Light-dependent Phosphorylation of the Drosophila Transient Receptor Potential Ion Channel*‡§

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The Drosophila phototransduction cascade terminates in the opening of an ion channel, designated transient receptor potential (TRP). TRP has been shown to become phosphorylated in vitro, suggesting regulation of the ion channel through post-translational modification. However, except for one phosphorylation site, Ser982, which was analyzed by functional in vivo studies (Popescu, D. C., Ham, A. J., and Shiell, B. H. (2006) J. Neurosci. 26, 8570–8577), nothing is known about the role of TRP phosphorylation in vitro. Here, we report the identification of 21 TRP phosphorylation sites by a mass spectrometry approach. 20 phosphorylation sites are located in the C-terminal portion of the channel, and one site is located near the N terminus. All 21 phosphorylation sites were also identified in the inaC mutant, indicating that phosphorylation of TRP at these sites occurred independently from the eye-enriched protein kinase C. Relative quantification of phosphopeptides revealed that at least seven phosphorylation sites were predominantly phosphorylated in the light, whereas one site, Ser936, was predominantly phosphorylated in the dark. We show that TRP phosphorylated at Ser936 was located in the rhabomere. Light-dependent changes in the phosphorylation state of this site occurred within minutes. The dephosphorylation of TRP at Ser936 required activation of the phototransduction cascade.

Reversible protein phosphorylation is a well established mechanism for regulating the activity of ion channels. Typically, the pattern of phosphorylation of ion channels is complex, involving several phosphorylation sites with consensus sequences for a number of protein kinases, such as protein kinase C (PKC), protein kinase A, calcium/calmodulin-dependent protein kinase, or casein kinase, which phosphorylate serine and threonine residues, as well as kinases phosphorylating tyrosine residues. For example, in the major delayed rectifier K⁺ channel Kv2.1, expressed in most central neurons, 16 phosphorylation sites have been identified by mass spectrometry, a subset of which contributes to graded modulation of voltage-dependent gating (2).

Transient receptor potential (TRP) channels constitute a protein family of about 30 unique homologs that are assigned to seven subfamilies on the basis of sequence homology: canonical TRPC, vanilloid TRPV, melastatin TRP, polycystin TRPP, mucolipin TRPML, and ankyrin transmembrane proteins TRPA and NOMPC-like TRPN (3, 4). The founding member of this protein family is the Drosophila TRP channel, which, together with its homolog TRPL-like (TRPL), is located in the rhabdomeral photoreceptor membrane of the fly compound eye and represents the major light-sensitive ion channel in this photolipase C-mediated visual transduction cascade (5).

Phosphorylation of several TRP channels has been described. Among the vertebrate TRPC channels, TRPC3 and TRPC6 are inhibited by phosphorylation events mediated by protein kinase C and protein kinase G (6–8). In contrast, Src kinase activity is required for the activation of TRPC3 by diacylglycerol (9), and Fyn kinase phosphorylates and thereby increases the activity of TRPC6 (10). Abolition of the putative protein kinase C phosphorylation site Thr635 in the S4/S5 linker region of TRPC3 by mutation results in increased channel activity and was found to underlie the phenotype of moonwalker mice, which is caused by loss of Purkinje cells (11). The regulation of the capsaicin- and heat-sensitive TRPV1 channel through phosphorylation of serine residues by protein kinase C is also well established (12–14). Phosphorylation of TRPV1 sensitizes this channel to capsaicin, heat, and other agonists. Besides protein kinase C, calcium/calmodulin-dependent kinase and protein kinase A were implicated in phosphorylation of TRPV1 (15, 16).

The first TRP channel shown to become phosphorylated again was the Drosophila TRP channel. This channel is part of a signaling complex assembled by the INAD scaffold protein together with phospholipase C and an eye-enriched protein kinase C (eye-PKCC) encoded by the inac gene. It was shown initially for the larger fly Calliphora vicina and later also for Drosophila that the addition of ATP to the isolated signaling complex resulted in phosphorylation of TRP and INAD, suggesting that these two proteins of the signaling complex are targets of the associated protein kinase C (17–19). A detailed in vitro analysis of TRP phosphorylation by Popescu and colleagues (1) revealed a PKC target site in TRP, Ser982. This site...
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was confirmed as a bona fide phosphorylation site in vivo by mass spectrometry. Mutation of Ser982 to Ala resulted in a defect in deactivation of the phototransduction cascade, suggesting that an increase in intracellular Ca\(^{2+}\) may regulate the dephosphorylation of this site.

EXPERIMENTAL PROCEDURES

Fly Stocks—The following strains and mutants of Drosophila melanogaster were used: w Oregon R, w;tru\(^{P443}\) (20), w;trup\(^{P365}\) (21), yw;inaE\(^{17}\) (22), w;Goa\(^{q}\) (23), w;norP\(^{A224}\) (24), and winaC\(^{P209}\) (25). Flies were reared at 25 °C. For all experiments, 1–5-day-old flies were used. Flies were illuminated with an 18-watt fluorescent lamp, 2400 lux, unless noted otherwise. For mass spectrometry experiments, flies were illuminated with white light or were kept in the dark overnight before they were subjected to immunoprecipitation. For analyses of the TRP phosphorylation state at Ser\(^{936}\) in different mutants, flies were kept in the dark for 12–18 h and were then illuminated with white light for 1 h and vice versa before they were subjected to Western blot analyses.

To analyze the action spectrum of TRP dephosphorylation at Ser\(^{936}\), flies were kept in the dark for 12–18 h and were then illuminated with white light (2400 lux; see above), blue light (acrylic glass wide band filter transmitting light between 310 and 490 nm, 30 lux), green light (acrylic glass wide band filter transmitting light between 460 and 610 nm, 140 lux), orange light (acrylic glass cut-off filter transmitting light >560 nm, 1300 Lux), or red light (acrylic glass cut-off filter transmitting light >630 nm, 270 lux) or were kept in the dark for 1 h before they were subjected to Western blot analyses. For investigation of the time course of TRP phosphorylation and dephosphorylation at Ser\(^{936}\), flies were kept in the dark or were illuminated with white light 12–18 h before the experiment and were then subjected to the opposite light condition for different periods of time. For immunolocalization of TRP or Ser(P)\(^{936}\), TRP, flies were illuminated with white light, kept in the dark for 12–18 h, or illuminated with white light plus UV (150-watt xenon high pressure lamp, 20,000 lux) for 1 h before they were subjected to immunocytochemistry. Dark-kept flies were dissected under dim red light (Schott RG 630, cold light source KL1500), whereas flies kept in white or colored light were dissected under these light conditions.

Immunoprecipitation of TRP from Fly Heads—Fly heads from wild type or inac\(^{P209}\) flies were separated from bodies by freezing in liquid nitrogen and vigorous vortexing. Heads were passed through a 45-mesh sieve holding back the bodies and were collected on a 25-mesh sieve (Neolab). Approximately 300 wild-type or inac\(^{P209}\) heads were homogenized in 0.5 ml of extraction buffer supplemented with protease and phosphatase inhibitors (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 50 μM (4-aminophenyl)-methanesulfonyl fluoride hydrochloride monohydrate, 10 μg/ml aprotinin, leupeptin, and pepstatin A, 10 mM benzamidine, 10 mM sodium fluoride, 1 mM orthovanadate, 10 mM β-glycerophosphate, 500 mM cantharidine, 10 mM sodium pyrophosphate) using a microp啜stle (Roth). Head homogenates were extracted on ice for 30 min. The mixture was centrifuged for 10 min at 12,000 × g, and the supernatant was used for immunoprecipitation. Immunoprecipitation of the INAD signaling complex from Drosophila head extracts was performed with a monoclonal α-TRP antibody (MAb83F6, Developmental Studies Hybridoma Bank, University of Iowa). Head extracts were incubated with 50 μl of protein G-agarose beads (Pierce) and 6 μg of α-TRP antibody for 1 h at 4 °C. The beads were collected by centrifugation at 10,000 × g for 1 min at 4 °C and washed four times, with 0.5 ml of ice-cold extraction buffer. Precipitated proteins were eluted from protein G-agarose beads with 25 μl of 2× SDS sample buffer (150 mM Tris, pH 6.8, 1.2% (w/v) SDS, 30% (w/v) glycerol, 15% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromphenol blue) at 80 °C for 10 min. The eluate was subjected to SDS-PAGE, and protein bands were visualized by colloidal Coomassie Blue staining (Roth).

Mass Spectrometry Analysis—Proteins were in-gel digested using either trypsin or chymotrypsin (Roche Applied Science) according to Shevchenko et al. (26). For chymotryptic digests, a modified incubation buffer was used (100 mM Tris-HCl, pH 7.6, 10 mM CaCl\(_2\)). After digestion, the gel pieces were extracted with 50% acetonitrile (ACN), 50% 0.1% formic acid (FA) (v/v) for 15 min. The supernatant was collected, and the gel pieces were covered with 5% FA for 15 min before the same volume of ACN was added. After incubation for 10 min, the supernatant was collected. The pooled supernatants were then dried in a vacuum centrifuge and stored at −20 °C. Dried samples were dissolved in 0.1% FA. Nano-LC-ESI-MS/MS experiments were performed on an ACQUITY nano-UPLC system (Waters) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific). Tryptic and chymotryptic digests of TRP were concentrated and desalted on a precolumn (2 cm × 75 μm, BEH 130 C\(_{18}\) reversed phase column) and separated on a 20 cm × 75 μm BEH 130 C\(_{18}\) reversed phase column (1.7 μm particle size, Waters). Gradient elution was performed from 1% ACN to 50% ACN in 0.1% FA within 60 min. The LTQ-Orbitrap was operated under the control of XCalibur 2.0.7 software. Survey spectra (m/z = 300–1800) were detected in the Orbitrap at a resolution of 60,000 at m/z = 400. Data-dependent tandem mass spectra were generated for the five most abundant peptide precursors in the linear ion trap. For all mea-
measurements using the Orbitrap detector, internal calibration was performed using lock-mass ions from ambient air as described in Ref. 27.

**Mass Spectrometry (MS) Data Analysis**—Mascot™ 2.2 (Matrix Science) and Sequest (Thermo Fisher Scientific) search engines were used for protein identification. Spectra were searched against the Drosophila subset of the NCBI protein sequence data base downloaded as FASTA-formatted sequences from the NCBI FTP site. Search parameters specified trypsin or chymotrypsin as the cleaving enzyme, allowing for missed cleavages (including cleavage before Pro), a 3 ppm mass tolerance for peptide precursors, and 0.5 Da tolerance for fragment ions. Carbamidomethylation of cysteine residues was set as a fixed modification, and Ser, Thr, and Tyr phosphorylation, methionine oxidation, and N-terminal acetylation of proteins were allowed as variable modifications. Phosphopeptide MS/MS spectra sequence assignments and phosphorylated residues were verified manually.

**Quantification of Light-dependent TRP Phosphorylation by MS**—Quantification of light-dependent TRP phosphorylation sites was accomplished by a label-free nano-HPLC-MS method. For each light condition (light or dark), five (tryptic digests) or three (chymotryptic digests) independent experiments and a technical replicate for each experiment were performed. The commercially available SIEVE 1.2 software (Thermo Fisher Scientific) was used for alignment of chromatographic peaks as well as detection, deisotoping, and peak area determination of MS1 features (peptides) over multiple LC-MS/MS runs. The SIEVE software uses the ChromAlign algorithm (28) to perform chromatographic alignment between LC-MS/MS runs (e.g. see supplemental Fig. S1). After completion of the alignment step, peptide peak areas were calculated by SIEVE based on extracted ion chromatograms (XICs) of each MS feature (peptide). Peak areas of TRP phosphopeptides were extracted from the SIEVE result table for normalization. Phosphopeptide identifications were based on (a) accurate mass and retention time and (b) assignment of phosphorylated residues in the corresponding MS/MS spectra of the Mascot and Sequest search results. Only phosphopeptides that were present in all biological and technical replicates of at least one light condition (light or dark) were considered for quantification. Normalization of peak areas in all tryptic or chymotryptic LC-MS/MS runs was achieved by using unphosphorylated TRP peptides as omnipresent internal standards as described (29, 30). Normalization peptides (reference peptides) were chosen by the following criteria: (a) peptides were present in every LC-MS/MS run, (b) they were not prone to miscleavages and oxidation, and (c) they showed peak area variations among different LC-MS/MS runs that reflected the general variations of the majority of the peptides (30). Four reference peptides (NNYEILK, YILAPDSEGAK, GIDPYFPR, and DIFSSLAK) were used for normalization of phosphopeptides derived from tryptic digests, and two reference peptides (QIISERADTEW and ILEEYQGTDFK) were used for the chymotryptic digests. To obtain normalization factors for each experiment, the peak area of a reference peptide was divided by the mean of the peak areas of this reference peptide in the sample set. The peak area of the phosphopeptides of each experiment was then divided by each of the four (tryptic digests) or two (chymotryptic digests) normalization factors, and the resulting values were averaged to obtain the normalized peak areas of the phosphopeptides. The mean of normalized peak areas for every phosphopeptide over all LC-MS/MS runs of one light condition (light or dark) was calculated, and the higher value was set to 100%. An unpaired t test was performed for statistical evaluation of the quantification results.

**Generation of a Phosphospecific Antibody against Ser(P)936-TRP**—A synthetic phosphopeptide, NH₂-CADEVpSLADD-CONH₂, including Ser(P)⁹³⁶, was synthesized and injected into rabbits in order to produce antisera (Pineda Antibody Service, Germany). For affinity purification, the phosphopeptide was conjugated to epoxy-activated Sepharose 6B (GE Healthcare) (10 mg of phosphopeptide per 0.5 g of Sepharose) overnight in 2 ml of coupling buffer (0.2 M NaHCO₃/Na₂CO₃, pH 10). After conjugation, the resin was washed once with 2.5 ml of coupling buffer, and residual active groups were blocked with 3 ml of 1 M ethanolamine, pH 8.0, overnight. The resin was washed five times with 2.5 ml of 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), pH 7.0, and packed into a C10/10 column with adaptor AC 10 (GE Healthcare). Unspecifically bound protein was washed off with 15 ml of 1× PBS, pH 7.0, 10 ml of 1× PBS, pH 3.0, and 15 ml of 1× PBS, pH 7.0. 15 ml of antiserum diluted in 30 ml of 1× PBS, pH 7.0, were passed over the column for 2 h, the column was washed with 10 ml of 1× PBS, pH 7.0, and bound antibody was eluted with 1× PBS, pH 3.0. Eluted fractions were adjusted to pH 7.0. The resulting antibody recognized TRP phosphorylated at Ser⁹³⁶ (Ser(P)⁹³⁶-TRP) and hereafter is referred to as α-Ser⁹³⁶-TRP.

**Western Blot Analyses**—For Western blot analyses, fly heads were homogenized in 1× SDS-PAGE extraction buffer (4% SDS, 1 mM EDTA, 75 mM Tris/HCl, pH 6.8), and extraction was carried out for 10 min at room temperature. The extracts were centrifuged at 16,000 × g at 22 °C for 10 min. Supernatants were subjected to SDS-PAGE according to Laemmli (31), using 8% polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad). The membrane was then blocked for 1 h with 5% skim milk in TBS-T (50 mM Tris/HCl, pH 7.3, 150 mM NaCl, 0.1% Tween 20). The ECL Plus Western blotting analysis system (GE Healthcare) was used for signal detection with x-ray film. The primary antibodies used for Western blot analysis were α-Ser⁹³⁶-TRP (MAb83F6, Developmental Studies Hybridoma Bank). When needed, the α-Ser⁹³⁶-TRP and α-TRP signals were quantified with ImageJ 1.41o (National Institutes of Health) by determining the integrated density (i.e. the sum of the values of the pixels in a selection) of each band. α-Ser⁹³⁶-TRP integrated density values were divided by α-TRP values for normalization.

**Immunocytochemistry of Fly Heads**—Immunocytochemistry was carried out as described elsewhere (32). Primary antibodies used for immunocytochemistry were α-TRP (MAb83F6, Developmental Studies Hybridoma Bank) and α-Ser⁹³⁶-TRP. Secondary antibodies were α-mouse Cy5 (Dianova), α-rabbit Cy5 (Dianova), α-mouse AlexaFluor 488 (MoBiTec), and AlexaFluor 546-coupled phalloidin (Invitrogen). The sections were examined with the AxioImager.Z1 microscope (objective,
RESULTS

Identification of in Vivo Phosphorylation Sites of the Drosophila TRP Ion Channel—To identify in vivo TRP phosphorylation sites, an MS-based analysis was carried out. Drosophila head extracts were prepared from either dark- or light-adapted flies. The TRP ion channel was purified by immunoprecipitation with a monoclonal α-TRP antibody and size fractionation by SDS-PAGE. The gel was stained with colloidal Coomassie Blue, and the TRP band at 150 kDa was excised and subjected to in-gel trypsin or chymotrypsin digestion. A representative gel is shown in Fig. 1A. TRP peptides from tryp tic or chymotryptic digests were analyzed by LC-MS/MS and identified by database searches using the Mascot and Sequest search engines, allowing protein modifications, such as oxidation of methionines, acetylation, and specifically phosphorylation. In total, a sequence coverage of 89% of the TRP protein was obtained. This includes 66% sequence coverage from tryp tic peptides and 74% sequence coverage from chymotryptic peptides (Fig. 1B). Nano-LC-MS/MS analysis revealed 20 phosphorylated serine and threonine residues that were located in the C terminus of the ion channel, including the previously identified phosphorylation site Ser982 (1). A single phosphorylated serine residue (Ser15) was identified in the N terminus of TRP (Fig. 1, B and D).

Phosphorylation of a Subset of the Identified Sites Is Regulated by Light—To investigate a possible physiological function of the identified TRP phosphorylation sites, we analyzed their light dependence. In order to obtain conclusive data on the light dependence of TRP phosphorylation sites, we quantified the amount of phosphopeptides present in samples isolated from flies kept in the dark or under white light illumination using a label-free mass spectrometry approach. Only phosphopeptides that were detected in all nano-LC-MS/MS runs of at least one light condition with a signal intensity of at least 10,000 counts were subjected to quantification. 17 of the 21 identified phosphorylation sites were located on phosphopeptides that met these criteria and were quantified. At least three independent nano-LC-MS/MS runs and three corresponding technical replicates were used for quantification. Alignment of nano-LC-MS/MS runs, construction of XICs, and calculation of peak

FIGURE 1. The TRP protein is highly phosphorylated. A, representative polyacrylamide gel stained with colloidal Coomassie Blue for TRP recovery after immunoprecipitation. PLC, phospholipase C; INAD, inactivation no afterpotential D. B, sequence coverage of TRP by nano-LC-MS/MS. Sequence fragments covered by tryp tic peptides are coded by red characters, and fragments covered by chymotryptic peptides are highlighted in yellow. Total sequence coverage was 89%. The phosphorylation sites identified by MS/MS are highlighted in red. Predicted transmembrane domains (39) are depicted by solid lines. C, LC-MS/MS spectrum of the doubly charged TRP phosphopeptide MAADEVpSLADDEGAPNEGEK phosphorylated at Ser936 obtained from dark-reared flies. The phosphorylation site was unambiguously assigned to Ser936 because of mass assignments of fragmentation ions y14 and y15. MS/MS spectra supporting the identification of the other 20 phosphorylation sites are shown in supplemental Fig. S2. D, schematic diagram of the TRP channel showing the positions of the TRP phosphorylation sites as identified by LC-MS/MS analysis. Gray circles, phosphorylation sites that do not contain a predicted kinase consensus sequence. White circles, phosphorylation sites that are embedded within a protein kinase C consensus sequence. Blue circles, phosphorylation sites that are embedded within a casein kinase I or II consensus sequence. Yellow circle, a phosphorylation site that is embedded within a protein kinase G consensus sequence. Green circle, a phosphorylation site that is embedded within a DNA protein kinase consensus sequence.
Phosphorylation sites, Thr849, Thr864, Ser872, Ser964, and Ser982, in the phosphorylation state of at least eight sites. Five phosphorylated amino acids are indicated as abundant phosphopeptide found in dark-reared (Fig. 2C). For each site, the most phosphorylated area was accomplished by SIEVE 1.2 software (Thermo Fisher Scientific), as described under “Experimental Procedures.” A representative XIC of a peptide comprising the Ser936 phosphorylation site is shown in Fig. 2A. Results of the relative quantification of tryptic and chymotryptic phosphopeptides are shown in Fig. 2, B and C, respectively. When possible, the most abundant peptide variant, phosphorylated at a single, unambiguously assigned site was chosen to determine the light dependence of the respective site. However, for some phosphorylation sites, the peptide variants that could be quantified reliably comprised more than one possible phosphorylation site. In most cases, these regioisomers could not be separated by nano-UPLC and were therefore quantified together. In these cases, the number of phosphorylations present on the respective peptides is given as nXP in Fig. 2, B and C, as well as in supplemental Fig. S3, A and B. Quantification revealed that there are significant light-dependent changes in the phosphorylation state of at least eight sites. Five phosphorylation sites, Thr849, Thr864, Ser872, Ser964, and Ser982, were present as unique sites on phosphopeptides that showed significant up-regulation in illuminated flies (Fig. 2, B and C).

One phosphorylation site, Ser936, could only be quantified as a doubly phosphorylated chymotryptic peptide (Fig. 2C). Because the other phosphorylation site on the corresponding tryptic peptide, Ser921, showed no significant light-dependent changes (Fig. 2B), we infer that Ser936 is predominantly phosphorylated in the light. A peptide containing either phosphorylated Ser961 or Thr963 was found at a significantly higher level in the light (Fig. 2C). Although these two sites were unambiguously identified by their fragmentation spectra, they could only be quantified together, and it is not possible to tell whether the phosphorylation of Ser961 or Thr963 or of both is up-regulated in the light. Finally, a single site, Ser936, was predominantly detected in its phosphorylated state in dark-adapted flies (Fig. 2, A and B).

*Phosphorylation of the Identified Sites Does Not Depend on Eye-PKC*—In order to determine which phosphorylation sites are substrates of eye-PKC, we isolated TRP from the eye-PKC null mutant inaC<sup>p209</sup> and analyzed its phosphorylation state by LC-MS/MS. Surprisingly, all phosphopeptides that were observed in TRP isolated from wild type flies were also identified in the inaC mutant, including the peptide comprising Ser936. Moreover, the light dependence of the phosphorylation of these sites observed in wild type flies was similar in the inaC mutant, as revealed by quantitative MS analysis (supplemental Fig. S2 and Fig. 2). This suggests that the phosphorylation sites identified here are not substrates of eye-PKC, or they are phosphorylated by at least one other kinase.

**TRP Phosphorylated at Ser936 Is Located within the Rhabdomeres**—In the following experiments, we focused on a more detailed characterization of the phosphorylation site Ser936 because it is the only site that is preferentially phosphorylated in the dark, whereas the other sites show preferential phosphorylation in the light or no light preference at all. To investigate TRP phosphorylation at Ser936 in more detail, a rabbit polyclonal antibody against a synthetic phosphopeptide containing phosphorylated Ser936 (Ser(P)<sup>936</sup>) was generated. Immunoaffinity purification of the crude antiserum against the phosphopeptide yielded an antibody, here referred to as α-Ser(P)<sup>936</sup>-TRP.
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but not Ser\(^{936}\) -TRP on Western blots and in immunocytochemistry (Figs. 3–5).

A possible physiological role of ion channel phosphorylation is the regulation of the intracellular trafficking of ion channels. For example, it has been shown that phosphorylation of the potassium channel Kv1.2 at clustered C-terminal sites correlates with its subcellular localization and therefore is likely to play a role in regulating the trafficking of this channel (33). We therefore were interested in determining the subcellular site at which Ser(P)\(^{936}\) -TRP is located. The localization of Ser(P)\(^{936}\) -TRP was studied by immunocytochemistry using α-Ser(P)\(^{936}\) and an α-TRP antibody detecting the TRP protein regardless of its phosphorylation state as a control. As expected, the control antibody revealed a TRP signal in the rhabdomeres (Fig. 3, A–C). The phosphospecific antibody α-Ser(P)\(^{936}\) also detected TRP in the rhabdomeres of photoreceptor cells R1–6 and R7 when the flies were kept in the dark (Fig. 3, D–F). When flies were illuminated with white light, the α-Ser(P)\(^{936}\) labeling of the rhabdomeres of photoreceptor cells R1–6 vanished. However, labeling of the rhabdomeres of R7 photoreceptor cells persisted (Fig. 3, G–J). Because the R7 photoreceptor cells express rhodopsins 3 and 4 absorbing maximally in the UV, we assumed that the fluorescent lamp we used for illumination did not emit a high enough intensity of the UV portion of the spectrum to trigger dephosphorylation of Ser(P)\(^{936}\) -TRP in R7 cells.

Illumination with a xenon lamp emitting UV light in addition to visible light indeed led to the disappearance of Ser(P)\(^{936}\) -TRP labeling of the rhabdomeres of R7 as well as R1–6 cells (Fig. 3, J–L). Thus, phosphorylation and dephosphorylation of TRP at Ser\(^{936}\) seems to occur in the rhabdomeres of different classes of photoreceptor cells.

Dephosphorylation of Ser\(^{936}\) Requires Activation of the Phototransduction Cascade—The phosphorylation state of TRP at Ser\(^{936}\) was found by quantitative LC-MS/MS to be light-dependent. In addition, as described above, dephosphorylation of Ser\(^{936}\) in R7 photoreceptor cells required illumination with UV light corresponding to the UV sensitivity of the rhodopsins Rh3 and Rh4 expressed in R7 cells. Thus, we inferred that the phosphorylation state of Ser\(^{936}\) depends on the phototransduction cascade. Hence, the dependence of the phosphorylation state on ambient light should be abolished in mutants affecting proteins of the phototransduction cascade. Blind mutants should mimic the effect of darkness, and constitutively active mutants should mimic the effects of light regardless of the light conditions applied. To test this assumption, flies harboring mutations in proteins involved in the phototransduction cascade were illuminated for 12–18 h and were then put into the dark for 1 h or vice versa before they were subjected to Western blot analyses with α-Ser(P)\(^{936}\) antibody (Fig. 4A). ninaE\(^{17}\) and norpA\(^{254}\) mutants are both visually impaired due to a null mutation in the rhodopsin 1 gene, encoded by ninaE (22, 34) or a null mutation in the norpA gene encoding the central effector enzyme of this signaling pathway, phospholipase Cβ (24). In both mutants, the pool of Ser(P)\(^{936}\) -TRP was as high as in wild type flies reared in darkness and, importantly, was independent of the light condition (Fig. 4A). Another mutant that shows a dramatic (~1000-fold) reduction in the photoresponse is Gqα\(^{1}\), a hypomorph that expresses only residual amounts of the visual G\(_{q}\) protein α subunit (23). As found in the ninaE\(^{17}\) and norpA\(^{254}\) mutants, TRP phosphorylation at Ser\(^{936}\) persists in the Gqα\(^{1}\) mutant exposed to light (Fig. 4A). These results indicate that the light-triggered dephosphorylation of the Ser\(^{936}\) residue in the TRP channel requires activation of the phototransduction cascade at least down to the effector enzyme phospholipase C. Activation of phospholipase Cβ in the Drosophila visual transduction cascade leads to production of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, which may affect protein kinases and phosphatases involved in the phosphorylation of Ser\(^{936}\) of TRP. Activation of phospholipase Cβ also triggers the opening of TRP and its homolog TRPL, resulting in Ca\(^{2+}\) influx into the photoreceptor cell. After activation of the phototransduction cascade, the major fraction of Ca\(^{2+}\) influx is mediated by TRP, which is about 10-fold more abundant and 25 times more Ca\(^{2+}\)-selective than TRPL (35). In order to test whether activation of TRP is sufficient for triggering dephosphorylation of Ser(P)\(^{936}\), we used a constitutively active TRP mutant, trp\(^{P365}\) (21, 36). Due to permanent Ca\(^{2+}\) influx, photoreceptor cells soon begin to degenerate in this mutant. However, in heterozygous flies kept in the dark, photoreceptors remain intact for 2 days post eclosion. To avoid loss of TRP due to photoreceptor cell degeneration and to circumvent the possibility that our α-Ser(P)\(^{936}\) antibody may not recognize the mutated TRP protein, we used heterozygous flies in our assay. In the trp\(^{P365}\)/+ fly, no Ser(P)\(^{936}\) -TRP was detected regardless of the light conditions (Fig. 4A). However, TRP was present in wild type amounts, indicating that photoreceptor degeneration did not yet com-

![Figure 3](https://example.com/fig3.png)
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To confirm our LC-MS/MS results, we also tested an inaC mutant that lacks eye-PKC (Fig. 4A). As expected, the absence of eye-PKC had no effect on the light-dependent phosphorylation of the TRP residue Ser\(^{936}\).

Finally, to analyze the action spectrum of TRP dephosphorylation, we illuminated wild type flies with different light qualities for 1 h. As a result, all light qualities that led to rhodopsin 1 to metarhodopsin conversion (white, blue, green, and orange), triggered Ser\(^{936}\) dephosphorylation. In contrast, red light illumination as well as darkness, which do not cause rhodopsin 1 conversion, did not trigger Ser\(^{936}\) dephosphorylation (Fig. 4B). These findings confirm the notion that the dephosphorylation of Ser\(^{936}\) requires the activation of the phototransduction cascade.

Light-dependent Changes in the Phosphorylation State of TRP at Ser\(^{936}\) Occur within Minutes—Using the \(\alpha\)-Ser(P)\(^{936}\) antibody, we determined the time courses of phosphorylation and dephosphorylation of Ser\(^{936}\). Flies were kept in the dark or under white light illumination for 12–18 h and were then switched to the opposite light condition for different amounts of time ranging from 1 min to 4 h. TRP phosphorylation at Ser\(^{936}\) was analyzed on Western blots probed with \(\alpha\)-Ser\(^{936}\), and the total amount of TRP present in the samples was determined by stripping and incubating the same blots with a monoclonal mouse antibody directed against Drosophila TRP (Fig. 5, A and B) (37). Although the total amount of TRP remained essentially unchanged in the light conditions tested, the pool of Ser\(^{936}\)-TRP changed significantly already 1 min after switching the flies to the new light condition. When switched from darkness to light, about 65% of Ser\(^{936}\)-TRP became dephosphorylated within 1 min. After 15 min in the light, only a residual fraction of Ser\(^{936}\)-TRP remained. In the opposite direction, rapid phosphorylation of TRP at Ser\(^{936}\) resulted in a more than 7-fold increase in the pool of Ser\(^{936}\)-TRP after 1 min in the dark, and the maximum amount of Ser\(^{936}\)-TRP was reached after 1 h of dark adaptation.

DISCUSSION

In the current study, we identified 21 in vivo phosphorylation sites of the Drosophila TRP channel, 20 of which are located in the C-terminal domain and one of which is located close to the N terminus. These 21 phosphorylation sites were unambiguously identified by their corresponding fragmentation spectra (MS/MS spectra). To obtain more insight into a possible physiological function of these phosphorylation sites, we analyzed their light dependence by a quantitative mass spectrometry approach.

An elegant and efficient method for protein quantification by mass spectrometry is a metabolic isotopic labeling approach like SILAC (stable isotope labeling by amino acids in cell culture) (38) because two different samples can be analyzed in a single LC-MS run. Technical variances of biochemical and mass spectrometric procedures affect both samples in the same way. Unfortunately, implementation of these metabolic labeling methods is difficult in multicellular organisms like Drosophila, and complete isotopic labeling of a whole organism is far from routine. Alternatively, an isotope label can be introduced chemically during sample preparation on the protein or peptide
Light-dependent Phosphorylation of TRP

**A**

![Graph A](Image_A)

**B**

![Graph B](Image_B)

**FIGURE 5.** Time course of TRP Ser(P)\textsuperscript{936} phosphorylation in the dark (A) and dephosphorylation in the light (B). TRP extracted from *Drosophila* heads of flies reared under the light conditions indicated was subjected to Western blot analysis with α-Ser(P)\textsuperscript{936} (upper panels). After recording the signals, the blots were stripped and reprobed with a monoclonal α-TRP antibody (lower panels). Black or white bars below the blots indicate light conditions (black, flies were kept in the dark for the given periods of time prior to extraction; white, flies were illuminated for the given periods of time prior to extraction). Molecular mass markers (in kDa) are indicated to the left. The graphs show a quantitative evaluation of Western blots from three experiments. The relative phosphorylation levels of Ser936 normalized to TRP contents are depicted. Phosphorylation in the dark (4 h) was set at 100%. Bars, mean of three independent experiments. Error bars, S.E.

level with reagents like ICPL (isotope-coded protein label), iTRAQ (isobaric tag for relative and absolute quantitation), or TMT (tandem mass tag). However, these methods often require additional purification steps that lead to loss of low abundance peptides (including phosphopeptides). Therefore, we used a label-free mass spectrometry method for quantification of TRP phosphorylation sites. This method is based on comparison of peak areas derived from XICs of each peptide and posttranslational modifications, including phosphorylation (30, 39–42).

To ensure maximal sequence coverage, we performed the quantitative analysis using two different proteases, trypsin and chymotrypsin, exhibiting different cleavage specificities. We decided to include only phosphopeptides in the quantification process that were detected in every LC-MS analysis. This comprised 17 of the 21 phosphorylation sites identified. Our quantitative analysis revealed that at least eight phosphorylation sites displayed significant differences in their phosphorylation state when TRP was isolated from dark- or light-reared flies, respectively. Interestingly, one site, Ser\textsuperscript{936}, was predominantly phosphorylated in the dark and became dephosphorylated in the light, whereas at least seven sites were found to occur more abundantly in the light in their phosphorylated state.

As analyzed by a phosphorylation site prediction tool (NetPhosK; available on the World Wide Web), most of the identified sites show consensus sequences for phosphorylation by PKC (Ser\textsuperscript{717}, Ser\textsuperscript{721}, Thr\textsuperscript{849}, Ser\textsuperscript{872}, Ser\textsuperscript{875}, Ser\textsuperscript{876}, Ser\textsuperscript{884}, Ser\textsuperscript{956} and Ser\textsuperscript{962}), three sites (Ser\textsuperscript{828}, Ser\textsuperscript{936}, and Ser\textsuperscript{964}) were predicted as casein kinase I or II sites, one site (Thr\textsuperscript{864}) was predicted as a protein kinase G site, one site (Ser\textsuperscript{881}) was predicted as a DNA-dependent protein kinase site, and seven sites (Ser\textsuperscript{15}, Ser\textsuperscript{726}, Ser\textsuperscript{861}, Thr\textsuperscript{863}, Ser\textsuperscript{980}, Ser\textsuperscript{1056}, and Ser\textsuperscript{1254}) were not predicted as phosphorylation sites at all. Because many of the TRP phosphorylation sites were predicted as PKC consensus sites, we hypothesized that eye-PKC that is associated with TRP in the INAD signaling complex might mediate phosphorylation of these sites. Moreover, eye-PKC was shown to phosphorylate TRP in vitro when the isolated INAD signaling complex is incubated with [γ-\textsuperscript{32}P]ATP (18, 19). Because eye-PKC is supposed to become activated by diacylglycerol and rising intracellular Ca\textsuperscript{2+} concentration following light activation of the photoreceptor cells, the observed in vitro phosphorylation of TRP by eye-PKC was suggested as a possible deactivation mechanism for the ion channel (1, 18, 19). However, all in vivo phosphorylation sites identified in the wild type were also present in the *inaC*\textsuperscript{P209} mutant, showing that phosphorylation of these sites can occur independently from eye-PKC. We cannot rule out that additional phosphorylation sites that escaped our mass spectrometric analysis are substrates of eye-PKC. Such sites may reside in hydrophobic regions of the channel or in the lysine- and proline-rich stretch near the C terminus that were not well covered in the mass spectrometric analysis. Alternatively, it is possible that a second protein kinase C, PKC53E(ey), which is also expressed in the eye (43), mediates phosphorylation of TRP sites *per se* or substitutes eye-PKC in the *inaC* mutant. Although the detected TRP phosphorylation sites are independent of eye-PKC, at least eight of these sites are phosphorylated light-dependently. These results show that besides eye-PKC, other light-activated protein kinases and/or phosphatases must be present in *Drosophila* photoreceptor cells.

To provide additional evidence for our mass spectrometry results by an independent method and to further investigate TRP phosphorylation, we analyzed the single site phosphorylated predominantly in the dark, Ser\textsuperscript{936}, in greater detail. Because both TRP regardless of its phosphorylation state (using the α-TRP antibody) and TRP phosphorylated at Ser\textsuperscript{936} (using the α-Ser(P)\textsuperscript{936} antibody) were found in the rhabdome, it is likely that phosphorylation and dephosphorylation of Ser\textsuperscript{936} take place in this photoreceptive cell compartment. Accordingly, the respective protein kinase and phosphatase should either reside in the rhabdome permanently or become recruited to the rhabdome quickly after a change of the light condition. As revealed by analysis of different mutants of the phototransduction cascade, the light-triggered dephosphorylation of Ser\textsuperscript{936} requires activation of the phototransduction cascade that results in Ca\textsuperscript{2+}-influx through TRP channels. Moreover, Ser\textsuperscript{936} is in its dephosphorylated state when a constitutively
active TRP is expressed in photoreceptor cells that mediates 
Ca\(^{2+}\)-influx without activation of the phototransduction cascade. Therefore, a possible trigger for dephosphorylation of Ser\(^{936}\) may be light-activated Ca\(^{2+}\) influx via TRP channels. The resulting rise in the intracellular Ca\(^{2+}\) concentration might, for instance, directly activate a Ca\(^{2+}\)-dependent protein phosphatase. Protein phosphatases that mediate dephosphorylation of phosphoserine or phosphothreonine residues can be grouped into four classes: protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B), and protein phosphatase 2C (PP2C). Although PP1 and PP2A are independent of divalent cations, PP2B and PP2C depend on the presence of Ca\(^{2+}\) and Mg\(^{2+}\), respectively (44). PP2A, which in Drosophila is encoded by the microtubule star (mts) locus, has been shown to dephosphorylate the photoreceptor protein INAD in vitro, and a mutation in the mts gene partially rescues the phenotype of the eye-PKC deficient inac mutant (45). In vitro studies on TRP phosphorylation and dephosphorylation using inhibitors of PP1 and PP2A, okadaic acid and microcystin, provided evidence for a possible role of these phosphatases in the dephosphorylation of TRP (19). However, this study used TRP that was phosphorylated in vitro by co-purified eye-PKC. According to our results, eye-PKC is not crucial for the phosphorylation of the 21 phosphorylation sites identified here, including Ser\(^{936}\). Whether or not a Ca\(^{2+}\)-dependent phosphatase like the Drosophila PP2B calcineurin is involved in the dephosphorylation of this site has yet to be determined.

 Whereas the phototransduction cascade is activated and deactivated within tens of milliseconds, we observed light-dependent changes in the phosphorylation state of TRP at Ser\(^{936}\) within minutes. It is therefore not likely that the light-triggered dephosphorylation of TRP at Ser\(^{936}\) regulates gating of the TRP channel. The dynamic change in the phosphorylation state of this site may rather be involved in processes that occur at a slower time scale, such as adaptation.

 It is striking that different TRP phosphorylation sites are regulated by light in opposite directions. Whereas several sites become phosphorylated in the light, a single site, Ser\(^{936}\), becomes phosphorylated in the dark. There are examples of other Drosophila phototransduction proteins that become either phosphorylated or dephosphorylated upon illumination. Rhodopsin and the regulatory protein arrestin2 that binds to rhodopsin and thereby inactivates the receptor quickly become phosphorylated when the eyes are illuminated (46–49). The recently characterized phosphoprotein retinophilin, which is required to suppress photoreceptor dark noise, becomes dephosphorylated when the flies are kept in the light (50). Another example for light-dependent dephosphorylation is Drosophila dMoesin (51). Here, dephosphorylation regulates the interaction of dMoesin with TRP and TRPL ion channels and its migration from the membrane to the cytoplasm. Because these proteins are all associated with the rhabdomeral photoreceptor membrane, it seems that separate kinases as well as phosphatases that become activated either in light or in darkness are present at or become recruited to this cellular compartment. Among the proteins studied, TRP so far is the only Drosophila phototransduction protein for which phosphorylation sites were identified that are regulated in opposite directions by light. It is possible that the different types of TRP phosphorylation serve different physiological functions. The identification of in vivo phosphorylation sites of the Drosophila TRP channel should facilitate mutagenesis studies that unravel the physiological role of these posttranslational modifications.

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