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Novel Mechanism of Massive Photoreceptor Degeneration Caused by Mutations in the *trp* Gene of *Drosophila*

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The *Drosophila trp* gene encodes a light-activated Ca^{2+} channel subunit, which is a prototypical member of a novel class of channel proteins. Previously identified *trp* mutants are all recessive, loss-of-function mutants characterized by a transient receptor potential and the total or near-total loss of functional TRP protein. Although retinal degeneration does occur in these mutants, it is relatively mild and slow in onset. We report herein a new mutant, Trp^{P365}, that does not display the transient receptor potential phenotype and is characterized by a substantial level of the TRP protein and rapid, semi-dominant degeneration of photoreceptors. We show that, in spite of its unusual phenotypes, Trp^{P365} is a *trp* allele because a Trp^{P365} transgene induces the mutant phenotype in a wild-type background, and a wild-type *trp* transgene in a Trp^{P365} background suppresses the mutant phenotype. Moreover, amino acid alterations that could cause the Trp^{P365} phenotype are found in the transmembrane segment region of the mutant channel protein. Whole-cell recordings clarified the mechanism underlying the retinal degeneration by showing that the TRP channels of Trp^{P365} are constitutively active. Although several genes, when mutated, have been shown to cause retinal degeneration in *Drosophila*, the underlying mechanism has not been identified for any of them. The present studies provide evidence for a specific mechanism for massive degeneration of photoreceptors in *Drosophila*. Insofar as some human homologs of TRP are highly expressed in the brain, a similar mechanism could be a major contributor to degenerative disorders of the brain.

Key words: TRP Ca^{2+} channel; photoreceptor degeneration; novel mechanism of neuronal cell death; semi-dominant trp *mutant; constitutive channel activity;* Drosophila

Phototransduction in flies is mediated by a phosphoinositide signaling cascade (Devary et al., 1987; Inoue et al., 1988; Selinger and Minke, 1988) with an absolute requirement for PLC_{β} (Bloomquist et al., 1988). A key participant in *Drosophila* phototransduction is the protein product of the *transient receptor potential (trp)* gene TRP (for review, see Hardie and Minke, 1993; Minke and Selinger, 1996; Montell, 1997; Scott and Zuker, 1998). Two classes of light-activated channels have been identified in *Drosophila*: a Ca^{2+}-selective class and a nonspecific cation class (Hardie and Minke, 1992). A growing body of evidence suggests that TRP is a subunit of the Ca^{2+}-selective class of channels (Hardie and Minke, 1992; Peretz et al., 1994a,b; Hardie, 1996). Two members of the TRP protein family, proteins encoded by the *trp* gene (Montell and Rubin, 1989; Wong et al., 1989) and a closely related gene, *trpl* (Phillips et al., 1992), appear to account for all light-activated channel activities in *Drosophila* photoreceptors. Thus, a double mutant *trpl*;*trp*, consisting of null alleles of *trp* and *trpl*, is totally unresponsive to light (Scott et al., 1997), suggesting that TRP and TRPL make up all light-activated channels or are required for their activation.

Mutations in the genes encoding proteins of the phototransduction cascade appear to be a major cause of hereditary retinal degenerations, which in turn are a major cause of human blindness. Among the phototransduction protein genes that have been identified in this role are the genes encoding rhodopsin and the α and β subunits of rod cGMP phosphodiesterase (for review, see Dryja, 1997). Likewise, mutations in the genes encoding phototransduction proteins of *Drosophila* have long been known to cause age-dependent retinal degenerations, such as those encoding rhodopsin (for review, see Bentrop, 1998) and phospholipase C_{β} (for review, see Pak, 1994).

The first *trp* mutant (trp^{CM}) was recovered as a spontaneously occurring mutant (Cosens and Manning, 1969), and several additional alleles were isolated in chemical mutagenesis that screened for defective electroretinograms (ERGs), the extracellularly recorded, light-evoked mass responses of the eye (Pak et al., 1969; Pak, 1979). All these *trp* mutants are characterized by a lack of the sustained component in the ERG elicited by a prolonged bright stimulus (Fig. 1*Ad*). The TRP protein in these mutants is either undetectably low in amount (Montell and Rubin, 1989; Wong et al., 1989) or nonfunctional (Reuss et al.,

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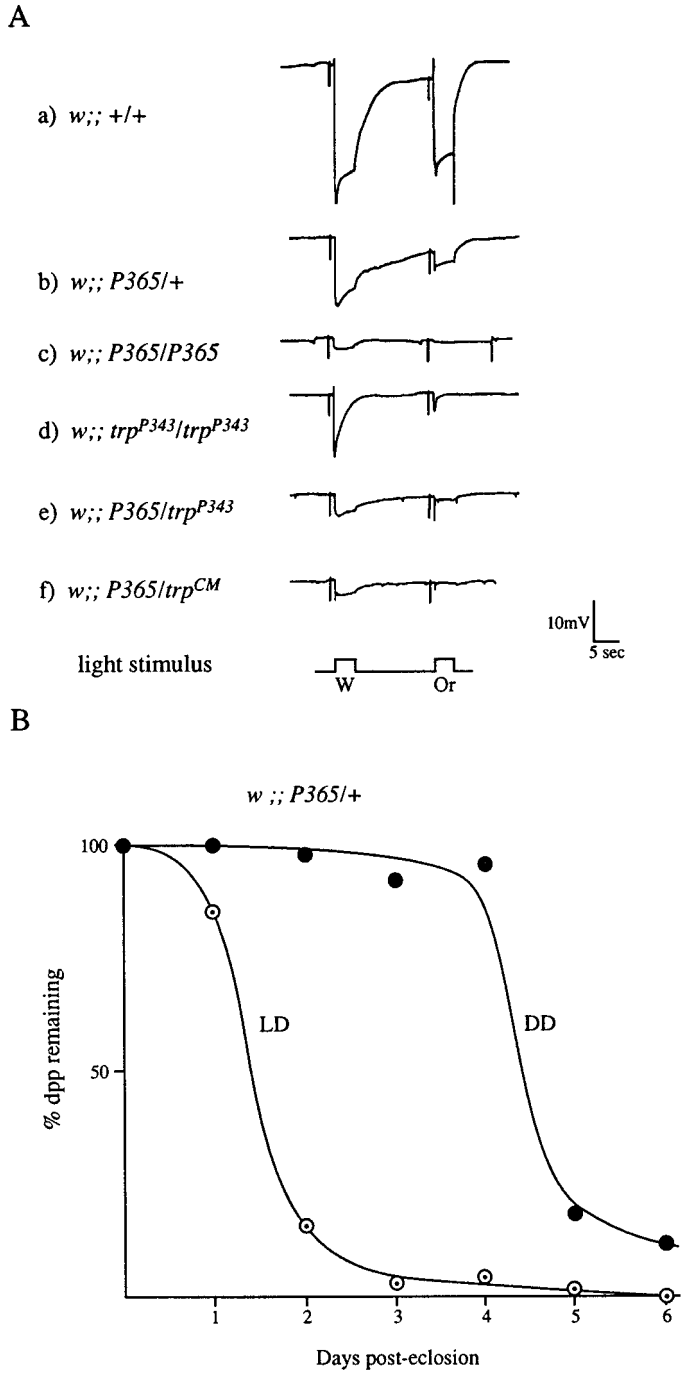

Figure 1. *A,* Comparison of the ERGs of *P365*-carrying mutants with those of wild type and *trp*. Representative ERGs recorded from wild type (*a*), *P365*/+ heterozygote (*b*), *P365*/*P365* homozygote (*c*), null *trp* mutant, trp^{P343}/trp^{P343} (*d*), and flies carrying heteroallelic combinations of *P365* with trp^{P343} (*e*) and trp^{CM} (*f*). All flies were marked with the mutation *w* (*white*) to remove the red screening pigments in the eye. All recordings both in this figure and Figure 4 were obtained at 7 d after eclosion. For each recording shown, a white stimulus and an orange stimulus, each of 4 sec duration, were presented 20 sec apart, after 1 min of dark adaptation (*bottom trace*). *B,* Time course of disappearance of the deep pseudopupil as a measure of photoreceptor degeneration in *P365*/+ heterozygotes. The fraction of flies that have not lost the deep pseudopupils was determined in sample populations raised in 12 hr light/dark cycles (*LD*) or in complete darkness (*DD*) as a function of age in days after eclosion. All flies were marked with *w* (*white*).

1997). These mutations have also been reported to cause retinal degeneration, although the degeneration is relatively mild and apparently does not become noticeable histologically until mutants are at least 2 wk after eclosion (Cosens and Perry, 1972; Stark and Sapp, 1989).

Among the many different classes of mutants isolated in the ERG-based mutant screen (Pak, 1995) is *P365*, which maps to the same region of the third chromosome as *trp*. Unlike the known *trp* alleles, however, *P365* is semi-dominant and causes rapid and massive photoreceptor degeneration but does not cause the familiar decaying receptor potential associated with *trp*. Here we show that, its unusual phenotypes notwithstanding, *P365* is a semi-dominant allele of *trp,* and we show further the basis of photoreceptor degeneration in *P365*, revealing a previously undescribed mechanism of photoreceptor degeneration.

MATERIALS AND METHODS

Materials. The wild-type strain used was Oregon R. Except where stated, all flies were marked with the mutation *w* (*white*) to remove the screening pigments in the eye. trp^{CM} is the original *trp* allele reported by Cosens and Manning (1969). All other mutants were generated on an Oregon R background by ethylmethanesulfonate mutagenesis in the laboratory of W.L.P.

Electron microscopy. The fixation procedure followed the protocol of Baumann and Walz (1989), as summarized in Leonard et al. (1992). Flies were perfused with freshly made fixative (4% paraformaldehyde, 3.5% glutaraldehyde, and 0.1 M sodium cacodylate, pH 7.3–7.4) by injection through a glass micropipette inserted into the thorax. Eyes were dissected out and fixed in the same solution 3–4 hr at room temperature and then overnight at 4°C after adding 1% tannic acid. They were washed for 15 min in 0.1 M sodium cacodylate, post-fixed for 2 hr in 2% OsO_4 and 0.1 M sodium cacodylate, and stained en bloc with 2% uranyl acetate overnight at 4°C after a 10 min water wash. They were then dehydrated in a cold ethanol and water series, infiltrated with Epox, embedded, and sectioned using a Diatome diamond knife. Sections of 0.1 μm were viewed on a Phillips 300 electron microscope.

Immunofluorescence confocal microscopy. Eyes were dissected from very young adult *P365* homozygous mutant or wild-type flies, <2 hr after eclosion, and fixed in PBS containing 4% paraformaldehyde and 0.3%Triton X-100 for 1 hr. They were incubated, in sequence, in PBS containing the following with washes in between: (1) 4% (v/v) normal goat serum to block nonspecific binding sites, (2) 3% (v/v) anti-TRP antiserum (obtained from Dr. Charles Zuker) and 4% normal goat serum at 4°C overnight, and (3) 2% (v/v) fluorescein-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) and 4% normal goat serum for 4 hr at 22°C. Phalloidin-TRITC (Sigma, St. Louis, MO) was added to the solution containing the secondary antibody to reach a final concentration of 3.8 mM. Signals were obtained from transverse optical sections of <1 μm thickness near the distal tips of rhabdomeres.

ERG. The ERGs were recorded as described in Larrivee et al. (1981), using glass microelectrodes filled with Hoyle's saline. The light stimuli originated from a tungsten halogen lamp (Bausch & Lomb) and were delivered to the preparation with a fiber optics light guide. The unfiltered intensity at the level of the fly was ~47 mW/cm^2. The light was used without filters for white stimuli and was filtered with a sharp-cut orange filter (Corning CS 2–73 or Schott OG 590) for orange stimuli.

Whole-cell recordings. Dissociated ommatidia were prepared from P15 pupae or newly eclosed adult flies (<1 hr after eclosion), using the procedures described by Hardie (1991a). Whole-cell patch-clamp recordings were performed as described in Hardie and Minke (1992) and Peretz et al. (1994b). Aliquots of isolated ommatidia were allowed to settle on a coverslip that formed the bottom of a chamber placed on the stage of an inverted microscope. The whole-cell, patch-clamp recording configuration was achieved using standard techniques. Recordings were made at 21°C using patch pipettes of 5–10 Ω pulled from fiber-filled borosilicate glass capillaries. Series resistance (7–14 Ω) was carefully compensated (>80%) during all experiments. Signals were amplified with an Axopatch-1D (Axon Instruments, Foster City, CA) patch-clamp amplifier, sampled at 900 Hz, and filtered at <5 kHz.

The bath solution contained (in mM): 120 NaCl, 5 KCl, 10 *N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid (TES), pH 7.15,

4 $MgSO_4$, and 1.5 $CaCl_2$. $CaCl_2$ was omitted whenever a low Ca^{2+} solution was used (see Fig. 6*D*). In parts of the experiments, 2 mM EGTA was added to reduce the Ca^{2+} level even further. The pipette solution included ions needed to block K^+ channel activity and contained (in mM): 120 CsCl, 15 tetraethyl ammonium (TEA) chloride, 2 $MgSO_4$, 10 TES, pH 7.15, 4 MgATP, 0.4 Na_2GTP, and 1 nicotinamide adenine dinucleotide.

Deep pseudopupil measurements. The deep pseudopupil (dpp) (Franceschini, 1972) was examined as a function of age in *P365*/+ heterozygotes, raised in either complete darkness (DD) or 12 hr light/dark (LD) cycles. All *P365*/+ heterozygotes used were marked with *white (w)*. For each illumination condition, a group of 40–50 flies was examined on each day after eclosion to determine the fraction of flies that had not yet lost the dpp. In the case of dark-reared flies, an effort was made to prevent the light used in dpp examinations from influencing the results of the following measurements. To this end, many groups of 40–50 flies were raised, and each group was aged for the desired number of days, examined just once at that age, and then discarded. Dpp measurements were also performed on some of the transgenic flies, but only under LD illumination conditions. These flies were not marked with w and therefore were red-eyed. The dpp was viewed under a dissecting microscope at a magnification of 20–25×. For white-eyed flies, it was viewed in reflected light, whereas for red-eyed flies, it was viewed in transmitted light.

Isolation and sequence analysis of the trp gene of P365. The 6.4 kb *Eco*RI genomic fragment, shown to rescue the *trp* mutant phenotype in P element-mediated germline transformation (Montell et al., 1985), was isolated from the *P365* mutant in two *Eco*RI subfragments, 4.8 and 1.6 kb. For the isolation of the 4.8 kb fragment, total genomic DNA prepared from *P365* mutant flies was size-fractionated, DNA fragments in the 4.8 kb size range were isolated and subcloned into the pGEM-Blue vector (Promega Biotec), and those containing the *trp* gene were identified by colony hybridization with *trp* cDNA. The 1.6 kb fragment was amplified by PCR using *P365* genomic DNA as template and primers designed from the published *trp* genomic sequences (accession number M34394): nucleotide positions 4876–4899 and 6443–6465 (complementary strand). The PCR-amplified DNA fragment was subcloned into the pGEM-T easy vector (Promega Biotec). The two subfragments were sequenced by the dideoxy chain termination method (Sanger et al., 1977), as modified in the Sequenase system (United States Biochemical Corporation).

The above analysis identified 11 nucleotide changes that could have altered the TRP protein sequence in *P365*. All 11 nucleotides were contained within four small regions of the *trp* gene, and these regions were sequenced in two wild-type strains, Oregon R and Canton S. They were amplified by PCR using total Oregon R or Canton S genomic DNA as template and the following four pairs of primers: (1) 1031–1050 and 1807–1826, (2) 2851–2870 and 3212–3231, (3) 4557–4576 and 4902–4921, and (4) 4876–4899 and 6443–6465. The amplified fragments were subcloned into the pGEM-T easy vector and sequenced as above.

Transgenic flies. Two classes of transgenic flies were generated: (1) those carrying a P element construct containing a wild-type copy of the *trp* gene, P[trp^+], in a *P365* mutant background and (2) those carrying a P element construct containing the *P365* mutant copy of the *trp* gene, P[*P365*], in a wild-type background.

To generate the first class of flies, we used the transgenic lines constructed by Montell et al. (1985) for transformation rescue of the *trp* mutant phenotype. These lines carried the wild-type 6.4 kb *trp* genomic fragment cloned into the P-element transformation vector, Carnegie 3, on the X or second chromosome in a homozygous trp^{CM} background. After suitable balancing of chromosomes and chromosomal rearrangements with *P365*, the following four classes of transgenic lines were generated: (1) P[trp^+]/Bal;*P365*/trp^+, (2) P[trp^+]/Bal;*P365*/*P365*, (3) P[trp^+]/P[trp^+];*P365*/trp^+, and (4) P[trp^+]/P[trp^+];*P365*/*P365*, where Bal denotes a balancer.

For the generation of the second class of transgenic flies, the 6.4 kb *Eco*RI genomic fragment was isolated from wild-type flies and subcloned into the pCaSpeR-3 transformation vector. The 4.8 kb *Eco*RI subfragment isolated from the *P365* mutant, as described above, was then swapped with the wild-type 4.8 kb fragment in the vector. The transformation vector thus contained a 4.8 kb fragment originating from *P365* and a 1.6 kb fragment originating from wild type. Although 11 nucleotide changes that could potentially alter the amino acid sequence had been detected in the *trp* gene of *P365*, seven of these were also found in two wild-type strains tested, suggesting that they are strain-specific polymorphisms (see Results). The remaining four mutations were all contained within the 4.8 kb subfragment. Standard methods were used to generate transgenic lines carrying one and two copies of the *P365* transgene in a wild-type background.

Western blot analysis. Total adult-eye or pupal protein homogenates were prepared as described in Pearn et al. (1996). They were boiled for 5 min in sample buffer (62.5 mM Tris-Cl, pH 6.8; 2% SDS; 10% glycerol; 2% β-mercaptoethanol; and 10 μg/ml bromophenol blue), fractionated by 6% SDS-PAGE, and electroblotted onto a nitrocellulose membrane. A monoclonal antibody generated against the TRP protein (Pollock et al., 1995) was used at 1:1500 dilution as the primary antibody, and immunolabeling was detected with alkaline phosphatase-conjugated anti-mouse secondary antibody (Boehringer Mannheim, Indianapolis, IN) and visualized by incubation with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Cytogenetic mapping of *P365*

The *P365* mutation was first localized by recombination mapping to the extreme distal end of the right arm of the third chromosome. Cytogenetic mapping of *P365*, performed using a series of deficiencies (Fisardi and MacIntyre, 1984), showed that the same two deficiencies, *ca-52* and *ca-165p*, used to determine the chromosomal position of *trp* (Wong et al., 1989), also identified the chromosomal position of *P365*, localizing both *P365* and *trp* to the same two-band region, 99C5–6.

ERG phenotype of *P365*

Cytogenetic colocalization of *P365* and *trp* prompted careful comparisons of their phenotypes. Figure 1*A, a–d,* compares the ERGs obtained from *P365*/+ heterozygotes and *P365*/*P365* homozygotes with those of wild-type and the null *trp* mutant trp^{P343} *(Scott et al., 1997) at 7 d after eclosion. P365* homozygotes have very little ERG responses remaining (Fig. 1*Ac*). ERG responses of the *P365* heterozygotes are intermediate between those of wild type and the *P365* homozygote in amplitude and waveform (Fig. 1*Ab*), i.e., the phenotype is semi-dominant. Unlike the response of trp^{P343} (Fig. 1*Ad*), however, responses of *P365* mutants are not "transient" but have a maintained component that persists throughout the duration of the stimulus (Fig. 1*Ab,c*). Even the small responses obtained from *P365*/*P365,* *P365*/trp^{P343}, or *P365*/trp^{CM}, none of which carries a wild-type *trp* allele, do not decay, but are maintained throughout the duration of the stimulus (Fig. 1*Ac,e,f*).

The *P365* mutation causes rapid photoreceptor degeneration

One of the striking features of the *P365* mutant is extremely rapid photoreceptor degeneration. To characterize this rapid degeneration, both *P365* homozygotes and heterozygotes were first examined for their dpp as a function of age, and, in addition, by transmission electron microscopy (EM) at selected time points. The dpp consists of superposed virtual images of rhabdomere tips in a group of neighboring ommatidia observed microscopically in the living fly (Franceschini, 1972). As photoreceptors degenerate, the superposition of rhabdomere tips can no longer be maintained, and the dpp disappears. In *P365*/*P365* homozygotes, no dpp was detectable even at eclosion, regardless of whether they were raised in DD or LD cycles (data not shown). In *P365*/+ heterozygotes, on the other hand, the dpp was intact in all flies in a sample population shortly after eclosion, but disappeared in a progressively larger fraction of the sample with age. Figure 1*B* plots the time course of disappearance of the dpp in *P365*/+ heterozygotes, marked with the mutation *white (w)* to remove the red screening pigments, under LD or DD illumination conditions. The dpp disappeared with a $t_{1/2}$ of ~1.5 d in LD illumination cycles, whereas it disappeared with a $t_{1/2}$ of ~4.5 d in DD

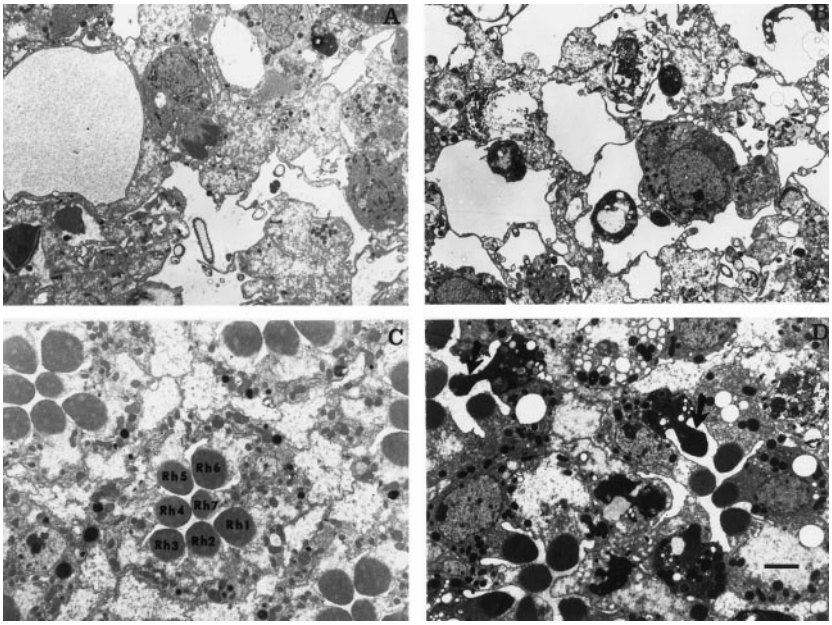

Figure 2. Electron micrographs of transverse sections of *P365* mutant retinas near the R7 and R8 rhabdomere boundary. Retinas of *P365* homozygotes raised in DD (*A*) and LD (*B*) at 0 d after eclosion. Retinas of *P365*/+ heterozygotes raised in DD (*C*) or LD (*D*) cycles at 2 d after eclosion. In *D*, some photoreceptors are missing, and some stain darkly (*arrows*). All flies were raised at 25°C. Rh1,.., Rh7: Rhabdomeres of R1,.., R7 photoreceptors. Scale bar, 2 μm.

illumination cycles (Fig. 1*B*). By contrast, no evidence of dpp deterioration was detectable in homozygous *w;; trp* mutants at such a young age, regardless of whether they were raised in LD or DD illumination cycles (data not shown). It should be noted, however, that the mutant ERG phenotype was already present at eclosion even in *P365*/+ heterozygotes and gradually deteriorated further with age.

Shown in Figure 2 are electron micrographs of transverse sections through the photoreceptor layer of *P365* homozygotes and heterozygotes at 0 and 2 d after eclosion, respectively, raised in DD or LD cycles at 25°C. In either illumination condition, the photoreceptors of *P365* homozygotes were already so severely degenerated at 0 d after eclosion that no recognizable rhabdomeres remained when viewed in sections from relatively proximal levels (~50–60 μm from the distal tips of rhabdomeres) (Fig. 2*A,B*). In *P365* heterozygotes raised in complete darkness, on the other hand, the photoreceptors appeared quite normal even at 2 d after eclosion (Fig. 2*C*). In *P365* heterozygotes of the same age raised in LD cycles, however, photoreceptors began to show evidence of degeneration. In many ommatidia, one or more rhabdomeres were missing (Fig. 2*D*), and some photoreceptors stained darkly, indicating that they were degenerating (Fig. 2*D, arrows*).

Substantial amounts of TRP remain in *P365*

Immunodetectable amounts of TRP in *P365* were also different from those in previously identified *trp* mutants. In striking contrast to all known *trp* mutants on which Western blot analysis has been performed (Montell and Rubin, 1989; Wong et al., 1989), a substantial amount of the TRP protein was present in young *P365* homozygotes. Figure 3 compares the immunodetectable TRP protein levels in *P365* homozygotes (lanes 3 and 6) with those in trp^{P301} (lanes 1 and 4) and wild-type (lanes 2 and 5) a day before eclosion (lanes 1–3) and within the first day after eclosion (lanes 4–6). In trp^{P301}, no TRP protein was detectable at either age, consistent with previous reports (Montell and Rubin, 1989; Wong et al., 1989; Pollock et al., 1995). In *P365* homozygotes, on the other hand, the TRP protein began to be detectable late in the pupal stage [5.7 ± 3.3% (n = 5) of wild-type level a day before eclosion] (Fig. 3, *lanes 2, 3*). By 0 d after eclosion, it was 22.4 ± 11.5% (n = 7) of the wild-type level (Fig. 3, *lanes 5, 6*).

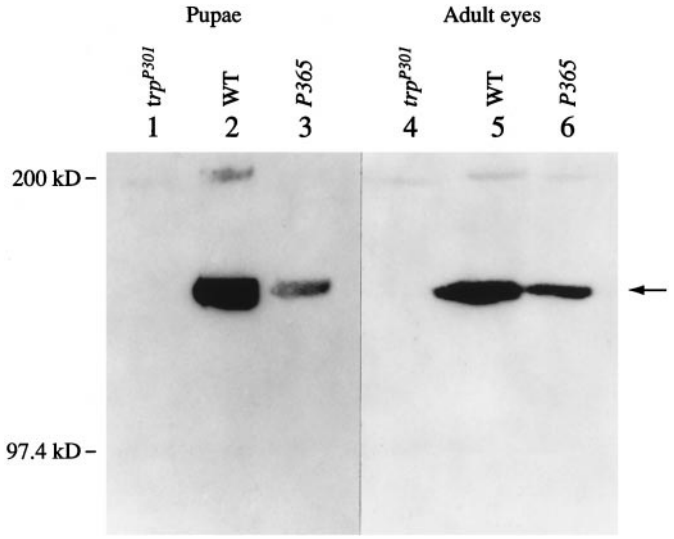

Figure 3. Western blot analysis of the *P365* mutant and controls (wild-type and trp^{P301}) at late pupal and early adult stages. *Lanes 1–3* were loaded with total pupal protein homogenates prepared from five pupae per lane harvested on the last day of pupal life, and *lanes 4–6* were loaded with total eye protein homogenates prepared from 10 eyes per lane dissected out within the first day after eclosion. *Lanes 1, 4,* trp^{P301}; *lanes 2, 5,* wild type; *lanes 3, 6, P365*. The blots were labeled with a monoclonal anti-TRP antibody.

Immunofluorescence confocal microscopy

Although electron microscopy has provided evidence that the rhabdomeres are all gone at 0 d after eclosion in *P365* (Fig. 2*A,B*), substantial amounts of the TRP protein are present in the same mutant at the same age under the same illumination conditions. Obvious questions are where the TRP protein remaining in *P365* is located and whether the TRP protein is correctly targeted to the rhabdomeres in *P365*, as in wild type.

Retinal degeneration in mutant fly eyes does not proceed uniformly throughout the length of the ommatidia. Instead, de-

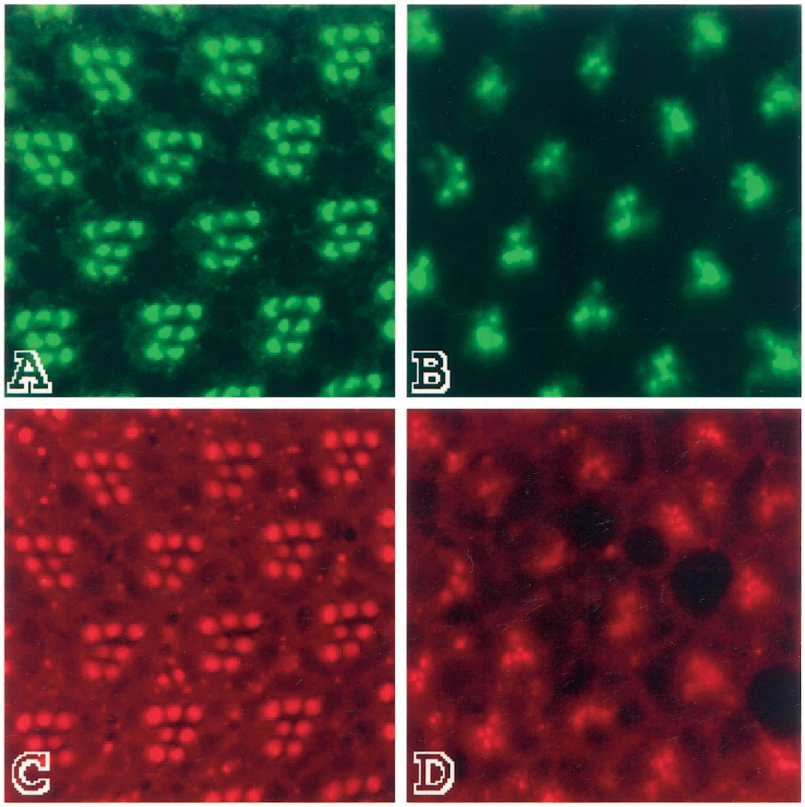

Figure 4. Localization of the TRP protein in *P365* and wild-type rhabdomeres by immunofluorescence confocal microscopy. *A, C,* Confocal micrographs of the same group of wild-type rhabdomeres double-labeled with a TRP antiserum and phalloidin and visualized for TRP labeling (*A*) and phalloidin labeling (*C*), respectively. *B, D,* Confocal micrographs of the same group of *P365* rhabdomeres double-labeled with a TRP antiserum and phalloidin and visualized for TRP labeling (*B*) and phalloidin labeling (*D*), respectively. All flies were marked with the mutation *white* to remove the screening pigments in the eye. The optical sections were <1-μm-thick near the distal tips of the rhabdomeres.

generation is more severe, at any given age, the more proximal the region of retina being examined (Leonard et al., 1992). Because the electron micrographs in Figure 2 were obtained from sections taken at a depth of about 50–60 μm from the distal tips of the rhabdomeres, it is possible that some residual rhabdomeres remain in the distal region of the ommatidia. Moreover, the large variations in the amount of the TRP protein among individual *P365* mutants (previous section) suggest that degeneration may be proceeding very rapidly at 0 d after eclosion, making it likely that even a few hours difference in age might make a large difference in the extent of degeneration.

Immunofluorescence confocal microscopy was performed to address the above questions making sure that very young flies (<2 hr after eclosion) were used and optical sections were taken near the distal tips of the rhabdomeres. Figure 4 compares confocal micrographs of the retinas of *P365* homozygotes (*B,D*) with those of wild type (*A,C*). The retinas were double-labeled with an anti-TRP antiserum (*A,B*) and phalloidin, which labels filamentous actin in the rhabdomeres (*C,D*). Extensive degeneration of rhabdomeres is evident in *P365* (*B,D*). However, it is also clear that some residual rhabdomere tips remain in virtually every ommatidium of *P365* at this age. Moreover, the TRP protein correctly localizes to the rhabdomeres in *P365* (*B*). It thus appears that the TRP protein detected in young *P365* homozygotes likely arises largely from the protein contained in the residual distal rhabdomere tips that still remain.

Are *P365* and *trp* allelic?

Genetic complementation tests

Although the phenotypes of *P365* and known *trp* mutants are very different, their cytogenetic colocalization raised the possibility that they might be allelic. To see if they are allelic, genetic complementation between *P365* and *trp* was tested by examining the ERG phenotypes of flies heterozygous for *P365* and several *trp* alleles (trp^{P301}, trp^{P343}, *and* trp^{CM}). ERGs recorded from all these heterozygous combinations of *P365* with *trp* alleles (shown for two in Fig. 1*Ae,f*) were much smaller in amplitude than those of *P365*/+ heterozygotes (Fig. 1*Ab* vs *e, f*) but somewhat bigger than those of *P365*/*P365* homozygotes (Fig. 1*Ac*). Results suggested that *P365* and *trp* do not complement, i.e., they are alleles of the same gene. However, the interpretation was complicated by the semi-dominance of *P365*. Therefore, the experiments detailed below and illustrated in Figure 4 were performed to obtain definitive answers.

Transgenic flies carrying P365 or trp^+ transgene

Two types of experiments involving transgenic flies were performed to determine whether or not *P365* is an allele of *trp*. In one, the *trp* gene isolated from the *P365* mutant was reintroduced into wild type, and in the other, the wild-type *trp* gene was introduced into the *P365* mutant.

P365 transgene in a wild-type background. Because *P365* is semidominant, a P-element construct containing the *trp* gene isolated from the *P365* mutant, designated P[*P365*], would be expected to induce a mutant phenotype in a wild-type background, if, and only if, *P365* is an allele of *trp*. Figure 5*A* shows the ERGs recorded from transgenic flies carrying one (Fig. 5*A, middle trace*) or two copies (Fig. 5*A, bottom trace*) of the P[*P365*] transgene in a wild-type background at 7 d after eclosion. Note that the former carries one copy of P[*P365*] and two copies of wild-type *trp* (trp^+), whereas the latter carries two copies of P[*P365*] and two copies of trp^+. One copy of P[*P365*] was sufficient to alter the ERG so that it had a slightly reduced amplitude and a much slower time course of decay at stimulus offset than wild type (Fig. 5*A, middle trace*). With two copies of P[*P365*], the ERG was very similar to that of *P365* heterozygotes both in amplitude and

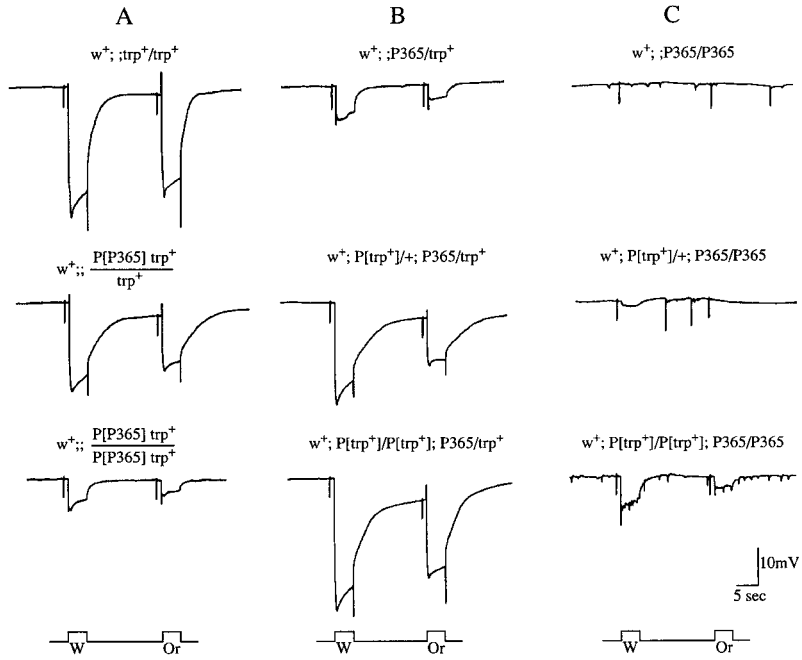

Figure 5. Induction of the *P365* ERG phenotype in wild type using a *P365* transgene and rescue of the *P365* ERG phenotype using a trp^+ transgene. *A*, Conversion of the wild-type phenotype (*top*) into a partial *P365* phenotype by the introduction of one (*middle*) or two (*bottom*) copies of the P element transformation vector carrying the *P365* allele of the *trp* gene P[*P365*] into a wild-type background. *B,* Rescue of the heterozygous *P365*/+ phenotype (*top*) using one (*middle*) or two (*bottom*) copies of the P element transformation vector carrying a wild-type allele of the *trp* gene, P[trp^+]. *C,* Partial rescue of the homozygous *P365*/*P365* phenotype (*top*) using one (*middle*) or two (*bottom*) copies of P[trp^+]. Unlike most other flies used in this work, these flies had normal red eyes. The Carnegie 3 vector used for the generation of the P[trp^+] transgene carries the *rosy* (*ry*) gene as a marker, which is detected in a ry^- and w^+ background. Consequently, all experimental flies in *B* and *C,* carrying this vector, had normal red eyes. The pCaSpeR-3 vector used for the generation of the P[*P365*] construct, on the other hand, carries a *mini-white* gene detected in a w^- background. Experimental flies in this case had eye colors of varying shades of red. To remove a possible source of variations in the ERG size or shape, these flies were made fully red-eyed by introducing the *w* gene (*A*). All control flies in the top row also had normal red eyes. The age of flies and stimulus protocol were as in Figure 1*A*.

waveform (Fig. 5, compare *A, bottom trace* with *B, top trace*). In both cases, the ratio of trp^+ to *P365* copy numbers was one.

trp^+ transgene in a mutant background. Conversely, we introduced wild-type copies of the *trp* gene into the *P365* mutant in an attempt to rescue the mutant phenotype. For this purpose, a transgenic line carrying a trp^+-containing P-element construct, designated P[trp^+], (Montell et al., 1985) was crossed with *P365* to generate transgenic lines carrying either one or two copies of the P[trp^+]-containing chromosome in either a *P365* heterozygous or homozygous background (see Materials and Methods). Results of ERG recordings from transgenic flies carrying P[trp^+] in heterozygous and homozygous *P365* backgrounds are presented in Figure 5, *B* and *C*, respectively. The ERGs of wild type, *P365*/+, and *P365*/*P365* are shown as controls in the top row of Figure 5. These look slightly different from those shown in Figure 1*A* because normal red-eyed flies were used for Figure 5 as opposed to white-eyed flies used for Figure 1*A* (see Fig. 5 legend).

Adding one copy of P[trp^+] to a *P365*/+ background substantially restored the ERG of the transgenic flies to that of wild type (Fig. 5*B, middle trace*). The ERG was remarkably similar to that obtained from transgenic flies carrying one copy of P[*P365*] in a wild-type background (Fig. 5*A, middle trace*) in that both ERGs tended to be smaller and to recover more slowly than that of wild type. Note that both classes of flies carried one copy of *P365* and two copies of trp^+. Adding two copies of P[trp^+] to a *P365*/+ heterozygous background (three copies of trp^+ and one copy of *P365*) virtually restored the ERG phenotype of the transgenic flies to that of wild type except for the slightly slower-than-normal return to baseline after the stimulus offset (Fig. 5, compare *B, bottom trace* with *A, top trace*).

In the experiments illustrated in Figure 5*C*, one or two copies of P[trp^+] were introduced into a homozygous *P365*/*P365* background. No response at all could be obtained from control *P365*/*P365* homozygotes (Fig. 5*C, top trace*). In contrast, transgenic flies carrying one copy of P[trp^+] in a homozygous *P365* background (one copy of trp^+ and two copies of *P365*) responded with very small ERGs (Fig. 5*C, middle trace*). If two copies of P[trp^+] were introduced into the same background (two copies of trp^+ and two copies of *P365*), ERG responses approaching those of *P365*/+ heterozygotes in size could be recorded (Fig. 5, compare *C, bottom trace* and *B, top trace*). Thus, the *trp* gene cloned from *P365* reproduces the *P365* mutant phenotype in transgenic flies, unequivocally demonstrating that the cloned *trp* gene harbors the molecular defect responsible for the mutant phenotype.

Deep pseudopupil measurements showed that the severity of the dpp phenotype varied in a manner consistent with the ERG phenotype in these transgenic flies, depending approximately on the trp^+/*P365* copy number ratio (data not shown). The above series of experiments established conclusively that mutations in the *trp* gene of *P365* are solely responsible for the *P365* mutant phenotypes. Thus, *P365* is a semi-dominant allele of the *trp* gene and, henceforth, will be designated Trp^{P365}, where *T* is capitalized to indicate that the mutation is dominant.

Molecular alterations in the Trp^{P365} gene

To identify mutations in the *trp* gene of Trp^{P365}, a 6.4 kb genomic DNA fragment that contains the entire *trp* gene (Montell et al., 1985) was isolated from the Trp^{P365} mutant and sequenced (Materials and Methods). Results identified 11 nucleotide differences predicted to cause amino acid differences between the Trp^{P365} coding sequence and the published wild-type *trp* sequence (Montell and Rubin, 1989; Wong et al., 1989). Seven of these 11 alterations were also found in the wild-type strains, Oregon R and Canton S. Thus, these probably represent strain-specific polymorphisms between the wild-type strain from which the published sequence was derived and the wild-type strain from which the Trp^{P365} mutant was isolated (Oregon R). The remaining four amino acid changes, Pro(500)Thr, His(531)Asn, Phe(550)Ile, and Ser(867)Phe, are shown schematically in Figure 6. The first two amino acid alterations, Pro(500)Thr and His(531)Asn, immediately flank the fourth transmembrane segment, S4, and the third change, Phe(550)Ile, is within the fifth transmembrane segment.

Single-cell functional analysis by whole-cell recordings

To investigate the mechanism underlying the Trp^{P365} phenotype, whole-cell patch-clamp recordings were performed on R1–6 pho-

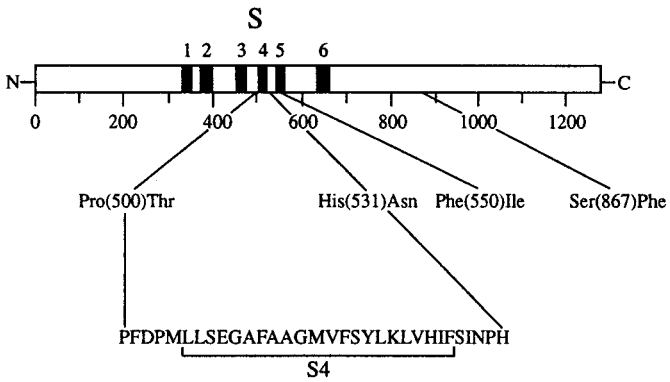

Figure 6. Deduced amino acid substitutions in the TRP protein of the Trp^{P365} mutant. The TRP protein is presented schematically at the *top*, with the transmembrane segments labeled S1 through S6 and amino acid positions labeled below (modified from Chevesich et al., 1997). The amino acid substitutions detected in Trp^{P365} are shown in the *middle*, with *thin lines* connecting the labels with the positions of the altered amino acids in the protein. The full amino acid sequence between the first two mutations is provided at the *bottom*. The fourth transmembrane segment sequence is labeled S4.

toreceptors (Hardie, 1991a,b; Ranganathan et al., 1991; Hardie and Minke, 1992) using dissociated ommatidial preparations of pupae and <1-hr-old adults of the following genotypes: Trp^{P365}/Trp^{P365} homozygotes, $Trp^{P365}/+$ heterozygotes, the heteroallelic combination, Trp^{P365}/trp^{CM}, and wild-type. The light-induced currents (LICs) were examined first. The LIC of the heterozygote $Trp^{P365}/+$ was indistinguishable from that of wild type at these ages (Fig. 7*A, left trace*). In contrast, photoreceptors of Trp^{P365}/Trp^{P365} homozygotes did not respond to light of any intensity at either age (Fig. 7*A, right trace*). In the case of Trp^{P365}/trp^{CM}, raised at 19°C and examined at P15 pupal stage (Hardie et al., 1993), a large subset of photoreceptors responded to light with smaller than normal responses (peak amplitude of 136 ± 92 pA vs 1518 ± 98 pA in wild type, in response to maximal intensity orange stimuli). The remaining subset (43%, $n = 7$) did not respond to light at all (data not shown). In <1-hr-old adults of Trp^{P365}/trp^{CM} raised at 19°C, however, the great majority (93%) of cells did not respond to light (Fig. 7*A, middle trace*) as in Trp^{P365}/Trp^{P365} homozygotes.

To investigate the reason for the inability of mutant cells to respond to light, membrane currents were recorded in the dark in response to voltage steps, given in 20 mV steps from a holding potential of −20 mV to obtain membrane potentials ranging between −100 and +80 mV, within 1 min after establishing the whole-cell configuration (Fig. 7*B*). In wild-type cells only small leak currents (75 ± 14 pA at −60 mV) were observed (Fig. 7*B, left traces*). Cells of $Trp^{P365}/+$ heterozygotes yielded similar results (data not shown). By contrast, in all light-insensitive mutant cells (Trp^{P365}/Trp^{P365} and Trp^{P365}/trp^{CM}) bathed in a medium with 1.5 mM Ca^{2+}, large outward currents with strong outward rectification and small but significant inward currents were recorded at positive and negative membrane potentials, respectively, (Fig. 7*B, middle* and *right traces*) from the moment the recording configuration was established. Recordings were difficult to obtain from photoreceptors of Trp^{P365}/Trp^{P365} homozygotes, presumably because most Trp^{P365}/Trp^{P365} cells had already degenerated by this age. However, all those Trp^{P365}/Trp^{P365} photoreceptors from which recordings could be made responded with large outward currents and small but significant inward currents (Fig. 7*B, right trace*). The inward current in Trp^{P365}/Trp^{P365} cells had an amplitude of 199 ± 33 pA at a holding potential of −60 mV. This current was significantly larger than the passive leak current which remained after the application of La^{3+} either in wild type or the mutant (97 ± 7 and 116 ± 26 pA, respectively, at −60 mV). The La^{3+}-sensitive component of this current, representing the constitutive TRP current, thus, was ~83 pA in Trp^{P365}/Trp^{P365}. A La^{3+}-sensitive component of similar magnitude was also found in Trp^{P365}/trp^{CM}. Thus, all photoreceptors that did not respond to light did respond to voltage steps with large outward currents and significant inward currents. These currents had properties very similar to those of the run down current (RDC) of wild-type photoreceptors, except that they were observed from the moment the recordings began and in a normal medium.

The RDC appears as a spontaneously occurring noisy inward current during prolonged whole-cell recordings of wild-type photoreceptors, presumably as a result of loss of regulation caused by metabolic exhaustion (Hardie and Minke, 1994a,b). It appears in almost every wild-type photoreceptor during prolonged (>10 min) recordings in a Ca^{2+}-free medium (Pollock et al., 1995; Arnon et al., 1997). It exhibits both inward and outward rectifications in a Ca^{2+}-free medium (Fig. 7*D, left trace*) but only the outward rectification and a small inward current in the presence of Ca^{2+} (Hardie and Minke, 1994a,b). Thus, a family of current–voltage curves obtained for the RDC at a normal external concentration of Ca^{2+} is very similar to those of the constitutive currents of Trp^{P365}/trp^{CM} or Trp^{P365}/Trp^{P365} shown in Figure 7*B*. La^{3+}, a potent Ca^{2+} channel blocker that causes wild-type photoreceptors to mimic the *trp* phenotype (Hochstrate, 1989; Suss-Toby et al., 1991), completely blocks the RDC of wild-type photoreceptors (Hardie and Minke, 1994b), suggesting that the RDC reflects the activation of the TRP channels.

Similarities between the wild-type RDC and the constitutive mutant currents included current–voltage characteristics and reversal potentials (E_{rev}), determined from families of current–voltage relationships, at both normal and low Ca^{2+} levels (see Table 1 for E_{rev} values). At low Ca^{2+} levels, both the mutant currents and wild-type RDC displayed inward and outward rectifications and reversal potentials (E_{rev}) near zero (Table 1; Hardie and Minke, 1994b for properties of RDC). Increasing external Ca^{2+} reduced the inward rectification and shifted the E_{rev} to more positive values by similar magnitudes in both classes of currents (Table 1). The reversal potentials of the two classes of currents displayed, at either Ca^{2+} concentration, no significant difference at the 5% level in a *t* test (Table 1). Finally, application of 10 μM La^{3+} to either the Ca^{2+}-free or the Ca^{2+}-containing bathing medium blocked the RDC and mutant currents in a similar manner (Fig. 7*C*). The data indicate that the RDC of wild type and the constitutive current in Trp^{P365} are indistinguishable in their properties.

A question arises as to whether or not the constitutive current in the light-insensitive mutant cells is a secondary consequence of degeneration. To answer this question we searched for conditions in which dark currents were present but the cell degeneration had not yet begun. Because of technical reasons, whole-cell recordings could not be performed in preparations older than a few hours after eclosion, and we looked for preparations in which degeneration was just barely beginning at eclosion. Most photoreceptors in Trp^{P365}/trp^{CM} heterozygotes raised at 19°C have been shown to have the mutant currents in the dark shortly after eclosion (Fig. 7*B, middle traces, E*). We performed Western blot and EM anal-

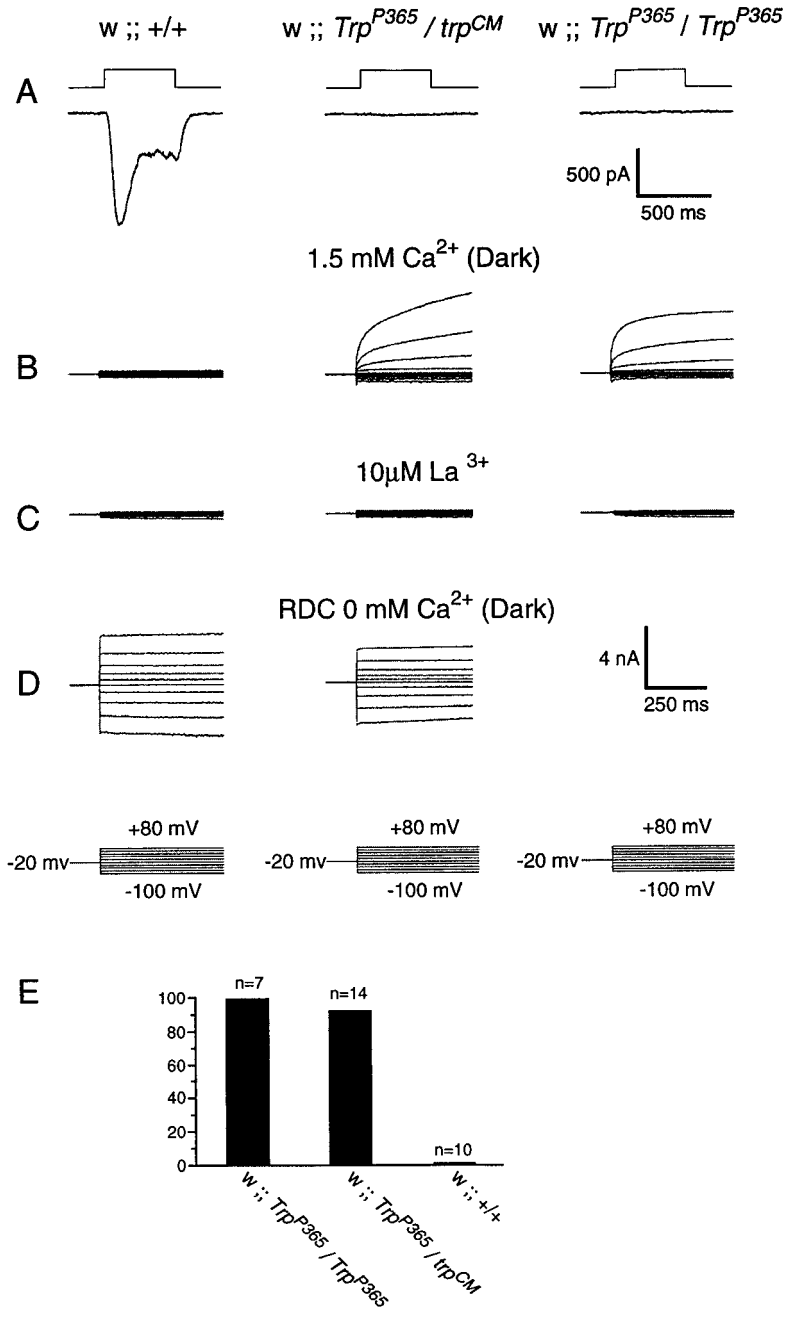

Figure 7. Single-cell functional analysis by whole-cell recordings. *A* shows a typical LIC of a wild-type cell (*left trace*) in response to an orange stimulus (OG 590 sharp-cut filter, 1 log unit neutral density filter) and the absence of any responses in Trp^{P365}/trp^{CM} and Trp^{P365}/Trp^{P365} (*middle* and *right traces*). The duration of the orange light stimulus is indicated above each trace. *B–D* compare families of current traces elicited by series of voltage steps from photoreceptors of wild type (*left column*), the light-insensitive cells of Trp^{P365}/trp^{CM} (*middle column*), and Trp^{P365}/Trp^{P365} homozygotes (*right column*). For each experiment, a series of nine voltage steps was applied from a holding potential of −20 mV in 20 mV steps (*D, bottom traces*). *B,* Membrane currents were recorded 30 sec after establishing the whole-cell configuration with physiological concentrations (1.5 mM) of Ca^{2+} in the bath. *C,* Application of 10 mM La^{3+} to the bath suppressed the membrane currents. *D,* Membrane currents obtained with 0 mM Ca^{2+} in the bath. In the case of wild type, the RDC is shown (*left traces*), which was allowed to develop by holding the cell in whole-cell configuration in a Ca^{2+}-free medium for 12 min. In the mutant, the constitutive currents were recorded as soon as the whole-cell configuration was established. The currents shown were recorded 25 sec after establishing the whole-cell configuration. *E,* A histogram summarizing the data illustrated in Figure 7*B* from several different flies. The number of cells with constitutive activity at the time of establishing the whole-cell configuration is shown for each genotype.

Table 1. Comparison of reversal potentials (E_{rev}) of mutant currents with those of wild-type RDC (in mV)

	Wild-type	Trp^{P365}/trp^{CM}	Trp^{P365}/Trp^{P365}
1.5 mM Ca^{2+}	6.98 ± 0.83 (n = 7)	5.26 ± 0.67 (n = 10)	8.68 ± 1.30 (n = 6)
~0 mM Ca^{2+}	−0.52 ± 0.52 (n = 4)	−0.39 ± 0.39 (n = 13)	

yses on these mutants to look for any evidence of photoreceptor degeneration. The amount of TRP in the Trp^{P365}/trp^{CM} heteroallelic mutants detected by Western blot at eclosion (Fig. 8*A, lanes 3, 4*) was ~35% less than that of wild type (lane 1) or $Trp^{P365}/+$ (lane 7) whether the mutants were raised at 19 or 24°C (Table 2). In Trp^{P365}/Trp^{P365} (Figs. 3, 8*A, lane 2*), on the other hand, the amount of the TRP protein was substantially reduced compared to wild type (Table 2). The larger amount of TRP in Trp^{P365}/trp^{CM} relative to Trp^{P365}/Trp^{P365} could not be attributed to the presence of trp^{CM} because trp^{CM}/trp^{CM} homozygotes, raised at either temperature, had a highly reduced level of TRP (Fig. 8*A, lanes 5, 6*; Table 2). The above results thus suggested that degeneration is responsible for the reduced TRP level in Trp^{P365}/Trp^{P365} homozygotes, and the relatively normal amount of TRP in Trp^{P365}/trp^{CM} reflected the absence of degeneration in this mutant. Consistent with this interpretation, EM sections showed that, unlike Trp^{P365} homozygotes (Fig. 8*C*), newly eclosed Trp^{P365}/trp^{CM} showed virtually no signs of photoreceptor degeneration (Fig. 8*B*) when both were raised at 19°C, even though the TRP channels of most mutant cells had already developed the

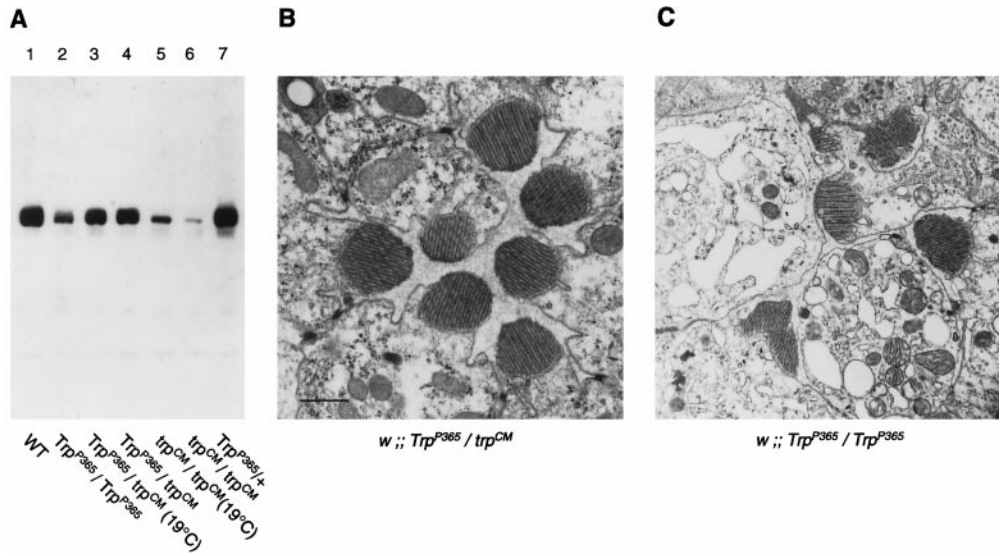

Figure 8. Western and EM analyses of Trp^{P365}/trp^{CM} and controls. *A,* Western blot analyses of the heteroallelic mutant Trp^{P365}/trp^{CM} (*lanes 3, 4*) and controls: wild type (*lane 1*), Trp^{P365}/Trp^{P365} homozygotes (*lane 2*), $Trp^{P365}/+$ heterozygotes (*lane 7*), and trp^{CM}/trp^{CM} (*lanes 5, 6*). For Trp^{P365}/trp^{CM} and trp^{CM}/trp^{CM}, one of the two lanes contained samples prepared from flies raised at 19°C (so indicated). All other samples were from flies raised at 24°C. *B, C,* Electron micrographs of transverse sections through the ommatidial layer (at the level of R7 photoreceptor nuclei) of Trp^{P365}/trp^{CM} (*B*) and Trp^{P365}/Trp^{P365} (*C*), both raised at 19°C. All samples were obtained from newly eclosed adult flies, and all flies were marked with *w*. Scale bar, 1 μm. The Trp^{P365}/Trp^{P365} mutant retina appears less degenerated in *C* above than in the sections shown in Figure 2, *A* and *B*. The main reason is that the flies used for the above micrograph were raised at 19°C, whereas those used for Figure 2 were raised at 25°C. In addition, sections shown in Figure 2 were obtained from much more proximal levels of the retina than the one shown above, and, in fly eye, degeneration proceeds from the proximal to distal direction.

Table 2. TRP protein levels in wild type and flies carrying various *trp* allele combinations (% of wild type)

Wild type	Trp^{P365}/Trp^{P365}	Trp^{P365}/trp^{CM} (19°C)	Trp^{P365}/trp^{CM}	trp^{CM}/trp^{CM} (19°C)	trp^{CM}/trp^{CM}	$Trp^{P365}/+$
100	38.2 ± 13.8	64.8 ± 7.2	65.1 ± 7.7	30.4 ± 8.8	15.1 ± 5.9	102.5 ± 3.9

constitutive current. Several hours later, the mutant cells began showing membrane shedding at the tips of some microvilli, suggesting that degeneration had begun (data not shown).

DISCUSSION

The following lines of evidence established conclusively that Trp^{P365} is an allele of the *trp* gene: (1) Trp^{P365} maps to the same two-band region, 99C5–6, as the previously identified *trp* mutations; (2) Complementation tests between Trp^{P365} and previously identified *trp* alleles yielded results consistent with their being allelic; (3) A wild-type *trp* transgene, when introduced into the genome of the Trp^{P365} mutant, rescues the mutant phenotype in a trp^{+}/Trp^{P365} ratio-dependent manner; (4) A Trp^{P365} mutant transgene, when introduced into the genome of a wild-type fly, induces the mutant phenotype in the transgenic fly in a trp^{+}/Trp^{P365} ratio-dependent manner; (5) Mutations that would alter the TRP protein sequence are present in the coding region of the *trp* gene of the Trp^{P365} mutant.

Two main conclusions that follow from the above lines of evidence are: (1) the transient receptor potential phenotype is not an obligatory consequence of mutations in the *trp* gene; and (2) a certain mutation(s) in a critical region of the *trp* gene or a combination of mutations in the gene can cause massive, semidominant degeneration of photoreceptors even though such degeneration has never been observed in previously identified *trp* mutants.

We first consider what might be the reason for the maintained responses seen in Trp^{P365} during light stimulus but not in any of the previously identified *trp* mutants (Fig. 1*A*). A key difference between the Trp^{P365} mutant and previously identified *trp* mutants appears to be in the amount of functional TRP channel protein. All of the previously identified *trp* mutants tested for the amount of the TRP protein have been reported to be either TRP protein null in Western blot analysis (Montell and Rubin, 1989; Wong et al., 1989) or drastically reduced in TRP content (Pollock et al., 1995). Moreover, the mutant that has some TRP (trp^{CM}) is functionally null when raised at a temperature of 25°C (Reuss et al., 1997). By contrast, the TRP protein level in Trp^{P365}/Trp^{P365} is a substantial fraction of the normal level at eclosion, and it is nearly normal in Trp^{P365}/trp^{CM} (Fig. 8). Moreover, the protein is functional, albeit abnormally (Fig. 7). Thus, the classical transient receptor potential phenotype, long considered a hallmark of *trp* mutants, may be characteristic only of a null or near-null subset of *trp* mutants.

We next consider the possible origin of severe photoreceptor degeneration in Trp^{P365}. A hypothesis most consistent with results to date is that one or more of the mutations in the *trp* gene of Trp^{P365} greatly increases the probability of spontaneous Ca^{2+} entry through the TRP channel by affecting the regulation of channel opening. For this hypothesis to be valid, the mutations need only render the channel regulation sufficiently unstable to

allow a higher probability of unregulated opening of the mutant channels than wild-type channels, with the probability rising as the number of Trp^{P365} copies increases or the fly ages. The ensuing accumulation of Ca^{2+} could then lead to cell death through the activation of proteases.

The following lines of evidence support the hypothesis. To begin with, two of the four mutations in the Trp^{P365} coding region, Pro(500)Thr and His(531)Asn, immediately flank the fourth transmembrane segment (S4) of the TRP channel (Fig. 6). In voltage-gated channels, to which the TRP channel has sequence homology (Phillips et al., 1992), the S4 segment is thought to be a voltage sensor for the activation of the channel and is characterized by the repeat motif containing the charged residues, Arg or Lys (e.g., Armstrong and Hille, 1998). Because the TRP channel is not voltage-gated, its S4 segment does not contain the Arg/Lys motif, but it retains amino acid sequence similarity to S4 domains of voltage-gated channels (Phillips et al., 1992). It is possible that the S4 domain of the TRP channel is also involved in the regulation of channel opening, and mutations that affect the structure of the domain, such as Pro(500)Thr, could alter the channel regulation.

Secondly, the results of whole-cell recordings are in strong support of the above hypothesis. In recordings performed shortly after eclosion, a constitutive current mediated through the TRP channel is present, under conditions in which the RDC is not expected, only in mutants carrying the Trp^{P365} allele, Trp^{P365}/Trp^{P365} and Trp^{P365}/trp^{CM}. Moreover, since the constitutive TRP current is present but the photoreceptors have not yet degenerated in Trp^{P365}/trp^{CM} at the P15 pupal stage or immediately after eclosion (Fig. 8), the constitutive TRP current must precede photoreceptor degeneration, consistent with the idea that the constitutive TRP current is responsible for degeneration. Although the amplitude of the constitutive inward current under normal physiological conditions, i.e., normal external concentrations of Ca^{2+} and negative membrane potentials, is relatively small (Fig. 7*B*), it is significantly larger than the leak current and represents a sizable, steady Ca^{2+} influx ($\sim 10^9$ ions/sec; see Peretz et al., 1994b for quantitative estimate), sufficient to cause serious disruption of Ca^{2+} homeostasis.

Because the TRP channel is most likely to be a tetramer, the subunit composition of individual channels varies with *trp* allele combinations. We speculate that the probability of unregulated TRP channel opening very much depends on the wild-type/mutant subunit compositions of individual channels and that the probability in turn determines how early in development the constitutive currents appear. Thus, in Trp^{P365} homozygotes, all TRP channels consist of the mutant TRP(P365) protein subunits. The probability of unregulated opening is high, and the constitutive TRP activity begins early in the development of the phototransduction machinery. By the time of eclosion, essentially all photoreceptor cells are in an advanced state of degeneration. In Trp^{P365}/trp^{CM} raised at 19°C, on the other hand, the partially functional TRP(CM) protein also contributes to subunits of individual channels. The probability of unregulated channel opening is lower than in Trp^{P365} homozygotes. The constitutive TRP current is just developing at the time of recording, and photoreceptor degeneration has not yet begun or is only barely beginning. In $Trp^{P365}/+$ heterozygotes, in addition to Trp^{P365}, a wild-type allele of *trp* contributes to the subunit composition of individual channels. The probability of unregulated TRP channel opening is even lower than in Trp^{P365}/trp^{CM}. Indeed, the $Trp^{P365}/+$ phenotypes are less severe than those of Trp^{P365}/trp^{CM} both in terms of ERG (Fig. 1*Ab* vs *f*) and degeneration (data not shown), and the constitutive TRP current has not yet developed at the time of recording.

To obtain the kinds of information extracted from Trp^{P365}/trp^{CM} using $Trp^{P365}/+$ (Fig. 7*D*), it would have been necessary to carry out the recordings a day or so after eclosion, when the fraction of cells with constitutive TRP activity is substantial. However, because of technical reasons, whole-cell recordings had to be performed within an hour or so after eclosion. We therefore sought to identify heteroallelic combinations of Trp^{P365} with other *trp* alleles that would cause the constitutive current to just begin developing at eclosion. Of the several heteroallelic combinations tested, Trp^{P365}/trp^{CM} turned out to be the most informative.

Analysis of Trp^{P365} has provided evidence for a novel mechanism of massive retinal degeneration in *Drosophila* involving mutations in the *trp* gene. The TRP protein has been shown to be a member of a large family of related proteins conserved throughout animal phylogeny (for review, see Hardie, 1996; Minke and Selinger, 1996; Montell, 1997). Tissue distributions of mammalian TRP-related proteins have not yet been studied extensively. Available data indicate that a given TRP-related protein tends to be widely distributed with heavier expression in selected tissues (for review, see Montell, 1997). Most of the mammalian homologs on which information is available appear to be highly expressed in the brain (Wes et al., 1995; Zhu et al., 1995). Because certain mutational alterations of the TRP protein of *Drosophila* can result in the death of photoreceptor cells, it is not difficult to imagine that similar alterations in some of the mammalian TRP-related proteins may also result in the death of cells in which the proteins are expressed. In light of the heavy expression of some of the TRP-related mammalian proteins in the brain (Wes et al., 1995; Zhu et al., 1995), the brain could be a major site of vulnerability for such degenerative diseases.

In addition, the Trp^{P365} mutant should prove valuable in future studies of TRP channel function. For example, mutants especially informative for functional studies of the TRP channel are those bearing TRP proteins that are altered in function. Insofar as Trp^{P365} is the first identified *trp* allele that encodes a modified but functional TRP protein, it should prove valuable in exploring the essential mechanisms underlying the TRP-dependent light-activated conductance. Another possible use of the Trp^{P365} mutation is in screening for genetic modifiers of Trp^{P365}. A genetic modifier is a second-site mutation which, when generated on a Trp^{P365} background, induces a phenotype either more (enhancer) or less (suppressor) severe than that of Trp^{P365}. Isolation of such modifiers is important because they often identify genes encoding proteins that interact with TRP. Use of Trp^{P365}, rather than previously identified *trp* mutants, can considerably simplify modifier screening because of its degeneration phenotype, which can be assayed by examining the deep pseudopupil.

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