnD

            101                                                150
      Beta  VLQLLDKYLI PNA..TQPES KVFYLKMKGD YFRYLSEVAS GDNKQTTVSN
   Epsilon  ILDVLDKHLI PAA..NTGES KVFYYKMKGD YHRYLAEFAT GNDRKEAAEN
       Eta  VLALLDKFLI KNCNDFQYES KVFYLKMKGD YYRYLAEVAS GEKKNSVVEA
     Gamma  VLSLLDNYLI KNCSETQIES KVFYLKMKGD YYRYLAEVAT GEKRATVVES
      Zeta  VLSLLEKFLI PN..ASQAES KVFYLKMKGD YYRYLAEVAA GDDKKGIVDQ
 1433proseq vLslLdk.LI pn...tq.ES KVFYlKMKGD YyRYLaEvA. G..kktvven

            151                                                200
      Beta  SQQAYQEAFE ISKKEMQPTH PIRLGLALNF SVFYYEILNS PEKACSLAKT
   Epsilon  SLVAYKAASD IAMTELPPTH PIRLGLALNF SVFYYEILNS PDRACRLAKA
       Eta  SEAAYKEAFE ISKEHMQPTH PIRLGLALNF SVFYYEIQNA PEQACLLAKQ
     Gamma  SEKAYSEAHE ISKEHMQPTH PIRLGLALNY SVFYYEIQNA PEQACHLAKT
      Zeta  SQQAYQEAFE ISKKEMQPTH PIRLGLALNF SVFYYEILNS PEKACSLAKT
 1433proseq S.qAY.eAfe Isk.emqPTH PIRLGLALNf SVFYYEIlNs Pe.ACsLAKt

            201                                                250
      Beta  AFDEAIAELD TLNEESYKDS TLIMQLLRDN LTLWTSENQG DEGDAGEGEN*
   Epsilon  AFDDAIAELD TLSEESYKDS TLIMQLLRDN LTLWTSDMQG DGEEQNKEAL
       Eta  AFDDAIAELD TLNEDSYKDS TLIMQLLRDN LTLWTSDQQD EEAGEGN*
     Gamma  AFDDAIAELD TLNEDSYKDS TLIMQLLRDN LTLWTSDQQD DDGGEGNN*
      Zeta  AFDEAIAELD TLSEESYKDS TLIMQLLRDN LTLWTSDTQG DEAEAGEGGE
 1433proseq AFDdAIAELD TLnEeSYKDS TLIMQLLRDN LTLWTSdQg de...g.g..

            251
   Epsilon  QDVEDENQ*
      Zeta  N*
 1433proseq .DVEDENQ*
```

Overnight cultures were started for these 13 transformants and their plasmids were purified by methods of Sambrook et al., (1989). The insert size was checked by restriction digestion and those which had the correct insert size were used in the subsequent subcloning experiment. Only nine of the restriction digested transformants retained an insert of the correct size.

Plasmids were purified for these nine positive transformants and they were double digested with HindIII and NcoI (these restriction sites were designed into the original PCR primers). Plasmid DNA was also prepared for the pTrc99A vector (Pharmacia) which was subsequently digested with the same enzymes. The restriction products were separated on an agarose 1% gel and the vector fragment as well as the digested β and γ inserts were gel purified following the protocol given by BIO 101. The purified inserts were subcloned into the pure pTrc99A vector for expression. The pTrc99A constructs were transformed as described above with XL1-Blue cells. Antibiotic selection, restriction digestion and Southern analysis were used to select for transformants.

Bacterial Expression. The ATG start codons of the DNA encoding the β and γ isoforms are contained within an NcoI restriction site. The cDNAs had been cloned as 750 bp fragments into the EcoRI site plasmid pCR2.1 (Invitrogen), so the intact gene was contained in a 740 bp NcoI-HindIII fragment. This fragment was ligated into the expression vector pTrc99A (Pharmacia); induction of the trc promoter using 1mM IPTG produced a band of M_r 30,000 on SDS-PAGE (Fig 6). Western blot analysis using commercial antibodies revealed that antibodies bound the isolated expressed proteins (Fig 7).

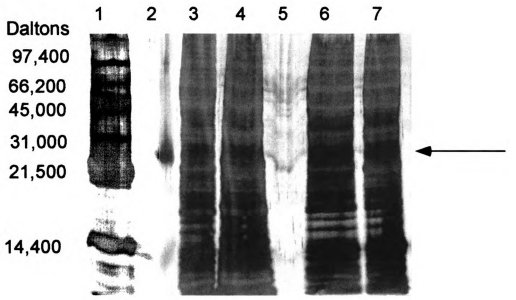

Figure 6. SDS PAGE of Expressed Proteins

A 12% SDS-polyacrylamide gel was used to fractionate 5 ul of crude lysates from induced and uninduced production of 14-3-3 Beta and Gamma proteins. Lane 1 is 5 ul of the Kaleidoscope Prestained Standards (BIO-RAD), lanes 3and 4 are 5 ul samples of un-induced and induced 14-3-3 Beta respectively, and lanes 6 and 7 are 5 ul samples of uninduced and induced 14-3-3 Gamma respectively. The arrow points out the doublet band about 30,31kDa..

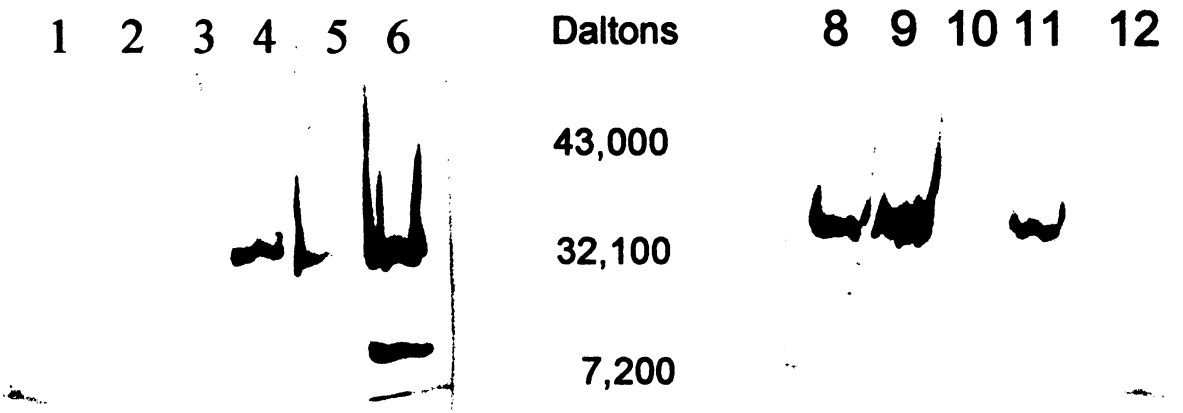

Figure 7. Western Blot Analysis of Expressed Proteins

The 14-3-3 Beta and Gamma proteins expressed using the pTrc99A vector were fractionated on a 12%-SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes by standard western blotting techniques. Immunoassays using commercial 14-3-3 Beta and Gamma antibodies were performed following a BIO RAD protocol.The crude lysates from expression were diluted 1:10; lanes 1,7,and 12 contained 5 ul of the Kaleidoscope Prestained Standards (BIO-RAD), lanes 3 and 4 are the uninduced expression of 14-3-3 Beta 1and 5 ul of diluted lysate respectively, lanes 5and 6 are the induced expression of 14-3-3 Beta 1 and 5 ul of diluted lysate respectively, lanes 8 and 9 are the induced expression of 14-3-3 Gamma 1 and 5 ul of diluted lysate respectively, lanes 10 and 11 are the uninduced expression of 14-3-3 Gamma 1 and 5 ul of diluted lysate respectively.

DISCUSSION

14-3-3 Isoforms In Bovine Photoreceptors. 14-3-3 is a multifunctional protein found in a diversity of tissues from yeast to mammals. In our laboratory we have found all seven of the known mammalian 14-3-3 isoforms in the rod photoreceptor cells, most if not all in the ROS [Pinke et al, 1997; Wei et al., 1997]. The actual functions of these retinal forms of 14-3-3 are still unknown but they appear either to exhibit an intrinsic PLC activity or to copurify with an authentic hyperactive phosphatidylinositol hydrolyzing protein [Pinke et al., 1997]. Attempts in our laboratory and others to isolate a typical X,Y-type eukaryotic PLC from purified ROS have been unsuccessful. Computer comparisons of published sequences of every downloadable PLC from national and international sequence banks to the array of 14-3-3 peptide sequences identified by Pinke et al, (1997) showed no significant sequence similarity. However a striking structural similarity between 14-3-3 and certain bacterial PLCs was observed when they were compared by hydrophobic cluster analysis (HCA) using a computer graphics program [Gaboriaud et al, 1987; Lemesle-Varloot et al, 1990]. Recombinant expression studies with the eta, zeta, and epsilon isoforms of 14-3-3 failed to produce an active PLC. Some possible involvement in the PLC signaling pathway would not be surprising considering many signaling proteins have been shown to interact with one or more isoforms of 14-3-3, PLC γ recently included. The actual interaction between these signaling proteins and

14-3-3(s) is specific to phosphoserine-containing motifs and speculated to be related to their ability to dimerize. 14-3-3 N-terminus mediates the formation of its hetero- or homodimers. It is believed that this dimerization would allow the protein to act as an adaptor between two molecules and thereby modulate their activity in a phosphoserine regulated manner [Jones et al., 1995]. The dimerization of 14-3-3 forms a hydrophobic pocket which may work to stabilize and bring two compounds into proximity in order to react. In pursuit of this possibility recent studies applying the original purified dimeric protein of 31,33 kDa to an HPLC heparin column used by Rhee's group to separate a variety of PLCs was done by Pinke et al, (1997). The column separated the 31,33 protein into a major peak (assessed by A_{280}) containing an assortment of 14-3-3 isoforms as denoted by western blot but no inherent PLC activity and a very small peak containing remarkably high activity estimated from A_{280} at 100 μmol/min/mg protein, but no 14-3-3 immunoreactivity. These results by Pinke et al, (1997) appear to strengthen the theory of co-purification of 14-3-3 and PLC, but an additional experiment running the smaller peak generated by.heparin purification on a SDS PAGE revealed a strong band less than 100 kDa in size, and two faint bands about the same size as the 31,33 kDa 14-3-3 dimer. There remains the possibility that the enzymatically active smaller peak is a derivative of 14-3-3 which does not immunoreact with the commercial antibodies, mapping mostly to the carboxy terminus of the different isotypes. Yet another theory is that 14-3-3s act as chaperones for degraded hyperactive PLC fragments. Their role as chaperones may be to protect or enhance the active sites of degraded PLCs. Since X and Y regions are presumed to comprise the active site of eukaryotic PLCs, degradation of N- and C-termini could greatly enhance the activity of ROS PLCs, as long as critical regions are

protected. This would be consistent with the striking structural similarity between certain PLCs and the putative chaperones. It is known that PLC activity is found in both membrane and particulate fractions [Rhee et al., 1989]. Perhaps the role of 14-3-3 is to promote the transition of PLCs from the particulate form to the soluble form.

A Focus For Future Research. The future of this line of research lies in studying the binding and localization of ROS 14-3-3s with respect to PLCs. The 14-3-3 clones can be used as bait in yeast-two hybrid screenings of a bovine retinal cDNA library and/or flag-epitope tagged to study in vitro and in vivo binding interactions of ROS 14-3-3s and PLCs. Reconstitution studies using the 14-3-3 clones and purified ROS PLCs may prove valuable in understanding 14-3-3s possible regulation of PLC activity. Is it possible to inactivate the PLC by removing the tightly bound 14-3-3 ?

Preliminary localization studies done by the author, using an FITC-labeled antibody to 14-3-3 β exhibited uniformly distributed fluorescence of ROS under confocal microscopy. Laurie Molday, a collaborator at UBC Vancouver, examined an ε-antibody by electron microscopy and found it distributed in the interior of both inner and outer segments. More extensive localization studies by Laurie Molday will be managed using a pool of commercial 14-3-3 antibodies recently obtained by our lab. It is our belief that cloning of these various 14-3-3 isoforms confirms expression in the rod photoreceptor cells and will bring us closer to understanding their possible function in the PLC signal transduction pathway.

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