The Second PDZ Domain of INAD Is a Type I Domain Involved in Binding to Eye Protein Kinase C

MUTATIONAL ANALYSIS AND NATURALLY OCCURRING VARIANTS*

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INAD is a scaffolding protein containing five PSD95/dlg/zonular occludens-1 (PDZ) domains that tether NORPA (phospholipase Cβ), the TRP calcium channel, and eye-PKC in Drosophila photoreceptors. We previously showed that eye-PKC interacted with the second PDZ domain (PDZ2) of INAD. Sequence comparison with a prototypical type I PDZ domain predicts that PDZ2 is the best candidate among the five PDZ domains to recognize eye-PKC that contains a type I PDZ ligand, Ile-Thr-Ile-Ile, at its carboxyl terminus. Replacement of Ile319 in eye-PKC with charged residues resulted in a drastic reduction of the PDZ2 interaction. Substitution of a conserved His with Arg at the second α-helix of PDZ2 led to a reduced binding; however, a Leu replacement resulted in an enhanced eye-PKC association. We isolated and sequenced the InaD gene. The coding sequence of InaD contains nine exons spanning 3 kilobases. Translation of coding sequences from three wild-type alleles revealed three SNPs affecting residues, 282, 319, and 333 of INAD. These polymorphisms are localized in PDZ2. Interestingly, we found two of three PDZ2 variants displayed a greater affinity for eye-PKC. In summary, we evaluated the molecular basis of the eye-PKC and PDZ2 association by mutational analysis and concluded that PDZ2 of INAD is a type I domain important for the eye-PKC interaction.

The InaD (inactivation-no-after potential D) gene is preferentially expressed in the compound eye and was isolated by subtractive hybridization (1). Molecular characterization of InaD indicates that it encodes a protein of 674 amino acid residues that contains five distinct PSD95/dlg/zonular occludens-1 (PDZ) domains (2, 3). PDZ domains are protein-protein interaction motifs of 80–100 residues and are implicated in clustering and localization of receptors and channels (4, 5). In Drosophila photoreceptors, INAD has been shown to interact with carboxyl-terminal sequences of three key components of the visual cascade leading to the formation of a macromolecular signaling complex (6–11). These INAD-interacting proteins include TRP (transient-receptor-potential), NORPA (no-receptor-potential A), and eye-PKC. Eye-PKC is involved in a negative regulation of visual transduction (12, 13), a G protein-coupled phospholipase Cβ-mediated process that converts the light leading to depolarization of photoreceptors (14, 15). Negative modulation of visual signaling by eye-PKC in vivo is dependent on its interaction with INAD (11). Previously we showed that eye-PKC associated with PDZ2 of INAD (11). Other reports have implicated PDZ3 or PDZ4 in the eye-PKC interaction as well (9, 16).

Structural studies of PDZ domains in PSD95, human homologues of dlg, and calcium/calmodulin-dependent serine protein kinase revealed that PDZ domains consist of six β-sheets and two α-helices forming a six-strand β-sandwich structure (17–19). With some exceptions (6, 20), most PDZ domains bind to the last 3–4 amino acids at the carboxyl-terminal tail of target proteins (21). Based on the target or ligand sequences, PDZ domains can be subdivided into two classes: type I and type II (21). Type I PDZ domains recognize ligands that contain either a Ser or Thr at the corresponding position (19, 21). Almost all PDZ-interacting ligands have a hydrophobic residue (Ile, Leu, or Val) at the carboxyl terminus (position 2) (2–5, 8). X-ray crystallographic studies revealed that the tetrapeptide ligand anchors to a groove formed between the second β-strand and the third α-helix of the PDZ domain (17, 19). Each PDZ domain appears to recognize a unique carboxy-terminal sequence (21).

Because the carboxy-terminal of eye-PKC (11) contains a type I PDZ ligand, we sought to identify a type I domain in INAD. Sequence alignment with the third PDZ domain (PDZ3) of PSD95, a type I domain, indicates that PDZ2 is the only type I domain in INAD. PDZ3 of PSD95 interacts with a tetrapeptide, Gln-Thr-Ser-Val (17), and the side chains of Gln form hydrogen bonds with those of Ser and Asn in PDZ3 of PSD95. In contrast, the corresponding residue of Gln in eye-PKC is Ile³, a residue with a hydrophobic side chain. We explored how a different residue at the −3 position of the target may interact with the different type I PDZ domains. We also performed site-directed mutagenesis by modifying Ile³ of eye-PKC and investigated the contribution of this residue in the PDZ2 recognition. To explore the basis of the type I interaction, we mutated a conserved His in the second α-helix of PDZ2. This His has been implicated in the interaction with Ser/Thr at the −2 position (17). Interestingly, we found that substitution of His¹⁴⁰ with a Leu resulted in enhanced eye-PKC interaction, whereas replacement with Arg led to a reduction of association.

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The abbreviations used are: PDZ, PSD95/dlg/zonular occludens-1; GST, glutathione S-transferase; PCR, polymerase chain reaction; PSD95, post-synaptic density protein 95; RACE, rapid amplification of cDNA ends; SNPs, single nucleotide polymorphisms; UTR, untranslated region; PKC, protein kinase C; eye-PKC, eye-specific protein kinase C; PAGE, polyacrylamide gel electrophoresis.

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To gain insight into the regulation of InaD expression, we analyzed the genomic structure of InaD and mapped the transcription start site by RACE. The InaD gene contains nine exons. Interestingly, we found SNPs in the coding exons leading to substitutions in three residues of PDZ2. We analyzed these variant PDZ2 and show that two modified PDZ domains display an increase in eye-PKC binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ficoll, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium were obtained from Research Organic, Inc. (Cleveland, OH). Phenol and deoxynucleotides were purchased from U. S. Biochemicals. Chloroform and other chemicals were from Fisher. Nitrocellulose filters were from Schleicher & Schuell. DNA size markers were purchased from Life Technologies, Inc. Restriction enzymes and modifying enzymes such as T4 DNA ligase were obtained from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA), or Promega (Madison, WI). RNasin and pGEMEX1 were obtained from Promega. [32P]dCTP was from PerkinElmer Life Sciences.

**Genomic Library Screening**—A Drosophila genomic library prepared from Canton S strain in Charon 4 vector (22) was plated. Filter replicas of the library were probed with radioactively labeled InaD in a hybridization solution (5% SSC, 0.5% SDS, 10 mM EDTA, 5× Denhardt’s solution, 1% SDS). Subsequent washes were done at 65 °C for 2 h (23). Positive plaques were identified by autoradiography and purified. The EcoRI inserts containing the InaD gene were identified and subcloned into the plBluescript KS vector (Stratagene) for sequence analysis.

**DNA Sequencing**—The nucleotide sequence was determined either by the dideoxy chain termination method (24) or by automatic DNA sequencing using ABI PRISM BigDye™ Terminator Cycle Sequencing Ready reaction kit (PerkinElmer Life Sciences). For DNA obtained by PCR at least four to six independent subclones were sequenced.

**Isolation of Fly Genomic DNA**—Total genomic DNA was isolated as described previously (1). Briefly, 50 flies were gently homogenized in 3 mL of buffer containing (100 mM NaCl, 100 mM Tris, pH 7.6, 100 mM EDTA, 0.5% SDS). The homogenates were incubated at 65 °C for 30 min, and 8 mM potassium acetate (one sixth volume) was added. The mixture was incubated on ice for 20 min, and the supernatant was recovered following centrifugation. RNase A (50 μg) was added to the supernatant to hydrolyze RNA. The mixture was subjected to phenol/chloroform extraction to remove proteins. Ethanol was added to the supernatant to precipitate genomic DNA.

**Isolation of RNA and Synthesis of First Strand cDNA**—Fly heads from various strains were isolated, and total RNA were extracted according to Chomczynski and Sacchi (25). Total RNA was precipitated by ethanol and quantified by spectrophotometry. To generate first strand cDNA, 20 μg of total RNA were added to a 25-μL reverse transcription reaction using the reverse transcription system (Promega). Following incubation at 42 °C for 1 h, the reaction was terminated by addition of 75 μL of 10 mM EDTA (pH 8.0). An aliquot of cDNA (1–2 μg of RNA equivalent) was used as templates for PCR analysis.

**Polymerase Chain Reaction**—PCR was used to amplify genomic DNA as well as cDNA using InaD-specific primers. A negative control containing no added templates was also performed to assure the specificity of amplifications. The amplified fragments were subcloned into pCR2.1 using the TOPO TA Cloning system (Invitrogen), and recombinant plasmids were purified and used for sequencing. The experimental conditions for PCR (30 cycles) were denaturation at 94 °C for 30 s and annealing at 50 °C for 30 s followed by extension at 72 °C for 2–3 min. The reaction mixture (50 μL) contained 50–100 ng of each primer, 1 μg of total genomic DNA (or first strand cDNA from 1–2 μg total RNA) as templates, 0.2 mM dNTP, and 2.5 units of Taq DNA polymerase (PerkinElmer Life Sciences), in a buffer containing 10 mM Tris (pH 8.3), 50 mM KCl, and 1.5 mM MgCl2. Primers for InaD genomic and cDNA amplification were primers b and d (see Fig. 4A). Primer sequences for the amplification of the eye-PKC carboxytail tail were TCT GGA TTC ATG GGA GGT (5′) and TAT GGA TCC TTA AAT GAT GGT TAT AAA CTC (3′).

**Rapid Amplification of cDNA Ends**—RACE was performed using the Marathon cDNA amplification kit (CLONTECH). Briefly, total RNA from Drosophila head was primed with a modified oligo(dT)12 primer to generate first strand cDNA by avian myeloblastosis virus reverse transcriptase. The first strand cDNA was then used as templates for the second strand cDNA synthesis mediated by RNase H, Erichtherich coli DNA polymerase, and E. coli DNA ligase. After ligating with an adaptor primer, the double-strand cDNA was used as templates for PCR using a gene-specific primer, b (see Fig. 4A) and AP1 primer that anneals to the adaptor primer. A second PCR reaction using the initial PCR mixture as templates and a different set of primers, primer a (see Fig. 4A) and AP1, was followed. Two antisense primers, a and b (see Fig. 4A), were used for the 5′ RACE. For the 3′ RACE, two sense primers, d and e (see Fig. 4A), were used for 3′ RACE. All four InaD-specific primers have melting temperatures of 72 °C. The DNA fragments obtained from the nested PCR amplification were subcloned and sequenced. Primer sequences for InaD are CCT TGG CAT GGC CAT CAT GTG AAT (primer a), CAG CAT CAG CAT GCC CCC TTT CTG CA (primer b), TGA AGC AGC GAT GAT GTC TCA GTT (primer d), and GGC ATG TGC TTC AAG CCC ATC AA (primer e).

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using the overlap extension method as described (26).

**Fusion Protein Expression in Bacteria**—Carboxyl-terminal tails of wild-type eye-PKC (641–700) and PDZ2 (206–363) of INAD were expressed as fusion proteins of glutathione S-transferase (GST). Briefly, recombinant pGEX4T1 plasmids were transformed into E. coli HMS174. Overnight cultures (1 ml) were prepared from a single colony and used to inoculate a 50-ml LB broth containing ampicillin. The cultures were grown at 37 °C for 2–3 h until the density of bacterial cultures (OD600) reached 0.6–0.7. The expression of fusion proteins was initiated by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration, 1 mM) after a 3-h induction. Following induction, and bacterial pellets were collected by centrifugation. The fusion lysates containing the fusion protein were prepared by resuspending the pellets in binding buffer (50 mM KPO4, pH 7.0, 150 mM KCl, 10 mM MgCl2, 1% glycerol, 1% Triton X-100 plus a mixture of protease inhibitors) followed by repeated sonication.

**Radio labeling of Proteins**—Recombinant plasmids containing target cDNA in pGEMEX1 (Promega) were constructed and used as templates for T7 RNA polymerase-dependent transcription. Incorporation of [35S]methionine into T7 gene 10 fusion proteins containing PDZ2 (206–363), the carboxyl-terminal tail of eye-PKC (562–700), and PDZ4 (485–577) was accomplished by in vitro transcription and translation using the TNT-coupled reticulocyte lysate system (Promega) (6). Briefly, 25 μl of reticulocyte lysates were added for a 50-μl reaction that contained [35S]methionine (40 μCi), circular plasmid templates (0.5–1 μg), RNase (40 μg), and T7 RNA polymerase (1 μl). In vitro translated radiolabeled proteins were analyzed by SDS/PAGE or used directly for pull-down assays.

**GST Fusion Protein Pull-down Assays**—Bacterial lysates containing similar amounts (5 μg) of GST fusion proteins or GST were incubated with radiolabeled target (10–20-fold excess) in binding buffer at 4 °C for 1 h. The reaction mixture (55 μl) was transferred to an Eppendorf tube containing 10 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech) prewashed with binding buffer. Incubation proceeded for 1 h at 4 °C with constant agitation for binding of GST fusion proteins to the beads. The supernatant of the mixture was removed, and an aliquot (5%) was analyzed by SDS/PAGE. The beads were washed twice with binding buffer (100 μl) three times to remove nonspecific binding. The GST fusion protein with bound radioactive proteins was eluted with SDS/PAGE loading buffer and analyzed on SDS/PAGE. Radioactivity was detected by autoradiography or with a PhosphorImager (445SI, Molecular Dynamics). Affinity of interaction was determined by the amount of bound radioactive probes. Nonspecific binding to GST was used as a negative control.

**Western Blot Analysis**—Western blotting was performed using alkaline phosphatase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). The presence of antigens was visualized upon staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Polyclonal anti-GST antibodies were purchased from Transduction Laboratories (San Diego, CA).

**RESULTS**

**PDZ2 is a Type 1 Domain and Interacts with an Xaa-(Ser/Thr)-Xaa-(Val/Leu/Ile) Sequence**—Previously our laboratory reported that PDZ2 interacted with the carboxy-terminal sequence of eye-PKC (11). However, Tsunoda et al. (9) showed that retinal eye-PKC associated with PDZ4 by pull-down assays. Xu et al. (16) suggested the involvement of PDZ3 and PDZ4 in the eye-PKC association by heterologous expression and co-immunoprecipitation. Questions remain as to which PDZ domains are important for the eye-PKC association. Importantly, both Xu et al. (16) and Adamski et al. (11) pointed out...
the involvement of the carboxyl tail of eye-PKC; a point mutation that converted the last residue of eye-PKC, Ile, to an Asp, led to a drastic reduction of the INAD interaction. To investigate which PDZ domain of INAD associates with eye-PKC, we examined the carboxyl-terminal tail of eye-PKC. Eye-PKC terminates with Ile-Thr-Ile-Ile, consistent with an interaction with a type I PDZ domain. The molecular basis of the interaction between a type I PDZ domain and a carboxyl-terminal sequence has been revealed in the x-ray crystallographic studies of PDZ3 of PSD95 and a target peptide (17).

We aligned PDZ3 of PSD95 with five PDZ domains of INAD and found that only PDZ2 resembles a type I domain (Fig. 1A) because several critical residues involved in target binding are conserved. In particular, the His residue located at the beginning of the second α-helix (Fig. 1B) is conserved in PDZ2 of INAD, His210. The N-3 nitrogen of His210 is likely to be involved in hydrogen bonding with the hydroxyl side chain of Ser/Thr at the corresponding position. Another conserved residue in PDZ2 includes Arg285 implicated in binding to the terminal carboxyl group of the target (Fig. 1). By analogy, the hydrophobic pocket for binding to the terminal hydrophobic residue of eye-PKC, Ile0, is contributed by Leu266, Leu262, Leu264, and Phe317 from PDZ2 (Fig. 1B). Overall, PDZ2 of INAD and PDZ3 of PSD95 share 28% sequence identity.

It is noteworthy that PDZ3 of PSD95 associates with a target sequence, Gln-Thr-Ser-Val. The polar side chain of Gln at the −3 position displays a reduction of the INAD association. A pull-down assay to examine PDZ2 binding to wild-type or mutant eye-PKC. Top panel, an autoradiogram showing the amount of 35S-labeled PKC retained following incubation with GST (lanes 1, 3, and 5) or GST-PDZ2 fusion protein (lanes 2, 4, and 6). Radioactive probes (5% of input) in the reaction are shown (middle panel). The amount of GST and GST-PDZ2 in the reaction was determined by Western blotting (bottom panel). B, a histogram depicting relative binding of two mutant eye-PKC affecting Ile−3 in the carboxyl terminus. The amount of radioactivity recovered was quantitated by PhosphorImager and relative level of binding plotted (n = 3). Wild-type eye-PKC exhibits a stronger interaction with PDZ2. In contrast, substitutions of Ile−3 with either Glu (Ile−3 → Glu) or Lys (Ile−3 → Lys) led to a drastic reduction of the association.

Site-directed Mutagenesis of Eye-PKC Tail Sequence—To investigate whether Ile−3 of eye-PKC is involved in the PDZ2 interaction, we generated and characterized two point mutants. The codon of Ile−3 was replaced with that of Glu (Ile−3 → Glu) or Lys (Ile−3 → Lys) by site-directed mutagenesis. Wild-type and modified eye-PKC cDNAs were used to generate radiolabeled fusion protein for PDZ2 binding. As shown in Fig. 2, we detected a drastic reduction of the PDZ2 interaction in these two mutants. In particular, the Glu substitution (Ile−3 → Glu) displayed a total loss of the association (Fig. 2A, lane 4, and Fig. 2B). These findings indicate that Ile−3 of eye-PKC is critically involved in the binding to PDZ2 of INAD.
involved in the PDZ2 interaction; substitutions with charged residues almost abolished the association.

Site-directed Mutagenesis of INAD PDZ2—As mentioned above, type I PDZ domains usually contain a basic residue, either His or Arg, at the beginning of αb (Fig. 1A) for binding to (Ser/Thr)-β of type I targets. In contrast, type II targets contain either a bulky hydrophobic or Tyr at the −2 position that interacts with a hydrophobic residue (e.g. Val) in the corresponding αb position of type II PDZ domains. We examined the involvement of His<sup>310</sup> in αb of PDZ2 by amino acid replacement followed by pull-down assays. When His was substituted by Arg (H310R), the interaction with wild-type eye-PKC was reduced to about 50% (Fig. 3). Interestingly, the Leu substitution (H310L) led to a 1-fold increase in the eye-PKC interaction. We also tested the eye-PKC binding to PDZ4 of INAD and showed a much weaker binding compared with that of PDZ2 (Fig. 3).

Both His and Arg have side chains capable of hydrogen bonding with (Ser/Thr)-β; however, Arg has an aliphatic side chain instead of an imidazole ring like His. It is likely that differences in the side chains at the corresponding αb position lead to a change in the eye-PKC interaction. When His<sup>310</sup> was substituted by Leu whose side chain does not support hydrogen bonding, an increased affinity toward eye-PKC was observed. We proposed that polar side chains from a neighboring residue of His<sup>310</sup>, such as Arg<sup>308</sup>, can engage in hydrogen bonding with the hydroxyl group of Thr-β of eye-PKC to stabilize the interaction in the H310L mutant. To investigate whether Arg<sup>308</sup> is important for the observed eye-PKC association, we substituted Arg<sup>308</sup> with Gly in the H310L background (R308G,H310L). We show that double mutants displayed a great reduction of the eye-PKC association (Fig. 3B), suggesting that Arg<sup>308</sup> is essential for the eye-PKC interaction in the H310L mutant.

Genomic Structure of the InaD Gene—We identified the InaD gene and investigated its genomic organization. Genomic clones were obtained either by screening a Drosophila genomic library or by gene amplification via PCR (Fig. 4A). The InaD gene was subjected to restriction enzyme mapping (Fig. 4A) and nucleotide sequencing. Comparison of the genomic DNA (accession number AF245280) with cDNA sequences provided the basis for the exon/intron organization as shown in Fig. 4A (middle panel). The coding sequence of InaD is contained within a 3- kilobase genomic fragment that has nine exons interrupted by eight intervening sequences. All introns are rather small in size ranging from 54 to 368 nucleotides and are flanked by conserved 5′ and 3′ consensus sequences (28) at each end. The InaD gene product is composed of five distinct PDZ domains (2, 3, 9, 11). To reveal whether each PDZ domain is encoded within an exon, we projected the location of introns in the translation product of InaD (Fig. 4A, bottom panel). We found both PDZ1 and PDZ2 are encoded by only one exon, whereas the remaining three PDZ domains are encoded by two adjacent exons (Fig. 4A).

To determine the transcription start site of the InaD gene, we employed 5′ RACE (Fig. 4B) followed by DNA sequencing. Similarly, the 3′-untranslated region (UTR) was also determined (Fig. 4B). The InaD cDNA has 157 nucleotides in the 5′-UTR and 67 nucleotides in the 3′-UTR. We also sequenced several InaD cDNAs from the expressed sequence tags collection generated by the Berkeley Drosophila Genome Project (29). Two of the longest cDNAs (accession numbers AA567782 and AA697690) contain an additional 23 nucleotides extending beyond our experimentally determined transcription initiation site (+1) (Fig. 4C). It is possible that multiple transcription start sites of InaD are used. Alternatively, premature chain termination by reverse transcriptase during the synthesis of cDNA may have also resulted in a shorter 5′-UTR.

The 5′-UTR and the adjacent upstream sequence of InaD are shown in Fig. 4C. We found stretches of TA-rich sequences upstream of the transcription initiation site (Fig. 4C) that could serve as binding sites for general transcription factors (30). Moreover, we also found sequences similar to the photoreceptor-specific cis-acting element, CTAATGGATT (31), at 40 nucleotides upstream of the putative transcription start site (Fig. 4C). This sequence may be important for controlling the photoreceptor-specific expression of InaD.

Polymorphisms in InaD—We compared nucleotide sequences of genomic DNA and cDNA and first noted an inconsistency in the codon of amino acid 319 (Table I). Genomic DNA obtained from the Canton S strain encodes a Ser (AGC) at 319 instead of an Asn (AAG) as seen in several InaD cDNAs. This discrepancy could be either post-transcriptional modifications such as editing or single nucleotide polymorphisms (SNPs). To distinguish between these two possibilities, we isolated and sequenced both genomic and cDNA sequences from three laboratory strains including two wild-type strains, W<sup>1118</sup> and Oregon R, and InaD<sup>2215</sup> (Table I). If editing occurs at a given codon, one expects that cDNA sequences will be different from genomic sequences obtained from the same strain. However, we found both genomic and cDNA sequences from each of these three strains are the same at codon 319. In both W<sup>1118</sup> and Oregon R, the codon (AAG) at 319 encodes Asn. In contrast, InaD<sup>2215</sup> and likely Canton S, Ser (AGC) was encoded at 319 (Table I). The corresponding amino acid in Calliphora INAD is Asn (32). This finding ruled out the possibility of post-tran-
Fig. 4. Genomic organization of the InaD locus. A, genomic structure of the InaD locus. Shown at the top is the restriction map of a genomic clone. The direction of transcription is indicated as an arrow above. The restriction map of the InaD cDNA is shown in the middle panel. The location of eight introns is indicated as triangles, and the sizes are indicated. The translation product of InaD is depicted below the cDNA, and each of the five PDZ domains is labeled as a filled square. The locations of amino acid residues that show polymorphism are marked with asterisks. RI, EcoRI; RV, EcoRV; bp, base pair; nt, nucleotides. B, ethidium bromide staining of a DNA gel containing DNA fragments obtained by RACE. Lane 1, initial 5' RACE PCR reaction using AP1 and b primers; lane 2, a nested PCR using the initial PCR mixtures as templates with AP1 and a primers resulted in a 0.6-kilobase 5' RACE product; lane 3, initial 3' RACE PCR reaction (using AP1 and d primers); lane 4, a nested PCR (using AP1 and e primers) gave rise to a 1.0-kilobase 3' RACE product. DNA molecular mass standards (lane M) are shown on the left. C, nucleotide sequence of the 5'-UTR and regulatory sequence of the InaD gene. Transcription initiation site of the InaD gene (+1) is localized about 157 nucleotides upstream of the translation initiation ATG. The translated exon sequence is shown in bold type. The putative cis element for photoreceptor-specific expression is boxed and is 40 nucleotides 5' upstream of the transcription initiation site. The underlined sequences are parts of the promoter sequences.

Additional SNPs were also observed. In Oregon R, we found nucleotide substitutions in residues 282 and 333. Pro282 is located in the second PDZ domain, and it is substituted by Leu in the Oregon R strain (Table I). The corresponding residue in Calliphora INAD is Thr282. Lys333 is also localized in PDZ2, and it is replaced by Gln in Oregon R. It is interesting to detect substitutions in PDZ2 because PDZ2 is critically involved in the eye-PKC interaction. It is likely that these changes may not lead to any significant alteration in the folding of PDZ2, and consequently the interaction with eye-PKC remains intact. Another possibility is that there are concomitant changes in the carboxyl-terminal tail of eye-PKC, which compensate for these modifications in INAD.

To investigate whether any polymorphisms in eye-PKC exist, we amplified and sequenced the genomic sequences encoding the last 139 amino acids of eye-PKC from both the Oregon R and the InaD strains. However, we did not observe any SNPs for eye-PKC (562–700) (data not shown).

Interaction between Eye-PKC and PDZ2 Domain Variants—We investigated whether polymorphic PDZ2 domains display different affinities toward eye-PKC. 35S-Labeled PDZ2 domains (N319S, P282L, and Q333K) were generated for pull-down assays. The eye-PKC interaction was compared with PDZ2 present in wild-type W1118 and quantitated. As shown in Fig. 5B, we detected a stronger eye-PKC interaction in both PDZ2 (N319S) and PDZ2 (P282L); particularly PDZ2 (N319S) displayed a 2-fold increase in the eye-PKC binding. However, PDZ2 (Q333K) displayed binding similar to wild-type PDZ2.

DISCUSSION

PDZ domains are protein-protein interaction motifs found in a variety of signaling molecules. Prototypical PDZ domains recognize a tetrapeptide ligand located at the carboxyl-terminal tail of the target protein (17, 19). The PDZ-target interaction has been implicated in subcellular localization and stabilization of receptors or other interacting proteins (4, 5, 19). INAD contains five distinct PDZ domains that are shown to associate with phospholipase Cb, eye-PKC, and TRP. Like INAD, proteins containing multiple PDZ domains are capable of tethering several signaling proteins leading to the formation of a signal transduction complex (transducisome or signalplex). This clustering of signaling proteins facilitates protein-protein interactions leading to fast kinetics of signaling processes. Furthermore, restricted distribution of proteins may contribute to specificity of signaling mechanisms, thereby preventing undesired cross-talks. Thus, INAD serves to illustrate the importance of scaffolding proteins in multi-component signaling processes such as G protein-coupled pathways.

PDZ2 Interacts with the Carboxyl-terminal Tail of Eye-PKC—We previously showed that the last three residues of
target binding. Surprisingly, we detected an enhanced eye-PKC interaction when His$^{310}$ was substituted with a Leu in PDZ2. As the side chain of Leu does not form hydrogen bonds, we speculated that a neighboring basic residue (Arg, His, or Lys) might serve as an alternative residue for anchoring Thr$^{-2}$ of eye-PKC. Further mutational analysis of Arg$^{308}$ confirmed this hypothesis.

How does Leu substitution of His$^{310}$ in PDZ2 lead to an increase in the eye-PKC interaction? As mentioned, the tail sequence of eye-PKC or ligand of PDZ2 is rather hydrophobic with three of the four residues being Ile. The presence of an additional hydrophobic residue in the binding pocket as in the Leu substituted PDZ2 may positively increase the eye-PKC association via additional hydrophobic interactions, provided that a neighboring basic residue, such as Arg$^{308}$, can be substituted for His$^{310}$. Based on these findings, we suggest that the ligand binding pocket of PDZ2 domains is flexible for allowing a slight rotation of ligand in the binding pocket for optimal interaction. The presence of Arg$^{308}$ is a unique feature of PDZ2 because none of the four PDZ domains of INAD contain a basic residue at the corresponding position.

We also examined the involvement of Ile$^{-3}$ of eye-PKC, because the residue at the $-3$ of the ligand also contributes to specificity of PDZ-target interactions. We show that replacement of Ile$^{-3}$ with charged residues such as Glu or Lys led to a drastic reduction of the PDZ2 association. However, substitution of Ile with a hydrophobic residue, Phe, was tolerated without significantly affecting the PDZ2 interaction as previously shown in the yeast two-hybrid assay (11). Additional evidence supporting the contribution of Ile$^{-3}$ was obtained by examining the structure of PDZ3 of PSD95. PDZ3 associates with a target sequence containing Glu at $-3$. The polar side chain of Glu$^{-3}$ forms hydrogen bonds with those of Asn$^{306}$ and Ser$^{308}$ in PDZ3 of PSD95. In contrast, eye-PKC has an Ile at the $-3$ position, which contains a hydrophobic side chain. Consistently, the projected residues for interacting with Ile$^{-3}$ in PDZ2 of INAD are Ala$^{263}$ and Ala$^{278}$, which have hydrophilic side chains. We conclude that the interaction between PDZ2 and the carboxyl-terminal of eye-PKC belongs to that of the type I PDZ domain and that basic residues flanking the critical His in the $a$-helix can be substituted for this critical His for anchoring (Ser/Thr)$^{-2}$ of the ligand. Moreover, our results indicate that PDZ2 of INAD prefers a hydrophobic residue at the $-3$ position of the target.

Amino Acid Polymorphisms in PDZ2—Nucleotide sequencing of several wild-type InaD alleles revealed SNPs in three residues of the InaD gene product. All three substitutions are in PDZ2 including Leu substitution for Pro$^{282}$ (P282L), Ser substitution at Asn$^{319}$ (N319S), and Gln substitution at Lys$^{333}$ (K333Q). K333Q is located beyond the sixth $\beta$-strand, N319S is in the linker region flanking the second $\alpha$-helix and the sixth $\beta$-strand, and P282L is located in the linker region between the third $\beta$-strand and the first $\alpha$-helix (Fig. 1A). These three residues have not been implicated in critical bonding with the target sequence, but substitutions may affect overall folding of PDZ2 leading to an alteration in the eye-PKC interaction.
Indeed, P282L and N319S display an increased affinity toward eye-PKC in pull-down assays. P282L was detected in Oregon R, whereas N319S was detected in the Canton S strain.

Both Canton S and Oregon R strains display wild-type electroretinograms similar to another wild-type strain, W1118. It is likely that a change of the INAD-PKC interaction results in subtle phenotype that is detected only in sensitive assays such as patch clamp recordings of dissociated photoreceptors. To date, there are no reports on comparison of various wild-type responses using the patch clamp analysis. It is also possible that the INAD-PKC interaction is optimal in W1118 and that a further increase in affinity does not result in any electrophysiological phenotype.

In summary, we evaluated the association between eye-PKC and PDZ2 and conclude that PDZ2 is a type I PDZ domain interacting with a type I PDZ ligand at the carboxyl tail of eye-PKC. The binding pocket of PDZ2 is unique; Leu substitution interacting with a type I PDZ ligand at the carboxyl tail of INAD and PDZ2 and conclude that PDZ2 is a type I PDZ domain

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