Mutation of Light-dependent Phosphorylation Sites of the *Drosophila* Transient Receptor Potential-like (TRPL) Ion Channel Affects Its Subcellular Localization and Stability*

**Background:** *Drosophila* TRPL is a cation channel of the phototransduction cascade that undergoes light-dependent subcellular translocation between cell compartments.

**Results:** *Drosophila* TRPL exhibits a light-dependent phosphorylation pattern required for its stable localization in the rhabdomere of photoreceptor cells.

**Conclusion:** Multiple phosphorylation sites control localization and stability of TRPL.

**Significance:** A member of the TRP ion channel family displays a complex phosphorylation pattern with functional relevance.

The *Drosophila* phototransduction cascade terminates in the opening of the ion channel transient receptor potential (TRP) and TRP-like (TRPL). Contrary to TRP, TRPL undergoes light-dependent subcellular trafficking between rhabdomeric photoreceptor membranes and an intracellular storage compartment, resulting in long term light adaptation. Here, we identified *in vivo* phosphorylation sites of TRPL that affect TRPL stability and localization. Quantitative mass spectrometry revealed a light-dependent change in the TRPL phosphorylation pattern. Mutation of eight C-terminal phosphorylation sites neither affected multimerization of the channels nor the electrophysiological response of flies expressing the mutated channels. However, these mutations resulted in mislocalization and enhanced degradation of TRPL after prolonged dark-adaptation. Mutation of subsets of the eight C-terminal phosphorylation sites also led to a reduction of TRPL content and partial mislocalization in the dark. This suggests that a light-dependent switch in the phosphorylation pattern of the TRPL channel mediates stable expression of TRPL in the rhabdomeres upon prolonged dark-adaptation.

Ion flux across cell membranes is controlled by regulating the transition between the open and closed state of ion channels and by adjusting the number of ion channel molecules in the plasma membrane. The latter is achieved by regulation of the subcellular trafficking and turnover of ion channels and has major impact on the physiological properties of neurons. For example, regulated subcellular translocation of AMPA-type glutamate receptors to synapses and NMDA receptor trafficking underlie the formation of long term potentiation and long term depression required for learning-related synaptic plasticity (1–3). Both gating and subcellular translocation of ion channels may be regulated by reversible protein phosphorylation, which often occurs in a complex pattern. For example, in the major delayed rectifier potassium channel Kv2.1 a mass spectrometry analysis identified 16 phosphorylation sites, a subset of which influences voltage-dependent gating of this channel (4). For another potassium channel, Kv1.2, a trafficking-dependent phosphorylation at multiple sites has been shown to regulate cell surface expression of the channel (5).

Transient receptor potential (TRP) channels, which are widely expressed in the animal kingdom and constitute a heterogeneous family of calcium permeable channel families, also display protein phosphorylation (6). TRP channels contain six transmembrane domains and cytosolic N and C termini. The founding member of the TRP protein family, the *Drosophila* TRP channel, together with its homolog TRPL (TRP-like) is expressed in photoreceptor cells of the fly compound eye and generates the receptor potential in the visual transduction cascade (7–9). A mass spectrometry analysis of posttranslational TRP modifications identified 21 phosphorylation sites (10). One of the light-dependent phosphorylation sites of TRP, Ser-982, was shown to be critical for the proper deactivation of the photoreceptor potential (11), whereas the physiological function of the other phosphorylation sites is still elusive. In contrast to TRP, TRPL undergoes subcellular trafficking in a light-dependent manner (12). In the dark, TRPL is located in the light-absorbing photoreceptor compartment, which is a microvillar structure in the apical portion of fly photoreceptor cells referred to as rhabdomere. Upon illumination, TRPL is first transported to the base of the rhabdomere within 5–10 min and then becomes internalized to a yet unknown intracellular compartment in the cell body via Rab5- and Rab4-dependent vesicle transport within several hours (13, 14). Unlike rhodopsin, which is degraded in the lysosome after its light-triggered internalization, TRPL does not enter the lysosomal pathway but is stored...
in the cell body and redistributed to the rhabdome when the flies are transferred from light to darkness (12, 15). Internalization of TRPL depends on a functional phototransduction cascade and is triggered by calcium influx through the TRP channel (16, 17). Hence, in mutants with severe defects in the phototransduction cascade or in the absence of TRP, TRPL fails to translocate from the rhabdome to the cell body upon illumination.

In the present study we used mass spectrometry and identified nine phosphorylation sites in the TRPL protein, one located near the N terminus of the protein and eight located in the C-terminal region of the channel. The phosphorylation pattern of these sites changed upon prolonged light or dark adaptation. We mutated the eight C-terminal phosphorylation sites or subsets of these phosphorylation sites and expressed the mutated channels in photoreceptor cells of transgenic Drosophila. Mutation of the eight C-terminal phosphorylation sites resulted in mislocalization and loss of TRPL protein from the rhabdome of dark-adapted flies. We propose a model in which the subcellular translocation of TRPL is associated with different phosphorylation states that regulate TRPL retention in the rhabdomeres and prevent its degradation.

EXPERIMENTAL PROCEDURES

Fly Stocks and Husbandry—The following strains of Drosophila melanogaster were used: yw, w[1118] Oregon R (here referred to as wild type), yw; trpl[1118] 1/81, bw, trpl[1118], trp[1118] (18), w[1118]· trpl[1118] 1/81, bw, trpl[1118], trp[1118] (19), yw; P[Rh1 > TRPL > eGFP,y+ ] (16). Transgenic flies expressing phosphorylation-deficient TRPL ion channels (yw; P[Rh1 > TRPL3x-eGFP,y+ ], yw; P[Rh1 > TRPL5x-eGFP,y+ ], yw; P[Rh1 > TRPL8x-eGFP,y+ ], yw; P[Rh1 > TRPL8xD-eGFP,y+ ], yw; P[Rh1 > TRPL5xD-eGFP,y+ ], yw; P[Rh1 > TRPL8xD-eGFP,y+ ] ) that were generated in this study were crossed into a trp[1118] or a trp[1118] mutant background using standard Drosophila genetics (20). Flies were raised at 25 °C on standard cornmeal food and used at an age of 3–5 days after eclosion for all experiments. For determining light-induced ion channel translocation, flies were kept in the dark for 4–5 days and were then illuminated with orange light (acrylic glass cut off filter transmitting light >560 nm, ~200 Lux) for 16 h. Dark-raised flies were dissected under dim red light (Schott RG 630, cold light source KL1500, Schott), whereas light-raised flies were dissected under white light.

Generation of TRPL Constructs—For mutagenesis of phosphorylation sites, a fragment containing the C-terminal region and part of the linker sequence between TRPL and eGFP (amino acids 546–1130) was PCR-amplified from the TRPL-eGFP construct generated by Meyer et al. (16) and was sub-cloned into pBluescript II SK (Stratagene). Mutagenesis was performed in pBluescript II SK using the QuikChange multi site-directed mutagenesis kit (Agilent). The mutated fragments were re-inserted in the pENTR-TRPL-eGFP vector using AvrII and AgeI restriction sites. For generation of the TRPL8x-construct without eGFP tag, the TRPL8x-eGFP construct in the pENTR vector was used as a template for PCR amplification of the C-terminal region. The 3’-primer was modified to reconstitute the original TRPL stop codon and to introduce a NotI restriction site. After AvrII/NotI digestion, the TRPL8x C-terminal fragment was cloned into pENTR-TRPL-eGFP vector, thereby exchanging the C-terminal region and the eGFP tag with the mutated TRPL-C terminus of TRPL8x. Using the Gateway system (Invitrogen), the mutagenized TRPL constructs in pENTR vector were recombined with a modified pYC4 vector containing a DEST cassette between the rhodopsin 1 minimal promoter (base pairs −833 to +67) and the 3’ untranslated region of rhodopsin (17). All clones were verified by DNA sequencing (Qiagen). P-element-mediated transformation of Drosophila was carried out as described previously (21). The constructs were injected into yw or yw; trpl[1118] embryos.

Immunoprecipitation of TRPL Channels—Protein extracts of 80 fly heads per sample for immunoprecipitation with α-GFP or 300 fly heads per sample for immunoprecipitation with α-TRPL were obtained as described (10). 2 μl of extraction buffer was used per fly head. In addition, the homogenates were subjected to sonication for 5 min before their extraction on ice for 30 min. Head extracts were incubated with 4 μg of α-GFP (Roche Applied Science) or 7 μg of α-TRPL antibodies (12) coupled to 35 μl of protein G (Roche Applied Science)- or protein A-agarose beads (Thermo Fisher Scientific), respectively, overnight at 4 °C. Wash and elution steps were performed as previously described (10) except that 0.1% instead of 1% Triton was used in the wash buffer. For subsequent mass spectrometry analysis the proteins bands were visualized by colloidal Coomassie Blue staining (Roth), whereas for multimerization studies the proteins were electropheretically transferred to PVDF membranes.

λ-Phosphatase Treatment—Protein extracts were generated as described for immunoprecipitation except that no phosphatase inhibitors were added to the extraction buffer. 200 units of λ-phosphatase, phosphatase buffer, and MgCl2 (New England Biolabs) were added to 25 μl of the supernatant. These samples and the untreated extracts were incubated at 30 °C for 1 h. Aliquots of the samples containing proteins equivalent to four Drosophila heads were subjected to immunoblot analysis using 5% polyacrylamide gels (Bio-Rad). Samples were not boiled before gel electrophoresis.

Gel Electrophoresis and Immunoblot Analysis—Protein extracts were generated as described (10). 4 μl of SDS extraction buffer was used per fly head, and extraction at room temperature was extended to 1 h. Immunoblot analysis was carried out as described (10) using anti-TRPL (12) and anti-β-tubulin antibodies (Developmental Studies Hybridoma Bank, University of Iowa). The ECL immunoblotting system was used for signal detection with the ChemiDocXRS+ Imaging system (Bio-Rad). For a quantitative analysis of TRPL expression, the TRPL and β-tubulin signals were quantified with Image Lab 4.0 (Bio-Rad) by determining the integrated density of each protein band, i.e. the sum of the values of the pixels in the protein band. For normalization, the values obtained for TRPL were divided by the anti-β-tubulin values of the same sample.

Mass Spectrometry—Mass spectrometry and data analysis was performed as described before (10), except that a longer gradient (90 min) was used for peptide elution from the HPLC column, survey spectra were recorded in the Orbitrap detector.
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in the m/z range of 250 to 2000, and data-dependent tandem mass spectra were generated for the seven most abundant peptide precursors in the linear ion trap. Mascot 2.3 (Matrix Science) and Sequest (Thermo Fisher Scientific) search results were loaded into Proteome Discoverer 1.3 (Thermo Fisher Scientific) for verification. Sequence assignments and phosphorylated residues were further verified by manual inspection of the corresponding MS/MS spectra.

Relative Quantification of Phosphorylation Sites—Relative quantification of TRPL phosphorylation sites was accomplished by a label-free nano-HPLC-MS method. Five to eight independent immunoprecipitation experiments for each light quality (“light” or “dark”) were carried out, and immunoprecipitated TRPL was digested with trypsin or chymotrypsin. A second set of five independent immunoprecipitations was performed with emphasis on the C-terminal multiple-phosphorylated peptides of TRPL. Note that the triple-phosphorylated forms of this C-terminal peptide were only detected in this second sample set. This might be due to the lower amount of protein in the first set of samples and the poor ionization efficiency that multiple-phosphorylated peptides exhibit in general. Thermo raw-files of the acquired nano-LC-MS/MS runs were processed and analyzed using Progenesis LC-MS software (Version 3.1.4, Nonlinear Dynamics). The LC-MS runs in each dataset were automatically aligned using one LC-MS run as reference. Two-dimensional maps (m/z versus retention time) of each run were generated, aligned to the reference run, and used to find a common set of feature outlines (signals defined by m/z ratio and retention time). Peptide abundance values were subsequently calculated as the sum of the peak areas within the isotope boundaries of the feature. Abundance values for each feature were normalized to account for different amounts of sample loading or run to run variations. The normalization process implemented in the Progenesis LC-MS software is based on the assumption that most features show no change in abundance in a given experiment. The software calculates for each feature a quantitative abundance ratio between the run being normalized and the reference run. The normalization factor for each run is then calculated from the distribution of the log of the abundance ratios using a recursive median approach. Further details about the calculation of peptide abundances and the normalization process in the Progenesis LC-MS software can be found at the Nonlinear homepage. Before quantification the number of features was reduced to signals with a charge state of +2, +3, +4, and +5. TRPL (phospho)peptides were identified by means of a Mascot generic file created by the Progenesis LC-MS software. For relative quantification of (phospho)peptide abundances, the mean (phospho)-peptide abundances from immunoprecipitations “in the light” and “in the dark” was calculated, and the higher value was set to 100%. If more than one phosphorylation site was present on a peptide these sites were quantified together. Five immunoprecipitations per light condition were performed for the chymotryptic peptides. Tryptic peptides were derived from eight immunoprecipitations except the ones containing phosphorylated S20 and double- and triple-phosphorylated STS1114–16 (n = 5). An unpaired Student’s t test was performed for statistical evaluation of the quantification results.

Electroretinogram (ERG) Measurements—Electroretinogram recordings were performed as previously described (14). For all ERG recordings, flies were light-adapted for 16 h with orange light followed by a subsequent 3-min dark adaptation before the first measurement. All ERG recordings were performed at room temperature. For quantitative analysis of the ERG recordings, oscillations were flattened using the low-pass filter at a cutoff frequency of 10 Hz. For calculating the inactivation during a 5-s orange light stimulus, the peak amplitude at the beginning of the light pulse (p) and the amplitude at the end of the light pulse (s) were determined. Inactivation was defined by the term (1 – s/p) × 100% according to Wang et al. (22).

Fluorescence Microscopy—Subcellular localization of eGFP-tagged ion channels was analyzed by fluorescence microscopy of intact eyes using water immersion as previously described (16). Living flies were submerged in ice-chilled distilled water, and eGFP fluorescence was observed with an Axiosimager.Z1m microscope (Zeiss, Germany; objective: Achroplan 20×/0.5 water immersion). For the quantification of the relative fluorescence intensities in the water immersion images, images were captured with the AxioCam MrM (Zeiss) camera and the AxioVision 4.6/4.8 (Zeiss) software. Quantification of the fluorescence in the rhabdomere of the photoreceptor cells was carried out with the original (not-processed) images and was performed with the ImageJ 1.42 software using the formula Frh = Ir/Ib, where Frh is the relative fluorescence of R1-R6, Ir is the fluorescence in the rhabdomeres of the photoreceptor cells R1-R6, and Ib is the background fluorescence of the R7/R8 rhabdomere. Microscope and camera settings were identical for all captured images except for exposure times. Exposure times were chosen individually to be as long as possible without running into pixel saturation due to overexposure. Using identical exposure times was not possible as it would have resulted either in completely black images for samples with weak fluorescence or in strong overexposure of samples with high fluorescence. Therefore, the fluorescence in rhabdomeres R1–6 was normalized using the fluorescence in the R7 rhabdomere. The average fluorescence was calculated from three ommatidia per eye. Five flies were analyzed for each genotype and illumination state. Localization of proteins on cryosections through Drosophila eyes was carried out as previously described (14, 23). The eGFP-tagged ion channels were visualized by their GFP fluorescence, whereas AF546-coupled phalloidin (Invitrogen) was used for labeling of the rhabdomeres. Cryosections were observed with an Axiosimager.Z1m microscope using an ApoTome module (Zeiss, Germany; objective: EC Plan-Neofluar 40×/1.3 oil). All images were captured with the AxioCam MrM (Zeiss) camera and the AxioVision 4.6/4.8 (Zeiss) software.

RESULTS

Drosophila TRPL Is Phosphorylated at Nine Serine and Threonine Residues—We applied mass spectrometry to identify in vivo phosphorylation sites of Drosophila TRPL in dark- and light-adapted flies. The TRPL protein was purified from protein extracts of Drosophila heads by immunoprecipitation and subsequent SDS-gel electrophoresis. The Coomassie Blue-stained TRPL band at ~130 kDa was excised and in-gel-digested with
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The Phosphorylation Pattern of TRPL Changes upon Dark-and Light Adaptation—TRPL undergoes a light-dependent subcellular translocation that may affect the phosphorylation state of TRPL. Therefore, we isolated TRPL either from dark-adapted flies, in which TRPL is located in the rhabdomere, or after 16 h of illumination with orange light, resulting in TRPL localization in the cell body (13, 16). To determine if phosphorylation of specific TRPL sites was light-dependent, phosphopeptides present in these samples were relatively quantified by a label-free mass spectrometric quantification approach as previously described for the Drosophila TRP channel (10).

In this analysis the amount of a particular phosphopeptide in a sample was measured as the normalized abundance of its isotope signals as described under “Experimental Procedures.” Five (chymotrypsin) and five to eight (trypsin) independent immunoprecipitations per light condition were analyzed by LC-MS/MS. Typically, a particular phosphorylation site was detected on different peptide ions varying in charge, methionine oxidation state, or peptide length (due to miscleavages). For quantification we selected the most abundant peptide variant that was present in at least five LC-MS/MS runs of at least one light condition. In the case of multiple phosphorylated peptides, we quantified the same peptide variant for each phosphorylation state, namely the most abundant variant identified for the highest phosphorylation state. A list of the quantified peptides is provided in supplemental Table S1.

Our quantification approach revealed a complex light-dependent change of the TRPL phosphorylation pattern. The N-terminal phosphorylation site Ser-20 was predominantly phosphorylated in light-adapted flies as indicated by the corresponding tryptic peptide (Fig. 2A). Ser-730 showed no significant light-dependent change (Fig. 2B). Ser-927 and Ser-931 were covered by two different tryptic peptides that displayed a significantly up-regulated phosphorylation of Ser-927 in the dark but no significant difference in the phosphorylation of Ser-931 (Fig. 2A). Likewise, the corresponding single-phosphorylated chymotryptic peptide containing Ser-927 and Ser-931 was not regulated, whereas a chymotryptic peptide double-phosphorylated at Ser-927 and Ser-931 was significantly up-regulated in dark-adapted flies (Fig. 2B). The phosphorylation of Thr-989 was up-regulated in the light as both the tryptic and the chymotryptic peptides were detected more abundantly in the light (Fig. 2, A and B). Phosphorylation of Ser-1000 on the other hand was up-regulated in the dark as revealed by the respective tryptic and chymotryptic peptides that were significantly regulated (Fig. 2, A and B). Among the three phosphorylation sites Ser-1114, Thr-1115, and Ser-1116, the non-phosphorylated and single-phosphorylated peptides were more abundant in the light, whereas double-phosphorylated peptides showed no significant light-dependent change and the triple-phosphorylated peptide (Ser-1114/Thr-1115/Ser-1116) was up-regulated in the dark (Fig. 2, A and B). We assume that the low level of the single-phosphorylated peptides in dark-adapted flies is due to additional phosphorylation at the adjacent phosphorylation sites that results in a conversion of the single-phos-
Phosphorylated peptide to double- and especially triple-phosphorylated peptides. If this is the case, the amount of the corresponding non-phosphorylated peptide should decrease in the dark, which is what we observed (Fig. 2, A and B). We conclude that the overall phosphorylation at these three sites is up-regulated in dark-adapted flies. Taken together, our mass spectrometry data indicate a complex switch of the TRPL phosphorylation pattern upon prolonged light or dark adaptation of the flies (Fig. 2C).

To obtain additional evidence for a light-dependent change of the TRPL phosphorylation pattern we carried out a SDS band shift assay of TRPL isolated from dark- or light-adapted fly heads (Fig. 2D).
Phosphorylation of Drosophila TRPL heads (Fig. 2D). In this assay TRPL displayed a higher apparent molecular weight in dark-adapted flies compared with light-adapted flies. This shift in apparent molecular weight results from TRPL phosphorylation, as treatment of the extracts with \( \Phi_{H9261} \)-phosphatase abolished the molecular weight difference. The result of this experiment suggests that the overall phosphorylation of TRPL is higher in the dark than in the light.

Mutagenesis of the Eight C-terminal Phosphorylation Sites Does Not Affect TRPL Multimerization—The phosphorylation of TRPL might mediate the folding and proper multimerization of the channel subunits, the biophysical properties, or the stability and localization of TRPL. To gain insight into the function of TRPL phosphorylation, we focused on TRPLs eight C-terminal phosphorylation sites. We mutated these serine and threonine residues either to alanine (TRPLx-eGFP) or to aspartate (TRPLxD-eGFP) and generated TRPL-eGFP fusion constructs that were expressed as transgenes in Drosophila photoreceptor cells R1–6. We generated constructs in which all eight C-terminal phosphorylation sites were mutated to alanine (TRPL8x-eGFP) or aspartate (TRPL8xD-eGFP) and also constructs in which a subset of these phosphorylation sites was mutated, namely TRPL3x-eGFP/TRPL3xD-eGFP (Ser-1114, Thr-1115, and Ser-1116) and TRPL5x-eGFP/TRPL5xD-eGFP (Ser-927, Ser-1000, Ser-1114, Thr-1115, and Ser-1116) (Fig. 3A). In addition, we made a construct corresponding to TRPL8x-eGFP but without the eGFP tag (TRPL8x) and used a previously described wild type TRPL-eGFP construct (16).

To show successful expression of the transgenes and to determine the expression level of the alanine-substituted TRPL-eGFP constructs, extracts of flies that were exposed to 16 h of orange light illumination were subjected to immunoblot analysis (Fig. 3B). This analysis revealed that flies homozygous for the transgene displayed a higher TRPL expression level compared with native TRPL, whereas the expression level of heterozyously expressed transgenes was comparable with that of native TRPL (Fig. 3B). To avoid unspecific effects caused by overexpression, we decided to use flies heterozygous for the transgenes in the following experiments.

Functional TRP channels are formed by assembly of four subunits as demonstrated, for example, for the TRPV1 channel (24). To investigate whether or not the mutated TRPL channels form multimers, co-immunoprecipitation experiments were performed. For this analysis we used flies that expressed the eGFP-tagged TRPL constructs and native TRPL, immunoprecipitated the eGFP-tagged proteins, and asked whether the native TRPL proteins would co-immunoprecipitate (Fig. 3C).

As expected, native TRPL co-immunoprecipitated with wild type TRPL-eGFP, whereas no TRPL band was detected in the immunoprecipitate from control flies that did not express eGFP-tagged TRPL. This result shows that TRPL-eGFP inter-
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acted specifically with native TRPL due to formation of multimers. The alanine-substituted TRPL-eGFP proteins (TRPL8x-eGFP, TRPL5x-eGFP, and TRPL3x-eGFP) also interacted with native TRPL, demonstrating that the mutations did not interfere with multimerization of TRP ion channels (Fig. 3C). Like their alanine-substituted counterparts, the aspartate substituted TRPL-eGFP channels were able to interact with native TRPL (data not shown). Thus, phosphorylation of the C-terminal region of TRPL is not required for the assembly of subunits into tetramers.

Expression of Phosphorylation-deficient TRPL-eGFP Proteins in blind trp<sup>P302</sup>;trp<sup>P343</sup> Null Mutants Restores the Light Response—TRP and TRPL constitute the light-activated ion channels in Drosophila photoreceptors (9). Therefore, a double mutant that lacks both proteins is blind and shows neither a response to light in single cell recordings nor in ERG recordings that measure light-triggered changes of a transepithelial potential composed of the summed responses of retinal cells. Accordingly, transgenic expression of mutated TRP or TRPL channels in a trp<sup>P302</sup>;trp<sup>P343</sup> double null mutant can be applied to analyze the functional properties of these mutant ion channels without interference from the native channels. The trp<sup>P343</sup> mutant that expresses only TRP displays a transient receptor potential; that is, during a prolonged light stimulus (for example 5 s), the receptor potential returns to base line before the stimulus is terminated. This can be described by measuring inactivation of the response at the end of a light stimulus where 100% inactivation corresponds to a full repolarization of the potential to base line. A second characteristic of the trp phenotype is a long refractory period that may last for more than a minute before a second light stimulus again elicits a maximal response. In a trpl<sup>302</sup>;trp<sup>P343</sup> double null mutant, wild type TRPL-eGFP was demonstrated to generate a transient photosresponse closely resembling the signal of native TRPL in a trp<sup>P343</sup> mutant (16).

We carried out ERG recordings of flies that expressed either wild type TRPL-eGFP or the phosphorylation-deficient constructs TRPL8x-eGFP, TRPL5x-eGFP, or TRPL3x-eGFP in trpl<sup>302</sup>;trp<sup>P343</sup> mutant background and compared their responses to repeated orange light stimuli to those of wild type flies and of a trp<sup>P343</sup> mutant (Fig. 4). Immediately before the recordings the flies were dark-adapted for 3 min, but before that flies were kept in orange light for 16 h because in the dark TRPL8x-eGFP was not stably expressed in the rhabdomere (see below and Fig. 5, F–I). In contrast to wild type, in the genetic background (trpl<sup>302</sup>;trp<sup>P343</sup>) used for these experiments TRPL is located in the rhabdomere in the light (see Fig. 5, F–I). This is because TRP is required for the light-induced translocation of TRPL from the rhabdomere to the cell body (12, 13, 16). In the flies harboring the mutated TRPL channels, the general wave form of the ERGs, the inactivation of the responses after a 5-s orange light stimulus and the refractory periods were indistinguishable from ERGs of the trp<sup>P343</sup> mutant but differed markedly from wild type (Fig. 4, A and C–E). These results show that the mutated phosphorylation-deficient TRPL channels behaved essentially like wild type TRPL channels, suggesting that ion channel properties were not distorted. In addition, the TRPL3xD-eGFP, TRPL5xD-eGFP, and TRPL8xD-eGFP channels elicited electrophysiological responses identical to TRPL-eGFP and to their alanine-substituted counterparts (data not shown). In most recordings from flies expressing the mutated or non-mutated eGFP-tagged TRPL channels, we observed oscillations superimposed on the receptor potential during the light-response (Fig. 4B). Similar oscillations have been described for ERG recordings from trpl<sup>P302</sup> mutants that lack the TRPL channel (25). These oscillations do not seem to originate in the photoreceptor cells, as no oscillations were observed in single cell recordings from isolated ommatidia of trpl<sup>P302</sup> flies (26).

C-terminal Phosphorylation of TRPL Is Required for Stable Rhabdomeric Expression in Dark-adapted Flies—Because TRPL undergoes a light-dependent translocation between cell compartments and displays a light-dependent switch in the phosphorylation pattern (see Fig. 2), we next investigated a possible effect of eliminated TRPL phosphorylation on the subcellular localization and stability of TRPL. Water immersion microscopy of intact Drosophila eyes is an easy and efficient means to determine whether fluorescently labeled proteins are present in the rhabdomere or in the cell body (27) and has been applied to visualize the subcellular translocation of eGFP-tagged TRPL (16). Using this method, localization of TRPL-eGFP in the rhabdomeres appears as a distinct trapezoidal fluorescence pattern resembling the arrangement of rhabdomeres R1–6 in each single eye, whereas localization in the cell body results in a more diffuse fluorescence outside the rhabdomeres (Fig. 5A). We applied water immersion microscopy to study the localization of TRPL-eGFP and of the mutated TRPL constructs in long term light- and dark-adapted flies (Fig. 5B). As native TRPL formed multimers with the mutated TRPL-eGFP constructs (see Fig. 3C), the TRPL-eGFP constructs were analyzed in a trpl<sup>P302</sup> mutant background to avoid the effects of native TRPL on the localization of the transgenic TRPL-eGFP fusion proteins. Results in wild type background and trpl<sup>302</sup>;trp<sup>P343</sup> background are provided in Fig. 5, F–I. As reported before in wild type background (16), TRPL-eGFP was located in the rhabdomere in the dark, translocated to the cell body within 16 h of orange light-adaptation, and became redistributed to the rhabdomere within 4 h when the flies were kept in darkness again (Fig. 5B, upper panels). Upon longer dark-adaptation (24 h) no change in the localization of wild type TRPL-eGFP was observed. The subcellular localization of TRPL8x-eGFP in the dark differed clearly from TRPL-eGFP (Fig. 5B, second row). After the initial long term dark adaptation, TRPL8x was not localized in the rhabdomere. Instead, TRPL8x-eGFP was detected as a faint fluorescence in the cell body far less intense as compared with the fluorescence of TRPL8x-eGFP after 16 h light adaptation (Fig. 5Bf). After 16 h of light adaptation the fluorescence outside the rhabdomeres of TRPL8x-eGFP was comparable with that of TRPL-eGFP in the light (Fig. 5Bb). This result indicates that TRPL8x-eGFP was newly synthesized in the light and located, correctly, outside the rhabdomeres. Upon dark adaptation the pool of phosphorylation-deficient TRPL translocated back to the rhabdomere within 4 h (Fig. 5Bg) but, in sharp contrast to wild type TRPL, was not retained in the rhabdomeres for a longer period of time. Instead, after 24 h of darkness, weak TRPL8x-eGFP fluorescence was detected in the cell body (Fig. 5Bh), comparable with the fluorescence after the
initial 5-day dark adaptation. The phenotype of flies expressing TRPL with five (TRPL5x-eGFP) or three (TRPL3x-eGFP) mutated phosphorylation sites was less severe compared with TRPL8x-eGFP (Fig. 5B, third and fourth row). In these flies the TRPL channel was located partially in the rhabdomeres when inspected after the initial dark adaptation or after the second, prolonged (24 h) dark adaptation. Many rhabdomeres, however, appeared dark under these conditions, indicating that a fraction of the photoreceptor cells showed the same mislocalization and degradation of TRPL as observed in TRPL8x-eGFP. A quantification of the relative amount of eGFP-tagged TRPL constructs in the rhabdomeres was performed by dividing the fluorescence in the rhabdomeres of the outer photoreceptor cells (R1-R6) by the fluorescence in the rhabdomere of the inner photoreceptor cells R7 or R8 that did not express the eGFP-tagged proteins (Fig. 5C). After both the initial dark-adaptation (5 days) and the second, long-term (24 h) dark adaptation, the relative rhabdomeric fluorescence of all phosphorylation-deficient TRPL-eGFP constructs was significantly lower than in flies expressing wild type TRPL-eGFP. As revealed by comparison with wild type flies (Fig. 5C, black bar), flies expressing TRPL8x-eGFP showed no rhabdomeric fluorescence attribut-
able to eGFP after these prolonged dark adaptations, whereas the effect of the mutations was less severe in TRPL5x-eGFP- and TRPL3x-eGFP-expressing flies. After 4 h of dark adaptation the mutants showed eGFP fluorescence in the rhabdomeres that, compared with TRPL-eGFP, was significantly lower only in the case of TRPL8x-eGFP. Taken together, our results suggest that mutation of C-terminal phosphorylation sites of TRPL affects the retention of TRPL in the rhabdomeres rather than its initial trafficking to the rhabdomeres during dark adaptation.

We also performed water immersion microscopy of the aspartate-substituted TRPL-eGFP channels TRPL3xD-eGFP, TRPL5xD-eGFP, and TRPL8xD-eGFP in a trpl<sup>302</sup>-null mutant background (Fig. 5, D and E). The TRPL8xD-eGFP channel showed a similar phenotype as the respective alanine-substituted TRPL8x-eGFP channel, which was removed from the rhabdomere after prolonged dark adaptation (Fig. 5, D and E). Contrary to TRPL3x-eGFP and TRPL5x-eGFP, only the TRPL5xD-eGFP but not the TRPL3xD-eGFP channel displayed a slightly decreased rhabdomeric localization after prolonged dark adaptation (Fig. 5, D and E).

To investigate the mislocalization of phosphorylation-deficient TRPL-eGFP constructs in more detail, we compared...
The eGFP fluorescence in cryosections of the eyes from flies expressing TRPL-eGFP, TRPL5x-eGFP, and TRPL8x-eGFP (Fig. 6, A–C). In agreement with the results from the water immersion experiments, significant eGFP fluorescence was detected in the rhabdomeres of 24-h dark-adapted TRPL5x-eGFP flies (Fig. 6B, g–i), whereas rhabdomeres of 24-h dark-adapted TRPL8x-eGFP were devoid of eGFP fluorescence (Fig. 6C, g–i). Interestingly, 24-h dark-adapted TRPL5x-eGFP and TRPL8x-eGFP flies displayed labeling in distinct spots outside the rhabdomeres that was different from the diffuse labeling of the cell body observed in light-adapted flies. This finding suggests that mislocalized TRPL5x-eGFP and TRPL8x-eGFP in the dark is not distributed to the storage compartment that harbors TRPL after internalization in the light but that it is transported to another cellular compartment, maybe a compartment of the degradation pathway. After 4 h of dark adaptation, cryosections of TRPL8x-eGFP showed that the mutated channels are localized at the base of the rhabdomere and at the stalk membrane (Fig. 6C, d–f), whereas TRPL5x-eGFP (Fig. 6B, d–f) and especially TRPL-eGFP (Fig. 6A, d–f) fluorescence covered the entire rhabdomere at this time point. This indicates that before its removal from the rhabdomere, TRPL8x-eGFP is already mislocalized at the stalk membrane.

To further substantiate our observation that phosphorylation-deficient TRPL was not stable upon prolonged dark adaptation, quantitative Western blot analyses were carried out. Protein extracts were prepared from fly heads after 5 days of dark adaptation, after subsequent 16 h orange-light illumination, and after a subsequent second 24-h dark-adaptation (Fig. 7, A and B). The obtained results revealed a significantly smaller amount of TRPL8x-eGFP present in Drosophila head extracts in the dark as compared with the respective amount of the ion channel in the light (36% after initial darkadaptation and 35% after second dark adaptation) and also of TRPL5x-eGFP (58% after initial dark adaptation and 53% after second dark adaptation) (Fig. 7B). TRPL levels were not significantly different in light- and dark-adapted flies for native TRPL or for TRPL3x-eGFP. Interestingly, the eGFP-tagged wild type TRPL showed a significant higher expression level after the first dark incubation compared with its level in the light (283% after initial dark adaptation). To exclude the possibility that the observed effects on the stability of the phosphorylation-deficient TRPL were due to the eGFP tag, we investigated a TRPL8x construct without eGFP. As expected, light-dependent changes in the level of this TRPL construct were similar to those observed for TRPL8x-eGFP (49% after initial dark adaptation and 24% after second dark adaptation). In conclusion, our results show that phosphorylation-deficient TRPL is not stably retained in the rhabdomeres upon prolonged dark-adaptation. It becomes mislocalized to punctate structures in the cell body, and it is degraded. This phenotype is most severe when all eight C-terminal phosphorylation sites are mutated and milder when only five or three of these sites are missing.

**DISCUSSION**

In the present study we identified nine **in vivo** phosphorylation sites of the Drosophila ion channel TRPL and demonstrated that mutation of C-terminal phosphorylation sites interferes with a correct and sustained localization of TRPL in the rhabdomere of dark-adapted flies. Phosphorylation arguably is the most common post-translational modification of eukaryotic proteins. It has been estimated that ~30% of cellular proteins are modified by phosphorylation (28). Proteome-wide analyses of phosphorylation sites based on mass spectrometric approaches revealed, for instance, that the phosphoproteomes of Drosophila S2 cells or of HeLa cells comprise at least 4600 and 2244 phosphoproteins, respectively (29, 30). An equally
important result of these studies is the finding that a single protein typically is phosphorylated at multiple sites and may thus adopt several phosphorylation states (28, 30). Indeed, the combinatorial possibilities resulting from the fact that each site of a multiple-phosphorylated protein can exist in two states are manifold. This has been referred to as a “bar code” of phospho-proteins that may be the basis for different signaling capacities of the proteins (31). The latter notion has been studied for G protein-coupled receptors that change their phosphorylation pattern in response to a stimulus. For example, the V2 vasopressin receptor and angiotensin II type 1 A receptor are phosphorylated by various receptor kinases. RNAi knockdown of subsets of these receptor kinases differentially affected the binding of β2-arrestin and β-arrestin-mediated downstream signaling (32, 33). Multiple phosphorylation sites have been identified for ion channels, for example for the potassium channel Kv2.1 that contains 16 phosphorylation sites (4) or for the Drosophila TRP channel with 21 sites. In the TRP channel, at least seven sites become predominantly phosphorylated in the light, whereas one site is predominantly phosphorylated in the dark (10). Here we identified phosphorylation sites in Drosophila TRPL that change their phosphorylation pattern depending on the light condition in which the flies were kept (Fig. 2). This change is not a simple general enhancement or reduction of TRPL phosphorylation; instead, some phosphorylation sites show stronger, whereas other sites show weaker phosphorylation in a given condition.

With respect to functional studies, the complex light-dependent phosphorylation pattern makes it difficult to choose sites for a mutagenesis approach. The simplest strategy is to mutate all of the identified phosphorylation sites, although this might affect protein folding and render the mutated protein non-functional. In the case of TRPL we chose to mutate all identified C-terminal sites, whereas the N-terminal site was left intact. In addition, we generated constructs in which subsets of these phosphorylation sites were mutated to alanine. For these additional mutants we chose phosphorylation sites that displayed increased phosphorylation in the dark, namely the three adjacent sites close to the C terminus (Ser-1114, Thr-1115, Ser-1116), Ser-927, and Ser-1000. The generated mutants lacked either all five of these sites or the three C-terminal sites. We were able to show that the mutated TRPL channels formed multimers with native TRPL and, importantly, formed functional ion channels in a trplP302;trpP343 double null mutant background. The ERG recordings from flies expressing TRPL8x-eGFP, TRPL5x-eGFP, or TRPL3x-eGFP revealed no obvious differences from flies expressing wild type TRPL-eGFP. Thus, removal of the eight C-terminal phosphorylation sites or subsets of these sites is not obviously affecting the biophysical properties of TRPL. It is still possible that particular phosphorylation sites affect channel properties, and these alterations are compensated by the mutagenesis of other phosphorylation sites in a multimutagenesis approach. However, also TRPL5x-eGFP and TRPL3x-eGFP channels behaved similar to TRPL-
eGFP channels, making compensatory mutations rather unlikely.

The mutations we generated affected the rhabdomeric localization and stability of TRPL in the dark. Surprisingly, mutagenesis of all eight C-terminal phosphorylation sites to aspartate resulted in a similar phenotype as mutagenesis to alanine. The similar phenotype of substitution with alanine and aspartate could be explained by assuming that the loss of a serine or threonine residue per se causes an altered conformation or induces subtle changes in the C-terminal domain resulting in the observed phenotype. However, our observation that TRPL stability is affected in dark-adapted flies but not in light-adapted flies argues that this phenotype is not due to an unspecific effect of the mutations on the structure of TRPL. Alternatively, the darkness-induced change in the phosphorylation pattern of the TRPL C-terminal region resulting in down- and up-regulation of individual phosphorylation sites may determine the stability of the ion channel in the dark. Distortion in either direction (elimination of phosphorylation by alanine substitution or phosphomimetic mutation) may then change the dark-specific phosphorylation pattern and hence affect TRPL stability. In addition, although aspartate and glutamate are widely used as phosphomimetic amino acid substitutions, it is also documented that aspartate and glutamate are not always able to mimic the physiological effect of serine/threonine phosphorylation (34).

Protein phosphorylation has been reported to regulate subcellular translocation of vertebrate TRP channels. For example, the vertebrate TRPC4 channel translocates to the plasma membrane upon EGF stimulation in COS-7 cells (35). Thereby, Src family kinases phosphorylate two tyrosine residues in the cytosolic C-terminal region of the TRPC4 channel, and this promotes plasma membrane localization of TRPC4. Likewise, in the vertebrate TRPV1 channel, phosphorylation of an N-terminal tyrosine residue by Src kinase is essential for its stimulated translocation to the plasma membrane upon application of nerve growth factor to HEK-293 cells (36). Another well studied example for phosphorylation-dependent localization is the Kv1.2 potassium channel expressed in mammalian brain. For Kv1.2 a cluster of in vivo phosphorylation sites located in the C-terminal region was identified by mass spectrometry (5). The phosphorylation of some of these sites (Ser-440, Ser-441) is restricted to the cell-surface population of Kv1.2, i.e. the phosphorylation pattern of this channel is different when the channel is located at different subcellular sites (5). Moreover, mutation of Kv1.2 phosphorylation sites negatively impacted cell surface expression of the channel (5). Likewise, for Drosophila TRPL, we observed that phosphorylation-deficient TRPL became removed from the rhabdomeric plasma membrane and was degraded upon prolonged dark adaptation. A possible mechanistic explanation for these results would be as follows (Fig. 8). Upon TRPL translocation from the cell body to the rhabdomeres in the dark, the phosphorylation pattern in the C-terminal region of TRPL changes. The change in the phosphorylation pattern of the C terminus does not seem to be the initial trigger for TRPL translocation to the rhabdomeres in the
dark, as mutated TRPL was able to transiently translocate to the rhabdomere albeit being mislocalized to the base of the rhabdomere and the stalk membrane (Fig. 6C). Instead, the dark-specific phosphorylation pattern is required for stable retention of TRP in the rhabdomeres, possibly due to phosphorylation-dependent interaction of TRP with an anchoring protein. In the light, TRPL is removed from the rhabdomeres and transported to a storage compartment in the cell body (12). Concurrent with its translocation, the phosphorylation of TRPL changes to the light-specific phosphorylation pattern in a way that hinders the majority of TRP molecules to enter the degradation pathway. However, when mutated TRPL is internalized accidently in the dark, it can neither adopt the dark-specific nor the light-specific phosphorylation pattern. As a consequence, it becomes misrouted toward a different compartment in the cell body (see Fig. 6C) from where it is eventually degraded. In contrast to the rhabdomeric localization in the dark, stable retention in the storage compartment during illumination is not impaired in a phosphorylation-deficient TRPL mutant. This suggests that trafficking of newly synthesized TRP to the storage compartment and residence in the storage compartment does not strictly depend on a specific phosphorylation pattern of the eight C-terminal phosphorylation sites. All together, our detailed analysis of Drosophila TRPL phosphorylation identified phosphorylation sites in the C-terminal region of the channel that control the subcellular localization and stability of this member of the TRP ion channel family.

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