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Independent Anchoring and Assembly Mechanisms of INAD Signaling Complexes in *Drosophila* Photoreceptors

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In *Drosophila* photoreceptors the multivalent PDZ protein INAD organizes the phototransduction cascade into a macromolecular signaling complex containing the effector PLC, the light-activated TRP channels, and a regulatory PKC. Previously, we showed that the subcellular localization of INAD signaling complexes is critical for signaling. Now we have examined how INAD complexes are anchored and assembled in photoreceptor cells. We find that *trp* mutants, or transgenic flies expressing *inaD* alleles that disrupt the interaction between INAD and TRP, cause the mislocalization of the entire transduction complex. The INAD–TRP interaction is not required for targeting but rather for anchoring of complexes, because INAD and TRP can be targeted independently of each other. We also show that, in addition to its scaffold role, INAD functions to preassemble transduction complexes. Preassembly of signaling complexes helps to ensure that transduction complexes with the appropriate composition end up in the proper location. This may be a general mechanism used by cells to target different signaling machinery to the pertinent subcellular location.

Key words: INAD; signaling complex; transducisome; Drosophila*; phototransduction; subcellular localization; signal transduction; anchoring; assembly*

Every cell must sort through a vast array of external signals and transduce them into the appropriate intracellular responses. Because many intracellular signaling cascades share common downstream components, an important strategy for maintaining specificity within one pathway, while avoiding unwanted cross-talk between different pathways, is the organization of transduction pathways into distinct signaling complexes (Pawson and Scott, 1997; Tsunoda et al., 1998). Scaffold proteins function as organizers of transduction pathways, bringing together signaling molecules into physically defined signaling units. This strategy enables a cell to promote specificity and selectivity while maximizing the speed of signaling.

In *Drosophila* phototransduction, speed of signaling is critical for achieving the temporal resolution necessary for a flying organism. Phototransduction in *Drosophila* is the fastest known G-protein-coupled signaling cascade, taking just a few tens of milliseconds to go from light activation of rhodopsin to the generation of a receptor potential and <100 msec to terminate the response (Ranganathan et al., 1995). In this pathway, light stimulation of rhodopsin activates a G-protein, which then activates a PLC. PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG), leading to the eventual opening and modulation of the light-activated ion channels, transient receptor potential (TRP), and TRP-like (TRPL). Calcium-dependent regulatory processes involving the activation of an eye-specific protein kinase C (eye-PKC), calmodulin (cam), arrestin, and cam-kinase then mediate deactivation of the light response (Ranganathan et al., 1991; Smith et al., 1991; Hardie et al., 1993).

An important strategy used by *Drosophila* photoreceptors to attain an extremely high speed of signaling is the organization of signaling components into transduction complexes by the scaffold protein INAD (these complexes are referred to as transducisomes; Huber et al., 1996; Shieh and Zhu, 1996; Chevesich et al., 1997; Tsunoda et al., 1997). INAD contains five PDZ domains, each binding a component of the phototransduction cascade: PDZ1 and PDZ5 bind PLC (Shieh et al., 1997; Tsunoda et al., 1997; van Huizen et al., 1998), PDZ2 and PDZ4 bind eye-PKC (Tsunoda et al., 1997; Adamski et al., 1998), and PDZ3 binds TRP (Shieh and Zhu, 1996; Tsunoda et al., 1997). This organization brings signaling components into close proximity, thus promoting (1) efficient signaling and (2) the creation of small microdomains in which localized changes in the level of intracellular Ca^{2+} can exert exquisite modulation of the light response (Hardie, 1991; Ranganathan et al., 1991; Scott and Zuker, 1997; Scott et al., 1997).

In wild-type flies, the INAD transducisomes localize to the rhabdomeres, a subcellular compartment consisting of 60,000 microvilli that house the $\sim 10^8$ molecules of rhodopsin found in *Drosophila* photoreceptors. In *inaD* null mutants PLC, TRP, and eye-PKC are distributed randomly in the photoreceptors; this mislocalization leads to a near-complete loss of signaling (Tsunoda et al., 1997). These findings demonstrate that the localization of signaling components is dependent on the presence of the INAD scaffold protein and that it is not the mere presence of signaling molecules that is critical for effective signaling, but rather their location.

Studies in a number of other systems have validated the importance of PDZ proteins in the subcellular localization of signaling

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components and cellular signaling (Kim et al., 1995, 1996; Brenman et al., 1996; Dong et al., 1997; Tejedor et al., 1997; Zito et al., 1997; Torres et al., 1998) (for review, see Sheng, 1996; Kornau et al., 1997; Craven and Bredt, 1998; O'Brien et al., 1998; Tsunoda et al., 1998). For example, in *Caenorhabditis elegans*, the development of the vulva is dependent on a Ras signaling pathway that is mediated by the LET-23 EGF receptor. LIN-2, LIN-7, and LIN-10 are all PDZ-containing proteins essential for localizing the receptor to the basolateral membrane of epithelial cells; mutations in *lin-2*, *lin-7*, or *lin-10* lead to the mislocalization of LET-23 receptors and result in a vulvaless phenotype (Hoskins et al., 1996; Simske et al., 1996; Kaech et al., 1998). In *Drosophila*, the polarization of epithelial cells is dependent on the PDZ scaffold protein Disks Lost (Dlt), which interacts with Crumbs and Neurexin IV (Bhat et al., 1999). Similarly, the PDZ protein Bazooka interacts with the Inscutable protein and is required for the apical localization of Inscutable and proper asymmetric division in *Drosophila* neuroblasts (Schober et al., 1999). Target proteins also play a role in localizing scaffold proteins. For instance, when PSD-95 is transfected into rat pyramidal cells, it localizes only to the dendrites; however, when it is cotransfected with Kv1.4 potassium channel, it also localizes to the axons (Arnold and Clapham, 1999).

In this study, we show that the TRP ion channel is essential for the localization of the INAD scaffold protein and that the TRP–INAD interaction is required for maintaining transduction complexes in the rhabdomeres. We also examined how transduction complexes are assembled and show that signaling complexes are preassembled before they are targeted to the rhabdomeres. The preassembly of transduction complexes may emerge as a powerful strategy in the organization of cellular signaling.

MATERIALS AND METHODS

Immunostaining cross sections of photoreceptors. INAD, TRP, eye-PKC, PLC, rhodopsin, and Dgq were detected by using polyclonal antibodies as previously described (Smith et al., 1991; Scott et al., 1995; Niemeyer et al., 1996; Tsunoda et al., 1997). For immunostaining cross sections of photoreceptors, we first fixed fly heads in 3% paraformaldehyde in PBS for 1 hr on ice and infiltrated them with 2.3 M sucrose in PBS overnight; finally, they were frozen and cut into 1-μm-thick sections. Tissue sections were treated with a blocking solution of 1% BSA and 0.1% saponin in PBS (PBS-S) for 30 min and then incubated with antibody either at 4°C overnight (INAD, TRP, Dgq, rhodopsin) or for 2 hr at room temperature (eye-PKC, PLC). Antibodies were diluted 1:300 (INAD), 1:20 (eye-PKC), and 1:100 (rhodopsin and Dgq) in PBS-S; the TRP and PLC antibodies were preabsorbed first with a homogenate of *trp* or *norpA* mutant heads to reduce background staining and were used at a final dilution of 1:100 (TRP) or 1:500 (PLC). FITC and lissamine rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used at 1:500 (FITC) or 1:200 (rhodamine) for a 1 hr incubation at room temperature.

Electron microscopic localization of INAD. Fly heads were bisected longitudinally and fixed in periodate–lysine–paraformaldehyde (PLP) solution (McLean and Nakane, 1974) for 1.5 hr at room temperature. Then the specimens were dehydrated partially with 90% ethanol, embedded in LR White medium (Polysciences, Warrington, PA), and cut into 80-nm-thick sections. The sections were picked up on Formvar-coated nickel grids, etched with saturated aqueous sodium *meta*-periodate (Sigma, St. Louis, MO) for 15 min, treated with a blocking solution of 5% normal goat serum and 0.05% Tween 20 (Wako Pure Chemical Industries, Osaka, Japan) in PBS (5NGS/PBST) for 20 min, and then incubated with anti-INAD antibody diluted 1:100 with 5NGS/PBST at 6°C overnight. After several rinses with PBST the sections were incubated with 10 nm colloidal gold-conjugated secondary antibody (British BioCell International, Cardiff, UK) diluted 1:50 with 5NGS/PBST for 2 hr at room temperature. The sections were counterstained with 2% aqueous uranyl acetate and Reynolds' lead solution (Reynolds, 1963) and then examined in a JEM 1200EX electron microscope (Jeol, Tokyo, Japan).

DNA constructs and transgenic flies. For *inaD* PDZ mutants three amino acid substitutions were made in each PDZ domain: PDZ1 (phe28ala, ile30ala, ile32ala), PDZ2 (leu260ala, leu262ala, leu264ala), PDZ3 (leu375ala, ile377ala, val379ala), and PDZ4 (met499ala, val501ala, val503ala). Mutant *inaD* constructs were cloned into a P-element-mediated transformation vector containing five Glass-binding sites derived from the *ninaE* promoter (pGMR; Hay et al., 1994). For *hs-inaD* flies, *inaD* cDNA was cloned into a P-element transformation vector under the control of the heat shock promoter (Baker et al., 1994). For trp^{C34} flies a truncated *trp* (with coding deleted for the C-terminal 34 amino acids) cDNA was cloned into the pGMR transformation vector. *Drosophila* P-element-mediated transformations and further genetic manipulations were performed with standard techniques.

Expression and analysis of proteins in Chinese hamster ovary (CHO) cells. inaD and mutant *inaD* cDNAs were cloned into the pcDNA3 transfection vector (Invitrogen, San Diego, CA), which uses the cytomegalovirus immediate-early (CMV) promoter for expression. CHO cells were transfected with Lipofectamine reagent (Life Technologies, Grand Island, NY) and grown for 24 hr in 10% fetal bovine serum, 0.5 μg/ml amphotericin B, and 100 μg/ml gentamycin in MEM α media (Life Technologies) at 37°C; then they were grown for an additional 24 hr in 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in Leibovitz's L-15 media (Life Technologies) at room temperature. Cells were fixed with 2% paraformaldehyde in PBS for 40 min and stained similarly to tissue sections (described above). Confocal images were collected on a Nikon/Bio-Rad 1024 confocal microscope and imported into Photoshop 5.0 (Adobe Systems, Mountainview, CA) for presentation.

Immunoprecipitation. Frozen heads (500–2000) were homogenized in 1 ml of Buffer A [containing (in mM) 20 HEPES, 30 NaCl, and 1 EDTA, pH 7.5, plus protease inhibitors] with a glass–glass homogenizer. The homogenate was centrifuged at 4000 × *g* for 1 min to remove chitinous material. Membranes were isolated by centrifugation at 100,000 × *g* for 30 min at 4°C and resuspended to a final equivalent concentration of 2 heads/μl (wild-type, *inaD* mutants).

For the cross-linking anti-INAD antibody to protein A beads, every 50 μl of anti-INAD antibody was incubated with 30 μl of ImmunoPure-immobilized protein A beads (Pierce, Rockford, IL) in a final volume of 170 μl of ImmunoPure IgG binding buffer (Pierce) overnight at 4°C. Antibody and beads then were cross-linked in 20 mM dimethyl dimelimidate (DMP) and 0.2 mM sodium borate, pH 9.0, for 30 min. Reaction was stopped with 0.2 M ethanolamine, pH 8.0. Beads were stored in PBS.

For immunoprecipitations, the membranes (wild-type, *inaD* mutant, equivalent of membranes from 50 heads; *hs-inaD*, equivalent of 500 heads) were solubilized in 1% Triton X-100 and incubated with anti-INAD antibody cross-linked to protein A beads (bed volume of 20 μl) in a 100 μl total volume overnight at 4°C. Then the beads were washed in Buffer A, resuspended in SDS buffer, and boiled; the entire immunoprecipitate was fractionated by SDS-PAGE.

RESULTS

Subcellular localization of INAD signaling complexes requires the TRP channel

Given that the subcellular localization of signaling components is essential for proper signaling (Tsunoda et al., 1997), we set out to investigate how INAD signaling complexes are maintained (anchored) in the rhabdomeres. To test whether INAD is anchored through one of its known partners, we examined the subcellular distribution of INAD in mutants lacking individual partners. We used null alleles of *trp*, *inaC*, and *norpA* to eliminate TRP, eye-PKC, and PLC, respectively. Figure 1*A* shows that, much like in wild-type cells, INAD is localized correctly to the rhabdomeres in *inaC* and *norpA* mutant photoreceptors. In contrast, INAD is severely mislocalized in null *trp* mutants, with most of the protein found in the cell body instead of the rhabdomeres (Fig. 1*A*). Because INAD itself is essential for the localization of TRP, eye-PKC, and PLC (Tsunoda et al., 1997), we investigated whether eye-PKC and PLC are mislocalized in null *trp* alleles. Indeed, immunolocalization studies demonstrated that eye-PKC

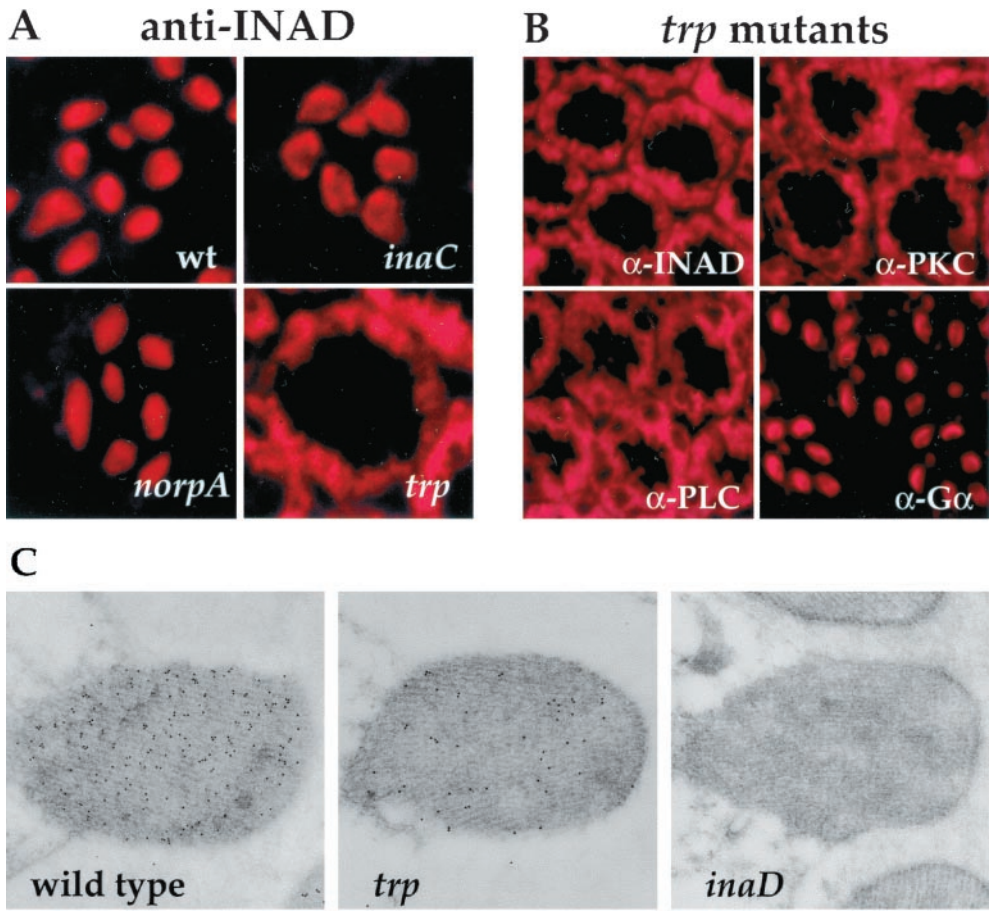

Figure 1. TRP is required for the localization of INAD signaling complexes. *A*, Anti-INAD immunofluorescent staining of cross sections (1 μm thick) of wild-type (*wt*), $inaC^{209}$, $norpA^{P41}$, and trp^{343} null mutant photoreceptors. Normal rhabdomeric localization of INAD is seen in $inaC^{209}$ and $norpA^{P41}$ mutants, whereas it is mislocalized severely in trp^{343} mutants. *B*, INAD, PLC, and eye-PKC also are mislocalized in *trp* mutants. Shown is immunofluorescent staining of cross sections of trp^{343} null photoreceptors. Note that Gα, the G-protein protein that shuttles between activated rhodopsin and transducisomes (Bahner et al., 2000), shows normal rhabdomeric localization. *C*, EM immunogold localization of INAD in wild-type and trp^{343} rhabdomeres, demonstrating that ~25% of the INAD seen in wild-type rhabdomeres still remains in *trp* mutant rhabdomeres. We cannot exclude the possibility that this small amount of INAD may be binding to TRPL channels in the rhabdomeres.

and PLC are mislocalized in *trp* mutant photoreceptors (Fig. 1*B*). Interestingly, eye-PKC and PLC are still coimmunoprecipitated with INAD in *trp* null mutants (data not shown), suggesting that complexes are stable even when they are mislocalized (see below).

To demonstrate that it is the specific interaction of INAD with TRP that is required for the rhabdomeric localization of INAD transducisomes, we generated two transgenic lines expressing mutant proteins predicted to disrupt the TRP–INAD interaction. In the first line, $inaD^{PDZ3}$, three conserved residues in the third PDZ domain of INAD (PDZ3; the site of TRP binding) were changed to alanines (leu375ala, ile377ala, val379ala). These mutations are expected to disrupt the interaction between PDZ domains and their targets (Doyle et al., 1996). The second transgenic line, trp^{C34}, expresses a mutant TRP protein that lacks its INAD binding site (a truncation of the C-terminal 34 amino acids; Shieh and Zhu, 1996). As predicted, TRP and INAD are mislocalized in $inaD^{PDZ3}$ and trp^{C34} mutants (Fig. 2). Because INAD is mislocalized in $inaD^{PDZ3}$ and trp^{C34} mutants, eye-PKC and PLC are mislocalized also (data not shown). Taken all together, these results substantiate the requirement for TRP in the maintenance of INAD complexes in the rhabdomeres and strongly suggest that part of the *trp* phenotype may be attributable to the mislocalization of transducisomes (see below).

INAD and TRP are targeted independently to the rhabdomeres

Because INAD is mislocalized in *trp* mutants and TRP is mislocalized in *inaD* mutants, we wondered about the relationship between TRP and INAD for targeting/anchoring to the rhabdomeres. For example, does one protein depend on the other for targeting to the rhabdomeres, and/or does one require the other for maintenance in the rhabdomeres? To investigate whether either TRP or INAD relies on the other for targeting to the rhabdomeres, we examined INAD and TRP at a time when rhabdomeres are completing their maturation and transduction proteins are first synthesized. Figure 3 shows that, in *trp* mutant photoreceptors, INAD initially is delivered and localized to the rhabdomeres of pupae but then becomes mislocalized by the time of eclosion. Similarly, in *inaD* null photoreceptors, TRP initially is targeted to the rhabdomeres of late pupae but becomes mislocalized in newly eclosed flies (Fig. 3). These results demonstrate that TRP and INAD do not depend on each other to be targeted to the rhabdomeres, but they need each other to remain in the rhabdomeres.

PDZ1 also is required for localization of INAD

Examination of the signaling properties of *inaD* and *trp* mutants reveals a salient difference between the two mutants. Although both *inaD* and *trp* null mutants show severe mislocalization of signaling components, light responses are robust in *trp* mutants but are extraordinarily poor in *inaD* mutants (Hardie and Minke, 1992; Tsunoda et al., 1997). Because signaling is fully dependent on the rhabdomeric localization of transduction components (Tsunoda et al., 1997), we reasoned that *trp* mutants may have a small amount of INAD signaling complexes remaining in the rhabdomeres (like the $inaD^{215}$ allele; see Tsunoda et al., 1997), and this may be sufficient to activate the TRPL channels (this is the light-activated channel remaining in *trp* mutants; Niemeyer et al., 1996). To test this postulate, we performed electron micro-

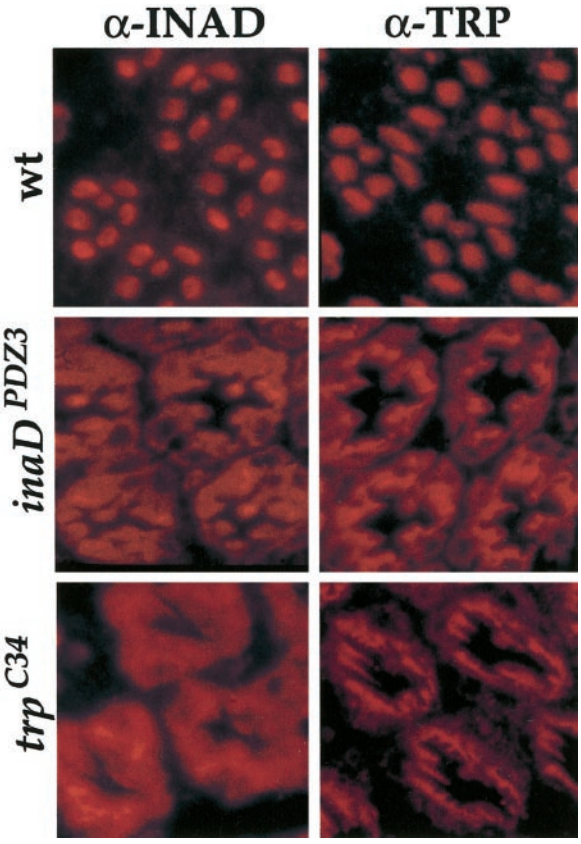

Figure 2. Mutations that disrupt the INAD–TRP interaction display mislocalization of INAD and TRP. Shown is immunofluorescent staining for INAD and TRP in cross sections (1 μm thick) of wild-type (*wt*), $inaD^{PDZ3}$, and trp^{C34} transgenic photoreceptors (as indicated). $inaD^{PDZ3}$ flies express an INAD protein containing three point mutations expected to disrupt the INAD–TRP interaction (see Materials and Methods). trp^{C34} flies express a truncated TRP protein that is missing its PDZ binding site (C-terminal 34 amino acids deleted). INAD and TRP both are mislocalized severely in $inaD^{PDZ3}$ and trp^{C34} transformants. Given that rhodopsin (Rh1) is not part of the INAD signaling complex (Tsunoda et al., 1997; Huber et al., 1998; B. H. Shieh, personal communication) and that Gα shuttles between Rh1 and the transducisome (Tsunoda et al., 1997; Bahner et al., 2000), we examined the localization of Rh1 and Gα as controls for normal rhabdomeric labeling. Indeed, rhodopsin and Gα are localized normally in *trp*, $inaD^{PDZ3}$, and trp^{C34} mutant backgrounds (data not shown).

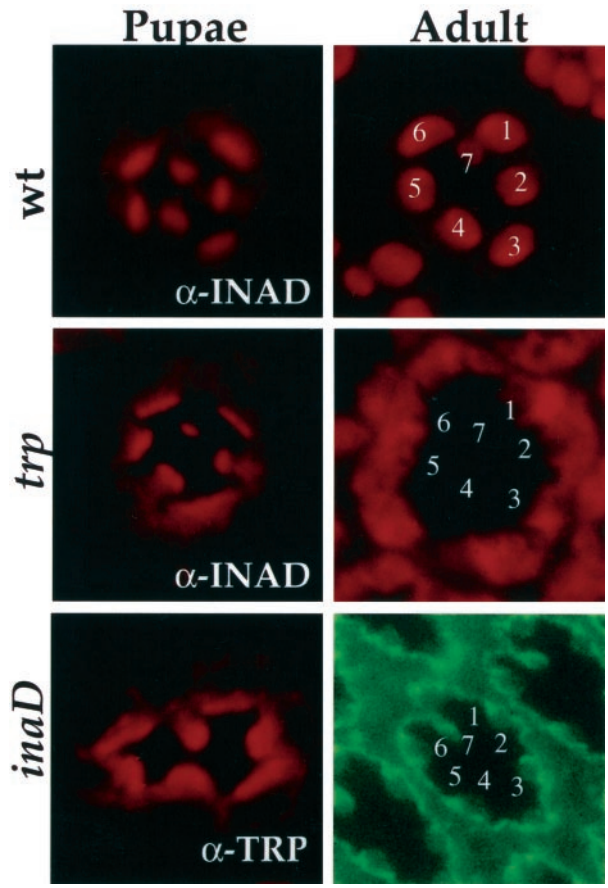

Figure 3. INAD and TRP are targeted independently to the rhabdomeres in pupae but require each other to be maintained in the rhabdomeres. Shown is anti-INAD and anti-TRP (as indicated) immunofluorescent staining of cross sections (1 μm thick) of wild-type (*wt*), trp^{343}, and $inaD^{1}$ null photoreceptors from pupae and adult flies. In wild type the INAD (*top row*) and TRP (data not shown) are targeted to the rhabdomeres in pupae and remain localized in the rhabdomeres of adult photoreceptors. In trp^{343} mutants (*middle row*) INAD is targeted to the rhabdomeres in pupae but becomes mislocalized in adult photoreceptors. Similarly, in $inaD^{1}$ null mutants (*bottom row*) TRP is targeted to the rhabdomeres in pupae but becomes mislocalized in adult photoreceptors. Rhabdomeres of individual R1–R7 photoreceptors are indicated by *numbers*.

scopic immunogold labeling (immunoEM) of INAD. Figure 1*C* shows that *trp* mutants indeed have ~25% of the level of rhabdomeric INAD of wild-type controls; this likely underlies the signaling seen in *trp* mutants.

Given that there is 25% of INAD in the rhabdomeres of *trp* mutants, we wondered whether INAD might be anchored to the rhabdomeric membrane through another one of its PDZ domains. We generated transgenic animals expressing INAD proteins each containing point mutations in one of the five PDZ domains and examined their localization. $inaD^{PDZ2}$ and $inaD^{PDZ5}$ mutants displayed normal rhabdomeric localization of INAD proteins (data not shown), suggesting that PDZ2 and PDZ5 do not play a role in anchoring. As expected, $inaD^{PDZ3}$ mutants showed a mislocalization of INAD protein (see Fig. 2). $inaD^{PDZ1}$ and $inaD^{PDZ4}$ transformants, however, did not produce stable proteins *in vivo* and we were unable to test them. To investigate the membrane association of these two PDZ mutants in a different system, we used a tissue culture assay to monitor membrane localization of INAD and its targets.

We expressed *inaD* in CHO cells and examined its subcellular localization with confocal microscopy. Wild-type INAD localizes to the periphery of the cell, in close proximity to the plasma membrane (Fig. 4*A,E*). Because this happens in the absence of TRP, this result suggests that membrane association does not depend on TRP–INAD interactions and perhaps would be expected to occur even in PDZ3 mutants. Indeed, when CHO cells are transfected with a PDZ3 mutant construct, they are nearly indistinguishable from wild-type controls (data not shown). These cells also show complex formation. eye-PKC exhibits punctate, perinuclear localization (Fig. 4*D*). However, when wild-type INAD and eye-PKC are coexpressed, eye-PKC now colocalizes with INAD to the periphery (Fig. 4*E*) and coimmunoprecipitates with INAD (data not shown). We next examined the localization of all five different PDZ mutants. In contrast to wild-type INAD and mutations in PDZ-2, PDZ-3, PDZ-4, and PDZ-5 domains (Fig. 4*C,G*), the PDZ1 mutant protein does not localize to the

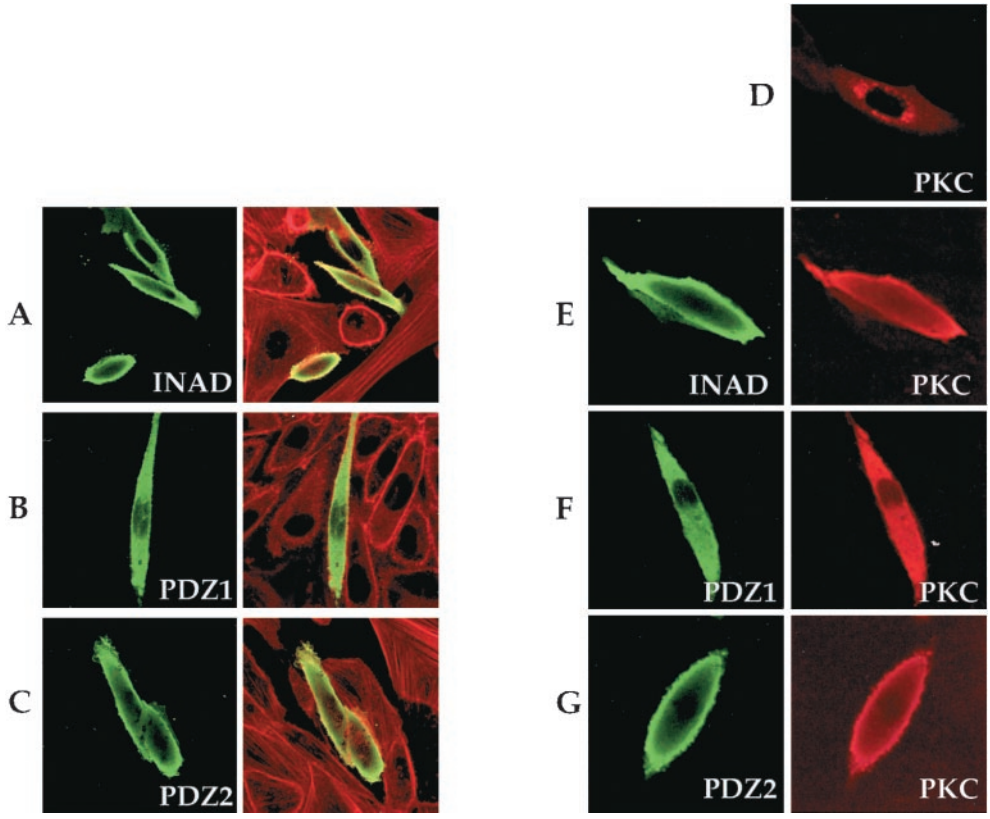

Figure 4. PDZ1 is required for localizing INAD at the membrane of CHO cells. *A–C*, Wild-type and mutant *inaD* constructs containing point mutations (see Materials and Methods) in PDZ1, PDZ2, PDZ3, PDZ4, or PDZ5 were transfected into CHO cells. Shown are confocal images of immunofluorescently stained CHO cells transfected with wild-type *inaD* (*INAD*; *A*), $inaD^{PDZ1}$ (*PDZ1*; *B*), and $inaD^{PDZ2}$ (*PDZ2*; *C*). *Left*, Anti-INAD staining. *Right*, Anti-INAD (*green*) superimposed with rhodamine-conjugated phalloidin staining (*red*). INAD shows membrane-associated staining, as seen in *A* and *E*, in 72.7% of transfected cells ($n = 414$), whereas PDZ1 shows membrane-associated staining in only 19.9% of transfected cells ($n = 272$). In most cells PDZ1 (*B, F*) is expressed diffusely throughout the cell. The percentage of transfected cells displaying membrane-associated localization was 63.8% for PDZ2 ($n = 315$), 74.8% for PDZ3 ($n = 302$), 42.4% for PDZ4 ($n = 85$), and 40.6% for PDZ5 ($n = 256$). *D–G,* Wild-type and mutant INADs redistribute eye-PKC when cotransfected. Shown is immunofluorescent staining of CHO cells cotransfected with *inaD* (*INAD*), $inaD^{PDZ1}$ (*PDZ1*), or $inaD^{PDZ2}$ (*PDZ2*) with eye-PKC. *Left*, Anti-INAD staining. *Right*, Anti-PKC staining. PKC transfected alone displays a punctate, perinuclear expression pattern (*D*). When cotransfected with *inaD* (*E*), $inaD^{PDZ1}$ (*F*), or $inaD^{PDZ2}$ (*G*), PKC is redistributed into an expression pattern like that of INAD, PDZ1, or PDZ2, respectively.

periphery. Instead, it is found expressed diffusely throughout the cell (Fig. 4*B,F*). To ensure that $INAD^{PDZ1}$ is a functional protein, we examined its interaction with eye-PKC. Figure 4*F* confirms that PDZ1 still can interact with and redistribute eye-PKC. These results indicate that PDZ1 is essential for membrane localization and suggest that INAD is anchored by interactions between PDZ3 and TRP and between PDZ1 and a membrane target.

Signaling complexes are assembled before they are targeted to the rhabdomeres

How are the "soluble" partners of INAD (eye-PKC and PLC) targeted to the rhabdomeres? To test whether eye-PKC and PLC could be targeted to the rhabdomeres in the absence of INAD, we examined their initial subcellular localization in wild-type, *trp*, and *inaD* animals from the earliest times that we could detect expression. Figure 5 shows that eye-PKC and PLC localize specifically to the rhabdomeres of wild-type and *trp* mutant pupae but remain mainly in the cell bodies of *inaD* mutant photoreceptors. These findings indicate that INAD is required for the targeting of PLC and eye-PKC and suggest that INAD may be preassembling signaling complexes before targeting to the rhabdomeres.

To determine whether INAD assembles its soluble partners into complexes before they are delivered to the rhabdomeres, we designed an experiment that would allow us to follow the fate of a pulse of newly synthesized INAD and its association with PLC and eye-PKC. We generated transgenic flies expressing INAD under the control of an inducible heat shock promoter (*hs-inaD*) and examined the formation of signaling complexes, rhabdomeric

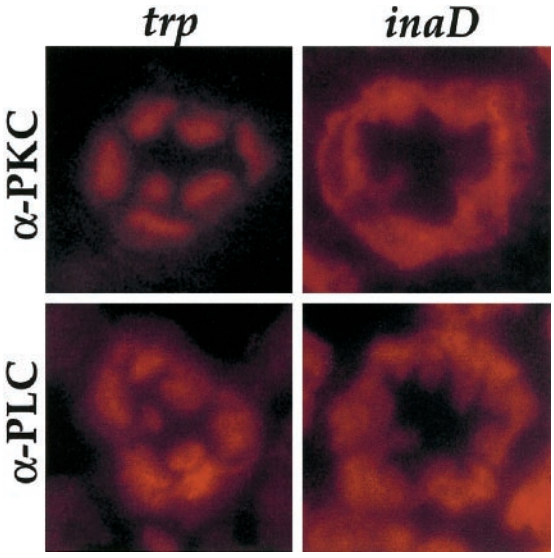

Figure 5. PKC and PLC require INAD to be targeted to the rhabdomeres. Shown is anti-PKC and anti-PLC immunofluorescent staining of cross sections (1 μm thick) of trp^{343} and $inaD^{1}$ pupal photoreceptors. Pupae were aged and sectioned at the earliest times of anti-PKC and anti-PLC detection. PKC and PLC are targeted to the rhabdomeres in *trp* mutants but were mislocalized at similar times in *inaD* mutants.

targeting, and electrophysiological recovery of light responses as a function of time after a pulse of INAD expression. Uninduced *hs-inaD* transgenic flies are virtually identical to *inaD* null mutants: TRP, eye-PKC, and PLC are unstable (Fig. 6) and mislo-

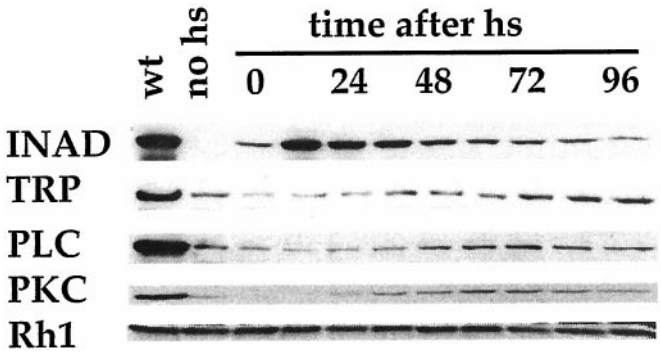

Figure 6. Induction of INAD expression *in vivo*. Shown is immunoblot analysis of transduction proteins in wild-type (*wt*) and *hs-inaD* transformant flies. *hs-inaD* flies without heat shock (*no hs*) did not express INAD, and levels of TRP, PLC, and eye-PKC were reduced dramatically (Tsunoda et al., 1997). *hs-inaD* flies were given a 2 hr heat shock (*hs*) at 37°C, and levels of transduction proteins were assayed at 12 hr intervals after heat shock (hs + 0, hs + 12 hr, etc.). Induction of INAD expression can be seen immediately after heat shock; this pulse of INAD protein expression was followed by a rise in the levels of TRP, eye-PKC, and PLC.

calized (data not shown), and light responses have severe defects with very poor amplitudes and kinetics of activation and deactivation (Fig. 7*A*; Tsunoda et al., 1997). A 2 hr heat shock at 37°C induces a pulse of INAD expression, which is followed by the restabilization of TRP, eye-PKC, and PLC (see Fig. 6). By 7 hr after heat shock INAD has reached the rhabdomeres, and ERG recordings begin to resemble wild-type responses (Fig. 7*A*).

To assay for preassembly, we immunoprecipitated INAD from heads of heat-shocked *hs-inaD* flies at 3 hr after heat shock, a time before INAD has reached the rhabdomeres and long before light responses have recovered (Fig. 7*A*). Figure 7*B* shows that INAD directly interacts with its soluble partners, eye-PKC and PLC, and that these complexes are preassembled. Control immunoprecipitations from uninduced flies, or *inaD* null mutants, showed only background levels of eye-PKC and PLC. As expected, rhodopsin is not found in the complex, although it is >1000-fold more abundant than any of these proteins. These results show that INAD preassembles eye-PKC and PLC into complexes and support a model in which signaling complexes, once assembled, are targeted as a whole to the rhabdomeres.

DISCUSSION

The strategic placement of the proper signaling machinery at the appropriate subcellular location is crucial for normal signaling and cellular function. For example, in neurons, ion channels and receptors are not distributed randomly throughout the cell membrane but are clustered at relevant subcellular sites: Na^+ channels at nodes of Ranvier, K^+ and Ca^{+2} channels at presynaptic terminals, and acetylcholine and glutamate receptors at postsynaptic sites. Similarly, the appropriate localization of receptors to the apical or basolateral membranes of epithelial cells is necessary for receiving and responding to various environmental cues.

In *Drosophila* photoreceptors, high sensitivity to light is achieved by having an extremely large number of light receptor molecules (~100,000,000 rhodopsins). All of these rhodopsin molecules are housed in a specialized subcellular compartment called the rhabdomere. Each rhabdomere consists of ~60,000 microvilli, which provide the huge membrane surface needed to accommodate the large number of receptor molecules and signaling components. INAD acts as a scaffold protein, organizing signaling components downstream of rhodopsin (TRP, PLC, eye-PKC; see introductory remarks) into discreet signaling complexes or transducisomes. Previously, we showed that INAD is essential for the rhabdomeric localization and organization of signaling complexes (Tsunoda et al., 1997). In this study we used INAD transducisomes as a model for examining how signaling complexes are assembled and anchored.

Anchoring of INAD signaling complexes

INAD, like many PDZ–scaffold proteins, is a soluble protein, yet it functions as an organizer of membrane-associated complexes. Thus, we wondered how INAD is anchored to the membrane. Because TRP is a transmembrane protein, we investigated whether TRP anchors INAD. Results from this study show that the interaction of INAD with the TRP channel indeed is required for anchoring signaling complexes in the rhabdomeres. TRP then may interact with the cytoskeleton, securing INAD and the whole complex to the membrane; ankyrin repeats on the N terminus of TRP could play a role in linking TRP to the cytoskeleton. Another possibility is that the INAD–TRP interaction reveals, or unmasks, sites on TRP or INAD that are important for membrane anchoring.

ImmunoEM studies showed, however, that ~25% of INAD remains in the rhabdomeres of *trp* null mutants, suggesting that TRP is not the only anchor of INAD. We have shown that PDZ1 of INAD may play a role in anchoring INAD to the membrane. PDZ1 has been proposed to bind an unconventional myosin III, encoded by the *ninaC* gene, that could link INAD to the actin cytoskeleton (Wes et al., 1999). INAD, however, is localized normally in *ninaC* mutants (Wes et al., 1999), suggesting that it is unlikely that NINAC anchors INAD. Possibly, PDZ1 interacts with a yet unidentified target protein that interacts with the cytoskeleton and anchors INAD. Notably, PDZ domains have been shown to bind directly to cytoskeletal-associated elements, such as α-actinin (Mandai et al., 1999), actin (Xia et al., 1997), protein 4.1 (Marfatia et al., 1997), neuroligins (Irie et al., 1997), and dystrophin (Brenman et al., 1996).

Assembly of INAD signaling complexes

How are signaling components initially targeted to the rhabdomeres? Where are signaling complexes assembled? We envision two potential scenarios: (1) signaling components are targeted independently to the rhabdomeres where they are assembled into complexes, and (2) signaling complexes are "preassembled" and subsequently targeted as a whole to the rhabdomeres. In this report, we present evidence supporting the latter strategy. Preassembly of INAD signaling complexes offers the advantage of minimizing the number of stray signaling components in the rhabdomeres while maximizing the number of complete, functional complexes. It is also worth noting that, in the absence of INAD, its targets TRP, PLC, and eye-PKC are very unstable and nearly undetectable (see Fig. 6 and Tsunoda et al., 1997). Interestingly, a pulse of INAD expression leads to their restabilization, likely via their assembly into transduction complexes. It will be of interest to determine whether this represents a regulatory strategy to ensure that "loose" signaling molecules do not wander about the cell.

Preassembly of macromolecular complexes has been documented for K_{ATP} channels (Zerangue et al., 1999) and mammalian T-cell receptor complexes (Klausner et al., 1990), as well as

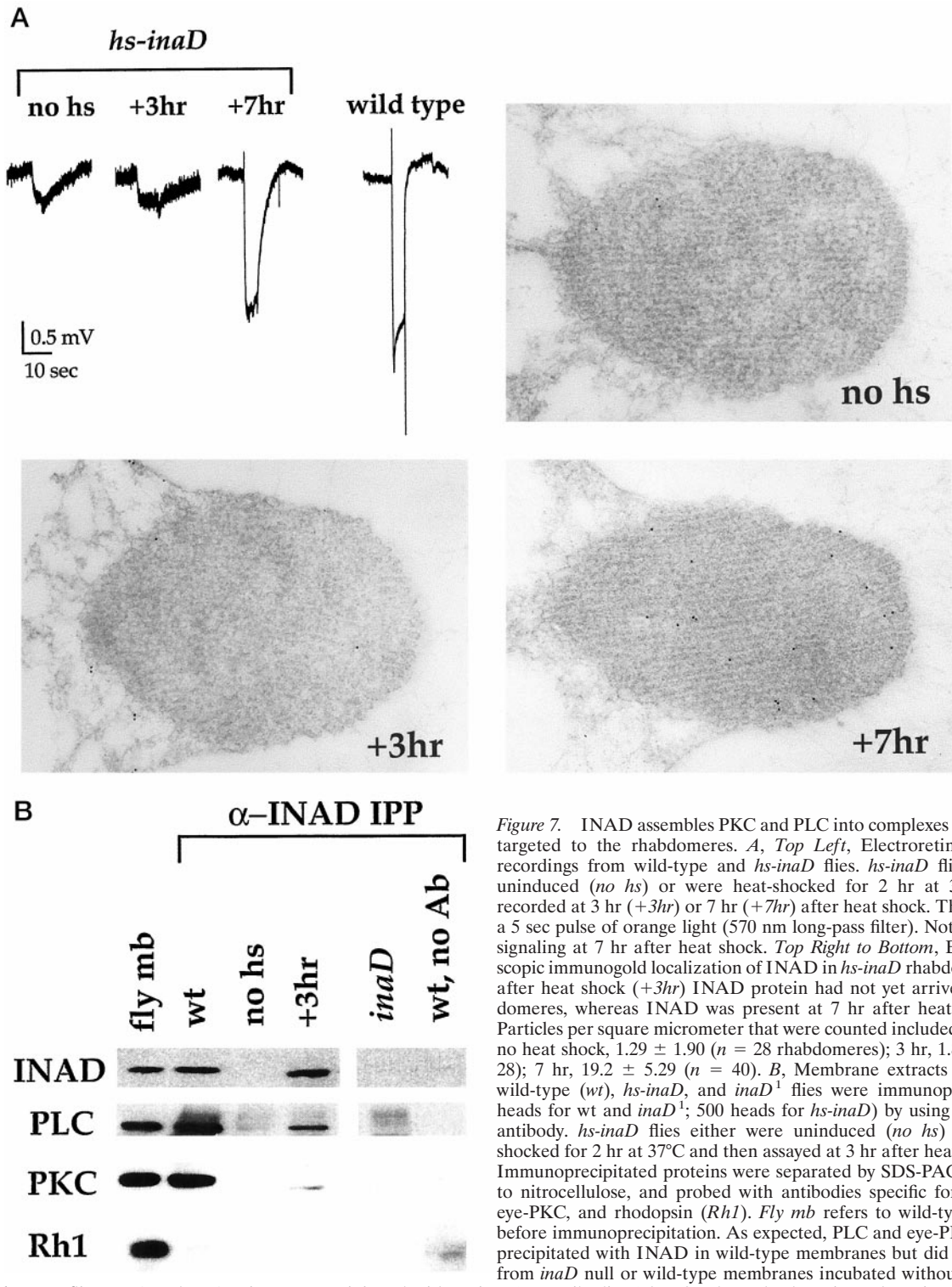

Figure 7. INAD assembles PKC and PLC into complexes before they are targeted to the rhabdomeres. *A*, *Top Left*, Electroretinogram (ERG) recordings from wild-type and *hs-inaD* flies. *hs-inaD* flies either were uninduced (*no hs*) or were heat-shocked for 2 hr at 37°C and then recorded at 3 hr (*+3hr*) or 7 hr (*+7hr*) after heat shock. The stimulus was a 5 sec pulse of orange light (570 nm long-pass filter). Note the rescue of signaling at 7 hr after heat shock. *Top Right to Bottom*, Electron microscopic immunogold localization of INAD in *hs-inaD* rhabdomeres. At 3 hr after heat shock (*+3hr*) INAD protein had not yet arrived in the rhabdomeres, whereas INAD was present at 7 hr after heat shock (*+7hr*). Particles per square micrometer that were counted included the following: no heat shock, 1.29 ± 1.90 (n = 28 rhabdomeres); 3 hr, 1.86 ± 2.30 (n = 28); 7 hr, 19.2 ± 5.29 (n = 40). *B*, Membrane extracts from heads of wild-type (*wt*), *hs-inaD*, and $inaD^1$ flies were immunoprecipitated (50 heads for wt and $inaD^1$; 500 heads for *hs-inaD*) by using an anti-INAD antibody. *hs-inaD* flies either were uninduced (*no hs*) or were heat-shocked for 2 hr at 37°C and then assayed at 3 hr after heat shock (*+3hr*). Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies specific for INAD, PLC, eye-PKC, and rhodopsin (*Rh1*). *Fly mb* refers to wild-type membranes before immunoprecipitation. As expected, PLC and eye-PKC coimmunoprecipitated with INAD in wild-type membranes but did not precipitate from *inaD* null or wild-type membranes incubated without antibody (*wt, no Ab*). From *hs-inaD* flies, PKC and PLC coimmunoprecipitated with anti-INAD antibodies 3 hr after heat shock, a time when signaling still is not restored and INAD has not yet reached the rhabdomeres (*A*); it should be noted that it is possible that immunoEM and immunoprecipitation may have different sensitivities. Rhodopsin, which is not a part of the signaling complex, did not coimmunoprecipitate with INAD in any genotype.

in *Chlamydomonas* and *Paramecium*, in which the outer dynein arms of the flagellum are preassembled in the cytoplasm before they are transported to the tip of the growing flagellum (Fowkes and Mitchell, 1998; Rosenbaum et al., 1999). Preassembly then may be a mechanism common to many cells for targeting different signaling machinery or macromolecular structures to their pertinent subcellular domains. Preassembly and the regulation of complex assembly/targeting may be particularly important in the nervous system where a single neuron must target distinct ion channel or receptor complexes, as well as the relevant regulatory machinery, to hundreds or thousands of different pre- and postsynaptic sites.

It will be important to understand where the complexes are assembled and how this assembly is performed. Strategies for enforcing preassembly may be similar to some immune receptors and K_{ATP} channel subunits that have been shown to contain endoplasmic reticulum (ER) retention signals that are concealed when subunits are assembled, allowing whole receptor or channel complexes to be targeted to the membrane, whereas individual subunits are degraded or retained in the ER (Bonifacino et al., 1990a,b; Zerangue et al., 1999). Although we have shown that INAD is required for targeting complexes to the rhabdomeres, virtually nothing is known about how targeting is accomplished. Genetic screens that track the localization of INAD may provide a fruitful means for identifying components involved in assembly and targeting. With the identification of proteins that play a role in these processes, we may begin to build a picture of how signaling complexes are assembled, how assembly is regulated, and how signaling complexes are targeted to the proper location.

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