

Two distantly positioned PDZ domains mediate multivalent INAD–phospholipase C interactions essential for G protein-coupled signaling

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***Drosophila* INAD, which contains five tandem protein interaction PDZ domains, plays an important role in the G protein-coupled visual signal transduction. Mutations in *InaD* alleles display mislocalization of signaling molecules of phototransduction which include the essential effector, phospholipase C- β (PLC- β), which is also known as NORPA. The molecular and biochemical details of this functional link are unknown. We report that INAD directly binds to NORPA via two terminally positioned PDZ1 and PDZ5 domains. PDZ1 binds to the C-terminus of NORPA, while PDZ5 binds to an internal region overlapping with the G box-homology region (a putative G protein-interacting site). The NORPA proteins lacking binding sites, which display normal basal PLC activity, can no longer associate with INAD *in vivo*. These truncations cause significant reduction of NORPA protein expression in rhabdomeres and severe defects in phototransduction. Thus, the two terminal PDZ domains of INAD, through intermolecular and/or intramolecular interactions, are brought into proximity *in vivo*. Such domain organization allows for the multivalent INAD–NORPA interactions which are essential for G protein-coupled phototransduction.**

Keywords: G protein/INAD/PDZ/PLC

Introduction

In the nervous system, elaborate membrane domains or compartments, such as synapses of neurons and microvilli in photoreceptors, are dedicated to specialized signal transduction. It is therefore essential to organize the signaling proteins at the functional sites with defined composition and stoichiometry. Many molecules involved in signaling contain small protein–protein interaction modules including Src-homology domains 2 (SH2) and 3 (SH3) (Pawson, 1994; Schlessinger, 1994), SEC7 domain (Stevens *et al.*, 1982; Kolanus *et al.*, 1996), phosphotyros-

ine domain (PTB) (Kavanaugh *et al.*, 1995; van der Greer and Pawson, 1995; Harrison, 1996) and PDZ domains (Woods and Bryant, 1991, 1993; Cho *et al.*, 1992; Kennedy, 1995; Sheng, 1996). Thus, proteins with multiple modular protein interaction domains are well suited for recruiting different functional components to organize the macromolecular complexes, thereby achieving signaling sensitivity, specificity and selectivity (see review by Pawson and Scott, 1997).

Modular PDZ domains, which have also been called ‘GLGF repeats’ and ‘disks-large homology repeats’ (DHRs), consist of ~80–100 amino acids. These domains were first identified as repeated sequences in the neuron-specific post-synaptic density protein (PSD-95/SAP-90), the *Drosophila* septate junction protein disks-large (dlg) and the epithelial tight-junction protein zona occludens-1 (ZO1) (Woods and Bryant, 1991, 1993; Cho *et al.*, 1992). PDZ domains are multifunctional protein interaction motifs that often bind to the carboxylated C-terminus of protein targets. For example, the three PDZ domains within PSD-95 were first shown to bind the C-terminal Ser/Thr-X-Val-COO⁻ motif found in certain *N*-methyl-D-aspartate (NMDA)-type glutamate receptors and in the Shaker-type potassium channel subunits with channel clustering activity (Kim *et al.*, 1995; Kornau *et al.*, 1995). In addition, PDZ domains have been found in a large number of diverse proteins which are localized in nuclear (Poulat *et al.*, 1997), cytoplasmic (see reviews by Kennedy, 1995; Sheng, 1996) and extracellular compartments (Bazan and Schall, 1996). This suggests that the PDZ–target protein interactions are involved in a variety of biological processes. Supporting this notion, *in vitro* selection of random peptide libraries has shown that, in contrast to other well studied protein interaction modules such as SH2 or SH3 domains, PDZ domains are capable of associating with diverse C-terminal carboxylated peptide sequences (Songyang *et al.*, 1997; Stricker *et al.*, 1997). One can switch the binding specificity by making specific amino acid substitutions in either the PDZ domain or the protein target (Stricker *et al.*, 1997). Based on these data and their amino acid sequence homology, it is thought that distinct individual PDZ domains fold similarly but display different specificity in their binding pockets, reminiscent of the similarly folded Fab domain of immunoglobulins with distinct antigen-binding specificity. Therefore, identification of the optimal binding sequence for orphan PDZ domains has allowed for a sequence comparison to identify candidate interacting proteins, thereby providing an entry point to the understanding of the molecular function of PDZ-containing proteins (Stricker *et al.*, 1997).

Signal transduction mediated by guanine nucleotide-binding proteins (G proteins) usually involves five biochemical steps starting sequentially from receptor, G

protein, effector, intracellular message and, finally, to target proteins such as ion channels. A current view of G protein function in cells includes random collisions between proteins with a high specificity at the sites of protein–protein interaction. Recent evidence has suggested that receptor, G protein and effector are restricted in specific subcellular membrane domains (see reviews by Rodbell, 1992; Neubig, 1994). In addition, G proteins were reported to interact directly or co-localize with cytoskeleton proteins including tubulin, actin, myosin and fodrin (Rasenick and Wang, 1988; Bourguignon *et al.*, 1990; Wang and Rasenick, 1991). Thus, an organized interaction of the receptors and G proteins with their effectors and cell membrane machinery appears to play an important role in their function. A detailed molecular and biochemical characterization of proteins that mediate the organization of these receptor systems is essential for a full understanding of G protein-coupled signal transduction.

The phototransduction cascade is one of the fastest known G protein receptor coupling systems. In *Drosophila*, the high temporal resolution is evidenced by the <20 ms latency between photon excitation and photoreceptor cell depolarization (Ranganathan *et al.*, 1991). Molecular understanding of this cascade would provide important insights into the general mechanisms of the G protein-coupled receptor signaling. Genetic studies of *Drosophila* phototransduction mutants have shown that this signaling pathway consists of several protein components that are essential for transducing and tuning the signals. The known proteins includes rhodopsin (RH1) (O'Tousa *et al.*, 1985; Zuker *et al.*, 1985), G proteins (DGQ) (Lee *et al.*, 1994; Scott *et al.*, 1995), norpA (no receptor potential A) phosphatidylinositol-specific phospholipase C- β (NORPA) (Bloomquist *et al.*, 1988), a protein homologous to store-operated ion channels (TRP; Montell *et al.*, 1985), an eye-specific protein kinase C (INAC) (Smith *et al.*, 1991) and the INAD protein encoded by the inactivation no afterpotential D locus (Pak, 1979; Shieh and Niemeyer, 1995). Mammalian homologs have been found corresponding to all proteins in the cascade, except INAD. In contrast to other members in this cascade, the primary sequence of INAD displays no significant similarity to other known signaling receptors or enzymes in G protein-coupled signaling pathways. Instead, it is almost completely occupied by five tandem PDZ domains, suggesting that it may function by linking other signaling molecules to form macromolecular complexes. Both missense mutations (*InaD*^{P215} in PDZ3 and *InaD*² in PDZ5) and a nonsense mutation that truncates the INAD at amino acid 270 in the middle of PDZ2 display dramatic changes in function or subcellular localization of several signaling molecules including TRP, NORPA and INAC (Shieh and Zhu, 1996; Chevesich *et al.*, 1997; Tsunoda *et al.*, 1997). These genetic studies combined with co-immunoprecipitation evidence (Huber *et al.*, 1996; Chevesich *et al.*, 1997) suggest that INAD plays a critical role in signal transduction, presumably by functioning as a scaffold to organize signaling proteins. At the biochemical level, however, little is known about the molecular mechanisms of the INAD–target protein interactions.

One current model suggests that the five tandem PDZ domains have distinct binding specificity. In principle, the

linear arrangement of the five modular domains allows for independent interactions with five individual target proteins. Although there is no information regarding PDZ domain organization of INAD, this model nicely explains the cell biological defects of two genetic mutant alleles in INAD: *InaD*^{P215} and *InaD*². These two mutations, one in PDZ3 and one in PDZ5, appear to affect selectively either TRP or NORPA localization, suggesting that PDZ3 and PDZ5 interact with TRP and NORPA independently (Chevesich *et al.*, 1997; Tsunoda *et al.*, 1997). The genetic mutations of INAD have provided strong evidence for INAD to function as an organizing scaffold protein. The mechanism of INAD–target interaction requires more detailed studies since a given genetic mutation may lead to both local and distant changes of protein function. Furthermore, because of insufficient biochemical data and limited genetic mutant alleles, the interaction proteins with PDZ1, PDZ2 and PDZ4 of INAD are currently unknown.

In this study, we demonstrate that INAD binds directly to NORPA via two distantly positioned PDZ domains, PDZ1 and PDZ5. The two clustered INAD-binding sites in NORPA have been mapped to the last three residues of the C-terminus for PDZ1 and an internal region for PDZ5. Deletion of one or both INAD-binding sites in NORPA does not impair the basal phospholipase C (PLC) activity of NORPA in transformed flies. However, flies expressing these mutated proteins show reduced or mislocalized expression of NORPA in rhabdomeres in *Drosophila* eyes and display severe defects in light-evoked responses. The combination of biochemical, cellular and electrophysiological evidence supports a model whereby the PDZ1 and PDZ5 domains of INAD are present in proximity resulting from intramolecular and/or intermolecular domain arrangements. Such a domain arrangement allows for multivalent INAD–NORPA interactions that are essential for the *in vivo* signaling.

Results

In vitro selection of optimal binding peptides for PDZ1 of INAD

Based on the primary structure, it is thought that INAD consists of five modular PDZ domains which occupy most of the protein (Figure 1A). Structural studies of PSD-95 have inferred several regions and residues that are involved in determining peptide-binding specificity. For example, the His372 in PDZ3 interacts with the side chain of serine at the –2 position of the co-crystallized carboxylate peptide (Doyle *et al.*, 1996). A mutation of the corresponding position of neuronal nitric oxide synthase (nNOS) PDZ from tyrosine to histidine converts the peptide-binding specificity from DXV-COO[–] of nNOS to TXV-COO[–], a sequence consensus that is normally specific for the PSD-95 PDZ domains (Stricker *et al.*, 1997). Comparison with the corresponding residues in INAD (boxed residues in Figure 1A) has revealed that the five PDZ domains have distinct residues at this position, suggesting that they each may recognize different peptide sequences (Figure 1A).

To determine the peptide-binding specificity for PDZ1 of INAD, we expressed this domain (amino acids 10–112) as a glutathione *S*-transferase (GST) fusion (denoted as GST–PDZ1) in *Escherichia coli*. Using the affinity-

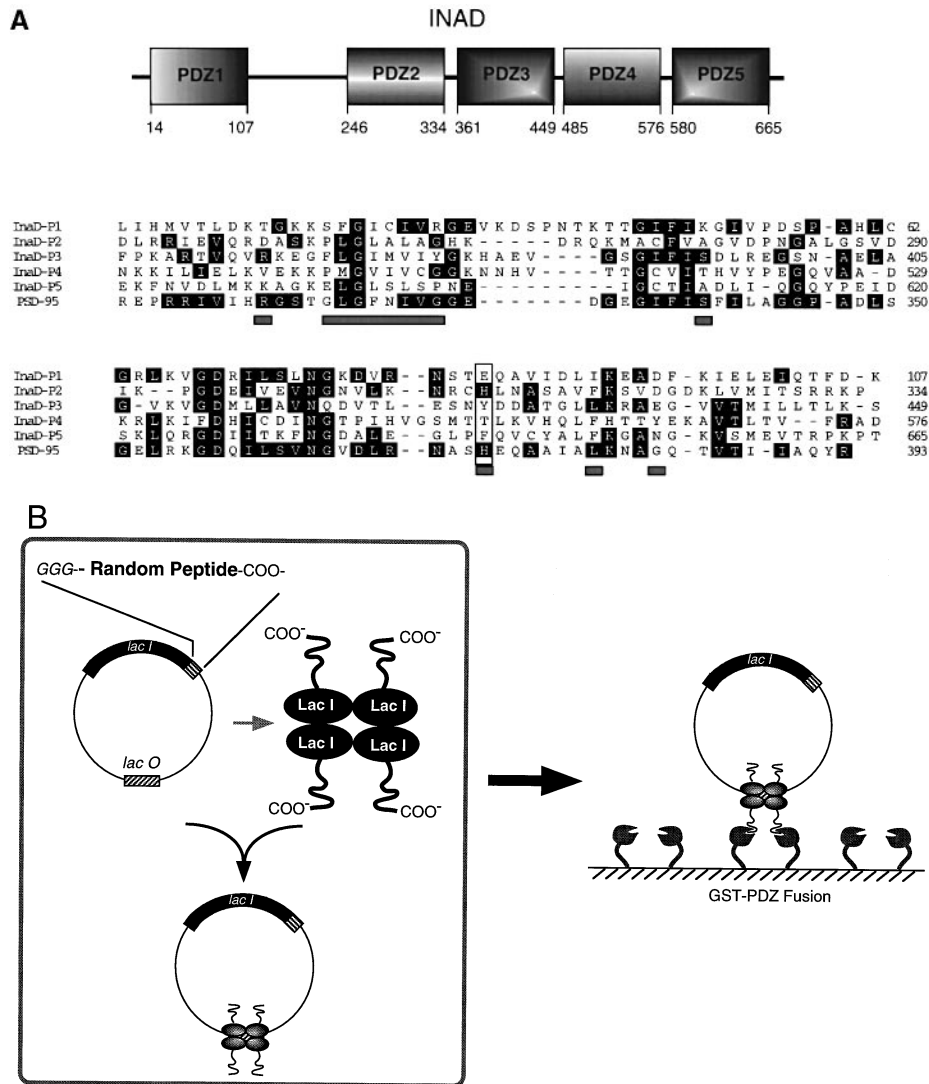


Fig. 1. The INAD domain arrangement and a method of peptide isolation. (A) Upper panel: schematic diagram of INAD. The shaded boxes indicate five tandem PDZ domains, named PDZ1–PDZ5. The amino acid positions for each PDZ domain are indicated below. Lower panel: amino acid alignment of the five PDZ domains against the sequence of PDZ3 of PSD-95. Highlighted residues in INAD are identical to those in PSD-95. The black bars below the PSD-95 sequence indicate regions or residues that are involved in determining the binding specificity of peptides (Doyle *et al.*, 1996). The boxed residues are thought to determine the side chain preference at the –2 position of the bound peptide (Doyle *et al.*, 1996; Stricker *et al.*, 1997). The numbers indicate the amino acid positions in INAD or PSD-95. (B) LacI repressor-based random peptide selection strategy. A random peptide library is constructed by inserting a random oligonucleotide of 15 codons at the C-terminus of the LacI repressor to form chimeric proteins with random C-terminal amino acid sequences. The LacI–random peptide chimeras are synthesized as a result of expression of individual LacI plasmids in *E. coli*. Since each expression plasmid also contains the LacI-binding sites (*LacO*), the chimeras bind to the corresponding plasmid DNA and are released from *E. coli* as protein–plasmid complexes after gentle lysis of bacteria. Affinity selection and recovery of plasmids allow for deduction of the peptide sequence on the basis of their corresponding DNA sequences.

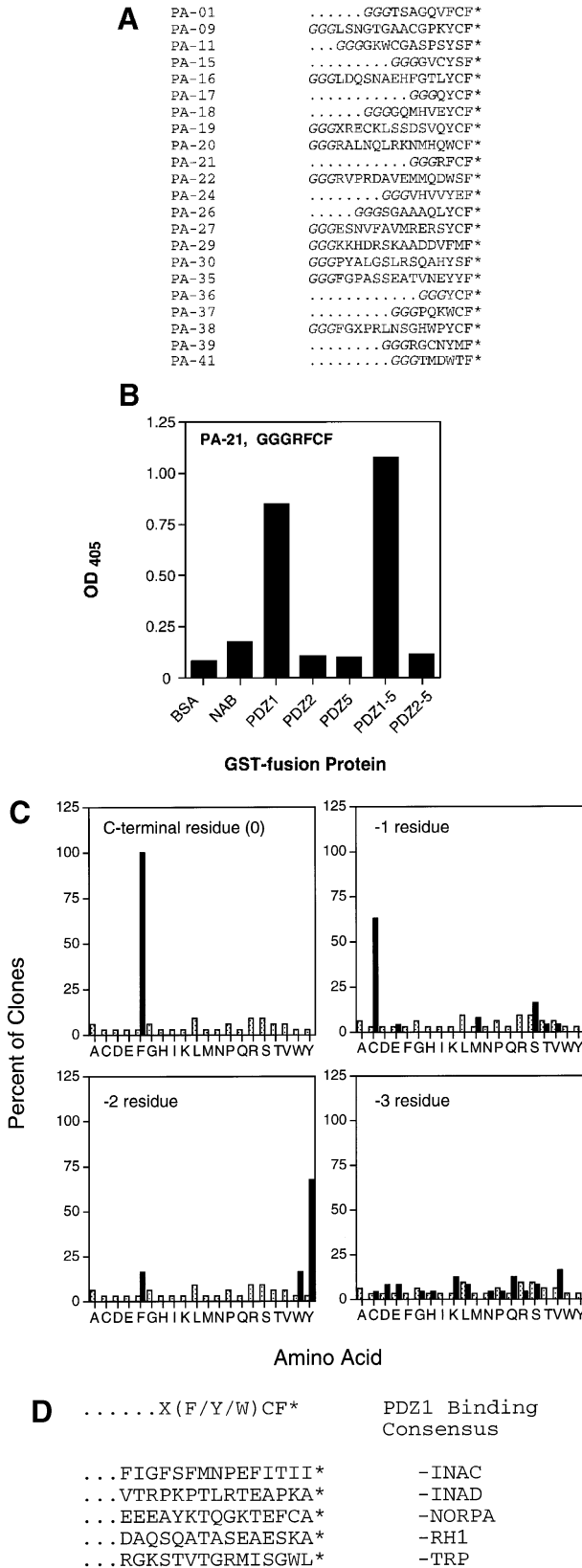
purified GST–PDZ1, we screened a total of 1.3×10^{10} random C-terminal peptides for specific GST–PDZ1–peptide association (Figure 1B; Stricker *et al.*, 1997). The complexity of the random C-terminal peptide library is sufficient to cover all possible sequences of the last four C-terminal residues, a conventional peptide length recognized by PDZ domains (Doyle *et al.*, 1996).

After four repeated rounds of affinity panning, a 628-fold enrichment was obtained, and individual peptides were used for the enzyme-linked immunosorbent assay (ELISA) test to confirm the specificity for the PDZ1 domain (see Materials and methods). The clones that bound to GST–PDZ1 but not to GST alone or bovine serum albumin (BSA) were isolated. The corresponding amino acid sequences were deduced by DNA sequencing

of the *lacI*-expressing plasmids. Figure 2A shows an amino acid alignment of 22 independent clones that bind specifically to GST–PDZ1.

To test the specificity of the PDZ1-binding peptides further, we expressed GST fusion proteins both individually as the PDZ2 and PDZ5 domains and in tandem forms as PDZ1 through PDZ5 (GST–PDZ1–5) and PDZ2 through PDZ5 (GST–PDZ2–5). Using the purified fusion proteins, ELISA binding studies showed that the PA-21 peptide (GGGRFCF–COO[−]) binds specifically to GST–PDZ1 and GST–PDZ1–5, but not to the other INAD PDZ domains (Figure 2). In addition, the PA peptides did not bind to the PDZ domain of nNOS or PDZ3 of PSD-95 (data not shown). This confirms the restricted specificity of PA peptides to PDZ1 of INAD.

Based on sequence comparison, particularly the sequences of the clones PA-21 and PA-36, PDZ1 of INAD recognizes three residues at the C-terminus. Calculation of the amino acid abundance in each position from the C-terminus (denoted as 0 position) indicates that PDZ1



prefers a C-terminal residue of phenylalanine (Figure 2C). The -1 position shows >60% cysteine. For the -2 position, only aromatic residues are found. The remaining upstream positions display no obvious sequence preference. Thus, the optimal peptide consensus for PDZ1 is X(Y/F/W)CF-COO⁻, which differs from the other known consensus sequences, such as DXV-COO⁻ for the nNOS PDZ domain (Stricker *et al.*, 1997) or (T/S)XV-COO⁻ for PSD-95 (Kim *et al.*, 1995; Kornau *et al.*, 1995).

Genetic and co-immunoprecipitation studies have implied that INAD interacts with several proteins, including RH1, TRP, INAC, calmodulin and NORPA (see Introduction). These proteins are candidates for interacting with PDZ1 of INAD. To test this, we compared their C-terminal sequences with the PDZ1-binding consensus. NORPA is the only protein containing a putative PDZ1-binding sequence (Figure 2D). This suggests a possible direct interaction between PDZ1 and the C-terminus of NORPA, the effector molecule that is essential for *Drosophila* phototransduction.

Specific association of the INAD PDZ1 with the NORPA C-terminus

To test for a potentially direct interaction between PDZ1 of INAD and NORPA, we expressed the full-length NORPA cDNA as an N-terminal HA-tagged fusion. After transfecting COS-7 cells, we prepared total cell lysates, separated the proteins by SDS-PAGE and probed with an anti-HA tag antibody. The monoclonal anti-HA antibody binds to the full-length NORPA protein and several smaller molecular weight species which presumably are degraded N-terminal fragments. The mock transfection showed no detectable binding signal (Figure 3A, left panel, lanes 1 and 2). When an identical duplicate filter was probed with the GST-PDZ1 fusion protein, we detected a binding signal corresponding to the size of the NORPA full-length polypeptide in transfected cells (Figure 3A, right panel, lanes 1 and 2). To confirm further the binding of PDZ1 to NORPA, we constructed two point mutants changing the FCA C-terminus of wild-type to FCF (NORPA-FCF) or FSA (NORPA-FSA). Overlay binding with PDZ1 to these mutated proteins showed strong binding to NORPA-FCF consistent with the panning results (Figure 2A), but

Fig. 2. Isolation of optimal peptides interacting with the PDZ1 domain of INAD. (A) A total of 48 colonies were selected after four rounds of affinity panning and their binding specificity was tested individually by the LacI ELISA (Stricker *et al.*, 1997; see also Materials and methods). The PDZ1-specific clones were selected and sequenced. Independent amino acid sequences in single letter codes are aligned. The asterisk indicates the stop codon. The italicized 'GGG' is part of the linker sequence which separates LacI from random peptides in the vector. (B) Specificity of peptide binding to various PDZ domains of INAD. One peptide clone, PA-21, was tested for specificity of binding to various soluble PDZ domains of INAD by LacI ELISA. The horizontal axis indicates GST fusion proteins used to coat the wells. PDZ3 and PDZ4 were not used because the expressed GST fusions were not soluble. NAB is a fusion protein serving as a negative control (see Materials and methods). The vertical axis shows the binding signals in optical density at 405 nm wavelength. (C) The amino acid abundance at each position was determined and is shown in a histogram. The horizontal axis indicates amino acids in single letter codes. Black bars show the actual abundance in percentage and empty bars indicate the expected abundance. (D) Amino acid sequence alignment of the C-termini of INAC, INAD, NORPA, RH1 and TRP, which shows that the C-terminus of NORPA shares some sequence similarity with the peptides that interact with PDZ1.

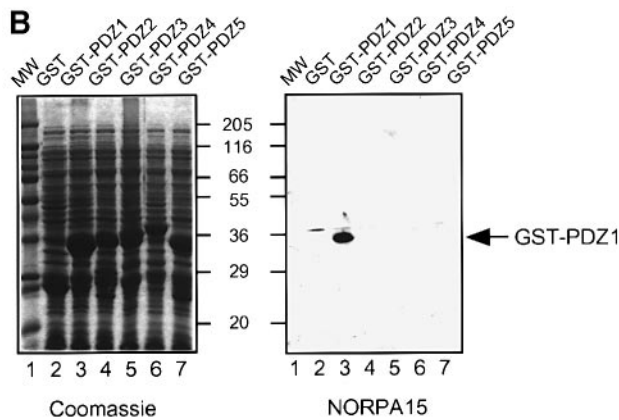
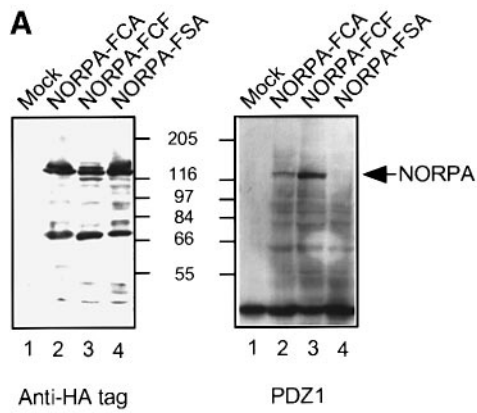


Fig. 3. Direct binding of PDZ1 to the NORPA C-terminus. **(A)** PDZ1 binds specifically to the NORPA C-terminus. Duplicate sets of total COS cell lysates 48 h after transfection were separated by SDS-PAGE and transferred onto nitrocellulose membranes. One membrane was probed with the anti-HA tag antibody (left) and the other was first probed with GST-PDZ1 followed by detection of an anti-GST antibody (right). Lanes 1, mock-transfected; lanes 2, NORPA-FCA; lanes 3, NORPA-FCF; lanes 4, NORPA-FSA. Molecular weight standards are marked between the two panels in kDa. **(B)** The NORPA C-terminal 15 amino acids bind specifically to PDZ1. Duplicate sets of total bacterial lysates after IPTG induction were separated by SDS-PAGE. One was stained with Coomassie and the other was transferred to nitrocellulose membrane and probed with NORPA15 (maltose-binding protein fusion containing the last 15 amino acids of NORPA). The binding of NORPA15 was detected with an anti-MBP antibody. Lanes 1, molecular weight standards; lanes 2, GST only; lanes 3, GST-PDZ1; lanes 4, GST-PDZ2; lanes 5, GST-PDZ3; lanes 6, GST-PDZ4; lanes 7, GST-PDZ5. The amino acid positions for each GST-PDZ fusion are listed in Materials and methods.

no binding to NORPA-FSA (Figure 3A, lanes 3 and 4; also see Discussion). This result is in complete agreement with the data of the *in vitro* selection experiments and demonstrates that PDZ1 is capable of binding to the C-terminus of the NORPA protein. One interesting observation based on the result of this binding experiment is that NORPA-FCF appears to interact more tightly than the native NORPA-FCA with PDZ1. Such a difference could have physiological relevance (see Discussion).

Conversely, to test whether the NORPA C-terminus binds specifically to PDZ1, we separated with SDS-PAGE the bacterial lysates containing various GST fusions corresponding to the five individual PDZ domains of

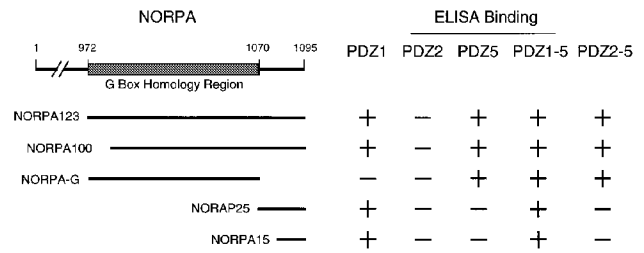


Fig. 4. PDZ5 of INAD binds to an internal region of NORPA. ELISA binding of soluble INAD-GST fusions to the C-terminus of NORPA. Left: a schematic diagram of NORPA marked with the G box homology region and numbers indicating amino acid positions. Deletion constructs expressing various MBP fusions are indicated. The ability of various MBP-NORPA fusions to bind to INAD was tested and is illustrated on the right. '+' ELISA binding is positive; '-' ELISA binding is negative.

INAD (Figure 3B, left panel). The interaction of NORPA with individual PDZ domains was tested by protein overlay binding with a maltose-binding fusion protein (MBP) which carries the last 15 amino acid residues of NORPA (NORPA15). Indeed, NORPA15 bound only to PDZ1 but failed to interact with other PDZ domains under the same conditions (Figure 3B, right panel, lane 3). Using glutathione beads bound with GST fusions of individual PDZ domains, we carried out 'pull-down' experiments to test the association of INAD with the purified NORPA15 fusion protein. Consistently, we found that only PDZ1 was capable of binding the NORPA15 fusion (data not shown). Considering the fact that both PDZ3 and PDZ5 produced in bacteria are able to bind to their corresponding ligands (Shieh and Zhu, 1996; Tsunoda *et al.*, 1997), the above data support the restricted physical association of NORPA15FCA-COO⁻ with PDZ1 of INAD.

Multiple contacts of NORPA with PDZ1 and PDZ5 of INAD

To test further the NORPA-INAD binding, we expressed various soluble forms of GST-INAD fusions including one that carries all five PDZ domains. The fusion proteins of INAD were tested for their association with various purified NORPA terminal fragments, including NORPA15 and NORPA123 (MBP fusion corresponding to the C-terminal 15 and 123 residues of NORPA). We found that not only does NORPA123 bind GST-PDZ1-5, but that it also binds to GST-PDZ2-5 which does not interact with NORPA15 apparently due to the lack of PDZ1 (Figure 4). This demonstrates that there is an additional interaction site present in the last 123 amino acids but away from the carboxylated C-terminus. Further tests showed that PDZ5 of INAD is sufficient to interact with an internal region of NORPA. To map the region in NORPA that is sufficient for the binding to PDZ5, we constructed additional deletion mutants of NORPA and tested for interaction with INAD. The binding site was mapped to an internal fragment corresponding to amino acids 972-1070 of NORPA, which shares amino acid homology with the corresponding region (known as the G box) of human PLC- β 1, a putative G protein-binding site (see Discussion). Thus, the PDZ5 domain of INAD binds to an internal region of NORPA, independent from the PDZ1-NORPA interaction.

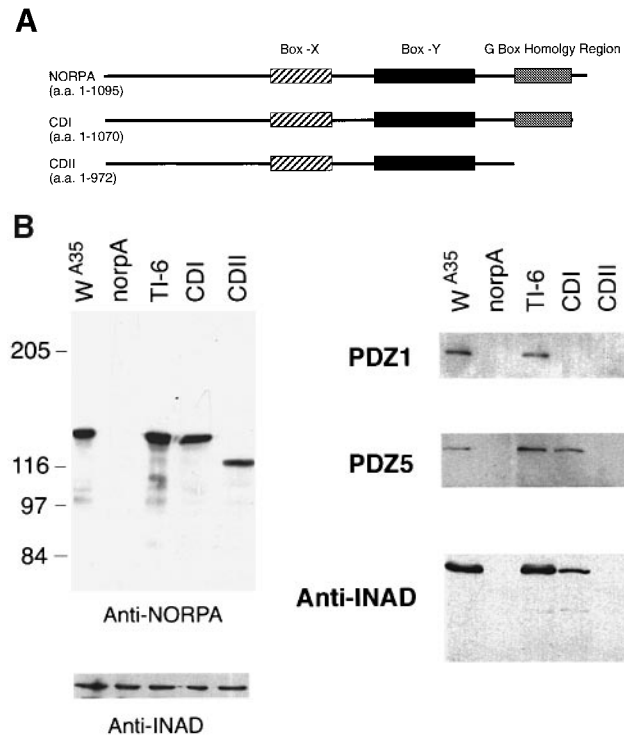


Fig. 5. Expression of NORPA lacking an INAD-binding site in *norpA^{p24}* null flies. (A) Schematic diagram of various deletion constructs used to generate transgenic flies. CDI deletes the last 25 amino acids lacking the PDZ1-binding site; CDII deletes the last 123 residues, which removes both the PDZ1- and PDZ5-binding sites. (B) Differential PDZ1 and PDZ5 binding to NORPA protein in transgenic flies. Protein extracts were prepared from fly heads of *w^{A35}*, *norpA^{p24}*, TI-6, CDI and CDII. Left panel: immunoblot detection of NORPA and INAD with corresponding anti-NORPA antibody (top) and anti-INAD antibody (bottom). The fly strains are indicated on the top. Molecular weight standards are marked in kDa. Right panels are anti-NORPA immunoblots of proteins pulled down by PDZ1 (top) or PDZ5 (middle) of INAD, or by anti-INAD antibody (bottom).

Deletion of INAD-binding sites in NORPA does not affect basal PLC activity

The ability of two distantly located PDZ domains to bind NORPA suggests several possible functional roles of the INAD–NORPA interactions, including localization and/or modulation of the NORPA PLC activity. To delineate the potential *in vivo* function of the INAD–NORPA interaction, we generated fly transformant lines that express NORPA mutants in a *norpA^{p24}* null background lacking either one (CDI) or both (CDII) INAD-binding sites (Figure 5A, and Materials and methods). Three independent lines for each construct were isolated: CDI-1; CDI-2 and CDI-3; and CDII-1, CDII-2 and CDII-3. The expression of these truncated proteins was detected by immunoblot with anti-NORPA antibody. Specific NORPA polypeptides corresponding to the predicted sizes were detected. The expression of CDII mutant protein was reduced, while no detectable difference in INAD expression was observed in these mutants (Figure 5B, left panel). The ability of the truncated proteins to interact with PDZ1 and PDZ5 was tested by affinity binding or ‘pull down’ experiments using either the purified GST–PDZ1 or GST–PDZ5 fusion protein. Crude protein extracts were prepared from fly heads of *w^{A35}* (control), *norpA^{p24}* (a null mutant), TI-6 (a *norpA^{p24}* line rescued with a full-length *norpA*

gene) (McKay *et al.*, 1995), CDI and CDII. The PDZ1 fusion protein can precipitate NORPA protein specifically in *w^{A35}* and TI-6, but failed to do so from the *norpA^{p24}*, CDI or CDII extracts. Consistently, PDZ5 only brought down NORPA protein from *w^{A35}*, TI-6 and CDI, since the expressed proteins all contain the PDZ5-binding site (Figure 5B, right panel). The ability of INAD and NORPA to associate *in vivo* was tested by immunoprecipitation, which also supports that INAD interacts with NORPA via two binding sites (Figure 5B, right panel).

To test whether either the recombinant INAD PDZ1 or PDZ5 domain could modulate the NORPA PLC activity, crude protein extracts were prepared from wild-type fly heads and the PLC activity was determined in the presence of the purified interacting PDZ1 and PDZ5 domains or a non-interacting PDZ2 domain (data not shown, and Materials and methods). The PLC activity showed no dosage-dependent or PDZ-specific changes. Thus, the recombinant PDZ domains of INAD failed to confer any additional modulatory activity on PLC in crude fly head extract.

Removal of the PDZ-binding site in NORPA produces truncated proteins, which prevents the INAD–NORPA association. Previous studies have shown that expression of the NORPA cDNA driven by the *ninaE* promoter rescues the *norpA^{p24}* null mutant as determined by the recovery of PLC activity and electrophysiological properties, including electroretinograms (ERGs) and the prolonged depolarizing afterpotential (PDA) (McKay *et al.*, 1995). This demonstrates that the overall approach is capable of producing functional NORPA protein *in vivo*. To test whether the truncations are detrimental to NORPA enzymatic activity, crude extracts were prepared from fly heads of TI-6, CDI and CDII. The basal PLC activity of NORPA was determined. The PLC activity in CDI and CDII is comparable with that observed from an extract of TI-6 (data not shown). Thus, judging by the basal PLC activity, the CDI and CDII proteins are indistinguishable from the TI-6 full-length rescued transformant. However, the ability of CDI and CDII to couple with G protein could also be compromised (see Discussion).

Reduction and mislocalization of the NORPA protein lacking INAD-binding sites

To test whether INAD functions by recruiting the NORPA protein to specific subcellular locations in proximity with the signaling complex, we compared the spatial localization of the NORPA protein in CDI and CDII transformants with that in the full-length NORPA TI-6 line.

There are ~800 ommatidia in *Drosophila* compound eyes. Each ommatidium consists of 20 cells including eight photoreceptor cells. Each photoreceptor cell projects a specialized microvillar structure, referred to as a rhabdomere, that houses the protein components for phototransduction. Within the eight photoreceptor cells (R1–R8), R1–R6 contain large rhabdomeres that extend the full depth of the retina. The remaining R7 and R8 cells are positioned tandemly in the center to occupy distal and proximal regions of the retina respectively. Thus, in a healthy ommatidium, one should observe six large rhabdomeres (in photoreceptors R1–R6) surrounding one small rhabdomere (in either photoreceptor R7 or R8) in a given cross-section.

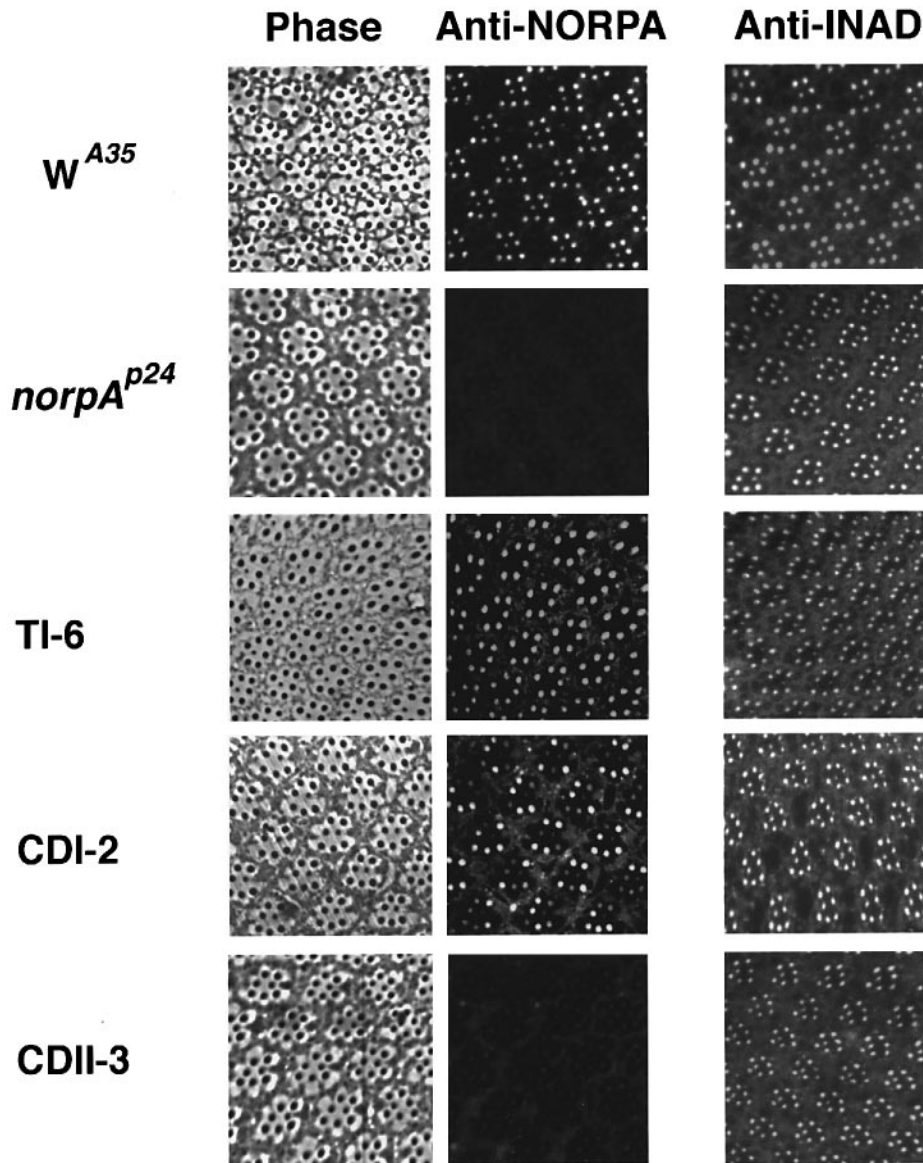


Fig. 6. Immunolocalization of NORPA in ommatidia of control and transgenic flies. Adult eyes (2–4 days after eclosion) of various strains were cross-sectioned and stained with anti-NORPA antibody with phase images of the corresponding areas. The same fly strains were also stained with anti-INAD antibody (right panels; the corresponding phase images are not shown). The labels on the left identify the strains for each triplet of images.

NORPA expression was found in rhabdomeres of all R1–R6 cells and R7 or R8 cells in control (w^{A35}) flies, as detected by affinity-purified anti-NORPA antibody (Figure 6). In the $norpA^{p24}$ null flies, no PLC activity could be found (McKay *et al.*, 1995; Pearn *et al.*, 1996). Consistently, immunostaining of $norpA^{p24}$ mutants with anti-NORPA antibody fails to detect the presence of the NORPA protein (Figure 6, also see McKay *et al.*, 1995). In the TI-6 line, NORPA expression in R1–R6 cells was restored as driven by the *ninaE* promoter. We found that all R1–R6 cells show a comparable fluorescence staining signal (Figure 6). In CDI transformants, the densely stained NORPA signal in rhabdomeres of all three lines was reduced, and in CDI-2 in particular we found that the staining pattern was significantly altered. More specifically, the NORPA staining signal was missing or significantly reduced in one or more cells (Figure 6). The rhabdomere

with the much reduced signal appears to be distributed randomly in a given view of an ommatidia cross-section. To test whether the CDI protein was distributed evenly among R1–R6 cells, we stained apical–basal sections and found that the expression of CDI protein was distributed discontinuously as patches along the microvilli (data not shown). In the CDII mutants, the dense staining of NORPA in rhabdomeres was completely abolished. It is known that the $norpA^{p24}$ null mutants exhibit retinal degeneration. The failure of NORPA expression in rhabdomeres was not due to the loss of rhabdomeres, since phase images show little variation among different ommatidia, and the rhabdomeric expression of INAD is similar (Figure 6). Thus, the deletions of the NORPA C-terminal regions, which have removed either PDZ1 or PDZ1 and PDZ5 interaction sites, cause significant reduction in the NORPA rhabdomeric expression.

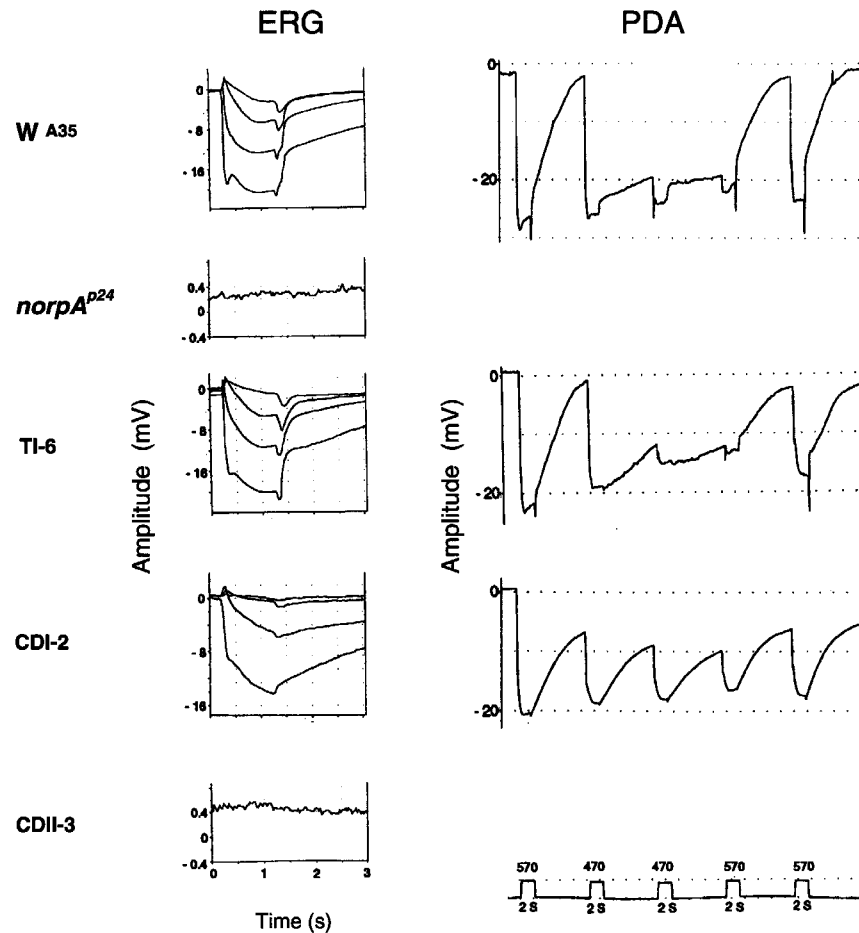


Fig. 7. Phototransduction defects of NORPA CDI and CDII mutants. Electroretinograms (ERG, left panel) and prolonged depolarizing afterpotentials (PDA, right panel) are recorded from w^{A35} , $norpA^{p24}$ null mutant, TI-6 transformant, CDI-2 transformant and CDII-3 transformant. Shown are typical results from adult females (4 days after eclosion). All ERG stimuli were at 470 nm for 1 s with intensities of 9.13, 10.10, 11.33 and 13.27 log quanta/cm²/s. The corresponding PDA recordings for these strains are shown in the right column. The PDA stimulation protocol is shown at the bottom (see also Materials and methods).

The INAD-binding sites in NORPA are essential for *Drosophila* visual signaling

To examine the functional consequences in visual signal transduction, we recorded the ERG and PDA from flies containing the truncated NORPA proteins (designated CDI and CDII). As previously reported, $norpA^{p24}$ null flies completely lack a depolarizing photoreceptor response (Hotta and Benzer, 1970; Pak *et al.*, 1970). The expression of a full-length NORPA protein in $norpA^{p24}$ results in restoration of the NORPA protein in the retina as well as a concomitant restoration of PLC activity and light-evoked responses (ERG and PDA) in photoreceptor cells (McKay, *et al.*, 1995; Figure 7).

In the three independent CDI transformant lines (CDI-1, CDI-2 and CDI-3), 74% of transformants ($n = 31$) varying in age from 1 to 4 days displayed ERGs and the rest showed no ERGs. The ERGs recorded from CDI flies also showed various degrees of abnormality (Figure 7, CDI-2). When these ERG-positive flies were subjected to PDA recording, most of the PDA responses were abolished (D.-M.Chen and W.S.Stark, unpublished results). Analyses in response to light intensity showed that CDI mutants exhibited up to a 100-fold reduction in ERG amplitude compared with that of TI-6 at a given light intensity (data not shown). Consistently, similar phenotypic changes were

also observed as a result of the lower expression of $G_{q\alpha}$ (Scott *et al.*, 1995).

In the three CDII lines (CDII-1, CDII-2 and CDII-3), the deletion of two INAD-binding sites completely abolished the ERG response ($n = 68$) (Figure 7). Taken together, both PDZ1–NORPA and PDZ5–NORPA interactions appear to be important. Perturbation of either interaction causes changes in the subcellular localization of NORPA protein, thereby resulting in defects in or complete loss of the light-evoked signal transduction.

Discussion

In this report, we provide biochemical evidence indicating that INAD directly binds to NORPA via two binding sites. The multivalent NORPA–INAD interactions are mediated by two distantly positioned PDZ1 and PDZ5 domains, suggesting a complex domain organization of the INAD PDZ modules. Genetic and electrophysiological data demonstrate that both binding sites are important for the rhabdomeric NORPA expression *in vivo* and optimal fly visual signal transduction.

INAD binding to target proteins

INAD is comprised of five tandem PDZ domains and, in principle, can bind five independent target proteins through

the PDZ and C-terminus association. Although there are more than five proteins that have been implicated to interact with INAD either directly or indirectly (see Introduction), so far the NORPA C-terminus (FCA-COO⁻) is the first one found to bind INAD PDZ1. A similar approach potentially is applicable to the studies of the other known and unknown interacting partners.

Most recently, Shieh *et al.* (1997) have reported that mutations in the C-terminus of NORPA affect both activation and deactivation of phototransduction, which is in general agreement with our data of NORPA CDI and CDII mutants. In contrast to their conclusion from overlay binding studies, we show that it is PDZ1 that binds to the NORPA C-terminus; instead the PDZ5 domain alone is sufficient to interact with NORPA through binding to an internal region.

Interestingly, our *in vitro* selection results suggest that the optimal binding sequence for INAD PDZ1 is YCF instead of FCA native to NORPA. In fact, results of our competition ELISA experiments with the last 15 amino acids of NORPA confirm that the FCF terminus does have 5- to 10-fold higher affinity than that of the native NORPA FCA terminus (R.van Huizen and M.Li, unpublished data). Additionally, we found that substitution of FCA by FSA completely abolished the association of PDZ1 with NORPA, consistent with the idea that the affinity of the FCA peptide is moderate and probably close to the threshold of protein overlay detection. Thus, a slight change in side chain properties results in loss of the binding (Figure 3). Collectively, considering the amino acid sequence consensus of the *in vitro* selected peptides, results of the site-directed mutagenesis studies and evidence from cell biological/electrophysiological studies, we conclude that PDZ1 interacts with the C-terminus of NORPA primarily through the last three residues.

The strength of the non-bias *in vitro* peptide selection for optimal binding consensus has also revealed a potential discrepancy. Namely, NORPA does not possess the optimal sequence for higher affinity association with PDZ1 (Figure 3). Why did evolution not select the optimal sequence? Teleologically, one could argue that the specific association with moderate affinity may be essential for certain regulatory purposes, i.e. evolution pressure may select variants with certain functional advantages. This notion has been supported by several lines of evidence. For example, mutations of proteins do not always result in a decrease or loss of activity. On the contrary, some mutations have produced variants with higher activity, as evidenced from *lacI* mutants which bind better than wild-type (Barkley and Bourgeois, 1980) and point mutations that have resulted in a variant of β -lactamase with much improved enzymatic activity (Stemmer, 1994). Although the precise functional connection between the affinity of the NORPA-INAD interaction and NORPA activity remains to be tested, one can speculate that with moderate affinity, the FCA-PDZ1 association would be more sensitive to modest conformational changes of INAD caused by a post-translational modification or protein-INAD interaction. Since there are two INAD-NORPA interactions, and if one assumes that the two contacts are completely independent, the compound association constant (i.e. avidity) between INAD and NORPA in fact is likely to be quite high, presumably in the nanomolar range. By

using C-terminally mutated NORPAs with a discrete increase or decrease of affinity to test their rescue ability, one may obtain important information as to how a change of binding affinity between PDZ1 and NORPA is coupled in signaling.

To gain a full understanding of the bipartite INAD-NORPA interaction, a critical question would be the functional role of the PDZ5-NORPA interaction. To achieve this goal, the first set of experiments would be to test whether PDZ5 and G_{qα} compete for the same site. Previous reports have suggested that the mammalian G box is an α -helix, and positively charged residues on one side of the helical wheel are involved in conferring the sensitivity to activated G protein (Wu *et al.*, 1993; Jiang *et al.*, 1994; Kim *et al.*, 1996). If PDZ5 and G_{qα} are not competitive, it would be possible and desirable to obtain point mutants that specifically affect the PDZ5-NORPA binding. The mutants lacking the ability to interact with PDZ5 not only provide tools to test the functional role of the PDZ5-NORPA interaction, but they also offer the possibility of determining whether the increased affinity of the PDZ1-NORPA(FCF) interaction could rescue NORPA localization and function.

A PDZ domain interacts with its target protein via one of two modes. The common mechanism is to bind to the 4-7 residues at the carboxylated C-terminus. Alternatively, PDZ can interact with an internal region of a target protein, such as PDZ-PDZ binding (Brenman *et al.*, 1996). Interestingly, INAD uses both modes to interact with NORPA. What would be the benefit? On the basis of the deduced PDZ1 binding consensus X(Y/F/W)C(F/A)-COO⁻, and assuming that the last three residues were selected randomly, there will be at least one protein with a matching C-terminal sequence in every 50 000 proteins. In the estimated 12 000-14 000 genes of the *Drosophila* genome (Gabor-Miklos and Rubin, 1996), the chance of having compatible C-termini for INAD interaction is low. However, the C-termini of cellular proteins are unlikely to be determined randomly. In fact, several *Drosophila* proteins including 15a ribosomal protein (FFF), NADH-ubiquinone oxidoreductase (FMF) and gooseberry distal protein (FGF) all contain C-termini compatible with INAD PDZ1 binding. How could a specific NORPA-INAD interaction be established? Besides temporal and/or spatial expression which could prevent non-functional INAD-protein interactions, the multivalent contacts between NORPA and INAD may be necessary for establishing the functional specificity.

Functional significance of the INAD-NORPA interactions

Much of our current understanding of INAD function comes from genetic experiments which have shown that mutated alleles of INAD cause profound cellular and functional perturbation of signaling molecules in phototransduction (Shieh and Zhu, 1996; Chevesich *et al.*, 1997; Tsunoda *et al.*, 1997). A mutation in INAD could either produce a discrete change within a given PDZ domain or lead to both local and distant structural changes.

In order to understand the biochemical and functional role of INAD-NORPA interaction, a systematic binding study was performed by testing which PDZ domain(s) of INAD is involved in mediating INAD-NORPA interaction.

By assuming that removal of one or two INAD-binding sites is likely to severely reduce or prevent the NORPA–INAD interaction, the functional roles of these interactions were tested by investigating cellular and phototransduction phenotypes of mutant NORPA proteins that lack the INAD-binding sites. In addition to modest changes of absolute protein expression in NORPA CDI and CDII mutants, when anti-NORPA antibody was used to stain horizontal sections of the adult retina, the wild-type rhabdomeres were stained throughout the whole length from apical to basal portions; *norpa* null gave background staining. Interestingly, under the same staining conditions, CDI mutants showed non-continuous NORPA staining along rhabdomeres; CDII mutants exhibited overall reduced expression of NORPA as judged by the immunofluorescence signal and the fact that the normal dense stain was lost. This result, combined with the CDI mutant phenotype, demonstrates that the ability to interact with INAD is linked directly to the rhabdomeric localization and expression of NORPA. The binding of PDZ5 to an internal region raises the question as to whether it interacts with a small peptide sequence. By deleting further into the G box homology region, we found that resultant fusion proteins became very ‘sticky’ and lost binding specificity (R.van Huizen and M.Li, unpublished results). In terms of physiological phenotypes, the C-terminal 123 residues of NORPA contains two INAD-binding sites and a G box homology region that potentially interacts with $G_{q\alpha}$ (see below). Thus, the physiological phenotype of CDII, which lacks the last 123 residues, could be a compound result from both failure of NORPA–INAD and defective NORPA–G protein interaction. If G protein and PDZ5 do not compete for the same binding site, future studies using NORPA point mutants that separate the binding ability of G protein and PDZ5 would provide important insights into the function of NORPA–INAD interaction.

Domain organization of INAD PDZs

An increasing number of genes with multiple protein interaction domains have been identified. However, little is known about domain arrangement in proteins with tandem PDZ domains (Pawson and Scott, 1997). One recent study on PSD-95 of the MAGUK family suggests that stoichiometric disulfide bonding is essential to bring four PSD-95 molecules together, which assembles a total of 12 PDZ domains (Hsueh *et al.*, 1997). Their interaction with oligomeric ion channels would allow for the complex interactions with potassium channels, NMDA receptors and neuroligins (Kim *et al.*, 1995; Kornau *et al.*, 1995; Irie *et al.*, 1997). The target binding by a protein interaction module may also be regulated. An example of this came from a series of studies that showed that the binding of human immunodeficiency virus (HIV) Nef to the SH3 domain of the Src-family tyrosine kinase Hck causes a marked increase in kinase activity (Moarefi *et al.*, 1997). Thus, the five PDZ domains of INAD may be organized structurally and/or oligomerically, and such an organization may also be regulated. INAD binds to NORPA through multiple contacts with the C-terminal 123 residues. The functional interaction of NORPA with two distantly positioned PDZ domains provides evidence that the two terminal PDZ domains of INAD are present in proximity *in vivo*. This can be achieved through intramolecular and/

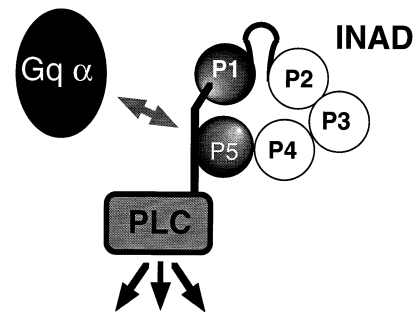


Fig. 8. A working model. The diagram represents one of the two postulated modes by which NORPA and INAD form the multivalent contacts. INAD can interact with NORPA by intramolecular arrangement (as shown) or INAD can simply form oligomers, which positions PDZ1 and PDZ5 in proximity to allow for multivalent contacts with the NORPA C-terminus. The model does exclude the possibility that both intramolecular and intermolecular interactions are involved in the domain arrangements. The multivalent interaction may provide structural constraints that allow for effective coupling with G protein.

or intermolecular molecular domain arrangements as a result of INAD oligomerization (Figure 8). We have tested biochemically for PDZ1–PDZ5 association. So far, no interactions can be detected with the purified PDZ1 and PDZ5 (R.van Huizen and M.Li, unpublished results), and presumably other PDZ domains of INAD may be involved in mediating the domain organization. It should also be noted that the evidence presented in this report cannot rule out the possibility that one INAD interacts with two NORPA molecules, nor can we rule out that two INADs bind to one NORPA molecule; both are interesting topics for future studies.

INAD, an organizing factor for G protein-mediated receptor signaling

A current view of G protein function in cells includes random collisions between protein with a high specificity at the sites of protein–protein interaction, which adequately and quantitatively explains vertebrate phototransduction in disk membrane (see review by Stryer and Bourne, 1986). In other systems, increasing evidence now supports the notion that receptor, G protein and effectors are spatially organized within cells (see reviews by Rodbell, 1992; Neubig, 1994), and a gene encoding INAD-like protein was reported recently (Philipp and Flockerzi, 1997). In *Drosophila*, rhodopsin has been suggested to be a component in the phototransduction complex organized by INAD, although no consensus has been reached on whether INAD binds directly to rhodopsin (Chevesich *et al.*, 1997; Tsunoda *et al.*, 1997). Since both NORPA and rhodopsin are capable of interacting with G protein, it is likely that G protein is also included in the INAD-organized signaling complex either by interacting with rhodopsin/NORPA or by direct binding to INAD. Interestingly, the PDZ5-binding site in NORPA overlaps with the G box homology region. In human PLC- β 1, the corresponding region has been shown to interact with G protein, which leads to the activation of PLC activity (Wu *et al.*, 1993; Jiang *et al.*, 1994; Kim *et al.*, 1996). On the basis of sequence homology, the G box homology region in NORPA is thought to interact with activated $G_{q\alpha}$ protein upon light stimulation. The binding of PDZ5 to the NORPA

G box homology region could be either competitive or synergistic with reference to NORPA–G protein coupling. After determination of affinity between purified INAD and NORPA protein, it would be interesting to test whether this PDZ5–NORPA interaction is involved in modulating the G protein–NORPA coupling in addition to its role in subcellular localization of NORPA.

The biochemical and functional evidence for INAD–PLC interaction reported here provide an entry point to begin more detailed molecular studies. The molecular understanding of how INAD organizes the signaling molecules would contribute important insights into the functional diversity of PDZ domains and the cellular mechanism of G protein-coupled receptor signaling.

Materials and methods

Construction of GST and MBP fusion expression plasmids

GST fusion constructs expressing various domains of INAD (PDZ1, 10–112; PDZ2, 233–340; PDZ3, 336–456; PDZ4, 471–595; PDZ5, 575–667; PDZ1–5, 10–667 and PDZ2–5, 233–667) were generated by subcloning PCR fragments into *SalI* and *NorI* sites of pGEX-4T2 (Pharmacia Biotech, Uppsala, Sweden). The maltose fusion constructs expressing peptides were obtained using a published protocol (Li *et al.*, 1997; Stricker *et al.*, 1997). To express various NORPA C-terminal MBP fusions, PCR fragments encoding various regions were cloned into a modified pELM3 with *SalI*–*NorI* restriction sites (pMBPsn). All primer sequences used are available upon request.

In vitro selection of PDZ-interacting peptides

Screening of the random 15mer peptide library for the INAD PDZ domains was carried out as described by Stricker *et al.* (1997). The random peptide library is a generous gift from Dr Peter Schatz at Affymax Research Institute. After four rounds of affinity panning, individual clones were tested by either Lacl or MBP ELISA, in which 0.5 µg of various GST–PDZ fusion proteins were coated on 96-well plates (Stricker *et al.*, 1997). In all ELISA experiments, GST–NAB_{HERG} was used as a negative control. NAB_{HERG} is a hydrophilic fragment of the HERG potassium channel. NAB_{HERG} forms a tetramer with high coating efficiency (Li *et al.*, 1997).

Protein overlay assay

Proteins were fractionated by SDS–PAGE and transferred onto a nitrocellulose membrane (Protran, Schleicher & Schuell, NH). Filters were first blocked in phosphate-buffered saline (PBS) supplemented with 5% Carnation milk for 2 h at room temperature. The binding to GST–PDZ fusions was initiated by incubating the filters in PBS with the following supplements: 5% Carnation milk, 0.2% Triton X-100 and 2 mg/ml of GST fusion protein. The binding reaction was carried out at 4°C for 16 h with constant agitation. After binding, the filters were washed with PBS supplemented with 5% dry milk and 0.2% Triton X-100, and incubated overnight at 4°C with a rabbit anti-GST antiserum (1:500 dilution) in the same buffer. The filters were then washed with PBS containing 0.2% Triton X-100 and incubated for 30 min with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories, CA). The binding of the HRP-conjugated secondary antibody was visualized by enhanced chemiluminescence (ECL, Amersham).

Fly strains

Drosophila melanogaster white (*w*^{A35}) was used as a positive control as it displays wild-type NORPA PLC activity and its genetic background is most similar to the *norpa* mutant fly employed in the experiments. For a negative control, the *norpa*^{P24/w} homozygous allele was used, which displays total blindness (Bloomquist *et al.*, 1988), exhibits no PLC activity in head homogenates and lacks detectable amounts of the NORPA protein (Zhu *et al.*, 1993). Flies were grown on yeast-supplemented Carolina instant media (Carolina Biological Supply) at 21°C in a 12 h light/dark diurnal cycle.

Construction of the NORPA mutations

The NORPA deletion constructs were engineered by the polymerase chain reaction (PCR), using the NORPA cDNA pG181-4 plasmid vector

as a template (Bloomquist *et al.*, 1988). A 5' internal oligonucleotide primer (5'-AACTACAGCGGCTCCACCACCAAC-3') was synthesized upstream from a unique restriction site (*NcoI*) in the *norpa* cDNA. The C-terminal primers were designed with an in-frame stop codon followed by an *XhoI* restriction site at the designated truncation regions [CDI (5'-CATCCGCTCGAGTTAGTCGGTACTGAATTCCTC-3') and CDII (5'-AACCCTCGAGTTATGCCTTCATGGCGTC-3')]. The amplified products were treated with Klenow polymerase, digested with *NcoI* and cloned into pG181-4 plasmid vector via *NcoI* and *SmaI* sites. The resultant deletion mutant constructs were confirmed by DNA sequencing. The remaining steps for producing the NORPA transgenic flies are identical to the published procedures of McKay *et al.* (1995).

To introduce point mutations into the NORPA C-terminal point mutation, the entire cDNA coding sequence of NORPA was amplified with a 5' primer of GGCTCTAGAAATGACCAAGAAGTACGAG and 3' primers of either GGCCCCGGGCTAAAAACAAAATTCGGTTTTCC for NORPA-FCF or GGCCCCGGGCTAGGCAGAAAATTCGGTTTTCC for NORPA-FSA. The PCR fragments were digested and subcloned into a modified pRC-CMV mammalian expression vector (Yu *et al.*, 1996). The wild-type and mutated *norpa* cDNAs were transiently expressed in COS-7 cells to produce the fusion proteins carrying an N-terminal 12CA5 (or HA) monoclonal tag (Xu and Li, 1997).

Phospholipase C activity assays

PLC activity in fly head homogenates was measured as described previously (McKay *et al.*, 1995). Briefly, 100 heads were ground in 1 ml of a buffer containing 50 mM Tris–HCl, pH 7.5, 250 mM KCl, 0.05% sodium deoxycholate, 0.1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, using a Teflon glass homogenizer on ice. These homogenates were then centrifuged at 12 000 g for 1 min to remove large particulate matter. The homogenates were then aliquoted and rapidly frozen at –70°C until used for the assay. Protein concentration was determined using the BCA protein assay (Pierce) with BSA as a standard. The PLC activity measurements of the crude homogenates were performed in a 0.1 ml volume of 50 mM Tris–HCl, pH 7.5, 10^{–7} M CaCl₂, 0.1 mg per ml BSA, 0.2 mM phosphatidylinositol, 44 000 d.p.m. of phosphatidyl-[³H]inositol 4,5-bisphosphate ([³H]PIP₂) and an appropriate amount of head homogenate. After 5 min, the reaction was quenched by precipitating non-hydrolyzed [³H]PIP₂ and diacylglycerol via the addition of 0.1 ml of 10% trichloroacetic acid and 0.05 ml of 10 mg/ml BSA. The samples were then incubated on ice for 15 min and centrifuged at 12 000 g to remove the precipitates. The amounts of [³H]inositol triphosphate in supernatant fractions were quantified by liquid scintillation.

Immunoblotting and immunolocalization

Immunoblotting was carried out according to published procedures (Yu *et al.*, 1996). Immunolocalization was performed on hemisected fly heads prepared from *w*^{A35}, *norpa*^{P24}, TI-6, CDI and CDII adults varying from 2 to 4 days old. Preparation of fly heads was carried out as described by Porter and Montell (1993) except that the tissue was dehydrated in an ethanol series (50, 70 and 90%) for 30 min per incubation. The sections were incubated for 2 h at room temperature with 50 µl of affinity-purified anti-NORPA antibody at 1:50 dilution.

Electrophysiological analyses

ERGs and PDA analyses were carried out on adult flies essentially as described by Chen *et al.* (1992). Briefly, fly compound eye was carefully located at the focal plane of an optical stimulator using 625 nm light at an average intensity of 16.43 log quanta/cm²/s. A glass micropipette was inserted into the retinal cell layer under 625 nm light. After the fly was dark adapted for 40 min, the eye was stimulated by 470 nm light and the ERG was recorded, amplified and fed into a MacLab/2e-Macintosh LC II computer system for storage and analysis. PDA was induced by two stimuli of 470 nm at ~16.04 log quanta/cm²/s for 2 s each and repolarized by two 570 nm stimulations at ~16.39 log quanta/cm²/s for 2 s each.

Acknowledgements

We thank Nicole Stricker and Jia Xu for their generous help with *in vitro* peptide selection and COS cell transfection, and Dr Craig Montell and members of the Montell laboratory for help and communicating unpublished data. We also thank M.Kathleen Loeffert for excellent technical assistance; Drs William Dower and Peter Schatz at Affymax Research Institute for support and peptide libraries; Drs Craig Montell,

King-Wai Yau and members of the Li laboratory for critical reading of the manuscript; and Robyne Butzner for help with the manuscript. This work is supported by grants from National Institutes of Health (to M.L., W.S. and Z.-C.L.), National Science Foundation (to R.S.), A.Sloan foundation and J.Klingenstein foundation (to M.L.).

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Received January 6, 1998; revised February 9, 1998;
accepted February 16, 1998