Biophysical Journal
Biophysical Letter

# Structurally Distinct Cation Channelrhodopsins from Cryptophyte Algae

Elena G. Govorunova,[1] Oleg A. Sineshchekov,[1] and John L. Spudich[1,*]
[1]Center for Membrane Biology and Department of Biochemistry and Molecular Biology, University of Texas Health Science Center at Houston, McGovern Medical School, Houston, Texas

ABSTRACT Microbial rhodopsins are remarkable for the diversity of their functional mechanisms based on the same protein scaffold. A class of rhodopsins from cryptophyte algae show close sequence homology with haloarchaeal rhodopsin proton pumps rather than with previously known channelrhodopsins from chlorophyte (green) algae. In particular, both aspartate residues that occupy the positions of the chromophore Schiff base proton acceptor and donor, a hallmark of rhodopsin proton pumps, are conserved in these cryptophyte proteins. We expressed the corresponding polynucleotides in human embryonic kidney (HEK293) cells and studied electrogenic properties of the encoded proteins with whole-cell patch-clamp recording. Despite their lack of residues characteristic of the chlorophyte cation channels, these proteins are cation-conducting channelrhodopsins that carry out light-gated passive transport of $Na^+$ and $H^+$. These findings show that channel function in rhodopsins has evolved via multiple routes.

Phototaxis receptors that depolarize the membranes of green (chlorophyte) algae (1) act as light-gated cation channels when expressed in animal cells (2,3). This unique property earned them the name "channelrhodopsins" and made them molecules of choice for optogenetic depolarization of the cell membrane and neuronal activation (4). In the phylogenetically distant cryptophyte algae, another family of channelrhodopsins with strictly anion selectivity was found and used to hyperpolarize the membrane and neuronal inhibition (5). Although these latter proteins, called anion channelrhodopsins, share some sequence homology with cation channelrhodopsins (CCRs) from green algae, their conduction mechanisms are clearly different (6–8).

*Guillardia theta*, the only cryptophyte organism the genome of which has been completely sequenced (9), is predicted to encode at least 53 microbial (type 1) rhodopsins (5). Except for the anion channelrhodopsins, few other rhodopsins from this organism have been investigated by heterologous expression (10). One cluster of *G. theta* rhodopsin protein models contains nine sequences, the closest homologs of which, besides similar proteins from other cryptophytes and uncharacterized fungal proteins, are haloarchaeal rhodopsins (Fig. S1 in the Supporting Material). Here we show that these proteins (which we designate as cryptophyte CCRs) are light-gated cation channels with distinctly different structures than those of previously known chlorophyte CCRs from green algae.

We synthesized human codon-adapted versions of three polynucleotides corresponding to the *G. theta* predicted transcripts 99928 (GenBank: KU761992), 120390 (GenBank: KU761994) and 162755 (GenBank: KU761993). The encoded polypeptides extend 150–200 residues beyond the seven-transmembrane-helix (rhodopsin) domain, but no other putative domains could be detected. We also included in our analysis a highly homologous protein from the cryptophyte alga *Proteomonas sulcata* (GenBank: KF992056) previously shown to generate photocurrents in neurons (11). We also synthesized a construct (GenBank: KU761991) corresponding to *G. theta* model 135937, but it was nonfunctional in our system.

An alignment of the rhodopsin domains showed a closer match of cryptophyte CCRs with haloarchaeal proton pumps than with chlorophyte CCRs (Fig. S2). Asp residues in the positions of the retinylidene Schiff base proton acceptor and donor (Asp-85 and Asp-96 in *Halobacterium salinarum* bacteriorhodopsin (*Hs*BR), respectively) are conserved in all four studied cryptophyte CCRs, whereas in the majority of chlorophyte CCRs Asp-85 is replaced with Glu, and Asp-96, with His (Fig. 1). Proton transport activity was not observed in a previously studied rhodopsin from the same organism, *Gt*R1, which also has Asp residues in both proton acceptor and donor positions, and a rhodopsin from the fungus *Neurospora*, NR, in which Asp-85 is conserved and Asp-96 is found as Glu (10,12).

*Submitted March 15, 2016, and accepted for publication May 2, 2016.*
*Correspondence: john.l.spudich@uth.tmc.edu
Editor: Andreas Engel.
http://dx.doi.org/10.1016/j.bpj.2016.05.001

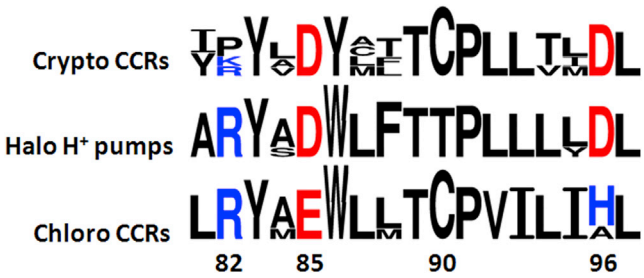



**FIGURE 1 Sequence logos of the transmembrane helix 3 generated with WebLogo 3 (17) from the alignment shown in Fig. S2. The numbering is according to the *Hs*BR sequence.**

None of the five conserved Glu residues in helix 2 of chlorophyte CCRs (highlighted *green* in Fig. S2) that contribute to their cation translocation pathway (13) is conserved in the tested cryptophyte proteins. However, cryptophyte CCRs share with CCRs from green algae a Cys residue in the position of Thr-90 in *Hs*BR (Fig. 1). Arg-82 (*Hs*BR numbering), conserved in most microbial rhodopsins, is replaced with Pro or Lys in *G. theta* CCRs. Modification of the Arg-82 homolog in an $H^+$-pumping rhodopsin from the green alga *Coccomyxa* converted it into an operational $H^+$ channel (14).

We expressed the constructs encoding the rhodopsin domains of four cryptophyte CCRs fused to enhanced yellow fluorescent protein in human embryonic kidney (HEK293) cells and tested their electrogenic function with whole-cell patch-clamp. Under our standard conditions (the holding potential ($E_h$) −60 mV, 150 mM NaCl in the bath, 126 mM KCl in the pipette, pH 7.4; for other details, see the Supporting Material), all four proteins generated inward currents in response to a light pulse. The mean amplitudes and half-decay rates of the photocurrents are shown in Fig. S3. Protein 99928 generated the largest currents, whereas the currents from the other three proteins were considerably smaller.

The action spectra of the photocurrents are shown in Fig. 2. In the genomes of most green algae, only two channelrhodopsin homologs have been found. According to a historical convention, the more red-shifted rhodopsin from the same organism is assigned the number *1*, and the more blue-shifted is assigned the number *2* (1).

As shown below, all three *G. theta* rhodopsins tested in this study are nonselective light-gated cation channels. Therefore, we assigned the abbreviation *Gt*CCR1 to the most red-shifted one (120390) with the maximal sensitivity at 520 nm; *Gt*CCR2, to the more blue-shifted rhodopsin (99928) with

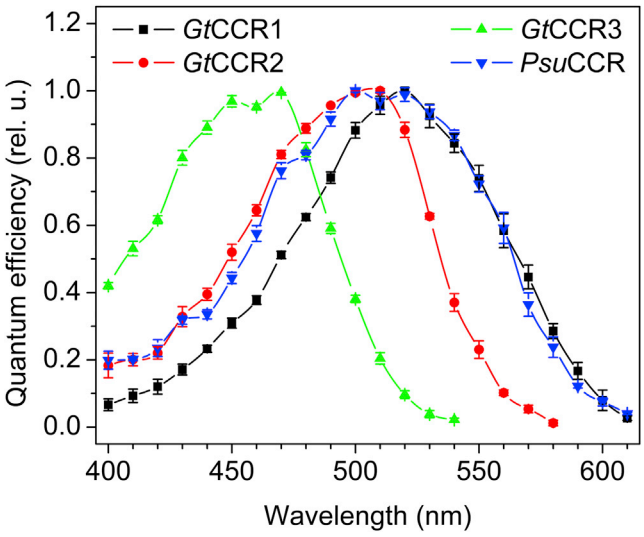



**FIGURE 2 The action spectra of photocurrents generated by cryptophyte CCRs in HEK293 cells.**

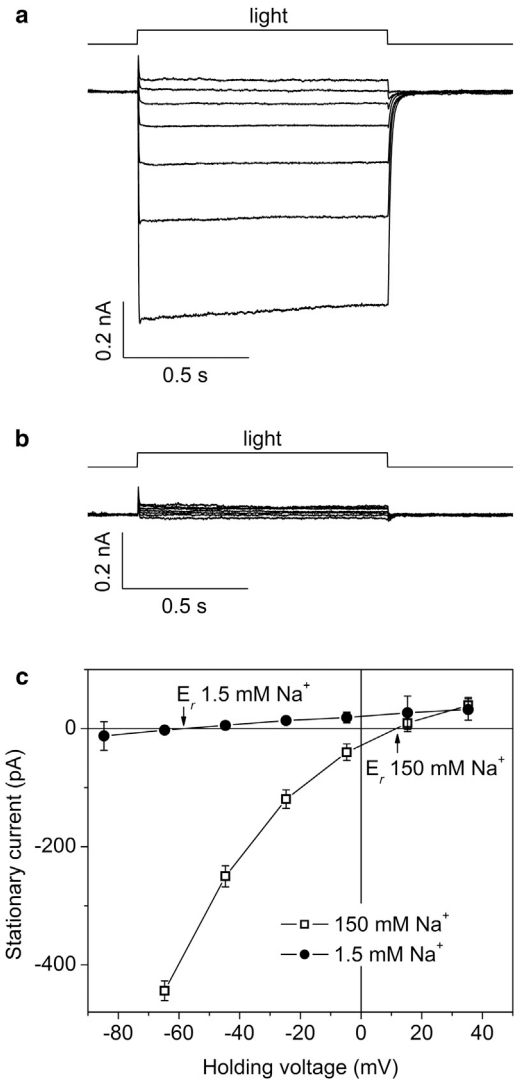



**FIGURE 3 Representative series of photocurrents generated by *Gt*CCR2 in a HEK293 cell at 150 (*a*) and 1.5 (*b*) mM $Na^+$ in the bath. $E_h$ was changed in 20-mV steps from −80 to 40 mV at the amplifier output (*bottom* to *top*). (*c*) The current-voltage relationship for the cell shown in (*a*) and (*b*). The data are the mean values mean ± SE ($n = 3$) corrected for liquid junction potentials.**

the peak at 505 nm; and *Gt*CCR3, to the most blue-shifted rhodopsin (162755) with peaks at ~460 nm. The spectral maximum of this latter protein corresponds to that of the *G. theta* photoaccumulation response (10).

Initially, when no information regarding its ionic selectivity was available, the homologous protein from *P. sulcata* was referred to as "*Ps*ChR2" (11). To avoid confusion with CCRs from the chlorophyte alga *Platymonas subcordiformis* (15), we will call it *Psu*CCR in this study. No number can be assigned to this rhodopsin as yet, because the spectral properties of its several other homologs from the same organism (Fig. S1) have not yet been determined.

Upon shifting $E_h$ to more positive values, the photocurrents generated by cryptophyte CCRs reversed their direction (Fig. 3 *a*). The current-voltage dependencies (IE curves) measured under standard conditions exhibited a significant inward rectification (Fig. 3 *c*, *open squares*).

To identify the nature of the transported ions, we determined reversal potentials ($E_r$) upon variation of the ionic composition of the bath solution. When $Na^+$ concentration in the bath was reduced 100-fold by partial replacement with *n*-methyl-D-glucamine ($NMG^+$), a dramatic inhibition of photocurrents was observed (Fig. 3 *b*). Moreover, for all tested proteins, $E_r$ shifted to more negative values (Figs. 3 *c*, *solid circles*, and S4), indicating that they passively transported $Na^+$ across the cell membrane.

A 100-fold reduction of the bath $H^+$ concentration (from pH 5.4 to pH 7.4) also led to an $E_r$ shift to negative values, although its magnitude was smaller than that for $Na^+$ (Fig. S4). Therefore, we concluded that cryptophyte CCRs transport both $Na^+$ and $H^+$, as do chlorophyte CCRs from green algae. Purely passive $H^+$ influx has also been demonstrated at low extracellular pH and negative membrane potentials for some rhodopsin $H^+$ pumps, such as that from *Gloeobacter violaceus* (16) i.e., a leaky-pump phenomenon. However, channel currents generated by cryptophyte CCRs cannot be explained by this mechanism, because at physiological conditions they are carried practically only by $Na^+$ ions (Fig. 3). $Na^+$ pumping can be excluded, because the direction of photocurrents depended on the Nernst equilibrium potential for $Na^+$.

Our results indicate that the microbial rhodopsin common ancestor of chlorophyte and cryptophyte CCRs converged on the same function through structurally different paths. Further research is needed to elucidate the mechanisms of cation conductance in cryptophyte CCRs, but it is already clear that their identification expands our current concepts about channelrhodopsins in an unexpected direction.

## SUPPORTING MATERIAL

Four figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)30274-0.

## ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants No. R01GM027750 and No. U01MH109146 and Endowed Chair No. AU-0009 from the Robert A. Welch Foundation.

## REFERENCES

1. Sineshchekov, O. A., K.-H. Jung, and J. L. Spudich. 2002. Two rhodopsins mediate phototaxis to low- and high-intensity light in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA*. 99:8689–8694.

2. Nagel, G., D. Ollig, …, P. Hegemann. 2002. Channelrhodopsin-1: a light-gated proton channel in green algae. *Science*. 296:2395–2398.

3. Nagel, G., T. Szellas, …, E. Bamberg. 2003. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc. Natl. Acad. Sci. USA*. 100:13940–13945.

4. Boyden, E. S., F. Zhang, …, K. Deisseroth. 2005. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* 8:1263–1268.

5. Govorunova, E. G., O. A. Sineshchekov, …, J. L. Spudich. 2015. NEUROSCIENCE. Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics. *Science*. 349:647–650.

6. Sineshchekov, O. A., E. G. Govorunova, …, J. L. Spudich. 2015. Gating mechanisms of a natural anion channelrhodopsin. *Proc. Natl. Acad. Sci. USA*. 112:14236–14241.

7. Govorunova, E. G., O. A. Sineshchekov, and J. L. Spudich. 2015. *Proteomonas sulcata* ACR1: a fast anion channelrhodopsin. *Photochem. Photobiol.* 92:257–263.

8. Sineshchekov, O. A., H. Li, …, J. L. Spudich. 2016. Photochemical reaction cycle transitions during anion channelrhodopsin gating. *Proc. Natl. Acad. Sci. USA*. 201525269.

9. Curtis, B. A., G. Tanifuji, …, J. M. Archibald. 2012. Algal genomes reveal evolutionary mosaicism and the fate of nucleomorphs. *Nature*. 492:59–65.

10. Sineshchekov, O. A., E. G. Govorunova, …, J. L. Spudich. 2005. Rhodopsin-mediated photoreception in cryptophyte flagellates. *Biophys. J.* 89:4310–4319.

11. Klapoetke, N. C., Y. Murata, …, E. S. Boyden. 2014. Independent optical excitation of distinct neural populations. *Nat. Methods*. 11:338–346.

12. Brown, L. S., A. K. Dioumaev, …, J. L. Spudich. 2001. Photochemical reaction cycle and proton transfers in *Neurospora* rhodopsin. *J. Biol. Chem.* 276:32495–32505.

13. Kato, H. E., F. Zhang, …, O. Nureki. 2012. Crystal structure of the channelrhodopsin light-gated cation channel. *Nature*. 482:369–374.

14. Vogt, A., Y. Guo, …, P. Hegemann. 2015. Conversion of a light-driven proton pump into a light-gated ion channel. *Sci. Rep.* 5:16450.

15. Govorunova, E. G., O. A. Sineshchekov, …, J. L. Spudich. 2013. Characterization of a highly efficient blue-shifted channelrhodopsin from the marine alga *Platymonas subcordiformis*. *J. Biol. Chem.* 288:29911–29922.

16. Vogt, A., J. Wietek, and P. Hegemann. 2013. *Gloeobacter* rhodopsin, limitation of proton pumping at high electrochemical load. *Biophys. J.* 105:2055–2063.

17. Crooks, G. E., G. Hon, …, S. E. Brenner. 2004. WebLogo: a sequence logo generator. *Genome Res.* 14:1188–1190.

**Biophysical Journal, Volume *110***

## Supplemental Information

# Structurally Distinct Cation Channelrhodopsins from Cryptophyte Algae

**Elena G. Govorunova, Oleg A. Sineshchekov, and John L. Spudich**

# Supporting Material

# Structurally distinct cation channelrhodopsins from cryptophyte algae

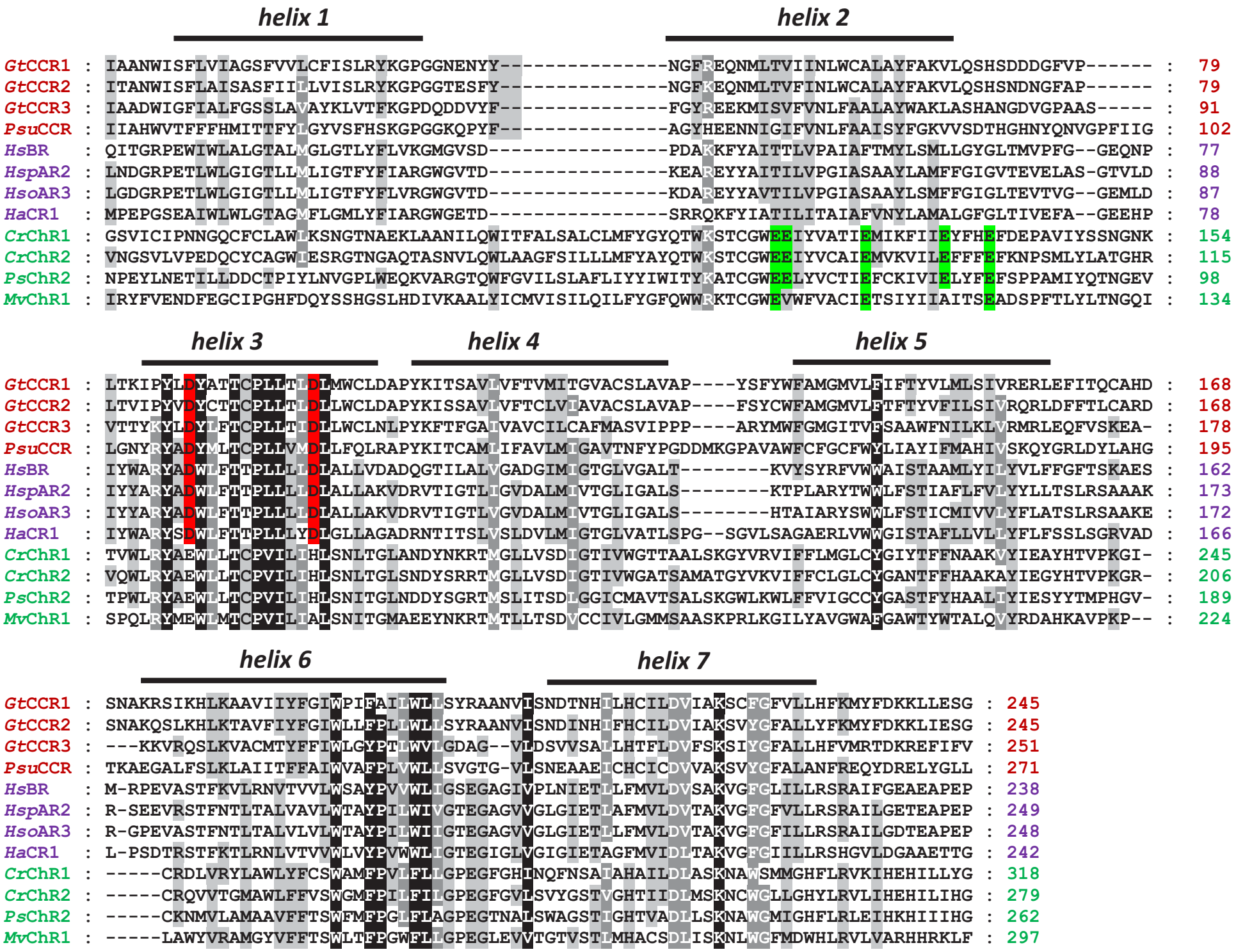
Elena G. Govorunova, Oleg A. Sineshchekov, and John L. Spudich

Center for Membrane Biology, Department of Biochemistry and Molecular Biology, University of Texas Health Science Center at Houston McGovern Medical School, Houston, Texas, USA

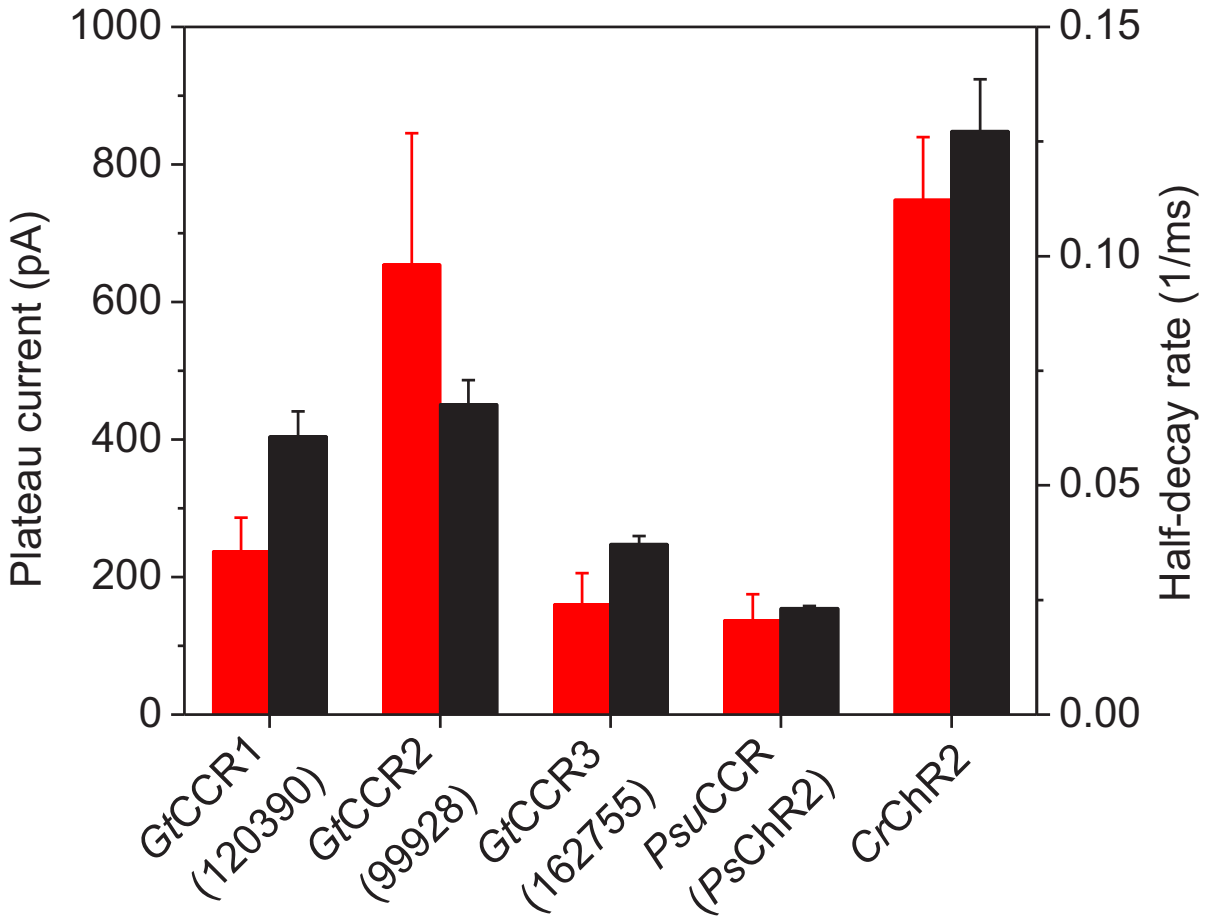
Address reprint requests and inquiries to John.L.Spudich@uth.tmc.edu


protein 110 [synthetic construct]
protein 89 [synthetic construct]
hypothetical protein GUITHDRAFT_159333 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_85745 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_52605 [Guillardia theta CCMP2712]
protein 72 [synthetic construct]
opsin 2 [Cyanophora paradoxa]
hypothetical protein GUITHDRAFT_86360 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_106800 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_99837 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_159252 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_152884 [Guillardia theta CCMP2712]
hypothetical protein [Nodosilinea nodulosa]
hypothetical protein [Kushneria aurantia]
hypothetical protein [Halomonas sp. PR-M31]
rhodopsin [Chroococcidiopsis thermalis]
rhodopsin [Gloeocapsa sp. PCC 7428]
hypothetical protein [Chlorogloeopsis fritschii]
hypothetical protein [Leptolyngbya boryana]
bacteriorhodopsin [Nostoc sp. NIES-3756]
bacteriorhodopsin [Chamaesiphon minutus PCC 6605]
hypothetical protein [Tolypothrix boutellieii]
rhodopsin [Natronomonas moolapensis]
bacteriorhodopsin [Natronomonas moolapensis 8.8.11]
bacteriorhodopsin [uncultured haloarchaeon]
rhodopsin [Halobacterium salinarium]
bacteriorhodopsin [Halorubrum coriense]
AR3-1-GFP [synthetic construct]
rhodopsin [Halorubrum sp. 8]
bacteriorhodopsin [uncultured archaeon A07HB70]
bacteriorhodopsin [uncultured haloarchaeon]
bacteriorhodopsin [uncultured haloarchaeon]
bacteriorhodopsin [uncultured archaeon]
bacteriorhodopsin [uncultured haloarchaeon]
bacteriorhodopsin [Synechocystis sp. PCC 7509]
rhodopsin [Leptolyngbya sp. NIES-3755]
bacteriorhodopsin [Chamaesiphon minutus]
rhodopsin [Hassallia byssoidea]
rhodopsin [Scytonema tolypothrichoides]
rhodopsin [Tolypothrix boutellieii]
rhodopsin [Tolypothrix campylonemoides]
hypothetical protein [Mastigocladopsis repens]
rhodopsin [Alterella atlantica]
hypothetical protein [Erythrobacter sp. KA37]
hypothetical protein [Sphingomonas sp. PAMC 26621]
hypothetical protein [Sphingomonas sp. Leaf38]
hypothetical protein [Sphingomonas sp. Leaf34]
hypothetical protein [Sphingomonas sp. ST-SE-2]
rhodopsin [Methylobacterium sp. GXS13]
sensory rhodopsin 1 [Haloarcula vallismortis]
bacteriorhodopsin [uncultured archaeon A07HR67]
rhodopsin [Halorubrum lacusprofundi]
sensory rhodopsin-2 [Halopiricum salinum]
sensory rhodopsin-2 [Haloferax mediterranei]
sensory rhodopsin-2 [Haloarcula sp. SL3]
sensory rhodopsin 1 [Haloarcula vallismortis]
sensory rhodopsin 1 [Haloarcula amylolytica]
sensory rhodopsin-2 [Haloarcula sp. GSL1127]
sensory rhodopsin 1 [Haloarcula argentinensis]
sensory rhodopsin 1 [Haloarcula californiae]
sensory rhodopsin-2 [Haloarcula hispanica]
sensory rhodopsin 1 [Haloarcula sinaiiensis]
sensory rhodopsin-2 [Haloarcula marismortui]
sensory rhodopsin-2 [Natronomonas pharaonis]
sensory rhodopsin-2 [Halobacterium hubeiense]
sensory rhodopsin II [Halobacterium hubeiense]
sensory rhodopsin-2 [Halobacterium salinarum]
family A G protein-coupled receptor-like protein [Phialocephala scopiformis]
hypothetical protein COCCADRAFT_26476 [Bipolaris zeicola 26-R-13]
hypothetical protein SBOR_9638 [Sclerotinia borealis F-4157]
hypothetical protein BC1G_13906 [Botrytis cinerea B05.10]
hypothetical protein SS1G_04339 [Sclerotinia sclerotiorum 1980]
hypothetical protein PV05_11623 [Exophiala xenobiotica]
hypothetical protein EPUS_05370 [Endocarpon pusillum Z07020]
hypothetical protein PV06_03044 [Exophiala oligosperma]
hypothetical protein PV08_05307 [Exophiala spinifera]
hypothetical protein G647_05224 [Cladophialophora carrionii CBS 160.54]
hypothetical protein A1O7_06308 [Cladophialophora yegresii CBS 114405]
hypothetical protein MYCFIDRAFT_211441 [Pseudocercospora fijiensis CIRAD86]
related to YRO2-putative plasma membrane protein
transcriptionally regulated by Hat1p [Melanopsichium pennsylvanicum 4]
hypothetical protein TREMEDRAFT_17826 [Tremella mesenterica DSM 1558]
hypothetical protein MELLADRAFT_39586 [Melampsora larici-populina 98AG31]
hypothetical protein SCLCIDRAFT_141768 [Sclerodermacitrinum Foug A]
hypothetical protein PFL1_01891 [Anthracocystis flocculosa PF-1]
protein of bacterial rhodopsin family [Rhodotorula toruloides NP11]
Rhodopsin
retinal binding site [Xanthophyllomyces dendrorhous]
hypothetical protein G7K_1936-t1 [Saitoella complicata NRRL Y-17804]
hypothetical protein PDE_04448 [Penicillium oxalicum 114-2]
hypothetical protein SNOG_00341 [Parastagonospora nodorum SN15]
hypothetical protein LEMA_P114110.1 [Leptosphaeria maculans JN3]
family A G protein-coupled receptor-like protein [Sphaerulina musiva SO2202]
hypothetical protein DOTSEDRAFT_75607 [Dothistroma septosporum NZE10]
FDD123 protein [Verticillium dahliae VdLs.17]
hypothetical protein BN1723_001539 [Verticillium longisporum]
hypothetical protein BN1708_017586 [Verticillium longisporum]
hypothetical protein BN1723_017257 [Verticillium longisporum]
putative bacteriorhodopsin [Colletotrichum sublineola]
bacteriorhodopsin [Colletotrichum simmondsii]
bacteriorhodopsin [Colletotrichum salicis]
heat shock protein 30 [Colletotrichum orbiculare MAFF 240422]
heat shock protein 30 [Colletotrichum gloeosporioides Nara gc5]
bacteriorhodopsin [Colletotrichum gloeosporioides Cg-14]
bacteriorhodopsin [Colletotrichum graminicola M1.001]
hypothetical protein 540285 08856 [Stachybotrys chlorohalonata IBT 40285]
hypothetical protein S7711 01519 [Stachybotrys chartarum IBT 7711]
hypothetical protein GGTG_05062 [Gaeumannomyces graminis var. tritici R3-111a-1]
Family A G protein-coupled receptor-like protein [Glarea lozoyensis ATCC 20868]
hypothetical protein M7I_6795 [Glarea lozoyensis 74030]
opsin-like protein [Marssonina brunnea f. sp. multigermtubi MB m1]
hypothetical protein AB675 5839 [Phialophora attae]
hypothetical protein HMPREF1541 00158 [Cyphellophora europaea CBS 101466]
hypothetical protein PV10 08466 [Exophiala mesophila]
hypothetical protein HMPREF1120 02861 [Exophiala dermatitidis NIH/UT8656]
Opsin-1 [Taphrina deformans PYCC 5710]
hypothetical protein Micbo1qcDRAFT_187824 [Microdochium bolleyi]
archaerhodopsin-2 precursor [Sphaerulina musiva SO2202]
bacteriorhodopsin [Aureobasidium pullulans EXF-150]
hypothetical protein DOTSEDRAFT_133285 [Dothistroma septosporum NZE10]
hypothetical protein PV11_03940 [Exophiala sideris]
hypothetical protein AC579_7431 [Pseudocercospora musae]
hypothetical protein AC578_1260 [Mycosphaerella eumusae]
hypothetical protein FAVG1_09553 [Fusarium avenaceum]
hypothetical protein FGSG_03064 [Fusarium graminearum PH-1]
hypothetical protein FG05_03064 [Fusarium graminearum]
hypothetical protein FPSE_02162 [Fusarium pseudograminearum CS3096]
opsin [Fusarium tangerinae]
opsin-like protein [Stemphylium lycopersici]
hypothetical protein PTT_09940 [Pyrenophora teres f. teres 0-1]
Rhodopsin bacterial [Macrophomina phaseolina MS6]
family A G protein-coupled receptor-like protein [Zymoseptoria brevis]
hypothetical protein MYCGRDRAFT_106573 [Zymoseptoria tritici IPO323]
hypothetical protein AC579_8497 [Pseudocercospora musae]
hypothetical protein AC578_3015 [Mycosphaerella eumusae]
hypothetical protein MYCFIDRAFT_56178 [Pseudocercospora fijiensis CIRAD86]
hypothetical protein FE78DRAFT_107577 [Acidomyces richmondensis]
hypothetical protein AUEXF2481DRAFT_3502 [Aureobasidium subglaciale EXF-2481]
family A G protein-coupled receptor-like protein [Aureobasidium melanogenum CBS 110.
family A G protein-coupled receptor-like protein [Aureobasidium pullulans EXF-150]
opsin-like protein [Aureobasidium namibiae CBS 147.97]
putative opsin-like protein [Eutypa lata UCREL1]
putative opsin-like protein [Roseilinia necatrix]
hypothetical protein PFICI_15171 [Pestalotiopsis fici W106-1]
hypothetical protein M422DRAFT_215320 [Sphaerobolus stellatus SS14]
hypothetical protein MELLADRAFT_50312 [Melampsora larici-populina 98AG31]
protein of unknown function [Taphrina deformans PYCC 5710]
Opsin-1 [Taphrina deformans PYCC 5710]
putative opsin 1 [Phaeomoniella chlamydospora]
hypothetical protein EPUS_00641 [Endocarpon pusillum Z07020]
Opsin-like protein [Acremonium chrysogenum ATCC 11550]
hypothetical protein [Podospora anserina S mat+]
hypothetical protein 540285 06423 [Stachybotrys chlorohalonata IBT 40285]
hypothetical protein 540288 06367 [Stachybotrys chartarum IBT 40288]
opsin-1 [Fusarium graminearum PH-1]
hypothetical protein FG05_07554 [Fusarium graminearum]
hypothetical protein COCC4DRAFT_158477 [Bipolaris maydis ATCC 48331]
opsin-1 [Sclerotinia sclerotiorum 1980]
hypothetical protein BC1G_02458 [Botrytis cinerea B05.10]
hypothetical protein Z520_10478 [Fonsecaea multimorphosa CBS 102226]
hypothetical protein PV07_05899 [Cladophialophora immunda]
hypothetical protein Z517_07125 [Fonsecaea pedrosoi CBS 271.37]
hypothetical protein A1O3_08726 [Capronia epimyces CBS 606.96]
hypothetical protein A1O1_01146 [Capronia coronata CBS 617.96]
hypothetical protein A1O7_01290 [Cladophialophora yegresii CBS 114405]
hypothetical protein G647_01353 [Cladophialophora carrionii CBS 160.54]
hypothetical protein PV04_00680 [Capronia semiimmersa]
Opsin-1 [Phialophora attae]
hypothetical protein G7K_4380-t1 [Saitoella complicata NRRL Y-17804]
rhodopsin-like 3 [Oxyrrhis marina]
hypothetical protein L869DRAFT_95897 [Mixia osmundae IAM 14324]
hypothetical protein M422DRAFT_149760 [Sphaerobolus stellatus SS14]
rhodopsin
a chromoprotein [Kledonobacter racemifer]
rhodopsin [Haloquadratum walsbyi]
bacteriorhodopsin II [Haloquadratum walsbyi C23]
rhodopsin [Haloquadratum walsbyi]
bacteriorhodopsin [Haloquadratum walsbyi...07HQW2]
rhodopsin [Pyrocystis lunula]
rhodopsin [Oxyrrhis marina]
rhodopsin [Oxyrrhis marina]
rhodopsin [Oxyrrhis marina]
hypothetical protein KNAG_0H01270 [Kazachstania naganishii CBS 8797]
hypothetical protein GUITHDRAFT_162755 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_148916 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_148915 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_135937 [Guillardia theta CCMP2712]
protein 100 [synthetic construct]
hypothetical protein GUITHDRAFT_164280 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_164579 [Guillardia theta CCMP2712]
ChCh2 [synthetic construct]
protein 107 [synthetic construct]
protein 109 [synthetic construct]
protein 94 [synthetic construct]
hypothetical protein GUITHDRAFT_107985 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_120390 [Guillardia theta CCMP2712]
hypothetical protein GUITHDRAFT_99928 [Guillardia theta CCMP2712]
proteorhodopsin [Candidatus Pelagibacter ubique]
bacteriorhodopsin [Candidatus Pelagibacter ubique]
proteorhodopsin [Candidatus Pelagibacter ubique HTCC1002]
proteorhodopsin [Candidatus Pelagibacter ubique]
bacteriorhodopsin [Candidatus Pelagibacter ubique]

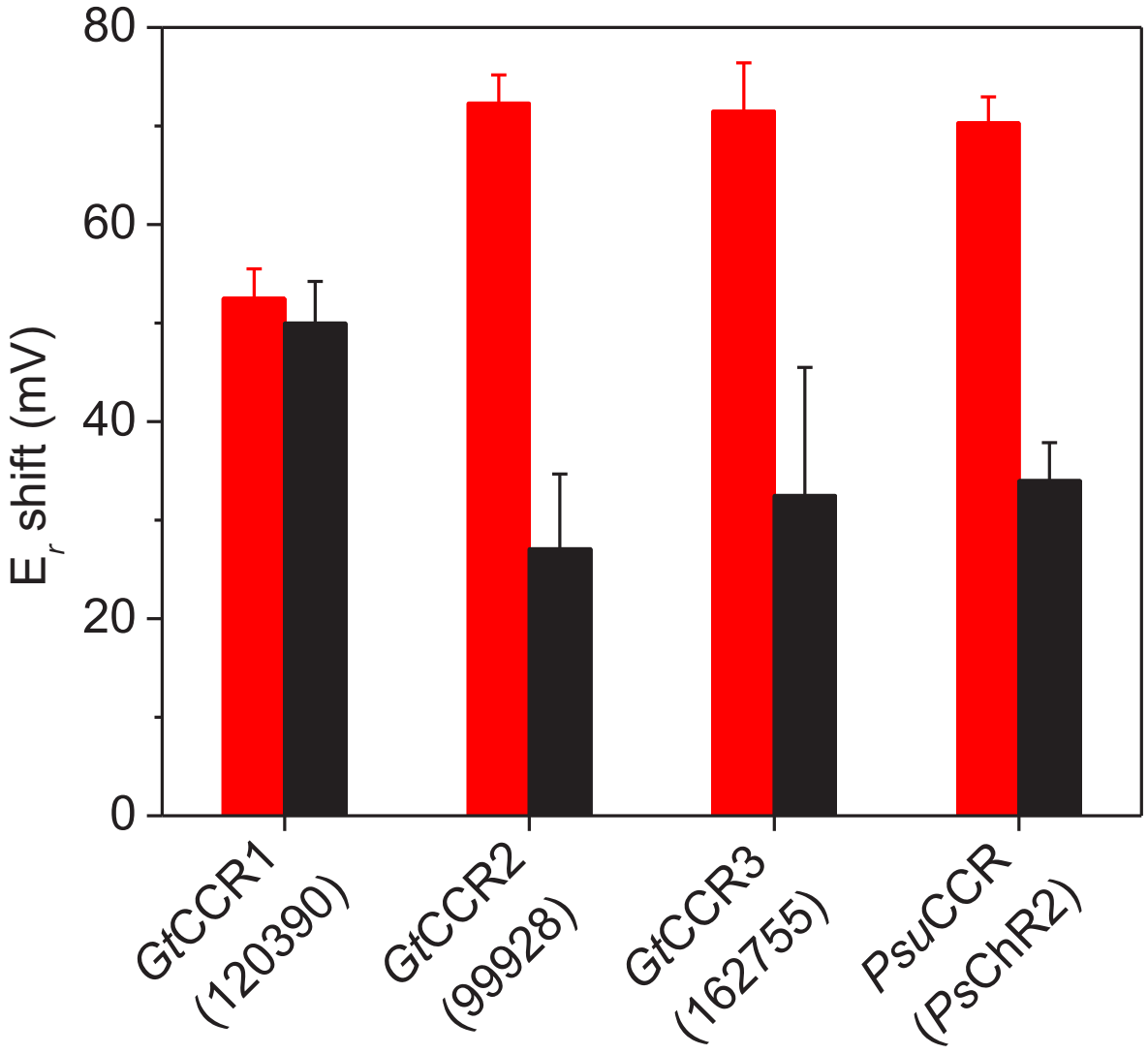
**Supporting figure 1.** A phylogenetic tree of 500 closest homologs of the rhodopsin domain of *Guillardia theta* protein model 99928 found in the NCBI non-redundant translated nucleotide database by Position-Specific Iterated BLAST. The hits are color-coded according to the phylogenetic position of their source organisms: orange, cryptophytes; yellow, fungi; olive, other eukaryotes; magenta, haloarchaea; cyan, cyanobacteria; pink, other prokaryotes; not colored, microbial rhodopsins from undefined source organisms. Proteins tested in this study are marked with the black arrows on the right. Some redundant entries have been manually removed by the Dendroscope 3 software (1). The tree was visualized using the EvolView server (2).


helix 1
helix 2
GtCCR1 : IAANWISFLVIAGSFVVLCFISLRYKGPGGNENYY----------NGFREQNMLTVIINLWCALAYFAKVLQSHSDDDGFVP------ : 79
GtCCR2 : ITANWISFLAISASFIILLVISLRYKGPGGTESFY----------NGFKEQNMLTVFINLWCALAYFAKVLQSHSNDNGFAP------ : 79
GtCCR3 : IAADWIGFIALFGSSLAVAYKLVTFKGPDQDDVYF----------FGYREEKMISVFVNLFAALAYWAKLASHANGDVGPAAS----- : 91
PsuCCR : IIAHWVTFFFHMITTFYLGYVSFHSKGPGGKQPYF----------AGYHEENNIGIFVNLFAAISYFGKVVSDTHGHNYQNVGPFIIG : 102
HsBR : QITGRPEWIWLALGTALMGLGTLYFLVKGMGVSD----------PDAKKFYAITTLVPAIAFTMYLSMLLGYGLTMVPFG--GEQNP : 77
HspAR2 : LNDGRPETLWLGIGTLLMLIGTFYFIARGWGVTD----------KEAREYYAITILVPGIASAAYLAMFFGIGVTEVELAS-GTVLD : 88
HsoAR3 : LGDGRPETLWLGIGTLLMLIGTFYFLVRGWGVTD----------KDAREYYAVTILVPGIASAAYLSMFFGIGLTEVTVG--GEMLD : 87
HaCR1 : MPEPGSEAIWLWLGTAGMFLGMLYFIARGWGETD----------SRRQKFYIATILITAIAFVNYLAMALGFGLTIVEFA--GEEHP : 78
CrChR1 : GSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWITFALSALCLMFYGYQTWKSTCGWEEIYVATIEMIKFIIEYFHEFDEPAVIYSSNGNK : 154
CrChR2 : VNGSVLVPEDQCYCAGWIESRGTNGAQTASNVLQWLAAGFSILLLMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHR : 115
PsChR2 : NPEYLNETILLDDCTPIYLNVGPLWEQKVARGTQWFGVILSLAFLIYYIWITYKATCGWEELYVCTIEFCKIVIELYFEFSPPAMIYQTNGEV : 98
MvChR1 : IRYFVENDFEGCIPGHFDQYSSHGSLHDIVKAALYICMVISILQILFYGFQWWRKTCGWEVWFVACIETSIYIIAITSEADSPFTLYLTNGQI : 134
helix 3
helix 4
helix 5
GtCCR1 : LTKIPYLDYATTCPLLTLDLMWCLDAPYKITSAVLVFTVMITGVACSLAVAP----YSFYWFAMGMVLFIFTYVLMLSIVRERLEFITQCAHD : 168
GtCCR2 : LTVIPYVDYCTTCPLLTLDLLWCLDAPYKISSAVLVFTCLVIAVACSLAVAP----FSYCWFAMGMVLFTFTYVFILSIVRQRLDFFTLCARD : 168
GtCCR3 : VTTYKYLDYLFTCPLLTIDLLWCLNLPYKFTFGAIVAVCILCAFMASVIPPP----ARYMWFGMGITVFSAAWFNILKLVRMRLEQFVSKEA- : 178
PsuCCR : LGNYRYADYMLTCPLLVMDLLFQLRAPYKITCAMLIFAVLMIGAVTNFYPGDDMKGPAVAWFCFGCFWYLIAYIFMAHIVSKQYGRLDYLAHG : 195
HsBR : IYWARYADWLFTTPLLLLDLALLVDADQGTILALVGADGIMIGTGLVGALT--------KVYSYRFVWWAISTAAMLYILYVLFFGFTSKAES : 162
HspAR2 : IYYARYADWLFTTPLLLLDLALLAKVDRVTIGTLIGVDALMIVTGLIGALS--------KTPLARYTWWLFSTIAFLFVLYYLLTSLRSAAAK : 173
HsoAR3 : IYYARYADWLFTTPLLLLDLALLAKVDRVTIGTLVGVDALMIVTGLIGALS--------HTAIARYSWWLFSTICMIVVLYFLATSLRSAAKE : 172
HaCR1 : IYWARYSDWLFTTPLLLYDLGLLAGADRNTITSLVSLDVLMIGTGLVATLSPG--SGVLSAGAERLVWWGISTAFLLVLLYFLFSSLSGRVAD : 166
CrChR1 : TVWLRYAEWLLTCPVILIHLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKGYVRVIFFLMGLCYGIYTFFNAAKVYIEAYHTVPKGI- : 245
CrChR2 : VQWLRYAEWLLTCPVILIHLSNLTGLSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVIFFCLGLCYGANTFFHAAKAYIEGYHTVPKGR- : 206
PsChR2 : TPWLRYAEWLLTCPVILIHLSNITGLNDDYSGRTMSLITSDLGGICMAVTSALSKGWLKWLFFVIGCCYGASTFYHAALIYIESYYTMPHGV- : 189
MvChR1 : SPQLRYMEWLMTCPVILIALSNITGMAEEYNKRTMTLLTSDVCCIVLGMMSAASKPRLKGILYAVGWAFGAWTYWTALQVYRDAHKAVPKP-- : 224
helix 6
helix 7
GtCCR1 : SNAKRSIKHLKAAVIIYFGIWPIFAILWLLSYRAANVISNDTNHLLHCILDVIAKSCFGFVLLHFKMYFDKKLLESG : 245
GtCCR2 : SNAKQSLKHLKTAVFIYFGIWLLFPLLWLLSYRAANVISNDINHIFHCILDVIAKSVYGFALLYFKMYFDKKLIESG : 245
GtCCR3 : ---KKVRQSLKVACMTYFFIWLGYPTLWVLGDAG--VLDSVVSALLHTFLDVFSKSIYGFALLHFVMRTDKREFIFV : 251
PsuCCR : TKAEGALFSLKLAIITFFAIWVAFPLVWLLSVGTG-VLSNEAAEICHCICDVVAKSVYGFALANFREQYDRELYGLL : 271
HsBR : M-RPEVASTFKVLRNVTVVLWSAYPVVWLIGSEGAGIVPLNIETLLFMVLDVSAKVGFGLILLRSRAIFGEAEAPEP : 238
HspAR2 : R-SEEVRSTFNTLTALVAVLWTAYPILWIVGTEGAGVVGLGIETLAFMVLDVTAKVGFGFVLLRSRAILGETEAPEP : 249
HsoAR3 : R-GPEVASTFNTLTALVLVLWTAYPILWIIGTEGAGVVGLGIETLLFMVLDVTAKVGFGFILLRSRAILGDTEAPEP : 248
HaCR1 : L-PSDTRSTFKTLRNLVTVVWLVYPVWWLIGTEGIGLVGIGIETAGFMVIDLTAKVGFGIILLRSHGVLDGAAETTG : 242
CrChR1 : -----CRDLVRYLAWLYFCSWAMFPVLFLLGPEGFGHINQFNSAIAHAILDLASKNAWSMMGHFLRVKIHEHILLYG : 318
CrChR2 : -----CRQVVTGMAWLFFVSWGMFPILFILGPEGFGVLSVYGSTVGHTIIDLMSKNCWGLLGHYLRVLIHEHILIHG : 279
PsChR2 : -----CKNMVLAMAAVFFTSWFMFPGLFLAGPEGTNALSWAGSTIGHTVADLLSKNAWGMIGHFLRLEIHKHIIIHG : 262
MvChR1 : -----LAWYVRAMGYVFFTSWLTFPGWFLLGPEGLEVVTGTVSTLMHACSDLISKNLWGFMDWHLRVLVARHHRKLF : 297

**Supporting figure 2.** A ClustalW partial protein alignment of four cryptophyte CCRs used in this study: *Gt*CCR1 *aka* 120390 (KU761994), *Gt*CCR2 *aka* 99928 (KU761992), *Gt*CCR3 *aka* 162755 (KU761993) and *Psu*CCR *aka Ps*ChR2 (KF992056). For comparison are also included haloarchaeal proton pumps: *Hs*BR, *Halobacterium salinarum* bacteriorhodopsin (V00474), *Halobacterium* sp aus-2 archaeorhodopsin-2 (S56354), *Halorubrum sodomense* archaeorhodopsin-3 (GU045593), *Haloarcula argentinos* cruxrhodopsin-1 (D31880), and chlorophyte CCRs: *Chlamydomonas reinhardtii* ChR1 (AF508965), *Chlamydomonas reinhardtii* ChR2 (AF508966), *Platymonas (Tetraselmis) subcordiformis* ChR2 (JX983143), and *Mesostigma viride* ChR1 (JF922293). Numbers in parentheses are GenBank accession numbers. Amino acid residue numbering and helical regions are according to the *Hs*BR sequence. The Glu residues conserved in helix 2 of chlorophyte CCRs but not found in cryptophyte CCRs or haloarchaeal rhodopsin proton pumps are highlighted green, and the Asp residues conserved in helix 3 of haloarchaeal rhodopsin proton pumps and cryptophyte CCRs are highlighted red.




**Supporting figure 3.** The mean amplitudes of stationary photocurrents (red bars, left axis) generated by cryptophyte CCRs in HEK293 cells at the saturating light intensity and the wavelengths of the maximal sensitivity for each protein (see Fig. 2 in the main text), and half-decay rates of these currents upon switching off the light (black bars, right axis). The data are the mean values ± SEM (*n* = 5-14 cells). Although the current amplitudes also reflect expression levels, expression yield was not the reason for the observed differences, because the intensities of the tag fluorescence of all tested constructs were similar. The half-decay rate of the current decay (half-closing of the channel) is the reciprocal of the time when the stationary photocurrent amplitude decreases 50% after switching off the light.




**Supporting figure 4.** The shifts of $E_r$ upon a 100-fold reduction of the $Na^+$ (from 150 mM to 1.5 mM; red bars) or $H^+$ (from pH 5.4 to pH 7.4; black bars) concentration in the bath determined for cryptophyte CCRs by measuring the IE curves as shown in Fig. 3 *c* in the main text. The data points are the mean values ± SEM (*n* = 3-5 cells).

## Supporting References

1. Huson, D. H., C. Scornavacca. 2012. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. *Syst. Biol.* 61:1061-1067.

2. Zhang, H., S. Gao, …, W. H. Chen. 2012. EvolView, an online tool for visualizing, annotating and managing phylogenetic trees. *Nucleic Acids Res.* 40:W569-572.