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# Phosducin interacts with the G-protein βγ-dimer of ciliate protozoan *Blepharisma japonicum* upon illumination

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**Summary**

**Immunological techniques and high-resolution FRET analysis were employed to investigate the *in vivo* colocalization and interaction of phosducin (Pdc) with the βγ-subunits of G-protein (Gβγ) in the ciliate *Blepharisma japonicum*. Immunological techniques revealed that illumination of cells resulted in a decrease in phosphorylation levels of Pdc and its colocalization with Gβγ. The observed light-induced Pdc dephosphorylation was also accompanied by significant enhancement of Gβγ binding by this molecule. Possible formation of the Pdc–Gβγ complex in cells exposed to light was corroborated by FRET between these proteins. Treatment of cells with okadaic acid, an inhibitor of phosphatase activity, entirely prevented Pdc dephosphorylation by light, colocalization of this phosphoprotein with Gβγ and generation of the Pdc–Gβγ complex. Cell fractionation and immunoblotting revealed that in cells exposed to light, the formation of Pdc–Gβγ complex and its translocation into the cytoplasm occur simultaneously with a change in the gel migration of Gβ. Moreover, a 33 kDa immunoanalog of 14-3-3 protein was identified and we showed that this protein is bound by phosphorylated Pdc in a cell adapted to darkness. The results of this study provide additional detailed characterization of the functional properties of the ciliate Pdc. The likely functional role of Pdc in *Blepharisma* is discussed.**

Key words: *Blepharisma japonicum,* βγ-dimer, G-protein, phosducin, 14-3-3, protein, photophobic response, protein phosphorylation, Pdc–Gβγ interaction, translocation, confocal imaging, cell fractionation, FRET, immunological analysis.

## Introduction

The phototransduction cascade in vertebrate photoreceptor cells is a well-studied G-protein-mediated signaling pathway. Phosducin (Pdc), a phosphoprotein abundantly expressed in the retina, has been identified as a potentially important regulator of this cascade (Lee et al., 1987; Lee et al., 1992; Kuo et al., 1989). Pdc regulates the G-protein cascade by competing with the α-subunit of G-protein (Gα) for binding to the βγ-dimer (Gβγ) of transducin, retinal-specific G-protein. The binding of Pdc to Gβγ diminishes the membrane association of Gβγ and facilitates the Gβγ translocation to cytoplasm by electrostatic interactions (Murray et al., 2001). The affinity of Pdc for Gβγ is determined by its phosphorylation state. When serine residues of Pdc are phosphorylated by cyclic nucleotide-dependent protein kinases and/or $Ca^{2+}$-calmodulin-dependent protein kinase type II, Pdc no longer inhibits the reassociation of Gα with Gβγ that is essential for G-protein recycling with activated rhodopsin (Yoshida et al., 1994; Lee et al., 1990; Lee et al., 2004; Gaudet et al., 1999; Thulin et al., 2001). Regulated competition between Gβγ and another cytosolic protein, 14-3-3, for binding to Pdc has also been described (Thulin et al., 2001; Nakano et al., 2001). The 14-3-3 proteins have been strongly implicated in regulating the subcellular distribution of many phosphorylated target proteins (Bridges and Moorhead, 2004; Mackintosh, 2004). The interactions of both 14-3-3 protein and Gβγ with Pdc are dependent on the phosphorylation state of Pdc. The discovery of Pdc's role in retinal photoreceptor cells of mammals and the identification of a similar downregulation function for phosducin-like protein, such as PhLP1, in other tissues and lower eukaryotic cells (Lee et al., 1987; Reig et al., 1990; Danner and Lohse, 1996; Flanary et al., 2000; Kasahara et al., 2000; Blaauw et al., 2003) indicate that these highly conserved proteins, which belong to subgroup I of the phosducin protein family, play an important role as cytosolic regulators of G-protein functions. However, lately there is growing evidence that PhLP may also have other cellular functions. It has recently been suggested that these proteins from subgroups I–III (PhLP1, PhLP2, PhLP3) mediate tubulin folding processes in the cell and that they are essential for Gβγ assembly, as modulators of chaperonin CCT (cytosolic-chaperonin-containing-TCP1) functions (Lukov et al., 2005; Lukov et al., 2006; Humrich et al., 2005; Stirling et al., 2006; Stirling et al., 2007; Knol et al., 2005).

In the light-sensitive ciliate *Blepharisma*, there exist proteins displaying high homology to Pdc and PhLP (Fabczak et al., 2004). The ciliate Pdc (28 kDa) is dephosphorylated in a light-dependent manner, while the identified PhLP homologs (40 kDa and 93 kDa) exhibit no detectable alteration of phosphorylation

state upon illumination. As in other organisms, phosphorylation of *Blepharisma* Pdc is controlled by cyclic nucleotide-dependent kinases and $Ca^{2+}$ and calmodulin. In cells exposed to light, dephosphorylation of Pdc occurs as a result of the activation of protein phosphatases (Sobierajska et al., 2005). The dephosphorylation of the Pdc coincides with cell step-up photophobic response to increasing light intensity (light stimulus) (Kraml and Marwan, 1983; Fabczak et al., 1993). The light-avoiding response consists of a delayed cessation of cell forward movement, a short period of backward swimming (ciliary reversal), finally followed by restoration of forward movement, usually in a new direction. The ciliary reversal observed during photophobic response is mediated by a membrane action potential, which can be elicited by an early light-induced depolarizing receptor potential (Fabczak et al., 1993). So far, the cell photosensitivity has been ascribed to its endogenous photoreceptor systems, composed of numerous pigment granules located beneath the plasma membrane (Giese, 1981; Matsuoka, 1983). Each granule contains the hipericin-like pigment blepharismin, which is thought to function as the primary photoreceptor eliciting the cell photoresponses (Tao et al., 1994; Song, 1997; Maeda et al., 1997; Matsuoka et al., 1997; Matsuoka et al., 2000). Recent analysis of primary photoprocesses in *Blepharisma* has revealed, however, that blepharismin undergoes an initial photocycle in the picosecond regime, indicating that the chromophore reaction in these cells does not play any active role in the phototransduction chain (Plaza et al., 2005; Plaza et al., 2007). The possibility that another class of photoreceptors may be utilized by *Blepharisma* in its photophobic behavior results also from our preliminary investigations, which showed the presence of a presumed rhodopsin-related protein in the cell membrane (H.F., unpublished data), as has been reported for other ciliates (Podesta et al., 1994; Nakaoka et al., 1991; Shinozawa et al., 1996). The phototransduction system in *Blepharisma* has been recently shown to be coupled to its membrane potentials and motile alterations, as in the case of photoreceptor cells of higher organisms (Rayer et al., 1990), *via* a G-protein-mediated signaling pathway (Fabczak, 2000a; Fabczak, 2000b).

The present study was initiated to analyze, *in vivo*, the influence of phosphorylation levels of *Blepharisma* Pdc on its localization and the interaction of Pdc with Gβγ. In addition, the possible interaction of Pdc with a newly identified putative 14-3-3 protein was investigated. Finally, the influence of okadaic acid, a specific phosphatase inhibitor, on colocalization and interaction of Pdc with Gβγ or 14-3-3 proteins was also examined. To complete this study, a combination of immunoblotting, coimmunoprecipitation, immunocytochemistry and a high-resolution FRET (fluorescence resonance energy transfer) assay was employed.

## Materials and methods

### *Cell cultivation*

*Blepharisma japonicum* (Suzuki, 1954) was grown at room temperature in Pringsheim medium containing 0.5 mmol $l^{-1}$ $CaCl_2$, 1.0 mmol $l^{-1}$ $MgSO_4$, 1.0 mmol $l^{-1}$ $NaNO_3$, 0.1 mmol $l^{-1}$ $NaH_2PO_4$ and 1.0 mmol $l^{-1}$ Tris-HCl buffer to maintain pH 7.0–7.2, in semi-darkness (Fabczak, 2000a). Ciliates were fed twice a week with *Tetrahymena pyriformis* grown axenically at room temperature in a medium consisting of 1.0% proteose peptone with 0.1% yeast extract. For experiments, *Blepharisma* cells were collected by low-speed centrifugation and washed in an excess of fresh culture medium without nutritional components. Cells were then incubated in a fresh culture medium (referred to as control medium) or in test solutions of designed composition for use in assay procedures.

### *Chemicals*

Materials for electrophoresis and immunoblotting were purchased from Bio-Rad Lab. (Hercules, CA, USA). Proteose peptone and yeast extract were obtained from Difco Lab. (Detroit, MI, USA), and Protein A-Sepharose CL-4B beads and Enhanced Chemiluminescence (ECL) kit were from Amersham Biosciences (Uppsala, Sweden). Polyclonal antiserum directed against phosducin (Pdc) was kindly provided by Professor C. D. Thulin (Brigham Young University, Provo, UT, USA) and purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies raised against a peptide within a divergent domain of 14-3-3ϵ, (T-16) and a peptide mapping at the C-therminus,(T-20) or amino terminus (H-300) of Gβ were also purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Okadaic acid and monoclonal antibody raised against phosphoserine (PSER 4A9) were obtained from Alexis Biochemical Corp. (Lausen, Switzerland). Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Calbiochem (Darmstadt, Germany) or Santa Cruz Biotechnology, Inc. Secondary antibodies conjugated with AlexaFluor 350, AlexaFluor 488 and AlexaFluor 546 were obtained from Molecular Probes, Inc. (Leiden, The Netherlands). All other reagents were purchased from Sigma-Aldrich Co. (Munich, Germany).

### *Cell photostimulation*

For each test, samples of ciliates adapted to darkness were first allowed to rest for 15 min to remove the effect of any mechanical disturbances and were then exposed to illumination for the specified time. Light stimulation of cell samples was carried out using a system consisting of a fibre optic light source (model MLW; Medizinische Gerate, Berlin, Germany) and a programmable electromagnetic shutter (model 1 22-841; Ealing Electro-Optics, Co., Watford, UK).

### *Immunoprecipitation*

Immunoprecipitation was carried out according to a method described previously (Fabczak et al., 2004) with minor modifications. Briefly, samples of dark-adapted cells (control), cells exposed to light for 30 s, and cells exposed to okadaic acid (1 μmol $l^{-1}$) for 10 min prior to illumination were first solubilized in lysis buffer comprising 10 mmol $l^{-1}$ Tris, pH 7.4, 150 mmol $l^{-1}$ NaCl, 1 mmol $l^{-1}$ EDTA, 1% Triton X-100, 50 mmol $l^{-1}$ NaF, 2 mmol $l^{-1}$ phenylmethylsulfonylfluoride (PMSF), 1 μmol $l^{-1}$ okadaic acid, 10 μg $ml^{-1}$ aprotinin, 10 μg $ml^{-1}$ leupeptin, 10 μg $ml^{-1}$ pepstatin. Insoluble debris was then removed by centrifugation at 13 000 ***g*** for 15 min at 4°C. The supernatant was collected and protein concentrations determined (Bradford, 1976). Samples containing equal amounts of protein were pre-cleared by incubation with 50% (v/v) protein A-Sepharose CL-4B beads for 1 h with rotation at

4°C. The beads were removed by centrifugation at 13 000 ***g*** for 1 min at 4°C. The supernatant fractions were then incubated with serum containing antibody directed against Pdc for 90 min at 4°C. The resulting immunocomplexes were collected by rotation with 50% (v/v) protein A-Sepharose CL-4B beads for 1 h at 4°C followed by centrifugation for 1 min. Subsequently, the resin carrying immunocomplexes was washed four times in lysis buffer and once with the same buffer lacking Triton X-100. The beads, transferred to fresh tubes during the washing steps, were taken up in twofold-concentrated SDS (sodium dodecyl sulphate) sample buffer (Laemmli, 1970), heated for 3 min at 95°C and pelleted by spinning for 5 min in a Sigma centrifuge (10 000 ***g***). The samples were stored at –20°C prior to SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and western blot analyses.

### *Cell fractionation assay*

To demonstrate translocation of Gβγ under different light conditions, cytoplasmic and membrane fractions were prepared from samples of dark-adapted cells (control) and cells exposed to light for 30 s. The cells were washed and homogenized in a lysis buffer consisting of 20 mmol $l^{-1}$ Tris (pH 7.4), 150 mmol $l^{-1}$ NaCl, 1 mmol $l^{-1}$ EDTA, protease and phosphatase activity inhibitors (50 mmol $l^{-1}$ NaF, 2 mmol $l^{-1}$ PMSF, 1 μmol $l^{-1}$ okadaic acid, 1 μg $ml^{-1}$ aprotinin, 10 μg $ml^{-1}$ leupeptin, 1 μg $ml^{-1}$ pepstatin) by passing through a 25-gauge needle 20 times. The homogenates were then centrifuged at 500 ***g*** for 5 min to remove nuclei and debris, and the collected supernatants centrifuged again at 100 000 ***g*** for 1 h at 4°C to pellet membranes. The obtained cell fractions were then analyzed by SDS-PAGE and western immunoblotting with antibody against Gβ.

### *Protein electrophoresis and western immunoblotting*

Protein electrophoresis was carried out according to the method of Laemmli (Laemmli, 1970). Cell samples were mixed with fourfold-concentrated SDS sample buffer supplemented with protease and phosphatase activity inhibitors (as above) and then resolved on 10% SDS-polyacrylamide gels using a Hoefer Electrophoresis System (Amersham Pharm. Biotechnol., Little Chalfont, UK). The separated proteins were transferred to nitrocellulose membrane in transfer buffer (Towbin et al., 1979) using a Hoefer System (model TE 22; Amersham Pharm. Biotech., Piscataway, NJ, USA). Blots were blocked by incubation in Tris buffer solution (TBS) composed of 10 mmol $l^{-1}$ Tris (pH 7.5) and 150 mmol $l^{-1}$ NaCl with the addition of 0.1% Tween-20 (v/v) and 2% bovine serum albumin (BSA) (TBS-Tween-BSA) for 2 h at room temperature. For the detection of specified cell proteins, the blots were then incubated with primary antibodies diluted 1:1000 in TBS-Tween-BSA overnight at 4°C. After several washes in TBS with 0.1% Tween-20, the blots were incubated for 1 h at room temperature with IgG-HRP conjugate secondary antibody in TBS-Tween-BSA. Finally, the membranes were washed several times in TBS-Tween and then specific antibody binding was visualized using an ECL detection system (Amersham Pharm. Biotechnol.). The intensities of immunoreactive protein bands were quantified using a laser densitometer equipped with ImageQuant software (Bio-Rad Lab.). Protein molecular masses were estimated based on their relative electrophoretic mobility using pre-stained molecular mass standards. In a set of control experiments, incubation with primary antibody was omitted.

### *2-D electrophoresis*

Isoelectric focusing of lysate proteins obtained from dark-adapted *Blepharisma* was carried out on 7 cm immobilised pH gradient strips (3–10 pH gradient; Bio-Rad Lab.). In the beginning, the strips were rehydrated for 16 h with 0.25 mg of proteins in 125 μl of solubilizing solution (8 mol $l^{-1}$ urea, 4% CHAPS, 2 mmol $l^{-1}$ tributylphosphine (TBP), 40 mmol $l^{-1}$ Tris, 0.2% ampholytes and Bromophenol Blue). The focusing was carried out at 20°C for 10 000 V-h at a maximum of 4000 V in a Protean IEF Cell (Bio-Rad Lab.). The strips were then incubated with gentle shaking in the first equilibration solution [6 mol $l^{-1}$ urea, 2% (w/v) SDS, 375 mmol $l^{-1}$ Tris (pH 8.8), 20% glycerol and 2% DTT] and subsequently in the next equilibration solution [6 mol $l^{-1}$ urea, 2% (w/v) SDS, 375 mmol $l^{-1}$ Tris (pH 8.8), 20% glycerol and 2.5% iodoacetamide] for 20 min each time. The second dimension was run on 10% SDS-PAGE and analyzed by western immunoblotting with antibody raised against Gβ as described above.

### *Immunocytochemistry*

To study the influence of Pdc phosphorylation on the localization of Pdc and Gβγ in dark-adapted and illuminated ciliate cells, okadaic acid treatment was used and the cells examined by immunocytochemistry. Samples of cells adapted to darkness (control) and cells exposed to light for 30 s, with or without treatment with okadaic acid (1 μmol $l^{-1}$) for 10 min prior to illumination, were fixed as described previously (Fabczak et al., 2004). The cells were washed twice in a buffer containing 60 mmol $l^{-1}$ Pipes (pH 6.9), 25 mmol $l^{-1}$ Hepes, 10 mmol $l^{-1}$ EGTA, 4 mmol $l^{-1}$ $MgCl_2$ (PHEM buffer) supplemented with protease and phosphatase activity inhibitors (1 μmol $l^{-1}$ leupeptin, 1 μg $ml^{-1}$ aprotinin, 1 μmol $l^{-1}$ okadaic acid) and then permeabilized for 10 min at 4°C in the same buffer supplemented with 0.05% Triton X-100 (v/v). Following two washes in PHEM buffer, the preparations were incubated for 1 h at room temperature in the same buffer supplemented with 2% BSA (w/v) to block nonspecific binding. Subsequently, the cell preparations were reacted overnight at 4°C with primary antibody against Pdc or Gβ diluted 1:100 in TBS with 1% BSA (TBS-BSA). Following extensive washing in TBS, the preparations were incubated for 1 h at room temperature with donkey anti-goat IgG conjugated with AlexaFluor 350 (Pdc) or goat anti-rabbit IgG conjugated with AlexaFluor 488 (Gβ) diluted 1:300 in TBS-BSA. Finally, the cell samples were washed in TBS and then mounted in Citifluor (Citifluor Ltd, London, UK). Examination of the cell preparations was performed using a confocal microscope (Leica TCS-SP2; Leica Microsystems, Exton, PA, USA). Interfering red fluorescence resulting from an endogenous photoreceptor pigment (blepharismin) excitation by the light derived from the confocal microscope source was eliminated by the microscope analyzing system. Matching cell images showing fluorescence of the AlexaFluor 350 and FITC were captured independently, pseudo-colored green and red, respectively, and then superimposed using Leica software (Leica Microsystems). To

quantify the degree of protein colocalization, confocal cell images were analyzed with Leica software and Adobe Photoshop. Nonspecific fluorescence was determined in cell samples that were suspended in TBS-BSA lacking the primary antibodies during the immunostaining process.

### *FRET analysis*

Fluorescence (or Förster) resonance energy transfer (FRET) was used to determine whether proteins Gβγ and Pdc, which appear colocalized in an immunocytochemical assay in *Blepharisma*, interact with one another *in vivo*. The concept of FRET is based on the phenomenon where the excited state energy of a fluorescent dye, called the donor, can be transferred non-radioactively over a very short distance (in general <10 nm) to a light-absorbing molecule, called the acceptor (Förster, 1948; Stryer, 1978; Hink et al., 2002). Efficient energy transfer between these fluorophores – the so-called FRET pair – results in increased acceptor emission (FRET Sensitized Emission).

FRET analysis was performed using Leica confocal software for the FRET Sensitized Emission Method. It comprises three steps: (1) setting experimental conditions, (2) generating calibration measurements and the calibration itself and (3) calculation of FRET efficiencies and cell measurements. FRET values were automatically generated by the software, according to the formula described by Van Rheenen et al. (Van Rheenen et al., 2004):

$$E_A(i) = [B - Ab - C\,(c - ab)] / C\,,$$

where $E_A(i)$ is the apparent FRET efficiency; $A$, $B$ and $C$ correspond to the fluorescence intensities of donor, FRET and acceptor, respectively; $a=A/C$ is the calibration factor calculated for correction of acceptor cross-excitation crosstalk in the donor image; and $b=B/A$ and $c=B/C$ are the calibration factors calculated for corrections of donor crosstalk and acceptor cross-excitation in the FRET image, respectively.

For FRET assay, dark-adapted cells and cells illuminated for 30 s were fixed and blocked as described above. The cell samples were exposed to primary antibody against Pdc at a dilution of 1:100 in TBS-BSA, overnight at 4°C. Subsequently, they were reacted for 1 h at room temperature with donkey anti-goat IgG conjugated with AlexaFluor 546 (acceptor fluorophore) diluted 1:300 in TBS-BSA. The same cell samples were incubated for 24 h at 4°C with antibody against rabbit Gβ and then incubated with donkey anti-rabbit antibody conjugated with AlexaFluor 488 (donor fluorophore). After each incubation step, the cell samples were washed three times with TBS-Tween. As a control for the specificity of the secondary antibodies, similar procedures were applied except that the cell samples were not incubated with the primary antibodies. The fluorescence intensities of the donor (Gβ labeled with AlexaFluor 488), acceptor (Pdc labeled with AlexaFluor 546) and FRET signal were recorded using a confocal microscope (Leica TCS-SP2; Leica Microsystems GmbH, Wetzlar, Germany), and FRET values were then estimated with appropriate analytical software. The microscope used a He–Ne laser tuned to lanes at 488 nm (0.48 mW) and at 546 nm (0.1 mW) to excite both fluorophores. The samples were examined with a 10× 1.4-numerical-aperture objective.

## Results

### *Effect of Pdc phosphorylation on its interaction with Gβγ*

The effect of the phosphorylation level of ciliate Pdc on its interaction with Gβγ *in vivo* was examined in dark-adapted and illuminated *Blepharisma* cells. Cell lysates were immunoprecipitated with antibody selectively recognizing Pdc protein (28 kDa) (Fabczak et al., 2004) and the phosphorylation level of Pdc was estimated by immunoblotting using antibody against phosphoserine residues (Fig. 1A). In both dark-adapted and illuminated cells, immunoblot analysis demonstrated that Pdc antibody exclusively precipitated one phosphorylated protein of molecular mass 28 kDa (Fig. 1A, lanes 1 and 2). Illumination of cells caused a significant decrease in the phosphorylation level of Pdc (Fig. 1A, lane 2), while phosphorylation of this protein was markedly higher in cells not treated with light (Fig. 1A, lane 1). These experiments also showed that light-induced dephosphorylated Pdc was strongly coimmunoprecipitated with Gβ (Fig. 1B, lane 2), whereas cell adaptation to darkness resulted in a marked decrease in the amount of Gβ in the immunoprecipitate (Fig. 1B, lane 1). A result similar to that seen with dark-adapted cells was observed in cells incubated with 1 μmol $l^{-1}$ of okadaic acid (Fig. 1B, lane 3). Treatment with this serine or threonine phosphatase inhibitor resulted in a significant decrease in the amount of Gβ bound to Pdc, despite cell stimulation by light. These data are consistent with the observed effect of okadaic acid on the levels of Pdc phosphorylation in cells (Fig. 1A, lane 3). The phosphorylation level of Pdc in cells incubated with this phosphatase inhibitor and subsequently exposed to light was similar to that found in cells adapted to darkness (Fig. 1A, lane 3), and in both cases binding of Gβ by Pdc was lower (Fig. 1B, lanes 1 and 3). These results showed that the decrease in phosphorylation of ciliate Pdc in response to cell illumination correlates well with the significant increase in Gβ binding by this protein.

### *Light-induced translocation of Gβγ to the cytoplasm*

Western blot analysis of a whole-cell lysate from dark-adapted *Blepharisma* demonstrated that antibody against Gβ recognized two protein bands; one robust band with a molecular mass of 32 kDa, and a second, hardly visible, at 36 kDa (Fig. 1B, lane 4; Fig. 2, lanes 1, 2). However, the same antibody showed clear immunoreactivity with a 36-kDa protein band in the immunoprecipitate obtained with antibody against Pdc protein (Fig. 1B, lanes 1–3). This finding suggests that in this ciliate, two isoforms of Gβ may exist, one of 32 kDa bound to the cell membrane and a second 36-kDa protein located within the cytoplasm. To verify whether, in ciliates exposed to light, altered migration of Gβ on SDS-PAGE accompanies translocation of this protein within the cell, the subcellular localization of Gβ was examined by cell fractionation and immunoblotting.

The results of this experiment clearly showed that in dark-adapted cells, the 32-kDa Gβ form was predominantly localized in the membranous fraction (Fig. 2, lane 3), while in the cytoplasm only a small amount of the 36-kDa protein was present (Fig. 2, lane 5). In cells subjected to light, the 36-kDa form of Gβ was found mostly in cytoplasm (Fig. 2, lane 6) with only a negligible amount of 32-kDa protein in this fraction (Fig. 2, lane 4). These findings demonstrate that in light-

stimulated cells the formation of the Pdc–Gβγ complex (Fig. 1B) and its translocation into the cytoplasm occur simultaneously with a change in the gel migration of Gβ, possibly due to post-translational modification of this protein.

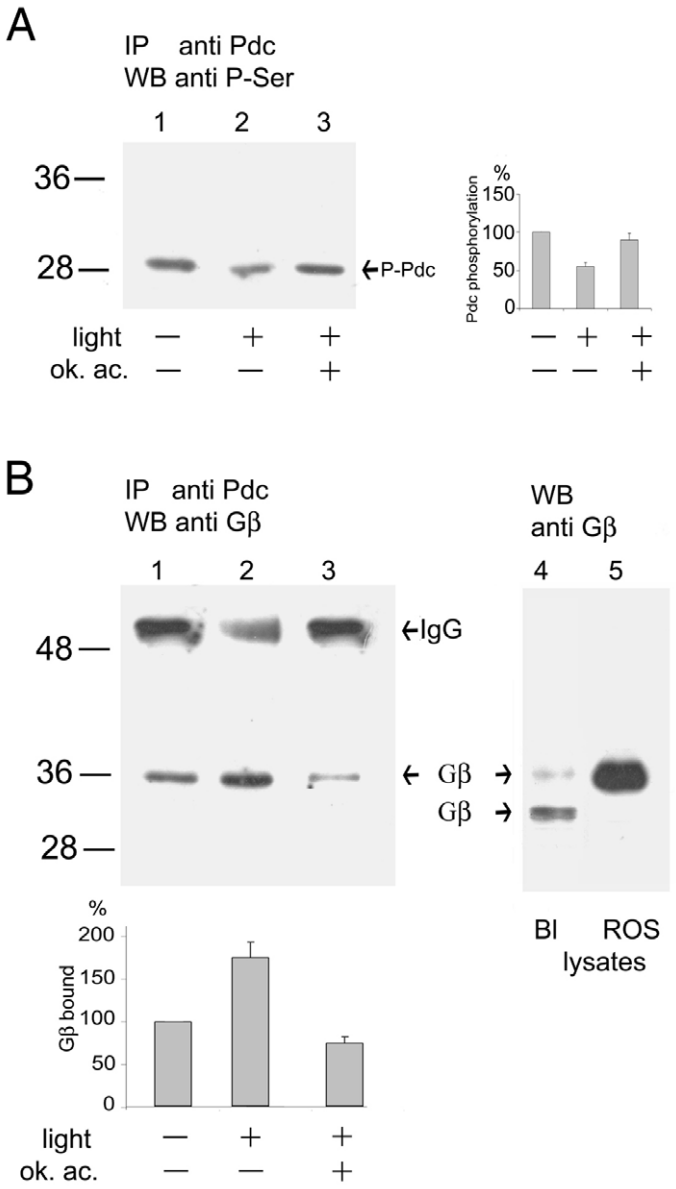


Fig. 1. Immunodetection of phosphorylation level of ciliate Pdc and interaction of Pdc with Gβγ in *Blepharisma*. The precipitated proteins with specific antibody against Pdc in lysates from dark-adapted cells (lane 1), light-stimulated cells (lane 2) and cells incubated with okadaic acid prior to illumination (lane 3) were analyzed by western immunoblotting with antibodies against phosphoserine (A) or Gβ (B). Lanes 4 and 5 show immunoblots identifying Gβγ in a whole cell lysate from *Blepharisma* and the rod outer segment (ROS) of bovine photoreceptor cells, respectively (B). The blots are representative of the results of three independent experiments. Inserted densitometric diagrams show the levels of Pdc phosphorylation (A) and bound Gβ (B) in lysates from dark-adapted cells and cells exposed to light or okadaic acid. The levels of Pdc phosphorylation or bound Gβ in lysate from the control dark-adapted cells were taken as 100%. Bars represent mean values ± s.e.m. of three experiments.

*2-D electrophoresis*

In order to prove that the antibody specifically recognizes the Gβ-subunit, proteins of dark-adapted *Blepharisma* lysate were separated on 2-D electrophoresis and then immunoblotted. The proteins of cell lysate, which cross-reacted with rabbit antibody raised against Gβ, resolved into two forms; one robust spot at a molecular mass of 32 kDa is well visible at an isoelectric point (pI) of about 6.0, and the second, in a more acidic quarter, is much weaker at a molecular mass of 36 kDa at about pI 5.4 (Fig. 3).

*Effect of light on the interaction of Pdc with 14-3-3 protein*

In retina cells, it has been shown that Pdc also interacts with a 14-3-3 protein, and that this interaction, like that with Gβγ, is highly dependent on the phosphorylation state of Pdc (Thulin et al., 2001; Nakano et al., 2001). Since photostimulation markedly affects Pdc phosphorylation in *Blepharisma*, experiments were performed to test the possibility that this ciliate possesses a 14-3-3 protein and to determine whether it

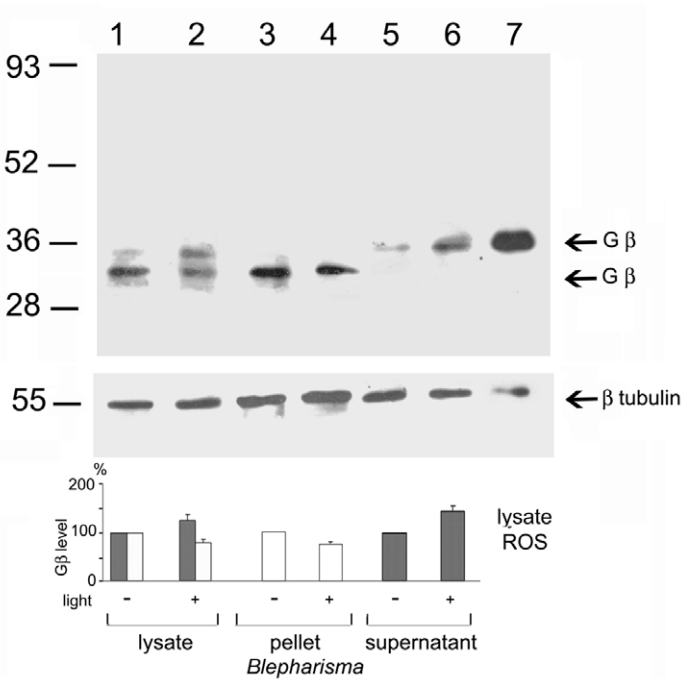


Fig. 2. Localization of Gβγ in dark-adapted and illuminated *Blepharisma* by cell fractionation and immunoblotting. Lysates from dark-adapted (lanes 1, 3 and 5) and illuminated (lanes 2, 4 and 6) cells were fractionated by centrifugation and Gβ was detected in the separate fractions by immunoblotting with specific antibody against Gβ. Results indicate interaction of the antibody with protein bands of ~32 kDa in the membranous fractions (lanes 3 and 4) and ~36 kDa in the cytoplasmic fractions (lanes 5 and 6). Lane 7 shows immunoblots identifying Gβ in whole cell lysate from the rod outer segment (ROS) of bovine photoreceptor cells. Blotting with β-tubulin antibody was used as a test of equal protein loading. Lower panel shows a densitometric quantification diagram of Gβ levels in lysate and cell fractions under different light conditions (filled and open bars correspond to 32 kDa and 36 kDa, respectively). The level of Gβ in lysate from the control dark-adapted cells was taken as 100%. Bars represent mean values ± s.e.m. of four experiments.

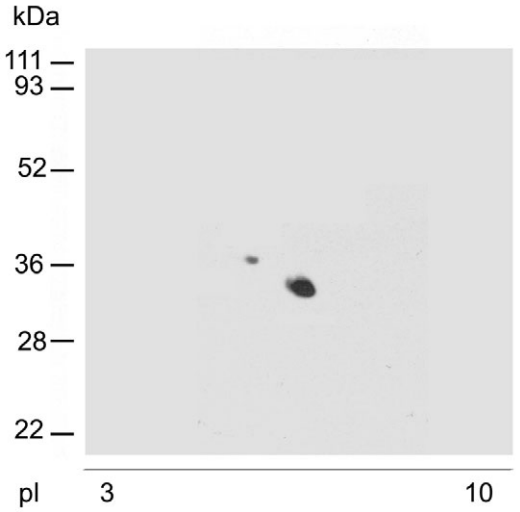


Fig. 3. Two-dimensional electrophoresis gel of dark-adapted *Blepharisma* lysate and reaction with antibody against Gβ. Results indicate that only polypeptides at 32 kDa and 36 kDa with a pI of ~6 and 5.4, respectively, are immunoreactive. This gel is representative of the results of three independent experiments.

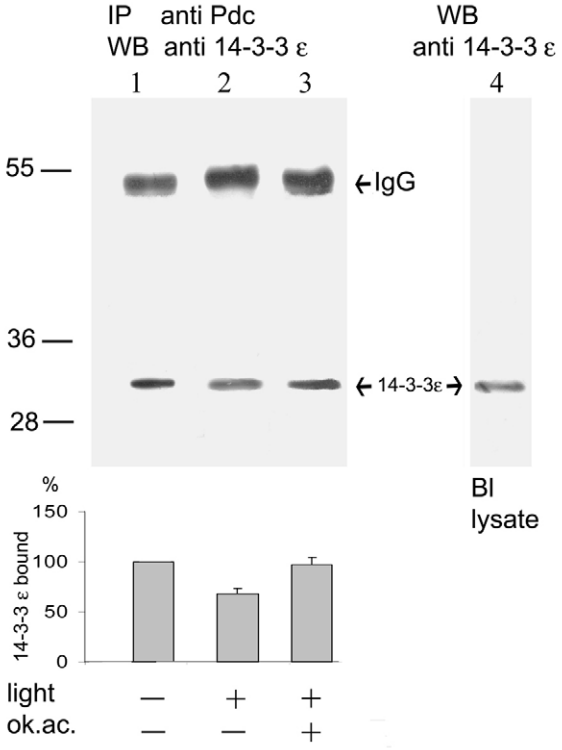


Fig. 4. Coimmunoprecipitation of Pdc and 14-3-3 protein in *Blepharisma*. Total cell lysates were subjected to immunoprecipitation with antibody against Pdc. The precipitated proteins in lysates from dark-adapted cells (lane 1), light-stimulated cells (lane 2) and cells incubated with okadaic acid prior to illumination (lane 3) were resolved by SDS-PAGE and immunoblotted for 14-3-3 ϵ protein. Arrows mark a coimmunoprecipitated protein of ~33 kDa (lanes 1–3). Western blot analysis confirmed that this polypeptide represents a putative 14-3-3 protein (lane 4). Lower panel shows a densitometric quantification diagram of 14-3-3 ϵ protein levels in cell lysate under different experimental conditions (lysate from dark-adapted or illuminated cells and cells incubated with okadaic acid prior to illumination, respectively). The level of 14-3-3 ϵ protein in lysate from the control dark-adapted cells was taken as 100%. Bars represent the mean values ± s.e.m. of three experiments.

can interact with Pdc. An immunoblot probed with a heterologous 14-3-3 protein antibody indicated that *Blepharisma* does indeed express a 14-3-3 protein immunoanalog of a molecular mass of 33 kDa (Fig. 4, lane 4). Coimmunoprecipitation experiments using the same antibody showed that this 14-3-3 protein does interact with ciliate Pdc (Fig. 4). In lysates from dark-adapted cells and illuminated cells that had been pretreated with okadaic acid, robust coimmunoprecipitation of 14-3-3 protein with Pdc antibody was observed (Fig. 4, lanes 1 and 3, respectively). By contrast, stimulation of cells by light resulted in a marked decrease in the amount of 14-3-3 protein in the obtained immunoprecipitate (Fig. 4, lane 2). These data indicate that the process of light-dependent protein phosphorylation can have a major effect on the binding of Pdc to 14-3-3 protein in this ciliate.

### *Colocalization of Pdc and Gβγ*

Since light activation of protein phosphatases in *Blepharisma* resulted in a decrease in Pdc phosphorylation levels and thus in generation of a Pdc–Gβγ complex (Fig. 1), the precise localization of these proteins in cells under different light conditions was examined by immunocytochemistry. In dark-adapted cells, Pdc was distributed uniformly within the cytoplasm (A1 in Fig. 5A,B), while Gβγ formed a thin layer located in the plasma membrane, and only faint cytoplasmic labeling was detected (A2 in Fig. 5A,B). Therefore, in cells not exposed to light, colocalization of Pdc and Gβγ was clearly not observed (A3 in Fig. 5A,B). Exposure of cells to light, however, resulted in the translocation of Pdc to a position close to the plasma membrane (B1 in Fig. 5A,B) where colocalization with Gβγ was observed (B3 in Fig. 5A,B). No significant differences were seen in the localization of Gβ in light-stimulated or dark-adapted cells (B2 in Fig. 5A,B). In ciliates pretreated with okadaic acid (1 μmol $l^{-1}$) before illumination, i.e. when Pdc phosphorylation was high (Fig. 1B, lane 3), Pdc translocation to the plasma membrane was inhibited (C1 in Fig. 5B) and no colocalization of Pdc with Gβγ occurred (C3 in Fig. 5B).

### *Energy transfer between Pdc and Gβγ*

Immunocytochemical investigations showed that Pdc was colocalized with Gβγ in illuminated *Blepharisma* (B3 in Fig. 5). To determine whether these two proteins directly interact under these conditions, FRET analysis was performed. These experiments revealed that only in cells exposed to light were significant levels of energy transfer between Pdc and Gβγ detected. The estimated FRET efficiency value between Pdc and Gβγ was about 20 times higher in ciliates adapted to light compared with control cells, i.e. cells adapted to darkness (Fig. 6). In dark-adapted cells, sub-membrane-localized FRET signals were not detected, indicating that close association of Pdc with Gβγ did not occur (A4 in Fig. 7). By contrast, in light-stimulated cells, the red color observed in the submembranous region suggested that a specific protein–protein interaction

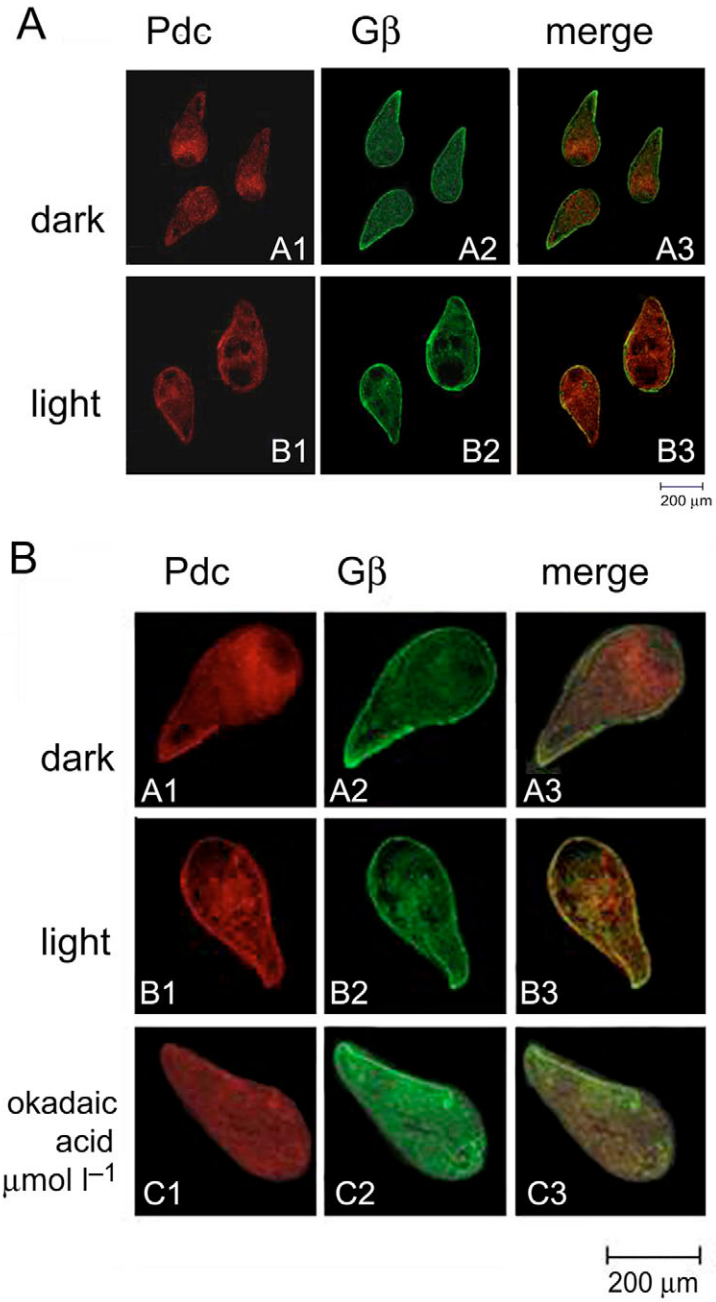


Fig. 5. Colocalization of Pdc and Gβγ related to light conditions in cells of *Blepharisma*. Distribution of Pdc (red) and Gβγ (green) and their colocalization (yellow) close to the plasma membrane identified by immunocytochemistry. (A,B) A1–A3, staining of Pdc and Gβ in dark-adapted cells; B1–B3, Pdc and Gβ in cells exposed to light. (B) C1–C3, cells after 10 min incubation with 1 μmol $l^{-1}$ okadaic acid followed by illumination.

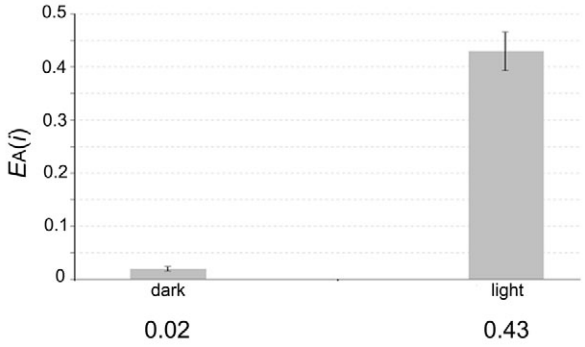


Fig. 6. Pdc and Gβγ interaction in *Blepharisma* examined by FRET. Cells were labeled and FRET values were generated as described in Materials and methods. Energy transfer efficiency was automatically calculated by the Leica software after subtracting the appropriate background value. The results are shown as the sensitized emission of the acceptor when the cells were excited at 488 nm.

occurred, not merely colocalization of Pdc and Gβγ in the same cellular compartment (B4 in Fig. 7).

## Discussion

It has been previously shown that the photosensitive unicellular eukaryote *Blepharisma japonicum* possesses a phosphoprotein of molecular mass 28 kDa localized within the cytoplasm (Fabczak et al., 2004; Sobierajska et al., 2005). This protein displays properties similar to those described for phosducin (Pdc), a highly conserved phosphoprotein first identified in the photoreceptor cells and pineal gland of mammals (Lee et al., 1987; Reig et al., 1990; Craft et al., 1991). Further studies have revealed, however, that it can be present in small amounts in various tissues of higher organisms (Boekhoff et al., 1997; Schulz, 2001), and other PhLP are found in lower eukaryotes (Flanary et al., 2000; Kasahara et al., 2000; Blaauw et al., 2003). In darkness, the ciliate phosphoprotein usually appears in a highly phosphorylated state, and in cells exposed to abrupt illumination it undergoes rapid and reversible dephosphorylation. The observed decrease in phosphorylation of this protein correlates with the motile photophobic response elicited by photoactivation of the specific cellular photoreceptor system present in *Blepharisma* (Tao et al., 1994; Maeda et al., 1997; Fabczak, 2000b; Matsuoka et al., 2000; Sobierajska et al., 2006). As reported recently, the deduced amino acid sequence of the ciliate phosphoprotein exhibits high levels of homology to phosducins of other organisms (Sobierajska et al., 2006). The *Blepharisma* Pdc homolog also contains an amino acid motif (gi_124020703; GenBank accession no. EF198414) displaying more than 80% identity to a previously characterized motif responsible for binding of Gβγ by Pdc (Gaudet et al., 1996).

The results of this study, obtained with standard methods combined with a more sophisticated experimental approach, provide further detailed characterization of the functional properties of Pdc in the protozoan *Blepharisma.* Immunological techniques revealed that the prominent decrease in the level of Pdc phosphorylation in response to illumination is followed by significant enhancement of Gβγ binding by the Pdc protein (Fig. 1A,B, lane 2). These observations were supported by the detection of a high-level energy transfer between Gβγ and Pdc using FRET

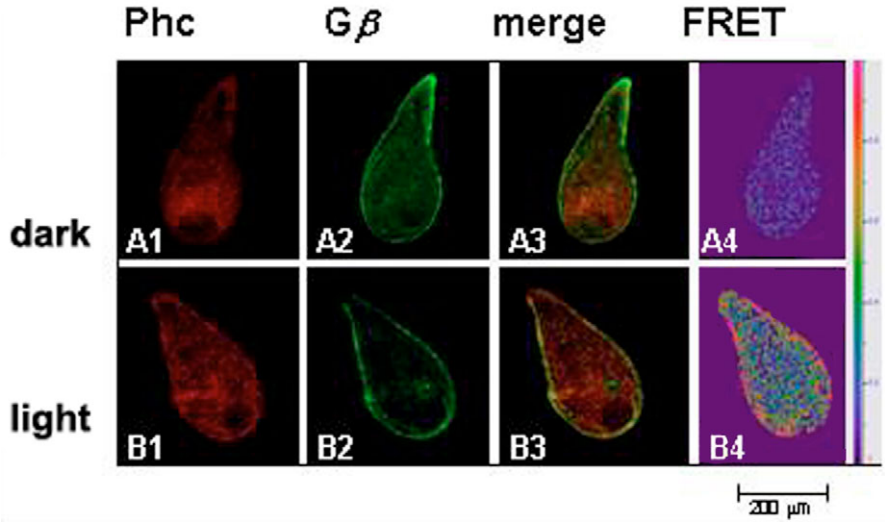


Fig. 7. Energy transfer between Pdc and Gβγ in *Blepharisma* detected by the FRET Sensitized Emission Method. Pixel by pixel FRET values between Pdc labeled with AlexaFluor 546 (A1, B1) and Gβ labeled with AlexaFluor 488 (A2, B2) in dark-adapted (A1–A4) and in light-stimulated cells (B1–B4). FRET images (A4, B4) are pseudocolored relative to the amplitude of FRET efficiency. The overlay is red where the overlap is significant (B4). The color code ranges between 0% (violet) and 100% (red).

analysis, indicating close interaction between these two proteins under illuminated conditions (Figs 6 and 7). The measured FRET efficiency value for Pdc and Gβγ interaction was about 20 times higher in light-stimulated cells than in cells adapted to darkness (Fig. 6). Both the light-evoked Pdc dephosphorylation (Fig. 1A) and the Pdc and Gβγ interaction identified by FRET were quite rapid, since they were completed in less then 30 s from the start of light stimulation. The similar time periods were necessary for colocalization of Pdc and Gβγ (Fig. 1B) and translocation of the Pdc–Gβγ complex to the cytoplasm (Fig. 2), as evidenced by the immunological investigations of *Blepharisma*. As has been recently reported, the dephosphorylation of Pdc in *Blepharisma* occurs in cells photoexcited for only a few seconds (Fabczak et al., 2004). Prolonged cell illumination leads to distinct rephosphorylation of Pdc during which no sequestration of Gβγ by Pdc is observed (data not shown). Comparing the rates of *in vivo* changes in Pdc phosphorylation upon illumination in the ciliate and in vertebrate photoreceptor cells of the intact retina, it is evident that phosphorylation changes in the former display appreciably faster kinetics (Lee et al., 2004). These observations have led to the suggestion that Pdc in *Blepharisma* not only contributes to the process of light adaptation, as it does in vertebrate photoreceptor cells (Chen et al., 2005), but this protein also controls in some way the process of signal transduction in ciliate cells.

This study also shows that disturbance of the proper functioning of protein phosphatase in *Blepharisma* significantly influences the aforementioned cellular events. Inhibition of protein phosphatase activity by the specific inhibitor okadaic acid greatly decreased the binding of Gβγ by the ciliate Pdc (Fig. 1B, lane 3). Consequently, not only was Gβγ sequestering ability lost, but the translocation of Pdc to the vicinity of the plasma membrane in dark-adapted cells was also abolished (Fig. 5). As in other organisms, the process of Pdc phosphorylation and dephosphorylation in *Blepharisma* is governed by the endogenous protein kinase-phosphatase system (Sobierajska et al., 2005). Pdc of this ciliate is a substrate of protein kinases A and G, and the level of its phosphorylation also depends on $Ca^{2+}$ and calmodulin. Potential phosphorylation targets of these enzymes are serine and threonine residues located close to the N-terminus of Pdc in *Blepharisma* (gi_124020703; GenBank accession no. EF198414). In this region of the protist Pdc, at least four serine residues may be phosphorylated under dark conditions (Fig. 8). In addition to these potential phosphorylation targets, the N-terminal region of Pdc also includes the amino acid motif RSXSXP (Fig. 8), which is preferred for the binding of 14-3-3 protein (Muslin et al., 1996; Bridges and Moorhead, 2004). To detect the existence of a *Blepharisma* 14-3-3 protein and determine whether it can bind to ciliate Pdc, immunological techniques were used. From the results of these investigations it was concluded that, as in photoreceptor cells, an immunoanalog of 14-3-3 protein is present (Fig. 4, lane 4), which can interact with phosphorylated Pdc (Fig. 4, lanes 1 and 3). 14-3-3 proteins constitute a family of eukaryotic proteins that are key regulators of a large number of processes ranging from mitosis to apoptosis (Rosenquist et al., 2000; Bridges and Moorhead, 2004). These proteins are implicated, among other things, in regulating the subcellular distribution of many phosphorylated target proteins (Muslin et al., 1996; Bridges and Moorhead, 2004; Mackintosh, 2004). An examination of 14-3-3 entries in the public databases reveals more than 150 isoforms (Rosenquist et al., 2000). The phylogenetic position of twelve 14-3-3 proteins from five protozoal species was tested relative to other eukaryotic 14-3-3 versions representing many of the previously described isoforms. The protozoal proteins, four from *Entodinium caudatum*, three from *Entamoeba histolytica*, four from apicomplexan parasites and one from *Tetrahymena piriformis*, formed clusters closer to the plant and animal epsilon isoforms (Rosenquist et al., 2000; Zhao et al., 1999; McEwan et al., 1999). There is high probability that 14-3-3 present in *Blepharisma* is also homologous to the epsilon isoform, and therefore antibody to this isoform was used (see Materials and methods). As shown previously, both the epsilon isoform and the zeta isoform have been found at high levels in the retina, and both interact with Pdc (Nakano et al., 2001; Thulin et al., 2001). In dark-exposed photoreceptor cells, when retinal Pdc is phosphorylated, 14-3-3 protein binds to phosphorylated serine residues of Pdc and effectively blocks its binding to Gβγ

(Nakano et al., 2001; Thulin et al., 2001; Lee et al., 2004). As has been recently reported, one of the functions of 14-3-3 protein is to sequester specific phosphoserine-containing proteins and anchor them and, in this way, prevent their binding to interacting partners (Thulin et al., 2001; Nakano et al., 2001; Bridges and Moorhead, 2004; Mackintosh, 2004). Such a function may be performed in *Blepharisma* by the 14-3-3 protein immunoanalog identified in this study.

Two-dimensional electrophoresis and cell fractionation experiments revealed that *Blepharisma*, like other cells (Chen and Lee, 2000; Jin et al., 2000), contains two different pools of Gβγ: one existing as a membrane bound fraction and the second dispersed throughout the cytoplasm (Figs 2 and 3). During cell illumination, the amount of ciliate Gβγ in the cytoplasm was shown to increase (Fig. 2, lane 6). An unexpected observation in these experiments was the reduced electrophoretic mobility displayed by Gβγ extracted from light-stimulated ciliates. In vertebrate photoreceptor cells, the translocation of Gβγ to the cytoplasm as a result of light stimulation has been reported, although a simultaneous shift in gel mobility was not observed (Yamamoto et al., 2007; Chen and Lee, 1997; Sokolov et al., 2004).

Fig. 2 shows that the pool of Gβ localized in the ciliate cytoplasmic fraction was represented by a 36-kDa polypeptide (see control bovine rod outer segment preparation in Fig. 2, lane 7); in contrast, membrane-bound Gβ migrated on SDS-PAGE as a 32 kDa protein. This result suggests that *Blepharisma* may possess two different isomers of Gβγ. However, the increase in the cytoplasmic pool of the 36-kDa polypeptide after light stimulation disproves this suggestion. Another possible explanation of this event is that cytosolic Gβ can undergo some modification that causes its electrophoretic mobility to change. Several lines of evidence support the conclusion that reversible protein post-translational modification underlies this mobility shift. In other cell systems, phosphorylation of a histidine residue of Gβγ has been described as an effect of cell stimulation (Kowluru, 2002; Mäurer et al., 2005). The shift of the 36-kDa protein to lower isoelectric pH values observed in Fig. 3 is indicative of a phosphorylation event because of the increased negative net charge due to the binding of phosphate groups (Kühn and McDowell, 1977). However, given the available evidence, it is difficult to state certainly that this phosphorylation occurs in *Blepharisma* or if such a modification can alter protein migration on SDS-PAGE. Long-term illumination of ciliate cells not only caused rephosphorylation of Pdc but also increased the amount of Gβγ in the membranous fraction at the cost of the cytoplasmic pool (data not shown).

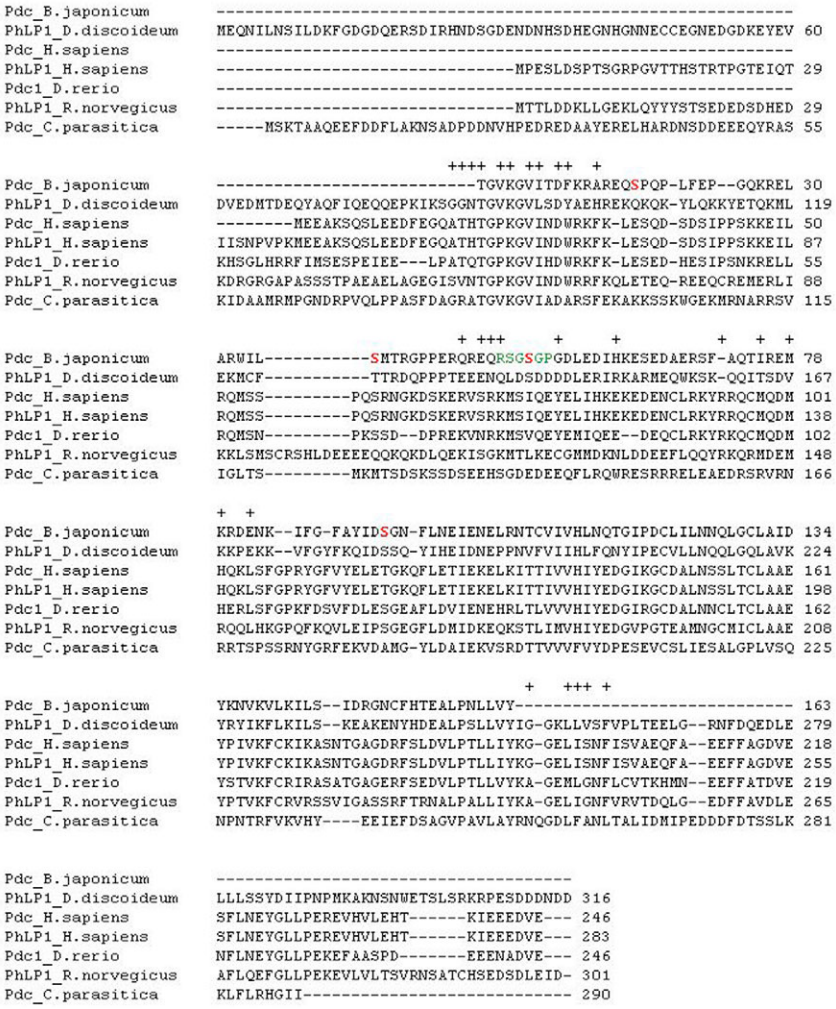


Fig. 8. Alignment of seven protein sequences (created by ClustalW; http://www.ebi.ac.uk/Tools/clustalw/index.html/) from the first subgroup of the phosducin family (Blaauw et al., 2003). In *Blepharisma*, serine residues of Pdc – the potential phosphorylation sites – are marked red, and residues highlighted in green form the characteristic amino acid motif (RSXSXP) that is believed to be involved in 14-3-3 protein binding to Pdc. Residues of human Pdc that contact Gβ are denoted by (+) above the aligned sequences. Protein accession numbers: *Blepharisma japonicum* Pdc, gi_124020703; *Dictyostelium discoideum* PhLP1, gi_33331889; *Homo sapiens* Pdc, gi_2967590; *Homo sapiens* PhLP1, gi_5430700; *Danio rerio* Pdc1, gi_38174547; *Rattus norvegicus* PhLP1, gi_66911418; *Cryptonema parasitica* Pdc, gi_6714949.

Further studies are necessary to determine what kind of Gβ modification occurs and whether Gβγ re-binds to the plasma membrane or becomes localized to some other cellular compartments, as has been described elsewhere (Azpiazu et al., 2006; Akgoz et al., 2004; Akgoz et al., 2006).

The findings of this study provide further detailed characterization of the functional properties of the Pdc of *Blepharisma*, an evolutionarily ancient lower eukaryote. Our results suggest that Pdc may not only contribute to the processes of light adaptation, as has been observed in vertebrate photoreceptor cells (Chen et al., 2005), but may also be involved in the control of light signal transduction preceding the motile photophobic response in ciliates, and perhaps in some other cell functions.

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