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Molecular Characterization of the Drosophila *trp* Locus: A Putative Integral Membrane Protein Required for Phototransduction

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Summary

Recent studies suggest that the fly uses the inositol lipid signaling system for visual excitation and that the Drosophila *transient receptor potential* (*trp*) mutation disrupts this process subsequent to the production of IP_3. In this paper, we show that *trp* encodes a novel 1275 amino acid protein with eight putative transmembrane segments. Immunolocalization indicates that the *trp* protein is expressed predominantly in the rhabdomeric membranes of the photoreceptor cells.

Introduction

The phototransduction cascade in flies appears to share many important features with signal transduction cascades that are initiated by a variety of stimuli such as neurotransmitters, hormones, and growth factors. As in many of these cascades, it appears that the fly's light-sensitive receptor, rhodopsin, activates a G protein (Blumenfeld et al., 1985; Paulsen and Bentrop, 1986). In flies, the effector for the G protein appears to be phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Devary et al., 1987; Inoue et al., 1988; Bloomquist et al., 1988). Diacylglycerol activates a serine/threonine phosphorylating enzyme referred to as protein kinase C (reviewed in Berridge, 1987); however, it is unclear whether DAG has any role in phototransduction. IP_3 has been shown to stimulate the release of Ca^{2+} from internal Ca^{2+} storage vesicles in Limulus ventral photoreceptors (Payne et al., 1986; Payne, 1986) and is presumed to similarly mobilize Ca^{2+} in Drosophila photoreceptor cells. Ca^{2+} in turn regulates a wide variety of cellular processes, including phototransduction (reviewed in Berridge, 1987). By analogy to work in Limulus, the release of Ca^{2+} from the intracellular stores in Drosophila is thought to result in the opening of the light-sensitive ion channels and depolarization of the receptor potential.

Phototransduction does not appear to be a strictly linear cascade, but is controlled by several feedback loops. One example of a feedback loop is the phosphorylation of photoactivated rhodopsin molecules by rhodopsin kinase. This phosphorylation turns off the rhodopsin, thereby preventing it from subsequently activating additional G proteins (transducins). Recent studies indicate that Ca^{2+} also may be important in feedback controls of both vertebrate and invertebrate phototransduction (Koch and Stryer, 1988; Payne et al., 1988).

Many of the genes and proteins important for signal transduction cascades have been identified from a variety of organisms. These include proteins required directly in the cascades, such as receptors (reviewed in Catterall, 1988; Barnard et al., 1987), G proteins (reviewed in Gilman, 1987), and PLC (Stahl et al., 1988; Mayer et al., 1988). Other genes and proteins required indirectly for signal transduction have also been identified. For example, the Ca^{2+}-ATPase of the muscle sarcoplasmic reticulum has been cloned (MacLennan et al., 1985; Brandl et al., 1986), and recently the Ca^{2+} release channel protein from rabbit muscle sarcoplasmic reticulum has been purified (Lai et al., 1988). Some of the genes encoding components required directly for phototransduction have been identified in Drosophila. These include four Drosophila opsins (O'Tousa et al., 1985; Zuker et al., 1985, 1987; Cowman et al., 1986; Montell et al., 1987; Fryxell and Meyerowitz, 1987) and PLC (Bloomquist et al., 1988).

During the last 20 years, Drosophila mutations that are defective in photoreception and processing of the light-initiated signal in photoreceptor cells have been identified (reviewed in Pak, 1979; Hall, 1982). These mutations provide a genetic approach to the identification and characterization of genes important in signal transduction that have not been identified previously in other organisms. An example of one such mutation is *transient receptor potential* (*trp*).

The *trp* locus is among the most analyzed of the Drosophila phototransduction mutations. The *trp* mutation was originally identified on the basis of a behavioral phenotype; under bright light conditions, *trp* flies behave as though blind (Cosens and Manning, 1969). The *trp* mutation is also characterized by an electroretinogram phenotype. Electroretinograms measure the change in potential due to extracellular current flow in the eye in response to light. Both wild-type and *trp* flies display a corneal negative electroretinogram in response to light. However, unlike wild-type flies, during continuous bright illumination, the receptor potential in *trp* flies quickly returns to baseline. The response of *trp* flies to a subsequent intense light stimulus is also abnormal. Wild-type flies always display a response to light regardless of the time interval between stimuli; however, *trp* flies require a 60 s dark recovery period after response inactivation and show no response to bright light after 2 s. Under conditions of dim illumination, the *trp* flies are indistinguishable from wild-type flies. Taken together, these data suggest that the *trp* phenotype may arise from depletion during intense illumination of a critical component required for phototransduction.

The *trp* locus appears to encode a protein important in a step intermediate between photoreception and

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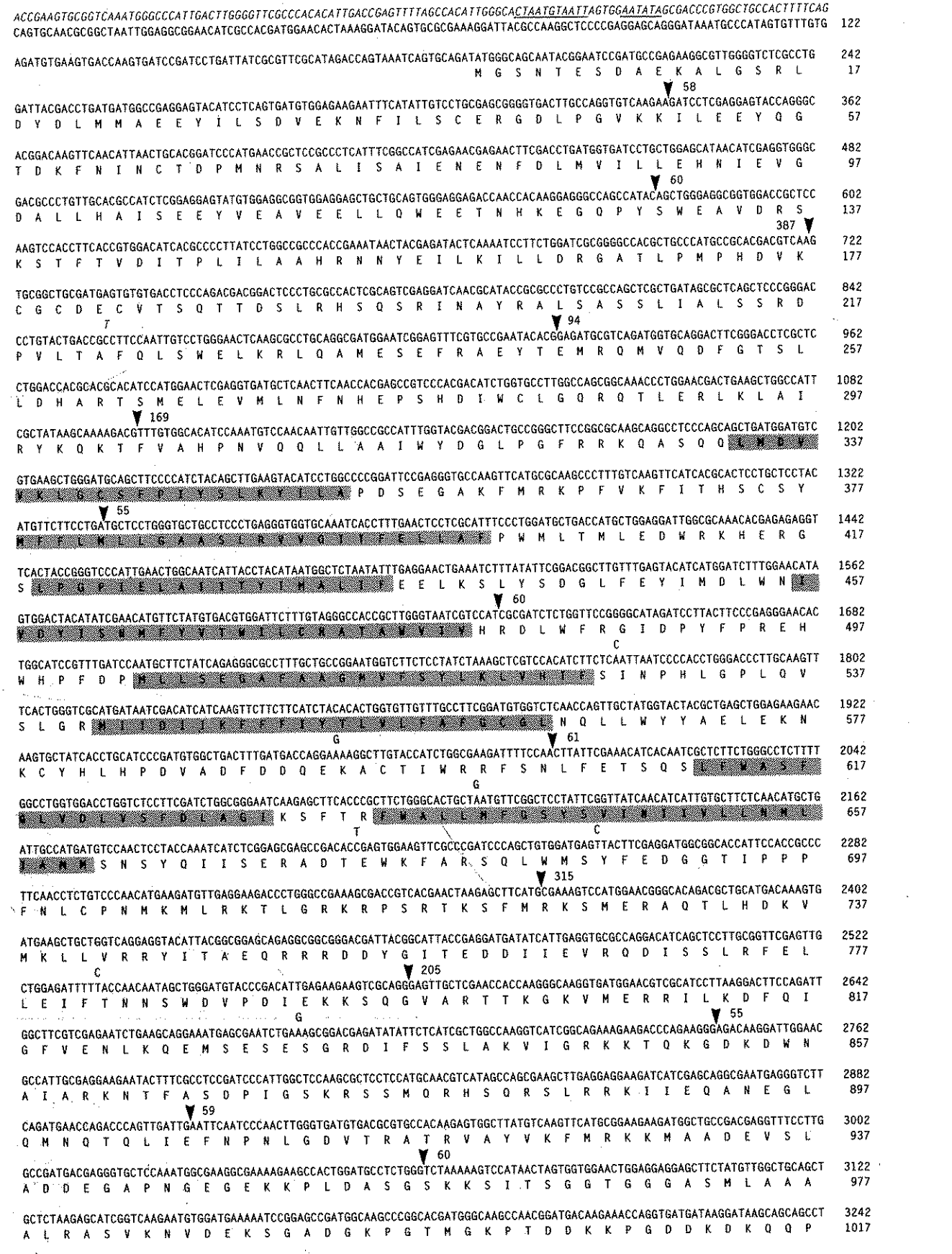
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Figure 1. DNA and Deduced Amino Acid of *trp*

Shown is the sequence of the 4.1 kb mRNA and the deduced amino acid sequence obtained from analyses of the cDNA clones c559-8, c*trp*-9, and c*trp*-7 (see Experimental Procedures). The longest cDNA, c*trp*-9 (nucleotides 165–4093), contains the entire protein coding region and is joined to a poly(A) tail at the 3′ end. The top and bottom lines indicate in italics the genomic DNA sequences flanking the 5′ and 3′ ends of the transcribed region. The initiation of transcription was determined by primer extension analysis. The running tally of nucleotides and amino acids encoded in the mRNA is shown on the right. The sizes of the introns are shown alongside the arrows indicating the positions of the intron–exon boundaries. The genomic DNA sequence (Canton S) is approximately 0.2% divergent at the nucleotide level from the cDNA sequences that were derived from cDNA libraries not made from RNA of the Canton S strain (see Experimental Procedures). These 8 nucleotide polymorphisms are indicated above the cDNA sequences. One of these polymorphisms, indicated in italics, results in an amino acid change. Some polymorphisms between the cDNAs isolated from the two different libraries were observed. At positions where a polymorphism exists, the Oregon R cDNA sequence is given. The underlined sequences include the CTAATGTAATT and AATATA sequences in the 5′ flanking region and the 3′ end processing signal, AATAAA. The AATATA sequence upstream of the transcription initiation site may serve the same function as a TATA box, and the CTAATGTAATT sequence is similar to the consensus sequence, CTAATTGRRTT (R denotes a purine), flanking the 5′ end of other Drosophila photoreceptor cell-specific genes (Mismer et al., 1988). The amino acid sequences corresponding to the eight putative membrane-spanning regions are shaded. The sequences have been deposited in the EMBL/Genbank data base (accession no. J04844).

the opening of the light-sensitive ion channels (Minke, 1982). The phenotype is not due to a defect in the rhodopsin, as the photopigment properties of *trp* mutants are normal (Minke, 1982). The light-sensitive channels also appear normal, as only the number but not the shape or size of the quantum bumps are affected in *trp* (Minke et al., 1975; Minke, 1982). (Bumps are small, discrete depolarization events generated by the absorption of single photons; they sum to produce the receptor potential.)

The *nss* mutation in the much larger fly, Lucilia, has a phenotype indicative of a defect in a protein very similar to *trp* (Barash et al., 1988). These studies strongly suggest that the *nss* gene is the Lucilia homolog of *trp*. The larger size of the Lucilia eye facilitates pharmacological studies that would be far more difficult in Drosophila. Chemicals, such as IP_3, affecting different steps in the phototransduction cascade were used to determine the step disrupted by the *nss* mutation (Suss et al., 1989). If the site of action of the *nss* defect is prior to the production of IP_3, then introduction of this chemical would be expected to elicit identical responses in the wild type and the *nss* mutant. However, IP_3 was shown to act synergistically with light to accelerate the decline of the *nss* receptor potential to baseline. These studies indicate that *trp*, by analogy to *nss*, has a role subsequent to the production of IP_3 (Suss et al., 1989).

In the current paper we describe the molecular characterization of the Drosophila *trp* gene, which had been identified by rescuing the mutant phenotype (Montell et al., 1985). We show that the 4.1 kb *trp* RNA encodes a 1275 amino acid protein. The *trp* protein appears to be a new component required in phototransduction, as it shows no significant similarity to any previously described protein. Analysis of the deduced amino acid sequence suggests that *trp* contains 8 transmembrane segments. Near the C terminus is a very hydrophilic 8 amino acid sequence that is repeated in tandem 9 times. Immunolocalization indicates that *trp* is expressed in the rhabdomeres of the photoreceptor cells. Rhabdomeres are specialized membranes of the photoreceptor cells composed of numerous microvilli containing rhodopsin and other components of the phototransduction cascade. The *trp* protein appears to be missing in each of the mutant alleles analyzed. Thus, the phenotype arises from absence of the protein rather than expression of a defective gene product.

Results

Isolation and Sequence Analysis of *trp* cDNAs

Two cDNA libraries prepared from mRNA expressed in the head of adult Drosophila were screened with the 6.5 kb *trp* genomic fragment previously shown to rescue the

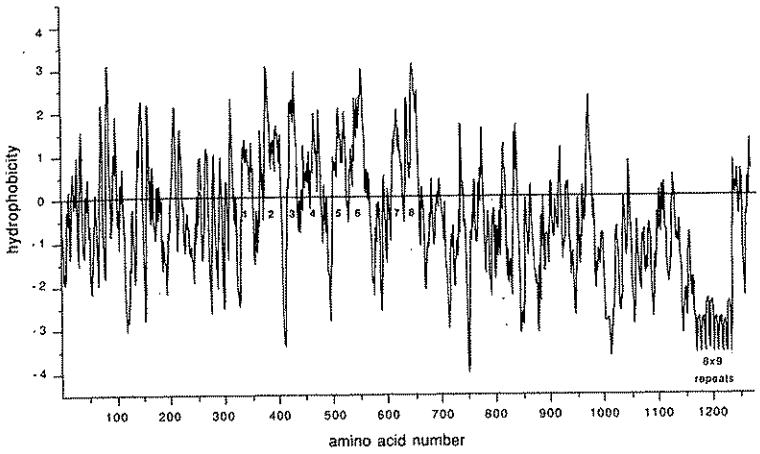


Figure 2. Hydrophobicity Plot of the *trp* Protein

Analyses were performed according the algorithm of Kyte and Doolittle (1982) using windows of 6 to 21 amino acids. Shown is the analysis using a 6 amino acid window that best illustrates the 8 × 9 repeats near the C terminus. Amino acid residues are plotted along the abscissa, and the hydrophobicity index is plotted along the ordinate. The eight putative transmembrane domains and the 8 × 9 repeats are indicated.

trp phenotype by germ line transormation (Montell et al., 1985). Among the positives, was a cDNA, λ*ctrp*-9 (3.9 kb), containing the entire protein coding region of *trp*.

Figure 1 displays the complete sequence of the *trp* coding region, obtained by DNA sequence analysis of several cDNAs including λ*ctrp*-9. Based on the DNA sequence data and primer extension analysis (see below), it appears that the *trp* mRNA is 4.1 kb. Assuming translation is initiated from the first AUG, *trp* encodes a protein of 1275 amino acids (or about 143 kd). Comparison of the deduced amino acid sequence with the protein sequence data bank indicated that *trp* does not fall into any class previously known to be required in signal transduction.

Based on hydrophobicity analysis, according to the algorithm of Kyte and Doolittle (1982), the *trp* protein can be divided into three domains: a 333 amino acid N-terminal domain with an overall neutral charge, a 228 amino acid central domain with as many as eight putative transmembrane segments, and a C-terminal domain of 614 amino acids with an overall hydrophilic character (Figure 2). The *trp* protein does not begin with an N-terminal hydrophobic signal sequence. Among the eight putative transmembrane regions in the central domain, segments 2, 3, 6, and 8 have hydrophobicity indices between 1.6 and 2.1 and are the most likely to be membrane-spanning regions. Segments 4, 5, and 7 are somewhat less likely, as their indices are about 1.3. Segment 1 has an index of only about 1.0 due to the presence of 3 charged and 4 polar residues. Using a two-dimensional wheel diagram (Schiffer and Edmundson, 1967), all 7 of these residues would be predicted to fall on the same face of the helix (data not shown). This type of clustering has been proposed to give rise to membrane-spanning amphipathic helices in a number of transport proteins that span the plasma membrane multiple times (reviewed in Catterall, 1988). A total of 15 charged residues are dispersed among the first seven putative membrane-spanning regions. However, the other putative transmembrane domains do not show as strong a clustering of charged and polar residues on one side. Although there are a few other regions with hydrophobicity indices greater than 1.0 (Figure 2), they are all less than 10 amino acids and are too short to be membrane-spanning regions.

The region between amino acids 980 and 1240 in the C-terminal domain is the most hydrophilic. Located near the C terminus is an 8 amino acid sequence, D-K-D-K-K-P-G/A-D, repeated in tandem 9 times (underlined in Figure 1 and illustrated in the hydrophobicity plot in Figure 2). Although 6 out of the 8 residues in each repeat are either acidic or basic, the overall charge is neutral. The tripeptide K-P-X (X is most commonly A or G) is repeated a total of 27 times in a 253 amino acid segment encompassing the 8 × 9 repeats.

Structure of the *trp* Gene

The intron–exon structure of the *trp* gene was determined by sequencing the 6.5 kb genomic region, which rescued the *trp* phenotype (Montell et al., 1985), and comparing this sequence with that of the cDNAs (Figure 3). The initiation of transcription was determined by

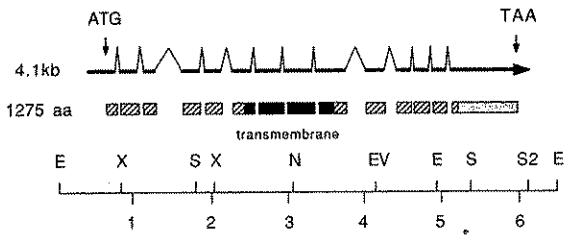


Figure 3. Structure of the *trp* mRNA

The bottom line represents the genomic DNA demarcated in kilobase pairs. The locations of the restriction sites EcoRI (E), EcoRV (EV), NarI (N), SmaI (S), SacII (S2), and XhoI (X) are shown. The bold horizontal lines joined by the caret symbols represent the exon and introns of the 4.1 kb mRNA. The direction of transcription is indicated by the arrowhead. The 1275 amino acid *trp* protein is represented by the boxes. The black boxes represent a hydrophobic region, the stippled box a very hydrophilic region, and the diagonally hatched boxes regions that are slightly hydrophilic or have an overall neutral charge.

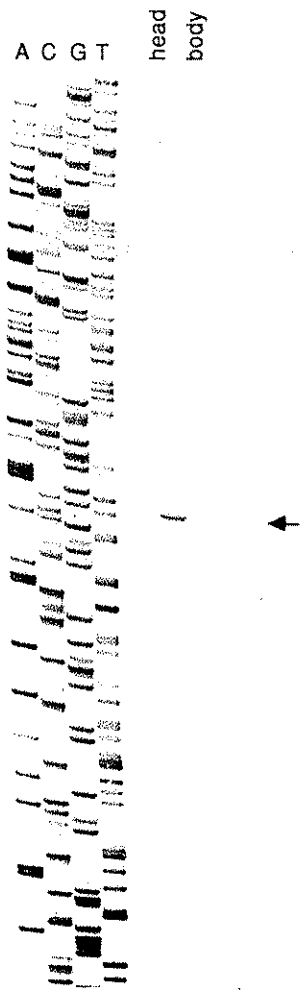


Figure 4. Primer Extension Analysis

Reverse transcription was carried out using 3 μg of poly(A)$^+$ RNA prepared from the heads or bodies of wild-type adults and a synthetic oligonucleotide (nucleotides 234–250; Figure 4) 5′ end–labeled with ^{32}P. The same oligonucleotide was used, in conjunction with a genomic clone spanning the 5′ end of *trp*, to generate a DNA sequencing ladder for size markers. The arrow indicates the major primer extension product. A minor primer extension product was also detected 2 bases 5′ of the indicated band.

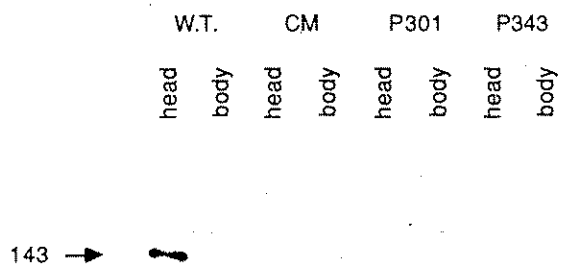


Figure 5. Detection of the *trp* Proteins in Wild-Type and Mutant Alleles by Protein Blot Analysis

Protein extracts prepared from the heads and bodies of wild-type flies and *trp* alleles, CM, P301, and P343, were fractionated on an SDS–8% polyacrylamide gel, transferred to nitrocellulose, and probed with the *trp* antiserum, αZE*trp*, followed by ^{125}I-labeled sheep anti-mouse immunoglobulin (Amersham). The size of the 143 kd protein band is based on the deduced amino acid sequence. This band migrated, relative to ^{14}C protein size markers (Amersham protein markers, CFA.626; data not shown), as a 145 kd protein.

primer extension analysis (Figure 4). The sequence at the 5′ end resembles the consensus sequence, ATCAG/TTC/T, which is found at the transcription initiation sites of many Drosophila genes (Hultmark et al., 1986). The *trp* mRNA initiates primarily at the third nucleotide in this consensus sequence with a minor start site at the first nucleotide. Near the 3′ end of the gene is a typical AATAAA sequence required for 3′ end formation (reviewed in Platt, 1986).

The 6.5 kb *trp* genomic fragment contains only 437 and 317 bp flanking the 5′ and 3′ ends of the transcribed region. Since this fragment rescued the mutant phenotype (Montell et al., 1985), it appears that less than 450 bp are required upstream or downstream of the transcribed region for proper expression of *trp*. Beginning 47 bp upstream of the start site of transcription is a perfect match to an 11 nucleotide consensus sequence, CTTAATTGRRTT (underlined in Figure 1), flanking the 5′ end of other photoreceptor cell–specific genes (Mismer et al., 1988). The sequence AATATA (underlined Figure 1), beginning 32 nucleotides from the site of transcription initiation, may serve the same function in *trp* as a TATA box.

Immunological Identification of the *trp* Protein

To localize the *trp* protein spatially on cytological sections of an adult fly head and to identify the *trp* protein on protein immunoblots, mouse polyclonal antisera were prepared to a β-galactosidase–*trp* fusion protein. The fusion protein, ZE*trp*, included an approximately 150 amino acid segment immediately N-terminal to the 8 × 9 tandem repeat. To identify the *trp* protein on immunoblots, extracts were prepared from the heads and bodies of wild-type flies, fractionated on a SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with the *trp* antisera (αZE*trp*). A single protein of the size predicted from the deduced amino acid sequence was detected in the extracts of wild-type heads, but not of bodies (Figure 5).

RNA and Protein Expression from *trp* Mutant Alleles

The *trp* RNA expressed from the three mutant alleles, CM, P301, and P343, is shown in Figure 6. The concentration of the *trp* RNA was reduced in each of the three alleles analyzed. The greatest decrease was observed in

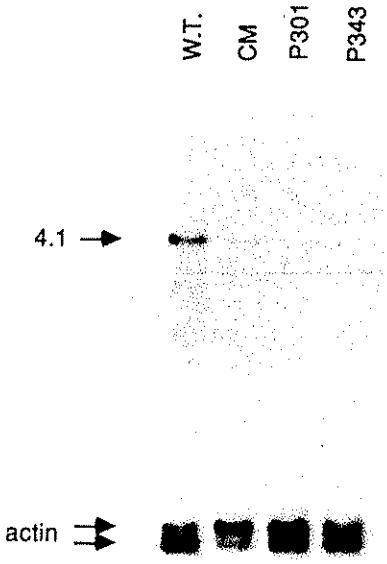


Figure 6. Analysis of *trp* RNA from Mutant Alleles

Poly(A)$^+$ RNAs were prepared from wild-type adults and three mutant alleles, CM, P301, and P343, fractionated on a 3% formaldehyde, 1.5% agarose gel (2 μg per lane), transferred to nitrocellulose, and probed with pc559-8 nick-translated with ^{32}P. The 4.1 kb size of the *trp* RNA, indicated in kilobases, is based on the sequences of the cDNAs and primer extension experiments. Taking into account the typical 200 base poly(A) sequence at the 3′ end of most mRNAs, the 4.1 kb size matches closely the 4.2 kb estimate relative to DNA size markers (Montell et al., 1985). The RNA blot was reprobed with a Drosophila actin gene, pDMA2 (Fyrberg et al., 1983), to demonstrate that equal concentrations of RNA were loaded onto each lane.

P343. The reductions in *trp* RNA levels were not due to poor RNA transfer, as the concentration of actin RNA was similar in the wild type and in each mutant (Figure 6).

Protein extracts were prepared from the heads and bodies of the same three *trp* alleles and analyzed by the protein immunoblot procedure (Figure 5). No evidence of the 143 kd band present in the heads of wild-type flies was detected in any of the *trp* mutants. These results strongly suggest that CM, P301, and P343 are all null alleles.

The *trp* Protein Is Spatially Localized to the Rhabdomeres

The compound eye of the fruit fly consists of approximately 800 repeat units referred to as ommatidia. Each ommatidium consists of photoreceptor cells, pigment cells, and corneal cells. The photoreceptor cells have a specialized portion of the plasma membrane, the rhabdomeres, consisting of tightly packed microvilli. The rhabdomeres contain high concentrations of rhodopsin and are the site of photoreception. To determine which cells within the compound eye express *trp*, we performed immunofluorescent localization. The mutant phenotype suggests that *trp* would be expressed in the photoreceptor cells.

Each ommatidial unit contains 8 photoreceptor cells. Two of the photoreceptor cells, R7 and R8, contain rhabdomeres that occupy the central distal and central proximal regions of the retina, respectively. The other 6 photoreceptor cells extend the full length of the retina and contain rhabdomeres closer to the periphery of the ommatidia. The rhabdomeres of the photoreceptor cells are spatially separated from each other. The photoreceptor cells are surrounded by pigment cells and covered by the cornea. If a protein is distributed evenly throughout the photoreceptor cells, then the immunofluorescence would be expected to appear as one central continuous bundle of staining in each ommatidium, since the photoreceptor cell bodies are contiguous. However, if a protein is expressed specifically or predominantly in the rhabdomeres, then the immunofluorescent pattern would not appear as one continuous bundle of staining in each ommatidium, since each rhabdomere is specially separated from the other rhabdomeres. Instead, the immunofluorescent pattern would appear as ribbons of staining extending the full length of the retina.

Figure 7 shows a 7 μm section of a wild-type adult fly

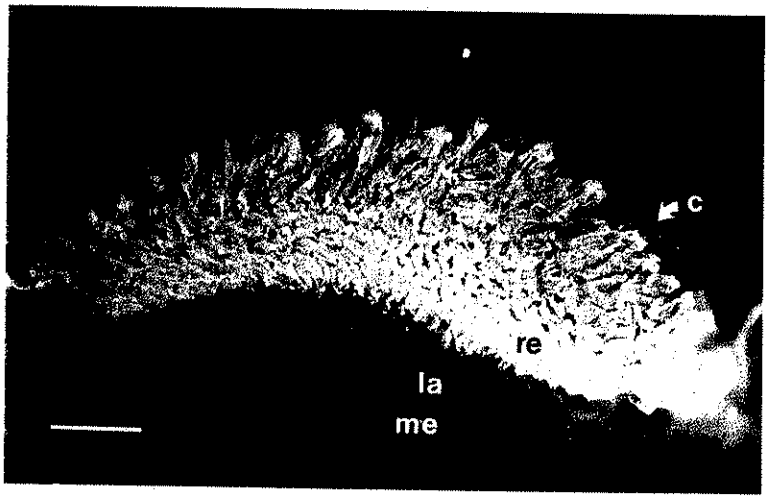
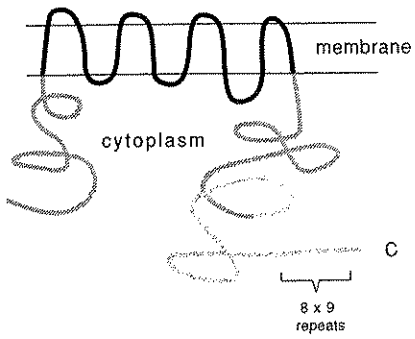


Figure 7. Immunofluorescent Localization of *trp*

A 7 μm tangential section of a wild-type (w^{1118}; trp^+) adult head was stained with αZE*trp* (first antibody), washed, and then stained with goat anti-mouse IgG (second antibody). Abbreviations: C, cornea; la, lamina; me, medulla; re, retina. The lamina and medulla are the regions in the optic lobes where the photoreceptor cells synapse. Bar, 50 μm.



3. Putative Structure of *trp* Protein

ısma membrane (rhabdomere) is represented by the two par-
ɔrizontal lines. The eight putative transmembrane segments
ɔnnecting regions are indicated by the black loops traversing
embrane. The strongly hydrophilic C-terminal region is repre-
d by the stipples, and the diagonal lines indicate regions that
lightly hydrophilic or have an overall neutral charge. The N and
rmini are oriented toward the cytoplasm, and the 8 × 9 repeat
on is indicated.

ead stained with αZE*trp*. The results suggest that *trp* is :xpressed predominantly in the rhabdomeres, as ribɔons of staining are observed. This localization is consistent with the proposal that *trp* is an integral membrane protein. No staining above background was observed in the pigment cells, the corneal cells, or the optic lobe (lamina and medulla).

Since *trp* is a putative membrane protein, it is possible that it is also spatially localized to vesicular membranes. The total mass of the vesicular membranes is lower and more dispersed than the rhabdomeres, and localization to these membranes cannot be excluded on the basis of the above immunofluorescent localization. Preliminary immunocytochemical localization using electron microscopy confirms that *trp* is expressed predominantly in the rhabdomeres and not in vesicles (Z. Selinger, C. Montell, G. Rubin, and B. Minke, unpublished data).

Discussion

In this report, we describe the molecular characterization of the *trp* gene, whose product appears to function in Drosophila phototransduction at a stage subsequent to the production of IP_3. The *trp* protein, which is not similar in sequence to any previously analyzed protein, is 143 kd, with eight putative transmembrane domains and a very hydrophilic 8 amino acid sequence repeated in tandem 9 times. Immunolocalization of *trp* indicates that it is concentrated in the rhabdomeres. Analyses of several mutant alleles indicate that the *trp* phenotype arises from the complete absence of protein rather than the production of a defective gene product.

The *trp* Protein Has Eight Putative Transmembrane Domains

A model for the *trp* protein is shown in Figure 8. This structure shares a number of general features with many receptor/transport proteins. For example, the Ca^{2+} channel has an even number of multiple transmembrane domains, one of which displays amphipathic character, and no hydrophobic N-terminal signal sequence (Tanabe et al., 1987). The $GABA_A$ receptor also has an even number of transmembrane domains and no N-terminal signal sequence (Schofield et al., 1987). These other integral membrane proteins fall into various classes. For example, voltage-activated channels, such as the K^+, Na^+, and Ca^{2+} channels, all have striking primary amino acid homology (reviewed in Catterall, 1988). The $GABA_A$, glycine, and nicotinic acetylcholine ligand-gated receptors form another family of highly related proteins (reviewed in Barnard et al., 1987). The individual members of each family show no significant amino acid homology with the members of another family. However, they all share the common features of an even number of transmembrane segments and the absence of a signal sequence. It is possible that *trp* is the first member of another general class of integral membrane proteins sharing these general features with receptor/transport proteins and having a role in signal transduction.

According to the model shown in Figure 8, the *trp* protein has an even number of transmembrane segments. If this model is correct, then the N and C termini must lie on the same side of either rhabdomere or vesicular membranes. The ends of the *trp* protein are depicted on the cytoplasmic side, as there is no N-terminal signal sequence. The localiztion of the *trp* protein to the membrane-rich rhabdomeres provides additional evidence that *trp* is an integral membrane protein.

The Structure and Localization of the *trp* Protein Supports Certain Possible Functions and Disfavors Others

The *trp* mutation is characterized by a rapid decay of the receptor potential during illumination with bright light and by a requirement for about a 60 s recovery period in the dark before a subsequent full response to light can be elicited. Electrophysiological and pharmacological studies suggest that *trp* has a role subsequent to the production of IP_3 (Suss et al., 1989). Indirect evidence suggests that the intracellular Ca^{2+} levels may be reduced in *trp* photoreceptor cells (Zuidervaart et al., 1979, Soc. Neurosci., abstract; Kirschfeld and Vogt, 1980; Lo and Pak, 1981; Minke, 1982). In addition, the *nss* and *trp* mutations appear to lower the activity of the PLC, suggesting that *trp* may be involved in feedback control of the phototransduction cascade (Suss et al., 1989). This latter effect may be a consequence of the decreased intracellular Ca^{2+} levels in *trp*, since the activity of PLC is dependent on Ca^{2+} concentration (Baer and Saibil, 1988). Based on these observations, there are many potential roles for *trp*. The results from the current paper lead us to disfavor most of these possibilities and focus on potential roles that are most consistent with the molecular analysis: a role in inositol phospholipid metabolism or possibly as a membrane protein that interacts with the light-sensitive ion channels.

Subsequent to the production of IP_3, other inositol phosphates such as IP_4, IP_5, and IP_6 are produced (re-

viewed in Downes, 1988). Little is known about the functions of these components or the proteins that synthesize them. Several studies have led to the suggestion that IP_4 may act as a second messenger. One proposal is that IP_4 facilitates Ca^{2+} entry into the cytoplasm by opening channels in the plasma membrane (Irvine and Moor, 1986). Another study has raised the possibility that IP_3 and IP_4 may act synergistically to regulate ion channels in the plasma membrane and evoke a full electrophysiological response (Morris et al., 1987). Consistent with the spatial localization and putative structure is the possibility that *trp* is the plasma membrane IP_4 receptor. Absence of the plasma membrane IP_4 receptor in *trp* could result in decreased intracellular Ca^{2+} levels, leading to lower Ca^{2+} levels in the internal storage vesicles. Rapid depletion of the small internal Ca^{2+} stores could account for the loss of receptor potential during bright illumination and the observation that the *trp* mutant has a defect subsequent to the production of IP_3. Lower total cytoplasmic Ca^{2+} levels could be responsible for the decreased PLC activity, since this enzymatic activity is affected by Ca^{2+} concentration.

In addition to IP_4, it appears that IP_5 and IP_6 also have physiological functions in the nervous system (Vallejo et al., 1987). It is possible that inositol phosphates, such as IP_4, IP_5, or IP_6, play a role in Drosophila phototransduction and that *trp* is important for production of one of these components. The *trp* protein might be an inositol phosphate kinase involved in the production of one of these higher phosphorylated forms of inositol. Although the *trp* protein has eight putative transmembrane domains, the majority of the protein, 333 and 614 amino acids at the N and C termini, is predicted to extend into the cytoplasm. An inositol kinase activity may be contained in these large terminal domains.

A recent study has demonstrated that IP_4 induces Ca^{2+} sequestration in rat liver cells (Hill et al., 1988). It is possible that IP_4 plays a similar role in Drosophila photoreceptor cells. Thus, failure to produce IP_4 could result in the *trp* phenotype either by limiting entry of Ca^{2+} into the photoreceptor cells or by impairing Ca^{2+} sequestration into the internal stores.

Alternatively, the *trp* protein could interact with the light-sensitive channels in the plasma membrane. There is evidence that the properties of channels can be modified by interaction with other proteins (see for example Ganetsky and Wu, 1986). The *trp* protein might have a role in reactivation of the light-sensitive channels in photoreceptor cells. Wild-type flies continually display a response to light and do not require a recovery period in the dark even after a very long and intense light stimulus. This is in contrast to the *trp* mutant, which quickly loses responsiveness to bright light and requires a 60 s dark recovery period before a subsequent intense light stimulus can elicit a full depolarization. It is possible that the loss of responsiveness during intense illumination and the 60 s dark recovery period are due to a requirement for the *trp* protein to quickly reset the light-sensitive channels before they can interact again with ligand and/or reopen. The *trp* protein may not be required under dim light conditions, as only a fraction of the light-sensitive channels are activated at any time and these could reset slowly as other channels are activated. However, the limitation of this model is that it does not account for the diminished PLC activity in *trp*.

The molecular analyses presented in the current paper argue against certain other possible functions for *trp*. For example, the *trp* protein might be an IP_3 receptor. Binding of IP_3 to IP_3 receptors has been shown to lead to the release of intracellular Ca^{2+} (Meyer et al., 1988). Alternatively, since *trp* is required subsequent to the production of IP_3 and the intracellular Ca^{2+} levels appear to be reduced, it is possible that *trp* has a role in storing Ca^{2+} (Suss et al., 1989). Therefore, *trp* might encode a Ca^{2+}–ATPase that pumps Ca^{2+} into the internal stores or a Ca^{2+} channel that releases Ca^{2+} from the storage vesicles. However, the *trp* protein is localized predominantly to the rhabdomeres, the specialized plasma membrane of the photoreceptor cells, and does not appear to be specifically localized to the membranes of any intracellular vesicle. Additionally, the *trp* protein is not homologous to the mammalian sarcoplasmic reticulum Ca^{2+}–ATPase (MacLennan et al.,1985; Brandl et al., 1986) and is much smaller than the 260 kd IP_3 receptor purified from rat brains (Supattapone et al., 1988) and the 400 kd Ca^{2+} release channel from the vertebrate sarcoplasmic reticulum (Lai et al., 1988).

Since *trp* is localized to the rhabdomeres, it is possible that it is a plasma membrane Ca^{2+} pumping ATPase. However, *trp* shows no homology with the recently reported C-terminal sequence of a plasma membrane Ca^{2+}–ATPase (Brandt et al., 1988). In particular, the *trp* protein does not contain any sequence that matches the 6 key residues in the 12 amino acid Ca^{2+} binding loops originally identified in calmodulin (Watterson et al., 1980) and subsequently in the plasma membrane and sarcoplasmic reticulum Ca^{2+}–ATPases.

An alternative proposal, consistent with the protein structure and localization, is that *trp* is the structural gene for the light-sensitive channels. However, electrophysiological analyses suggest that *trp* does not encode the light-sensitive channel (Minke, 1982). This conclusion is strongly supported by the protein immunoblot analysis of three *trp* alleles presented in the current report. Since the behavioral and electrophysiological response of *trp* flies is normal under conditions of dim light, the light-sensitive channels must be present. Therefore, if *trp* encodes the light-sensitive channel, then the protein must be defective rather than absent. The demonstration that the *trp* protein is completely missing in each mutant allele examined, indicates that *trp* is not the structural gene for the light-sensitive channel.

In conclusion, on the basis of the spatial localization, the putative protein structure, and the analysis of protein expression from several mutant alleles, it appears that many potential roles for *trp* can now be considered highly unlikely. Thus, it appears that *trp* is a protein with a role subsequent to the production of IP_3 that has not

been previously described. The results presented in the current paper are consistent with a role in the inositol phospholipid signaling system.

Experimental Procedures

RNA Analyses

Polyadenylated RNA was prepared from wild-type adult flies (Oregon R strain of D. melanogaster) and from the mutant alleles CM, P301, and P343 as described (Montell et al., 1985). CM is the original allele isolated (Cosens and Manning, 1969), and the P301 and P343 alleles were isolated by W. L. Pak. Polyadenylated RNA (2 μg of each) was fractionated on 3% formaldehyde, 1.5% agarose gels as previously described (Montell et al., 1985). The RNAs were transferred to nitrocellulose by blotting and then probed with pc559-8 (a *trp* cDNA; see below) nick-translated with ^{32}P.

Primer extensions were carried by hybridizing a synthetic oligonucleotide (nucleotides 234–250; Figure 4), 5′ end–labeled with ^{32}P, with 3 μg of head or body poly(A)$^+$ RNA from the Oregon R strain. Reverse transcription was carried out as described (Maniatis et al., 1982) for cDNA synthesis. The synthetic oligonucleotide was also used to obtain a DNA sequencing ladder (for size markers) after hybridization with an M13–*trp* genomic clone, m*trp*EX1. This M13 clone was created by inserting the 0.8 kb EcoRI–XhoI fragment encompassing the 5′ end of *trp* (Figure 3) between the EcoRI and SalI sites of M13mp18.

Isolation of cDNAs

A cDNA library prepared from D. melanogaster Oregon R strain adult head poly(A)$^+$ RNA (gift from B. Yedvobnick and S. Artavanis-Tsakonas) was screened with ^{32}P-labeled p559E1.7 (Montell et al., 1985). p559E1.7 consists of a 1.6 kb genomic EcoRI fragment from the 3′ end of *trp* (4.9–6.5 kb; see Figure 3). The filters were hybridized and washed as described (Montell et al., 1987). Among the cDNAs isolated, c559-8 was the longest (nucleotides 585–4066; Figure 1). DNA sequence analyses of several other *trp* cDNAs, as well as the genomic DNA, showed that c559-8 is missing 2 nucleotides (1631–1632; Figure 1).

A second Drosophila cDNA library, prepared from adult head poly(A)$^+$ RNA (Cowman and Rubin, unpublished data), was subsequently screened with p559C3A to isolate longer cDNAs. p559C3A contains the 6.5 kb *trp* genomic region that rescued the *trp* phenotype (Montell et al., 1985). One of the cDNAs isolated, *ctrp*-9 (nucleotides 165–4093), includes the entire protein coding region. The cDNA that extends closest to the 5′ end, *ctrp*-7 (3.8 kb), begins at nucleotide 77 but terminates approximately 100 nucleotides before the 3′ end. Each of the cDNAs mapped by in situ hybridization to polytene salivary gland chromosomes to the cytogenetic position of *trp*, 99C (Levy et al., 1982; Wong et al., 1985; Montell et al., 1985).

DNA Sequencing

DNA sequencing was carried out according to the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemicals), [^{35}S]dATP, and 60 cm buffer gradient gels (Biggin et al., 1983).

The plasmid pc559-8 was constructed by partial digestion of λc559-8 with EcoRI and subcloning the 3.5 kb fragment into the EcoRI site of pEMBL9+ (Dente et al., 1983). The λ*ctrp*-9 cDNA consists of a 2.7 and a 1.3 kb EcoRI fragment (the 1.3 kb fragment includes a 3′ poly(A) tail of about 100 bases). The λ*ctrp*-7 cDNA consists of a 2.8 and a 1.1 kb EcoRI fragment. Both λ*ctrp*-9 EcoRI fragments and the 2.8 kb λ*ctrp*-7 fragment were subcloned into the EcoRI site of Bluescript KS M13(+) (Stratagene) to create the plasmids pc*trp*-9-2.7R, pc*trp*-9-1.3, and pc*trp*-7R. The *trp* fragments in the plasmids pc*trp*-9-2.7R and pc*trp*-9-1.3 have the 5′ and 3′ ends (with respect to the orientation of transcription) closest to the universal primer. The 2.8 kb fragment is inserted in pc*trp*-7R with the 3′ end closest to the M13 −20 primer (with respect to the orientation of transcription). The plasmid p559C3A (Montell et al., 1985) consists of the 6.5 kb genomic fragment, which rescued the *trp* mutation, subcloned into the transformation vector Carnegie 3 (Rubin and Spradling, 1983).

Ten micrograms of pc559-8 and 10 μg of p559C3A were randomly sheared by sonication and repaired, and fragments in the 300–600 nucleotide size range were inserted into the SmaI site of M13mp10 as described (Montell et al., 1987). Recombinant M13 clones containing the cDNA inserts were identified by plaque hybridizations with the corresponding purified fragments nick-translated with ^{32}P. The inserts were sequenced using the universal primer (New England Biolabs #1211). Single-stranded pc*trp*-9-2.7R and pc*trp*-9-1.3 DNAs were prepared and sequenced using a series of chemically synthesized oligonucleotides spaced 300 nucleotides apart. The 5′ end of c*trp*-7 was sequenced using single-stranded pc*trp*-7R and the same oligonucleotide used for the primer extension (nucleotides 234–250).

Preparation of *trp* Antisera

The *trp* antisera were raised to a protein consisting of β-galactosidase joined at the C terminus to a fragment of the *trp* protein. The fusion protein was generated using the β-galactosidase expression vector, pUR292 (Rüther and Müller-Hill, 1983). To generate the plasmid pZE*trp*, pUR292 was digested with an excess of BamHI, partially digested with EcoRI, and ligated to a 0.35 kb BamHI–EcoRI fragment from M13c559-8-100 (beginning at nucleotide 3180; Figure 4). M13c559-8-100 was generated by random shearing of c559-8 and used in the DNA sequence analysis (see above). The fusion protein was expressed in E. coli K12 strain *BMH 71-18* and purified by electroelution from an SDS–polyacrylamide gel as described (Montell and Rubin, 1988). Samples (100 μl per mouse; ~50 μg of protein) were emulsified by sonication in 200 μl of Freund's complete adjuvant and introduced subcutaneously into two BALB/c female mice. The mice were subsequently injected after 3 and 6 weeks with similar quantities of the fusion protein emulsified in Freund's incomplete adjuvant. Seven days following the last boost, the animals were bled and sera (αZE*trp*) were collected.

Protein Blot Analysis

Sixteen milligrams of adult heads and bodies, separated in mass by agitation of frozen flies (Oliver and Phillips, 1970), was emulsified in 400 μl of 2× SDS sample buffer using a microfuge tube pellet pestle (Kontes Scientific) and a motor-driven homogenizer (Beilco Glass Inc.). The extracts were boiled for 2 min, and 10 μl of each protein extract was fractionated on an SDS–8% polyacrylamide gel. The proteins were transferred to nitrocellulose by electrophoresis in transfer buffer (20 mM Tris base, 150 mM glycine, 20% v/v methanol) without SDS. The nitrocellulose was rinsed briefly in PBS, incubated at room temperature for 4 hr in PBS–BSA (5% BSA dissolved in PBS; pH readjusted to 7.4), rinsed in PBS, incubated overnight at 4°C with αZE*trp* from mouse #8 (diluted 1:500 in PBS–BSA), washed for 10 min in PBS, washed twice for 10 min in PBS with 0.05% NP40, and rinsed once in PBS. The nitrocellulose was then incubated for 4 hr at room temperature with 0.2 μC/ml ^{125}I-labeled sheep anti-mouse immunoglobulin (Amersham) in PBS–BSA, washed as above, and exposed by autoradiography.

Light Microscopy Immunolocalization

Frozen sections (7 μm) of adult fly heads (w^{1118}) were mounted on slides previously dipped in 0.1 mg/ml poly-L-lysine, dried, fixed for 30 min with 2% formalin in 75 mM sodium phosphate (pH 7.0), and rinsed for 10 min in TBS (10 mM Tris–HCl [pH 7.4], 130 mM NaCl, 5 mM KCl, 1 mM EGTA). The fixed sections were covered for 30 min at room temperature with 50 μl of 50% fetal bovine serum (blocking serum) over a humidified chamber, rinsed for 10 min in TBS, covered overnight at 4°C with 50 μl of αZE*trp* (1:250 dilution in TBS), rinsed for 10 min in TBS, and covered for 30 min at room temperature with 50 μl of rhodamine-conjugated, affinity-purified goat anti-mouse IgG (Cappel Worthington) diluted 1:50 in TBS. The sections were then rinsed for 10 min in TBS, covered with 50 μl of mounting medium (90% glycerol, TBS, 25 mM propyl gallate) and a coverslip, and viewed by epifluorescence.

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