Differential Localizations of and Requirements for the Two Drosophila ninaC Kinase/Myosins in Photoreceptor Cells

Jeffery A. Porter,* Jennifer L. Hicks,† David S. Williams,‡ and Craig Montell*

*Department of Biological Chemistry and Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and †Department of Visual Sciences, Indiana University, Bloomington, Indiana 47405

Abstract. The ninaC gene encodes two retinal specific proteins (p132 and p174) consisting of a protein kinase domain joined to a domain homologous to the head region of the myosin heavy chain. The putative myosin domain of p174 is linked at the COOH-terminus to a tail which has some similarities to myosin-I tails. In the current report, we demonstrate that the ninaC mutation results in light- and age-dependent retinal degeneration. We also show that ninaC flies display an electrophysiological phenotype before any discernible retinal degeneration indicating that the electrophysiological defect is the primary effect of the mutation. This suggests that ninaC has a role in phototransduction and that the retinal degeneration is a secondary effect resulting from the defect in phototransduction. To examine the requirements for the individual ninaC isoforms, mutant alleles were generated which express only p132 or p174. Elimination of p174 resulted in a ninaC phenotype as strong as the null allele; however, elimination of p132 had little if any effect. As a first step in investigating the basis for the difference in requirements for p174 and p132 we performed immunolocalization at the electron microscopic level and found that the two isoforms display different subcellular distributions in the photoreceptor cells. The p132 protein is restricted primarily to the cytoplasm and p174 to the rhabdomeres, the microvillar structure which is the site of action of many of the steps in phototransduction. This suggests that the p174 myosin-I type tail is the domain responsible for association with the rhabdomeres and that the substrate for the p174 putative kinase may be a rhabdomeric protein important in phototransduction.

The Drosophila ninaC proteins are unique in that they consist of linked domains homologous to protein kinases and the myosin heavy chain head. The functions of many protein kinases and myosins have been described from a large variety of cell types and organisms. Protein kinases are regulatory molecules which modulate the activity of proteins by serine, threonine, or tyrosine phosphorylation (reviewed in Hanks et al., 1988; Blackshear et al., 1988). Myosins are mechanoenzymes that convert the chemical energy in ATP into force (reviewed in Warrick and Spudich, 1987; Korn and Hammer, 1988; Pollard et al., 1991). However, the physiological role of the combination of both a protein kinase and a myosin head domain joined in the same molecule, as in ninaC, is not clear.

Mutations in the ninaC locus have been identified, facilitating a genetic approach to address the role of ninaC in vivo (Matsumoto et al., 1987). These mutations were isolated on the basis of a defect in photoreceptor cell physiology. The ninaC locus is one of eight loci, mutations in which reduce the prolonged depolarization afterpotential (PDA)1 (Matsumoto et al., 1987). However, unlike the other nina mutants, the decrease in PDA in ninaC is very slight. The PDA results from the stable conversion of a substantial amount of rhodopsin to the light activated form, metarhodopsin, in response to blue illumination (Cosens and Briscoe, 1972; Hillman et al., 1972). Mutations that reduce the rhodopsin content result in a decreased PDA. In ninaC, the reduced rhodopsin content was shown to be due to a reduction in rhabdomeric volume. Rhabdomeres are the specialized photoreceptor cell microvillar structures containing the rhodopsin and are the site of action of a number of the steps in phototransduction subsequent to photoreception.

To facilitate a more thorough understanding of the role of ninaC, the gene has been cloned and characterized (Montell and Rubin, 1988). The locus is expressed as two retinal-specific proteins, 132 and 174 kD (p132 and p174) which encode an NH2-terminal domain homologous to protein kinases joined to a region homologous to the myosin heavy chain head. The p132 protein consists of an additional 82 amino acids at the COOH-terminus of the myosin-like domain. The p174 protein has a 448 amino acid COOH-terminal tail containing a basic domain with a similar overall positive charge found in the basic domain of myosin-I tails (reviewed in Pollard et al., 1991). Myosin-Is are single-
headed myosins which differ in primary sequence from double-headed myosin-IIs most notably in the COOH-terminal tail. Although the pI74 tail has some similarities to myosin-I tails, the ninaC proteins are a novel type of myosin, since they contain an NH2-terminal kinase domain.

To address the role of the ninaC kinase/myosins in photoreceptor cells, we set out to determine whether the reduced rhodoberic volume resulted from retinal degeneration or from a developmental defect and whether the ninaC mutation had any effect on phototransduction independent of the morphological phenotype. We also examined the requirements for the individual ninaC isoforms in the photoreceptor cells, by constructing site-directed mutations that result in expression of either pI32 or pI74. Finally, we investigated whether the two isoforms have different subcellular distributions by performing EM immunolocalization using antisera specific to each protein. In the current report, we show that ninaC flies undergo light- and age-dependent retinal degeneration and display an electrophysiological phenotype suggestive of a defect in phototransduction. Furthermore we demonstrate that the two ninaC isoforms have different subcellular localizations and the pI74 protein, which is predominantly in the rhabdomeres, is the primary ninaC isoform required in the photoreceptor cells. The data are consistent with the model that pI74 moves along actin filaments in the rhabdomeres, via the myosin domain, and modulates the activity of other rhabdomeric proteins important in phototransduction by phosphorylation.

Materials and Methods

Preparation of Fly Heads for Transmission EM

To determine the time course of retinal degeneration at the ultrastructural level, Canton S flies were reared in the dark or under a 12 h light/12 h dark cycle at 25°C. Flies were exposed to ~0.4 mW from a Philips F40CW bulb during the 12 h light period. To maintain dark-reared flies for longer than 9 d posteclosion at 25°C, it was necessary to transfer them to fresh vials every 7 d using a Kodak no. 1 safety light with a 15 W bulb. Heads were collected from young flies (30 min posteclosion) and after 3, 9, and 21 d every 7 d using a Kodak no. 1 safety light with a 15 W bulb. Heads were incubated for 3 h at 25°C, it was necessary to transfer them to fresh vials to prevent photolysis. The flies were exposed to ~0.4 mW from a Philips F40CW bulb.

Preparation of Rabbit Polyclonal Antisera

Rabbit polyclonal antisera specific to pI32, pI74, and pI74 were generated to E. coli fusion proteins consisting of portions of the COOH-terminal regions unique to pI32 and pI74 joined to β-galactosidase. To construct the β-galactosidase-174 KD fusion protein, a PvuII-Spe fragment was subcloned from pCninaC-15 (nucleotides 3,987-4,654 corresponding to the 4.8 kb cDNA; Montell and Rubin, 1988) to pBluescript KS+ (Stratagene) creating pSL2. The plasmid, pSL2, was then digested at the polylinker sites, Sall and Xbal, which flank the 5' and 3' ends of the ninaC fragment and the 0.7 kb insert was subcloned into the β-galactosidase expression vector pUR288 (Rüther and Müller-Hill, 1983). The fusion protein encoded by this plasmid, pZLAninaC, includes the COOH-terminal 221 out of the 420 amino acids unique to pI74. The β-galactosidase-132 KD fusion protein was constructed by first removing the sequences common to both ninaC mRNAs from pCninaC-62R (a cDNA corresponding to the 3.6 kb mRNA; Montell and Rubin, 1988) by digesting with BspMI (nucleotide 3366) and Smal (an enzyme in the polylinker flanking the 5' end of the cDNA), filling in the BspMI site with dTTP and Klenow and performing an intramolecular ligation. The resulting ninaC sequence in this clone, pCninaC-62Δ.3, encodes 53 out of the 54 amino acids specific to pI32 as well as the 3' untranslated region. The 0.2-kb ninaC segment was then excised from pCninaC-62Δ.3 with BamHI and HindIII (enzymes in the polylinker flanking the 5' and 3' ends of the ninaC insert) and subcloned into the expression vector pUR280 (Rüther and Müller-Hill, 1983). Rabbit polyclonal antisera that reacts with both ninaC isoforms, zBZ551, was raised to an E. coli fusion protein previously described (Montell and Rubin, 1988). The fusion proteins were grown in E. coli, purified by electroelution from acrylamide gels as described (Montell and Rubin, 1988) and introduced subcutaneously into New Zealand female rabbits.

Electron Microscopic Immunocytochemistry

The immunolocalizations of pI32 and pI74 were determined at the EM level using hemisected wild-type and ninaC2105 (negative control) Drosophila heads fixed in 2% formaldehyde, 0.5% glutaraldehyde, and 0.1 M phosphate (pH 7.4). The tissue was then dehydrated in a methanol series and stained en bloc with 2% uranyl acetate in 70% methanol. The eyes were then infiltrated overnight at 4°C, embedded in L.R. White (EM Science, Gibbstown, NJ) for 48 h at 55°C and thin sections were collected on nickel grids. To perform the following incubations, the grids were floated, section-side down, on a drop of each of the solutions. Sections were etched with saturated metaperiodate for 1 h, blocked with 4% BSA for 30 min, and then incubated overnight at 4°C with the rabbit polyclonal antisera, pI32 and pI74, or with nonimmune rabbit serum. The sections were then incubated for 30 min with biotinylated goat anti-rabbit IgG (Jackson Laboratory, Bar Harbor, ME) and then for 30 min with streptavidin conjugated to 15-nm colloidal gold (Amersham). Sections were stained with 2% uranyl acetate at 0.4% lead citrate. The percent distributions of colloidal gold particles in the cytoplasm and rhabdomeres were determined by tabulation of the number of particles from multiple sections from at least two animals. The values presented were adjusted to account for any non-specific background in areas on the grids free of tissue.

Protein Analyses

To check the specificity of the rabbit polyclonal antisera, pI32 and pI74, and expression of the ninaC proteins in the P[ninaC°t32] and P[ninaC°t74] transgenic transformant lines, extracts were prepared from heads and bodies as described (Montell and Rubin, 1988), fractionated on SDS-6% polyacrylamide gels, transferred to nitrocellulose, and probed with the rabbit antisera zBZ551 (1:1,000), pI32 (1:4,000) and pI74 (1:1,500) as previously described (Montell and Rubin, 1988) except that 125I-labeled protein A (1:1,000 dilution, 0.1 μCi/ml, 70-100 μCi/g; New England Nuclear, Boston, MA) was used as the secondary antibody.

Construction of Transformant Lines Expressing One ninaC Isoform

The mutations in the P[ninaC°t32] and P[ninaC°t74] transgenic transformant lines were generated by oligonucleotide-directed mutagenesis. The primer used to construct the Δt74 mutation, CCAAATACTAAAAAGGAAAAAAC- CGC, created a T-G transversion in the second base of the tenth intron of the 4.8 kb mRNA (see Fig. 3). The underlined nucleotide corresponds to the base altered by the mutagenesis. The Δt32 mutation was generated with a primer, GCGGTCCGATCTAAAAGGCCCTAGGATTCCGC-GAC, which hybridizes to the last 20 nucleotides of the tenth exon and the first 20 nucleotides of the 11th exon of the 4.8 kb mRNA. The underlined nucleotides correspond to the bases flanking the 5′2 nucleotide intron sequence deleted by this mutation.

The Journal of Cell Biology, Volume 116, 1992
The template DNA used to generate the Δ132 mutation, pBSKK1, was constructed by subcloning the 2.9 kb KpnI ninaC genomic fragment (coordinates 90-119 on the genomic map; Montell and Rubin, 1988) from A51 (Montell and Rubin, 1988) into the KpnI site of the phagemid pBluescriptKS+. Coordinate 90 is proximal to the T7 primer site in pBSKK1. To construct the Δ174 mutation, an Xbal-XhoI fragment (coordinates 6-10.2 on the genomic map), was subcloned between the Xbal and XhoI sites of pBluescriptKS+ to create pBSXX1. Single-stranded pBSKK1 and pBSXX1 DNAs were prepared by superinfection with R408 helper phage in a daf”, auxg” strain, E. coli CI236, according to methods described by Kunkel et al. (1987) which provides for a strong selection against the non-mutagenized strand in the duplex DNA. To perform the superinfection, 25 ml of 2YT broth was inoculated with 0.5 ml of cells, transformed with pBSKK1 or pBSXX1 and incubated for 2 h at 37°C before superinfecting with 5 x 10^6 helper phage. After 5 h incubation at 37°C, the phage were purified by precipitation with polyethylene glycol/NaCl and banding on a CsCl gradient and DNA extracted from the phage with phenol as described (Sambrook et al., 1989). The in vitro mutagenesis was performed as described (McClary et al., 1989) except that the primer-template hybridization mix was kept at room temperature rather than on ice for first 10 min of the second strand synthesis before shifting the temperature to 37°C. The intended mutations were identified by sequencing plasmid DNA prepared from ampicillin resistant colonies. The frequency of the Δ132 and Δ174 mutations were 5/6 and 4/10, respectively. The 2.9- and 4.0-kb ninaC fragments from pBSKK1 and pBSXX1 were sequenced completely demonstrating that there were no unintended additional mutations generated during the mutagenesis.

The 4.0-kb XbaI-XhoI fragment, containing the point mutation at the 4.8-kb mRNA 5’ splice junction, was subcloned from pBSXX1 into pGninaCAXb-Rl creating the clone pGninaCA51. The pGninaCAXb-Rl clone consists of the BamHI-XbaI fragment (ninaC genomic coordinates 3.4–6.2) and the 4.7-kb EcoRI fragment (ninaC coordinates 9.9-14.6) subcloned from A51, in the same orientation, into the BamHI-XbaI and EcoRI sites of pHSS7. The ninaC sequence in pGninaCASJ was excised with NotI and subcloned into the ry’ P-element transformation vector, pDM30 (Misseri and Rubin, 1987), creating pRGninaCASJ. The 2.9-kb KpnI fragment, containing the Δ132 mutation, was excised from pBSKK1 and used to replace the corresponding wild-type KpnI fragment in pHSS7, creating pRGninaCA132. The plasmid, pRGninaC”, consists of the wild-type ninaC genomic sequence (coordinates 3.4–14.6) subcloned from pHSS7 into the NotI site of pDM30.

The wild-type and mutated ninaC DNAs, pRGninaC”, pRGninaC- Δ132, or pRGninaCASJ (400 μg/ml) and pR257 (100 μg/ml) were injected into ∼500 ninaC”/ry M cytotype embryos as described (Spradling and Rubin, 1982; Rubin and Spradling, 1982). The 11.2-kb genomic sequence injected into the ninaC”/ry embryos (coordinates 3.4-14.6; Montell and Rubin, 1988) included 2.3- and 2.2-kb flanking the 5’ and 3’ ends of the transcribed region. This differed from the original sequence used to rescue the ninaC phenotype which included 4.5 kb of 5’ flanking sequence (Montell and Rubin, 1988). Eight independent ry’ transmitters were obtained with pRGninaCA132 and with pRGninaCASJ. Stocks homozygous for the insertions were generated and transformants with secondary chromosomal insertions were placed in a w+ background.

Electroretinogram Recordings

Electroretinogram (ERG) recordings were performed by applying glass electrodes, filled with Ringer’s solution, to small drops of electrode cream (Sigma Chemical Co., St. Louis, MO) placed on the surface of the compound eye and the thorax. The light source was a projector (model 765; Newport Electronics Inc., Santa Ana, CA) with a 100 W quartz tungsten-halogen lamp. The intensity of unfiltered light was ∼20 mW/cm². The ERGs were amplified using a WPI Dam 60 differential amplifier and recorded on a Macintosh SE using a MacLab analog-digital converter and the Chart 4 v3.1 program.

Results

ninaC Rhabdomeres Undergo Light- and Age-dependent Retinal Degeneration

It has been shown previously that the diameter of the rhabdomeres in ninaC is reduced relative to wild type (Matsumoto et al., 1987). To determine whether this phenotype is due to a developmental defect or to light- or age-dependent retinal degeneration, we examined rhabdomeres from a null allele, ninaC”, by transmission EM, at various ages after rearing in the dark or on a 12 h light/12 h dark cycle.

The Drosophila compound eye is composed of ~800 repeat units referred to as ommatidia. Each ommatidium contains 20 cells including eight photoreceptor cells. Six of the photoreceptors, R1-6, contain rhabdomeres that extend the full depth of the retina and occupy the periphery of the ommatidia. Rhabdomeres are the rhodopsin containing microvillus structures which are the Drosophila equivalent of the outer segments of the vertebrate photoreceptor cells. The rhabdomeres of the R7 and R8 cells occupy the central distal and proximal regions of the ommatidia, respectively. Consequently, only seven of the eight photoreceptor cells are present in any cross-section.

In wild-type flies there is no discernable retinal degeneration in response to light or with age. However, the rhabdomeres from ninaC” retinas undergo both light- and age-dependent retinal degeneration. The diameter of the rhabdomeres from young (<30 min posteclosion) wild-type flies reared in the dark (Fig. 1 A) were indistinguishable from 21-d-old flies reared on a light/dark cycle (Fig. 1 B). No photoreceptor cell degeneration was apparent in young ninaC” flies reared in the dark (Fig. 1 C). However, if the flies were reared on a light/dark cycle, a modest level of degeneration was observed immediately after eclosion (Fig. 1 D). Among these newly enclosed flies, the diameter of the rhabdomeres was consistently reduced ~20%. Retinal degeneration proceeded gradually until the rhabdomeres were almost completely gone after 21 d (Fig. 1 E). In some ommatidia, the central ultraviolet sensitive R7 rhabdomeres degenerated somewhat less than the six outer rhabdomeres (Fig. 1 F). The R7 cells may occasionally degenerate more slowly presumably because there is relatively little ultraviolet in the ambient light. The R8 rhabdomere, located directly below R7, degenerated to the same extent as the outer rhabdomeres (data not shown). Of primary significance here, the retinal degeneration was significantly reduced in flies reared in the dark. After 21 d in the dark, the degree of degeneration was comparable to the newly eclosed ninaC” flies reared on a light/dark cycle (Fig. 1 F). The rhabdomere diameter of the 21-d-old dark reared flies was consistently reduced only ~25%. These results demonstrate that the small size of the rhabdomeres in ninaC previously reported (Matsumoto et al., 1987) is due to light- and age-dependent retinal degeneration and not to a developmental defect.

ninaC Electroretinogram Is Defective Prior to Retinal Degeneration

To determine whether the slight decrease in PDA in ninaC results from retinal degeneration, we examined the PDA from ninaC” flies reared on a light/dark cycle and in the dark. We found that the decrease in the PDA in ninaC” flies correlates with retinal degeneration. The PDA is reduced in ninaC” flies reared on a light/dark cycle but not in young dark reared ninaC” flies which have not undergone retinal degeneration (data not shown). On the basis of this electrophysiological phenotype, it appeared that ninaC did not have a role in phototransduction since the elec-
The $ninaC$ mutation induces light- and age-dependent retinal degeneration. Tangential sections of wild-type and $ninaC^{P235}$ compound eyes at a depth of 25 $\mu$m viewed by transmission EM. A 2 $\mu$m scale bar is shown below A. The seven rhabdomeres corresponding to the six outer photoreceptor cells, RI-6, and the central photoreceptor cell, R7, are the seven large oval structures arranged in trapezoidal pattern. (A) Wild type (Canton S) reared in the dark <30 min posteclosion; (B) 21-d old wild-type reared under a 12 h light/12 h dark cycle; (C-F) $ninaC^{P235}$; (C) dark reared <30 min posteclosion; (D) dark reared under a 12 h light/12 h dark cycle and <30 min posteclosion; (E) 21-d old reared under a 12 h light/12 h dark cycle; (F) 21-d-old reared in the dark. During the 12 h light period, the flies were exposed to ~0.4 mW/cm$^2$ from a Philips F40CW bulb.

The trophophysiological phenotype appeared to be a secondary effect of the retinal degeneration.

To determine whether there is an ERG defect which is not a secondary effect of retinal degeneration, we examined young $ninaC^{P235}$ flies reared in the dark. We found that the $ninaC$ proteins may have a role in phototransduction since $ninaC^{P235}$ flies which have not undergone retinal degeneration, within the resolution of the analysis, still display an ERG phenotype. Shown in Fig. 2 are ERGs of wild-type and $ninaC^{P235}$ flies dark adapted for 60 s. Wild-type flies display a corneal negative ERG in response to light. Upon cessation of the light stimulus, the receptor component of the ERG quickly returns to the dark level (Fig. 2 A). The response of wild-type flies to a second pulse of light was indistinguishable from the first. This is in contrast to $ninaC^{P235}$, which was characterized by a larger response to the first light pulse than the second (Fig. 2 B). The amplitude of the first response is often but not always larger than that observed in wild type. In addition, the amplitude of the OFF transient is frequently but not always reduced relative to wild type. However, of primary significance here, the response of $ninaC^{P235}$ flies to the initial light stimulus is consistently followed by a slow return to the baseline after cessation of the light. The ERG waveforms were indistinguishable between young dark reared $ninaC^{P235}$ flies, which show no retinal degeneration, and older light/dark reared $ninaC^{P235}$ flies which display significant levels of retinal degeneration (data not shown). Therefore, the ERG phenotype shown in Fig. 2 B appears to be a primary effect of the $ninaC$ mutation, since it is observed before any discernable morphological degeneration, and suggests that $ninaC$ may have a role in phototransduction.

**Generation of $ninaC$ Alleles Expressing Just p132 or p174**

The myosin head domain of the p132 and p174 proteins are joined to tails of 82 and 448 amino acids that are identical for the first 28 amino acids and differ by the COOH-terminal 54 and 420 amino acids (Fig. 3). The 3.6- and 4.8-kb mRNAs encoding these two isoforms are synthesized from the same primary transcript and differ due to alternative RNA processing.

To determine whether one or both $ninaC$ proteins are required to prevent retinal degeneration and for normal electrophysiology, we generated mutants that express only p132 or p174. Two oligonucleotide-directed mutations were constructed which were designed to eliminate synthesis of either the 3.6- or the 4.8-kb mRNAs and consequently p132 and p174, respectively. To eliminate the 3.6-kb mRNA, the tenth intron specific to the 4.8-kb mRNA was precisely deleted (Fig. 3, bracket). This mutation removed the 3' end processing signal, AAUAAA, specific to the 3.6-kb mRNA and should therefore prevent synthesis of the 3.6-kb mRNA without affecting production of the 4.8-kb mRNA. To eliminate the 4.8-kb mRNA, we avoided constructing a deletion removing the unique portion of the 4.8-kb mRNA since this mutation might also affect sequences required for 3' end formation of the 3.6-kb mRNA. Instead, we constructed a mutation which should prevent RNA splicing of the 4.8- and not the 3.6-kb mRNA. Virtually all introns begin with the dinucleotide GU (Mount, 1982). To eliminate the 4.8-kb mRNA, a single T-G transversion was constructed in the second base of the tenth intron. This transversion would not affect p132 encoded by the 3.6-kb mRNA as it is the third position of the codon, GGT, encoding glycine. This mutation would change the codon to GGG which also encodes glycine.

The altered $ninaC$ genes and a wild-type control were introduced into $ninaC^{P235}$;ry embryos, by P-element-mediated germline transformation. Multiple ry' transformants were obtained for each construct and designated P[$ninaC^{173}$] and P[$ninaC^{174}$] for the mutations intended to eliminate p132.
and p174. An immunoblot probed with a polyclonal antiserum that recognizes both ninaC proteins demonstrated that P[ninaC°'32] expresses only p174 and P[ninaC°'1] only p132 (Fig. 4).

Requirements of the Individual ninaC Proteins for Normal Electrophysiology and to Prevent Retinal Degeneration

To determine whether one or both ninaC proteins are required for normal electrophysiology, we performed ERGs with P[ninaC°'32] and P[ninaC°'1] flies and found that only p174 is essential for a wild-type ERG. Shown in Fig. 5 are the ERGs obtained with young dark-reared flies that have not undergone retinal degeneration. The ERG waveform obtained with ninaC°'32, ry flies transformed with the wild-type ninaC gene was indistinguishable from wild-type flies (compare Figs. 2 A and 5 A). Therefore, all the sequences necessary for rescue of the ninaC phenotype are encoded within the 11.2-kb sequence used in the transformations. Elimination of p32 had no apparent effect on the ERG (Fig. 5 C). The ERG of P[ninaC°'32] flies did not differ with age or rearing on a light/dark cycle (data not shown). However, young P[ninaC°'1] flies reared in the dark elicited an ERG similar to the null mutant, ninaC°'1 (Fig. 5 D).

The effects of eliminating p32 and p174 on light- and age-dependent retinal degeneration during a light/dark cycle was determined by performing ultrastructural analyses on cross-sections of the adult retina (Fig. 6). The results demonstrated that p174 was the only ninaC isoform required to prevent light- and age-dependent retinal degeneration. As was observed with wild-type flies, ninaC°'1, ry flies transformed with the wild-type ninaC gene showed no degeneration after 21 d on a light/dark cycle (compare Figs. 1 B and 6 A). This was in contrast to P[ninaC°'1] flies which degenerate over the same time course as ninaC°'1. After 21 d on a light/dark cycle, the outer rhabdomeres in both P[ninaC°'1] and ninaC°'1 were nearly completely degenerated (Fig. 6, B and D). However, the effect of eliminating p32 was minor. After 21 d on a light/dark cycle, the rhabdomeral diameter in P[ninaC°'32] flies did not appear to be significantly reduced (Fig. 6 C).

p174 Is Localized to the Rhabdomeres and p132 to the Cytoplasm

As a first step in analyzing the basis for the difference in requirements for p174 and p32 in the photoreceptor cells, we addressed the question as to whether the two ninaC isoforms display different spatial localizations. Previous immunol...
Figure 4. Expression of ninaC proteins in wild-type and P[ninaC<sup>132</sup> and P[ninaC<sup>174</sup>]. Protein extracts prepared from wild type (Canton S), P[ninaC<sup>132</sup>], and P[ninaC<sup>174</sup>] heads were fractionated on a SDS–6% polyacrylamide gel, transferred to nitrocellulose, probed with a ninaC antiserum, αZB551 (Montell and Rubin, 1988), that reacts to both isoforms and then with 125I-labeled protein A. The upper and lower bands correspond to p174 and p132, respectively.

Localization studies demonstrated that the ninaC proteins are expressed specifically in the retina (Montell and Rubin, 1988). These studies were performed using light microscopy and an antiserum that recognizes both ninaC proteins and therefore could not resolve whether the ninaC proteins have the same or different spatial localizations.

Using rabbit polyclonal antisera specific to p132 and p174, respectively (Fig. 7), we performed EM immunolocalization and found that p174 was localized predominantly in the rhabdomeres (Fig. 8A) while p132 appeared to be restricted primarily to the extrarhabdomeral cytoplasm of wild-type photoreceptor cells (Fig. 8B). Tabulation of the number of colloidal gold particles from multiple sections showed that 87.5% of the immunoreactivity to p174 was in the rhabdomeres and 12.5% in the extrarhabdomeral cytoplasm. Conversely, only 2% of the p132 immunostaining was localized to the rhabdomeres and 98% to the extrarhabdomeral cytoplasm. Neither ninaC protein was localized to the photoreceptor cell processes which extend proximally from the retina (Montell and Rubin, 1988; data not shown). Thus, p132 and p174 appeared to be spatially localized to different subcellular regions within the photoreceptor cells.

Figure 5. ERG recordings on ninaC isoform alleles. The signal amplitude and time scale are the same as Fig. 2. (A) Wild-type transformant P[ninaC<sup>+</sup>]; (B) ninaC<sup>9235</sup>; (C) P[ninaC<sup>132</sup>] and (D) P[ninaC<sup>174</sup>].
Figure 6. Morphology of ninaC isoform alleles. Tangential sections of compound eyes, from 21-d-old flies raised on a light/dark cycle, at a depth of 25 μm viewed by transmission EM. A 2 μM scale bar is shown below A. (A) Wild-type transformant P[ninaC⁺]; (B) ninaC°t2; (C) P[ninaC°t2]; and (D) P[ninaC°t4].

The localization data were extended by the observation that p132 fractionates into the high-speed supernatant and p174 into the low-speed pellet after centrifugation of extracts prepared from wild-type heads. Nearly all of p174 was in the low-speed pellet even after extraction in buffer containing detergent and 1 M KCl (Fig. 9). This was in contrast to p132 which remained in the high-speed supernatant under a variety of extraction conditions including buffer containing 0.1 M KCl and no detergent (Fig. 9). Thus, the biochemical and immunoelectron microscopic data suggest that p132 is primarily free in the cytosol outside the rhabdomeres and p174 is primarily a rhabdomere-associated protein.

Discussion

The ninaC Mutation Induces Light- and Age-dependent Retinal Degeneration

The ninaC locus was originally identified on the basis of a PDA phenotype resulting from a reduced rhodopsin content. The reduced rhodopsin content was shown in ultrastructural studies to be due to smaller rhabdomeres in ninaC relative to wild type. In the current paper, we showed that the reduced rhabdomeric diameter in ninaC was due to light- and age-dependent retinal degeneration.

The majority of the retinal degeneration in ninaC was prevented by maintaining the flies in the dark. This demonstrated that the retinal degeneration was primarily a light-induced defect. The small amount of degeneration in old flies, maintained in the dark, could be due to age-dependent retinal degeneration or to a low amount of thermally induced activation of visual transduction which occurs in both vertebrate and invertebrate photoreceptors in the dark (Srebro and Behbehani, 1972; Yau et al., 1979; Aho et al., 1988). Alternatively, this low level of degeneration could be due to exposure to very low levels of ambient light during the maintenance or manipulations.

Although the mechanisms responsible for retinal degeneration have not been clarified, it appears that many mutations that have a profound effect on phototransduction induce some retinal degeneration. A point mutation in human rhodopsin has been shown to cause retinal degeneration as has mutation of the mouse rd gene which encodes the β subunit of the rod cGMP-phosphodiesterase (Dryja et al., 1990; Bowes et al., 1990). In Drosophila, mutations in any one of several genes required for phototransduction, such as ninaE which encodes the major opsin and norpA which encodes the retinal phospholipase C, cause some retinal degeneration (reviewed in Smith et al., 1991). However, retinal degeneration can also result from mutations in structural proteins which have no apparent role in phototransduction (Travis et al., 1991).

The four Drosophila visual mutants which display the most pronounced retinal degeneration phenotypes are rdgA, rdgB, rdgC, and norpA. The rdgA mutation results in age-dependent retinal degeneration and is deficient in diacylglycerol kinase activity (Inoue et al., 1989). Among the other Drosophila visual mutants, only rdgB, rdgC, and norpA display significant light-dependent retinal degeneration (Hotta and Benzer, 1970; Harris and Stark, 1977; Meyertholen et al., 1987; Steele and O’Tousa, 1990). The NH₂-terminal region of rdgB is homologous to phosphati-
dylinositol transfer proteins (Vihtelic et al., 1991; T. S. Vihtelic, M. Gable, J. E. O’Tousa, and D. R. Hyde, personal communication), the rdgC sequence is similar to class 1, 2A and 2B phosphatases (F. Steele and J. E. O’Tousa, personal communication) and norpA is homologous to phosphotidy-
inositol-specific phospholipase C (Bloomquist et al., 1988).
Figure 8. EM immunolocalization of p132 and p174. Thin sections embedded in L. R. White were probed with αp174 (A) or αp132 (B) and then with streptavidin conjugated to 15-nm colloidal gold. Bar, 0.75 μm. ECS, extracellular space; R, rhabdomere.
Thus, *ninaC* represents the fourth *Drosophila* mutation characterized by significant light-dependent retinal degeneration. It remains to be determined whether the basis of the retinal degeneration is similar between the four mutations that induce light-dependent retinal degeneration. One possibility is that some of these mutations may result in high levels of intracellular Ca++, since elevated levels of Ca++ is associated with degeneration in a variety of cells (Farber, 1981).

**Possible Role of ninaC**

The observation that *ninaC* displays an ERG phenotype prior to any discernable retinal degeneration, within the resolution of the analysis, indicates that the electrophysiological defect may be the primary effect of the mutation. This suggests that *ninaC* may have a role in phototransduction. Retinal degeneration may be a secondary effect resulting from the defect in phototransduction.

The *ninaC* ERG is characterized by a slow return to the baseline after cessation of the light stimulus and by a smaller receptor potential in response to a second light stimulus. Although the basis of this ERG is unclear, one possibility suggested by the slow return is that there is a defect in the quenching mechanism or turnoff of the response to light. The molecular basis for the turnoff of the receptor potential is not well understood. However, the mechanism which leads to the inactivation of rhodopsin appears to involve serine/threonine phosphorylation and interaction with arrestin (Thompson and Findlay, 1984; Wilden et al., 1986). It is possible that the role of the *ninaC* kinase is to turn off other phototransduction proteins through a mechanism involving serine/threonine phosphorylation. Consistent with this hypothesis, we have found that *ninaC* exhibits serine/threonine kinase activity and that obliteration of this kinase activity by a point mutation in the kinase domain results in a null phenotype (J. A. Porter and C. Montell, unpublished observations).

**Rhabdometric p174 Is the Most Important ninaC Isoform**

Two approaches were used to investigate the role of the individual *ninaC* proteins. The first was to create alleles that express just p132 or p174. Although there were no existing *ninaC* alleles that eliminated just p32, there were several that appeared to eliminate p174 (Matsumoto et al., 1987). However, it could not be ruled out that there were also one or more physiologically relevant amino acid substitutions in the 132-kD protein of these chemically induced alleles. We found that elimination of p174 resulted in a phenotype indistinguishable from the null allele. However, there was no significant effect from elimination of p132. A clue as to the basis for the difference in requirements for the *ninaC* isoforms and the potential role and substrates of p174 could be suggested by elucidation of the intracellular localizations of the individual proteins. One possibility is that the two *ninaC* proteins share the same spatial distribution and perform different functions in similar intracellular locations. Alternatively, p32 and p174 could be expressed in different photoreceptor cells or be spatially restricted to different locations within the same photoreceptor cells.

Therefore, the second approach that was used to investigate the roles of the two *ninaC* isoforms was to determine the intracellular localizations of p132 and p174. The results in the current paper showed that p174 and p132 were primarily restricted to different subcellular locations. The large isoform was predominantly in the rhabdomeres and the small isoform in the cytoplasm. One possible explanation for the apparent dispensability of p132 is that p174 might substitute for p32. Consistent with this proposal, there appears to be a slightly higher concentration of p174 in the cytoplasm of *P[ninaC][174]* relative to wild-type flies; however, p32 is still restricted to the cytoplasm of *P[ninaC][132]* flies (J. A. Porter, J. L. Hicks, D. S. Williams, and C. Montell, unpublished results). Alternatively, p32 may have some subtle role in the photoreceptor cells unrelated to phototransduction or retinal degeneration.

**Binding of p174 to Rhabdомерes Appears to be Mediated by the COOH-terminal Tail**

The only difference between the two *ninaC* proteins are the 54 and 420 COOH-terminal amino acids unique to p132 and p174. The COOH-terminal domain of p174 has some similarities to the ~400 amino acid tail regions of myosin-I's. These tail regions display considerable sequence diversity but share similar overall charge distributions. In particular, is a highly charged 250 amino acid segment with a net positive charge of +19 to +42 (Pollard et al., 1991). Since p32 and p174 differ by the presence or absence of this myosin-I...
type COOH-terminal tail, investigations of the individual roles of the two ninaC proteins may also contribute to elucidation of the roles of this myosin-I domain.

Since the only difference between the two ninaC proteins is in the COOH-terminal region, this strongly suggests that the rhodomer-e-binding region is in the COOH-terminal domain. This in vivo result is consistent with in vitro data suggesting that the COOH-terminal region of myosin-I may be involved in binding phospholipid vesicles (Adams and Pollard, 1986; Hayden et al., 1990).

Possible Function of the Rhodomeric p174

The results in the current paper suggest that ninaC may have a role in phototransduction. Therefore, it was intriguing that elimination of the rhodomeric p174 resulted in a null phenotype since the rhodomer-e are the site of photoreception and interaction with many of the important proteins in phototransduction. These include rhodopsin, the G-protein, phospholipase C, arrestin, and rhodopsin kinase. One model for p174 function is that it modulates the activity of other rhodomeric proteins important in phototransduction by phosphorylation. An alternative model is that p174 is involved in distal movement of the rhodomeral membrane as discussed previously (Arikawa et al., 1990). Previous in vitro studies with known myosin-IIs suggest that some of these molecules may have a role in movement of membranous organelles (reviewed in Pollard et al., 1991). However, the observation that the primary effect of the ninaC mutation may be an ERG phenotype supports the model that p174 plays a role in phototransduction.

The p174 protein may consist of linked kinase and myosin domains to enable the kinase domain to move along the actin filaments which have recently been shown to be present in the rhodomer-e (Arikawa et al., 1990). This could provide p174 with a mechanism to rapidly deactivate other rhodomeric proteins important in phototransduction by phosphorylation. The COOH-terminal domain may be required only for localization of p174 to the rhodomer-e. Since ninaC shows an ERG phenotype and retinal degeneration which is greatly accelerated in the light, it is possible that the p174 kinase activity is a light-dependent activity mediated by the Ca2+ flux which occurs during phototransduction. Consistent with this hypothesis, we have found that the ninaC proteins bind the calcium receptor protein calmodulin in vitro (J. A. Porter and C. Montell, unpublished observations). Important future experiments include a detailed description of the predicted enzymatic activities, identification of the in vivo substrate(s) for p174 and clarification of the mechanism regulating the p174 protein kinase.

We thank Dr. T. D. Pollard for helpful comments on this manuscript and Toyin Oyebade and Anju Thomas for excellent technical assistance.

This investigation was supported by grants from the National Eye Institute to C. Montell (EY08117) and to D. S. Williams (EY07042). C. Montell also thanks the National Science Foundation for a Presidential Young Investigator Award, the American Cancer Society for a Junior Faculty Research Award, and Merck Sharp and Dohme Research Laboratories for research support.

Received for publication 25 September 1991 and in revised form 29 October 1991.

References

Adams, R. J., and T. D. Pollard. 1986. Propulsion of organelles isolated from Acantthamoeba along actin filaments by myosin-I. Nature (Lond.). 322: 754-756.

Aho, A.-C., K. Donner, C. Hyden, L. O. Larsen, and T. Reuter. 1988. Low retinal noise in animals with low body temperature allows high visual sensitivity. Nature (Lond.). 334:348-350.

Arikawa, K., J. L. Hicks, and D. S. Williams. 1990. Identification of actin filament structures in the rhodomeral microvilli of Drosophila photoreceptors. J. Cell Biol. 110:1993-1998.

Blackshaw, P. J., A. C. Nairn, and J. F. Ku. 1988. Protein kinases 1988: a current perspective.FASEB (Fed. Am. Soc. Exp. Biol.) J. 2:2957-2969.

Bloomquist, B. T., R. D. Shortridge, S. Schneuwly, M. Perdue, C. Montell, H. Svitkine, G. Rubin, and W. L. Pak. 1988. Isolation of a putative phospholipase C gene of Drosophila, norpA, and its role in phototransduction. Cell. 54:723-733.

Bowes, C., L. Tiansen, M. Danciger, L. C. Baxter, M. L. Applebury, and D. B. Farber. 1990. Retinal degeneration in the rod mouse is caused by a defect in the β subunit of rod cGMP-phosphodiesterase. Nature (Lond.). 347: 677-680.

Cosenso, D., and D. Briscoe. 1972. A switch phenomenon in the compound eye of the white-eyed mutant of Drosophila melanogaster. J. Insect Physiol. 18:627-632.

Dryja, T. P., T. L. McGee, E. Reichel, L. B. Hahn, G. S. Cowley, D. W. Yandell, M. A. Sandberg, and E. L. Berson. 1990. A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. Nature (Lond.). 343:364-366.

Farber, L. 1981. The role of calcium in cell death. Life Sci. 29:1289-1295.

Hanks, S. K., A. M. Quinn, and T. Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science (Wash. DC). 241:42-52.

Harris, W. A., and W. L. Stark. 1977. Hereditary retinal degeneration in Drosophila melanogaster. A mutant defect associated with the phototransduction process. J. Gen. Physiol. 69:261-281.

Hayden, S. M., J. S. Wolenski, and M. S. Mooseker. 1990. Binding of a domain-specific protein to photoreceptor vesicles. J. Cell Biol. 111:443-451.

Hillman, P., S. Hochstein, and B. Minke. 1972. A visual pigment with two physiologically active states. Science (Wash. DC). 175:1486-1488.

Hotta, Y., and S. Benzer. 1970. Genetic dissection of the Drosophila nervous system by means of mosaics. Proc. Natl. Acad. Sci. USA. 67:1156-1163.

Inoue, H., T. Yoshioka, and Y. Hotta. 1989. Dicyaglycerol kinase defect in a Drosophila retinal degeneration mutant rdgB. J. Biol. Chem. 264:5990-6000.

Korn, K. D., and J. A. Hammer III. 1988. Myosins of nonmuscle cells. Ann. Rev. Biophys. Biophys. Chem. 17:23-45.

Kunkel, T. A., J. D. Robers, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. 154:367-382.

Matsumoto, H., K. Isono, Q. Pye, and W. L. Pak. 1987. Gene encoding cytoskeletal proteins in Drosophila rhodomer-e. Proc. Natl. Acad. Sci. USA. 84:985-989.

McClary, J. A., F. Whitney, and J. Geiselhardt. 1989. Efficient site-directed mutation of the visual pigments of Drosophila. BIophysics. 7:282-289.

Meyers, M. E., P. J. Porter, and J. A. Hamill. 1987. Studies of the Drosophila norpA phototransduction mutant. II. Photoreceptor degeneration and rhodopsin maintenance. J. Comp. Physiol. A. 161:793-798.

Mishner, D., and G. M. Rubin. 1987. Analysis of the promoter of the Rh2 opsin gene in Drosophila melanogaster. Genetics. 120:173-180.

Montell, C., and G. M. Rubin. 1988. The Drosophila ninaC locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. Cell. 52:757-772.

Mount, M. S. 1982. A catalogue of splice junction sequences. Nucleic Acids Res. 10:459-472.

Pollard, T. D., S. K. Dobberstein, and H. G. Zorc. 1991. Myosin-I. Annu. Rev. Physiol. 53:653-681.

Rubin, G. M., and A. C. Spradling. 1982. Genetic transformation of Drosophila with transposable element vectors. Science (Wash. DC). 218:348-353.

Rütter, U., and B. Müller-Hill. 1983. Easy identification of cDNA clones. EMBO (Eur. Mol. Biol. Organ.) J. 2:1791-1794.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Smith, D. P., M. A. Starnes, and C. S. Zuker. 1991. Signal transduction in the visual system of Drosophila. Annu. Rev. Cell. Biol. 7:161-190.

Spradling, A. C., and G. M. Rubin. 1982. Transposition of cloned P elements into Drosophila germ line chromosomes. Science (Wash. DC). 218:341-347.

Sretore, R., and M. Bodebohema. 1972. The thermal origin of spontaneous activity in the Limulus photoreceptor. J. Physiol. (Lond.). 224:349-361.

Steele, F., and J. E. O'Tousa. 1990. Rhodopsin activation causes rhodopsin degeneration in Drosophila rudC mutant. Neuron. 4:883-890.

Thompson, P., and J. B. Findlay. 1984. Phosphorylation of ovine rhodopsin. Identification of the phosphorylation sites. Biochem. J. 221:773-780.
Travis, G. H., J. G. Sutcliffe, and D. Bok. 1991. The retinal degeneration slow (rds) gene product is a photoreceptor disc membrane-associated glycoprotein. Neuron. 6:61–70.

Whitelic, T. S., D. R. Hyde, and J. E. O'Tousa. 1991. Isolation and characterization of the Drosophila retinal degeneration B (rdgB) gene. Genetics. 127:761–768.

Warrick, H. M., J. A. Spudich. 1987. Myosin structure and function in cell motility. Annu. Rev. Cell Biol. 3:379–421.

Wilden, U., S. W. Hall, and H. Kühn. 1986. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. Proc. Natl. Acad. Sci. USA. 83:1174–1178.

Yau, K.-W., G. Mathews, and D. A. Baylor. 1979. Thermal activation of the visual transduction mechanism in retinal rods. Nature (Lond.). 279:785–786.