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Short Communication

Light-induced Interaction of Putative Phosducin with Gβγ-subunits of G-protein in the Ciliate *Blepharisma japonicum*

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Summary. Immunoblot of whole-cell lysate from *Blepharisma japonicum* with a polyclonal antibody raised against the rat phosducin display one major protein band of molecular weight of 28 kDa. Immunoprecipitation of detected phosducin immunoanalogue from lysate of dark- and light-adapted ciliates with the anti-phosducin antibody and subsequent analysis of the precipitated protein with a monoclonal antibody raised against phosphoserine residues revealed also the presence of a 28 kDa protein phosphorylated on a serine residues. This phosphoprotein exists in a highly phosphorylated form in ciliates adapted to darkness and its dephosphorylation occurs in illuminated cells. An immunoblot assay of the precipitated phosducin-related protein with a rabbit polyclonal antibody directed against β-subunit of G-protein showed one major protein band of molecular weight of about 34 kDa in lysate obtained from ciliates exposed to light. In addition, based on partial cloning of the putative ciliate phosducin nucleotide sequence homology to the phosducin belonging to the subgroup I of phosducin protein family was deduced. The results obtained in this study confirm the previously reported hypothesis that in the ciliated protist *Blepharisma japonicum* phosducin does exist and resembles that observed in a wide variety of higher eukaryotes and also in some microorganisms.

Key words: *Blepharisma japonicum*, ciliate, G-protein, phosducin, protein phosphorylation, photophobic response, photosensory transduction.

INTRODUCTION

The family of large G-proteins plays an essential role in transducing extracellular signals from the cell-surface receptors to the intracellular effectors (Hamm 1998). G-protein-coupled signaling is regulated at the G-protein level by two different mechanisms. The slow intrinsic GTP-ase activity of α-subunit (Gα) of G-protein can be accelerated by a large family of G-proteins, the regulators of G- protein signaling (RGS) (Berman *et al*. 1996, Hepler 1999). The second mechanism prevents βγ-subunit (Gβγ) of G-protein from modulating its downstream effectors and reassociating with Gα-GDP to undergo another cycle of receptor-mediated activation. The regulatory protein carrying out this task is a cytosolic phosphoprotein, phosducin. It was first discovered in vertebrate retinas and developmentally related pineal glands (Lee *et al.* 1984, Reig *et al.* 1990). Nowadays, there is increasing evidence that phosducin and many phosducin-related proteins are widely expressed also in other tissues (Bauer *et al.* 1992, Danner and Lohse 1996). Existence of proteins of the phosducin

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family has recently been reported in some lower eukaryotic organisms as well. In the yeast *Saccharomyces cerevisiae*, two phosducin-like proteins, PLp1 and PLp2 were detected, that *in vivo* can bind and regulate Gβγ activity (Flanary *et al*. 2000). In the case of the fungus *Cryphonecteria parasitica*, one gene bdm-1 was identified and shown to encode phosducin-like protein involved in regulation of Gβγ function and accumulation of Gα (Kasahara *et al*. 2000). Moreover, three genes, designated as *phlp*-1, *phlp*-2 and *phlp*-3, encoding distinct phosducin-like proteins, phosducin-1, phosducin-2 and phosducin-3, were discovered in *Dictyostelium discoideum* and it was firmly established that at least one of them, phosducin-1, also plays a role in G-protein-coupled signaling control (Blaauw *et al*. 2003). As reported lately, a cytosolic protein of 28 kDa showing properties similar to those described for phosducin has also been detected in the lower eukaryote, the photosensitive ciliate *Blepharisma japonicum* (Fabczak *et al*. 2001, 2004; Sobierajska *et al*. 2005). This phosphoprotein was shown to be highly phosphorylated in dark-adapted cells and dephosphorylated in ciliates exposed to light. The light-dependent dephosphorylation of phosducin immunoanalogue matches the light-dependent motile behaviour of the cell (photophobic responses). The observed modification of the cell photobehavior results from activation of a specific cellular photoreceptor system (Giese 1973, Tao *et al*. 1994, Maeda *et al*. 1997, Matsuoka *et al*. 2000, Fabczak 2000a). The light harvesting system is coupled to the cell locomotory system *via* G-protein-mediated signaling and membrane potential changes (Fabczak *et al*. 1993; 1998; 1999; 2000a, b) as in the case of photoreceptor cells of higher organisms (Rayer *et al*. 1990).

The present study was initiated to identify the 28 kDa protein detected in the ciliate *Blepharisma japonicum* and to characterize its light-dependent changes in phosphorylation levels with immunoblotting, immunoprecipitation and PCR assays. Furthermore, examinations were attempted to show possible interaction of phosducin immunoanalogue with β-subunit of G-protein in ciliates exposed to light.

MATERIALS AND METHODS

Cell culture

Ciliates *Blepharisma japonicum* were grown as described elsewhere (Fabczak 2000b). Before each experiment, cells were collected by a low-speed centrifugation and then washed in an excess of fresh culture medium lacking nutritional components. Finally, the cell samples were used for biochemical assays following cell incubation in darkness or light in the fresh culture medium. Cell illumination was provided by a 150 W fiberoptic white light source (MLW, Germany), which was equipped with an electromagnetic programmable shutter (mod. l22-841, Ealing Electro-Optics, England).

Immunoblotting

Cell samples were mixed with sample buffer supplemented with protease and phosphatase inhibitors (50 mM NaF, 2 mM PMSF, 10 μM okadaic acid, 10 μg/ml aprotinin, 100 μg/ml leupeptin) to terminate reactions (Laemmli 1970), and then boiled for 5 min. Proteins from solubilized cells were separated by 10% SDS-PAGE with a Hoefer System (Amersham, USA) and transferred to nitrocellulose membranes (Bio-Rad, USA) for 60 min at 100 V in a transfer buffer solution (Towbin *et al*. 1979). Membranes were exposed to TBS-BSA-Tween blocking solution (150 mM NaCl, 10 mM Tris, pH 7.5, 2% bovine serum albumin (BSA), 0.2% Tween-20) for 2 h at room temperature followed by incubation with a polyclonal antibody raised against rat phosducin (kindly provided by Professor Craig Thulin from Brigham Young College in Provo, USA; Thulin *et al*. 1999) at 1:5000 dilution or a rabbit polyclonal antibody directed against β-subunit of G-protein (Santa Cruz Biotechnol. Inc., USA) at 1:1000 dilution in TBS-BSA-Tween solution overnight at 4°C. After several washes in TBS with 0.1% Tween-20, blots were incubated for 60 min at room temperature with secondary antibody, anti-rabbit IgG-horseradish peroxidase conjugate (Calbiochem, Germany) at a 1:10000 dilution in TBS-BSA-Tween solution. Finally, membranes were washed in TBS-Tween buffer and developed with an ECL detection system (Amersham, Sweden). Protein molecular weights were determined based on their relative electrophoretic mobilities with prestained molecular weight markers (Bio-Rad). In control set of experiments the incubations with primary antibody were omitted. A protein concentration in an individual cell sample was estimated with a method reported by Bradford (1976) using BSA as a standard.

Immunoprecipitation

Samples of dark- or light-adapted cells were solubilized in Triton buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 20 mM Tris, pH 7.4) and then centrifuged for 15 min at 13200*g* at 4°C. The supernatants with protease and phosphatase inhibitors (10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 1 μM okadaic acid, 5 mM NaF) were used for immunoprecipitation assays. The cell samples were subjected to preclearance for 1 h with 30% (v/v) protein A-Sepharose CL-4B (Amersham, Sweden) at 4°C. After this, the mixture was centrifuged for 1 min at 13200g at the same temperature. The supernatant fraction was incubated for 1.5 h at 4°C with serum containing anti-phosducin and then incubated again for 1.5 h with a new portion of protein-A-Sepharose. The resin was washed three times in washing buffer (150 mM NaCl, 20 mM Tris, pH 7.4) transferred to new tubes for the third wash then resuspended in sample buffer, boiled for 5 min and analyzed with SDS-PAGE according to the method of Laemmli (1970). Western blot analysis with the anti-phosphoserine residues (clone PSER-4A9 from Alexis, Switzerland) or anti-α-subunit of G-protein antibodies (Santa Cruz Biotechnol. Inc., USA) were carried out as described in previous immunoblotting section.

PCR analysis and cloning

Messenger RNA was isolated from *Blepharisma japonicum* cells using PolyATract System 1000 (Promega) and used as the template for cDNA synthesis by a Universal RiboClone cDNA Synthesis System (Promega). PCR reaction was performed in Minicycler TM (MJ Research) with synthesized primers homologous to a highly conserved Gβ-protein-binding motif of phosducin in N-terminal domain , Phd - F (5'ACGGG(C/T)CCAAA(A/G)GGGGTGAT- 3') and CRX domain, Phd - R (5'-GATAC(C/T)AA (A/T) AGGTTAGTA-3') characteristic for I subgroup of phosducin The amplification reaction was run for 30 cycles of the following sequence: 94°C (30 s), 36°C (45 s) and 72°C (60 s) and final extension step at 72°C for 10 min. The PCR products were analyzed by 1.5 % agarose gel electrophoresis. DNA was extracted from the gel by a NucleoSpin (Macherey-Nagel) and then ligated into pUC19 plasmid (Promega). *Escherichia coli* JM109 competent cells were transformed with this construct according to standard protocols (Sambrook and Russel 2001). Finally, plasmids were isolated from *Escherichia coli* cells by alkaline minilyses and the obtained constructs were cut by restriction enzymes. The reaction products were visualized in agarose gel stained with ethidium bromide. The obtained clone was sequenced and used to search the GeneBank with the BLAST algorithm.

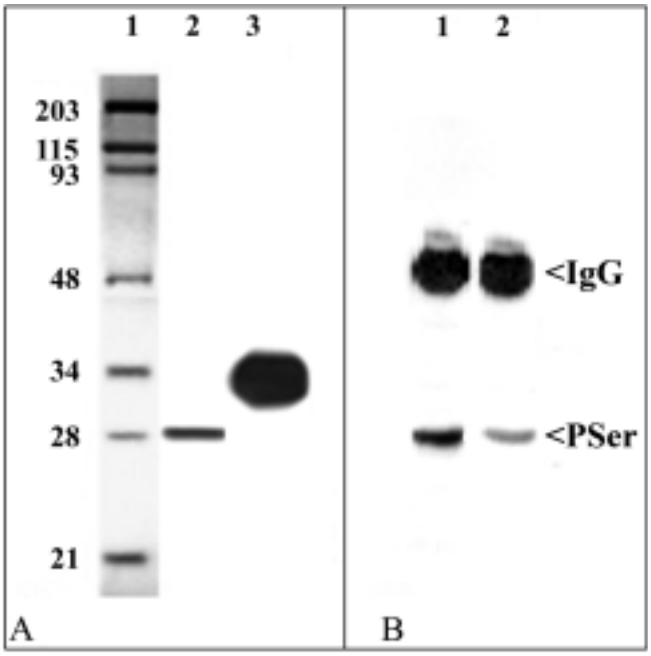

Fig. 1. Identification and light-induced changes in phosphorylation level of phosducin immunoanalogue in *Blepharisma japonicum*. **A** -Immunoblotting for identification of phosducin immunoanalogue in whole-cell lysate from ciliates and phosducin in homogenate from bovine rod outer segment used as a specificity control of the antibody applied. A polyclonal antibody raised against phosducin recognized protein band of 28 kDa in lysate from ciliates (lane 2) and 33 kDa protein band in homogenate from bovine rod outer segment (lane 3). Positions of molecular marker stained by Comassie Brilliant Blue are shown on the left (lane 1). **B** - Immunoprecipitation assay of putative phosducin in ciliates with anti-phosducin antibody. The precipitated proteins in lysates from dark-adapted (lane 1) and illuminated (lane 2) ciliates were analyzed by the anti-phosphoserine antibody.

RESULTS AND DISCUSSION

Immunoblotting of a whole-cell lysate from *Blepharisma japonicum* (Fig. 1A, lane 2) with a polyclonal antibody raised against rat retinal phosducin showed immunoreactivity with one protein of 28 kDa only. A clear immunoreactivity was also displayed with protein band of 33 kDa in bovine rod outer segment homogenate used for specificity control of applied antibody (Fig. 1A, lane 3). The results of this examinations indicated that labeled ciliate protein has molecular weight of 28 kDa similar to that of protein exhibiting light-dependent phosphorylation previously described (Fabczak *et al.* 2004).

To examine *in vivo* whether changes in phosphorylation levels of the identified ciliate phosducin immunoanalog can be induced by light, the whole-cell lysate prepared from dark-adapted and from light-exposed cells were immunoprecipitated with the anti-phosducin antibody. Subsequently, the precipitated proteins were analyzed by means of monoclonal antibody raised against phosphoserine residues. Under both experimental conditions, the anti-phosducin antibody precipitated exclusively one 28 kDa protein phosphorylated on phosphoserine residues (Fig. 1B). These experiments also corroborated previous observations that the illumination significantly decreased phosphorylation levels of the phosphoprotein in the cells (Fig. 1B, lane 2), while the phosphorylation level of this protein was much higher in untreated cells (Fig. 1B, lane 1) (Fabczak *et al.* 2004). These results showed that the tested organisms possess indeed the 28 kDa protein, highly analogous to phosducin from higher organisms, which shows enhanced phosphorylation in dark-kept organisms and becomes markedly dephosphorylated in cells exposed to light (Shulz 2001).

In successive experiments the immunoprecipitated proteins were probed by immunoblotting with rabbit polyclonal antibody raised against β-subunit (Gβ) of G-protein. As shown in Fig. 2A, in lysate from ciliates adapted to light a robust coimmunoprecipitation of the Gβ at protein band of 34 kDa was observed (lane 3), while in lysate prepared from dark-adapted cells this protein band was missing (lane 2). These data

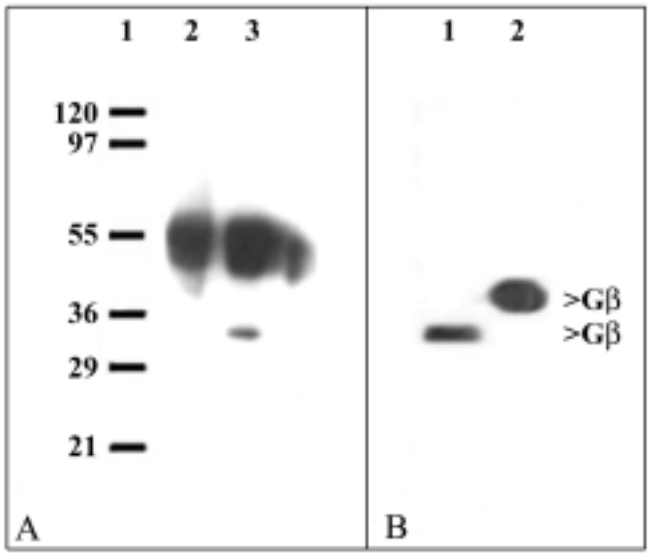

Fig. 2. Interaction between Gβ and phosducin immunoanalogue in ciliate *Blepharisma japonicum*. **A** - Co-immunoprecipitation of phosducin immunoanalogue in ciliates. Lysates from cells adapted to darkness and cells exposed to light, were immunoprecipitated with anti-phosducin antibody and immunoblotted with anti-Gβ-antibody. Bands at 55 kDa correspond to high chains of IgG. **B** - Immunodetection of Gβ immunoanalogue in whole-cell lysate from ciliates with anti-Gβ-antibody. Other details as in Fig. 1.

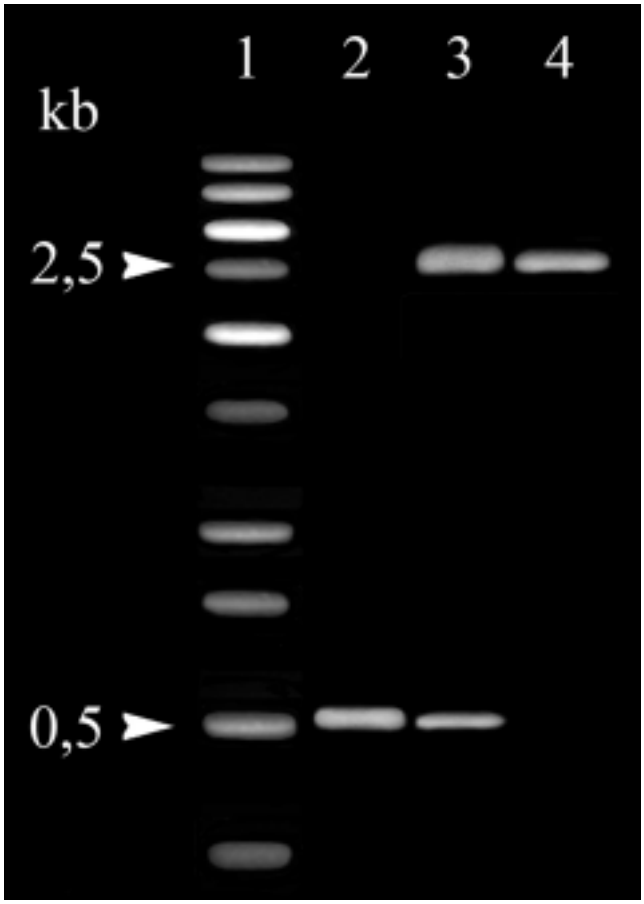

Fig. 3. PCR amplification and cloning of the putative phosducin in ciliate *Blepharisma japonicum*. Lane 1, molecular weight marker; lane 2, PCR product of 500 bp (bottom arrowhead) with synthesized primers homologous to highly conserved Gβ-protein-binding motif of the phosducin in N-terminal domain and CRX domain characteristic for I subgroup of phosducin protein family; lane 3, visualization of construct after alkaline minilysis isolation and restriction enzyme cutting; lane 4, pUC19 plasmid alone.

are consistent with the effect of the light on phosphorylation level of protein identified as the phosducin immunoanalogue in immunoprecipitation assay (Fig 1B). Additionally, immunoblotting of whole-cell lysate with anti-Gβ-antibody also revealed a major protein band of molecular weight of about 34 kDa (Fig. 2B, lane 1) as well as a protein band of 36 kDa in bovine rod outer segment homogenate (Fig. 2B, lane 2), confirming that the antibody possess sufficient selectivity for the Gβ. The ciliate protein had molecular weight of 40 kDa, similar to that showed in coimmunoprecipitation assay with the anti-phosducin antibody shown in Fig. 2A. The results of these experiments provide evidence that the ciliate phosducin immunoanalogue in its dephosphorylated form may translocate towards cell membrane and interact with a protein analogous to β-subunit of G-proteins.

Since the results of Western blot analysis suggested the presence of phosducin immunoanalogue in *Blepharisma japonicum* further characterization of this phosphoprotein was undertaken by a cloning procedure. When primers homologous to a highly conserved Gα-protein-binding motif of the phosducin in the N-terminal and CRX domain, characteristic for subgroup I of the phosducin protein family (Blaauw *et al.* 2003), were employed in PCR analysis, a single band of approximately 500 pb was generated (Fig. 3, lane 2). This is the appropriate molecular value for cDNA of the expected fragment of known phosducin. The deduced amino acid sequence displayed a significant degree of homology (~ 41%) to phosducin, which belongs to the subgroup I of phosducin protein family (Blaauw *et al.* 2003) (Fig. 4).

The results obtained in this study support the hypothesis that in the ciliate *Blepharisma japonicum* phosducin exists and highly resembles those found in a wide variety of higher eukaryotes (Shulz 2001) and also in some lower eukaryotic cells (Blaauw *et al.* 2003, Flanary *et al.* 2000, Kasahara *et al*. 2000). In these organisms

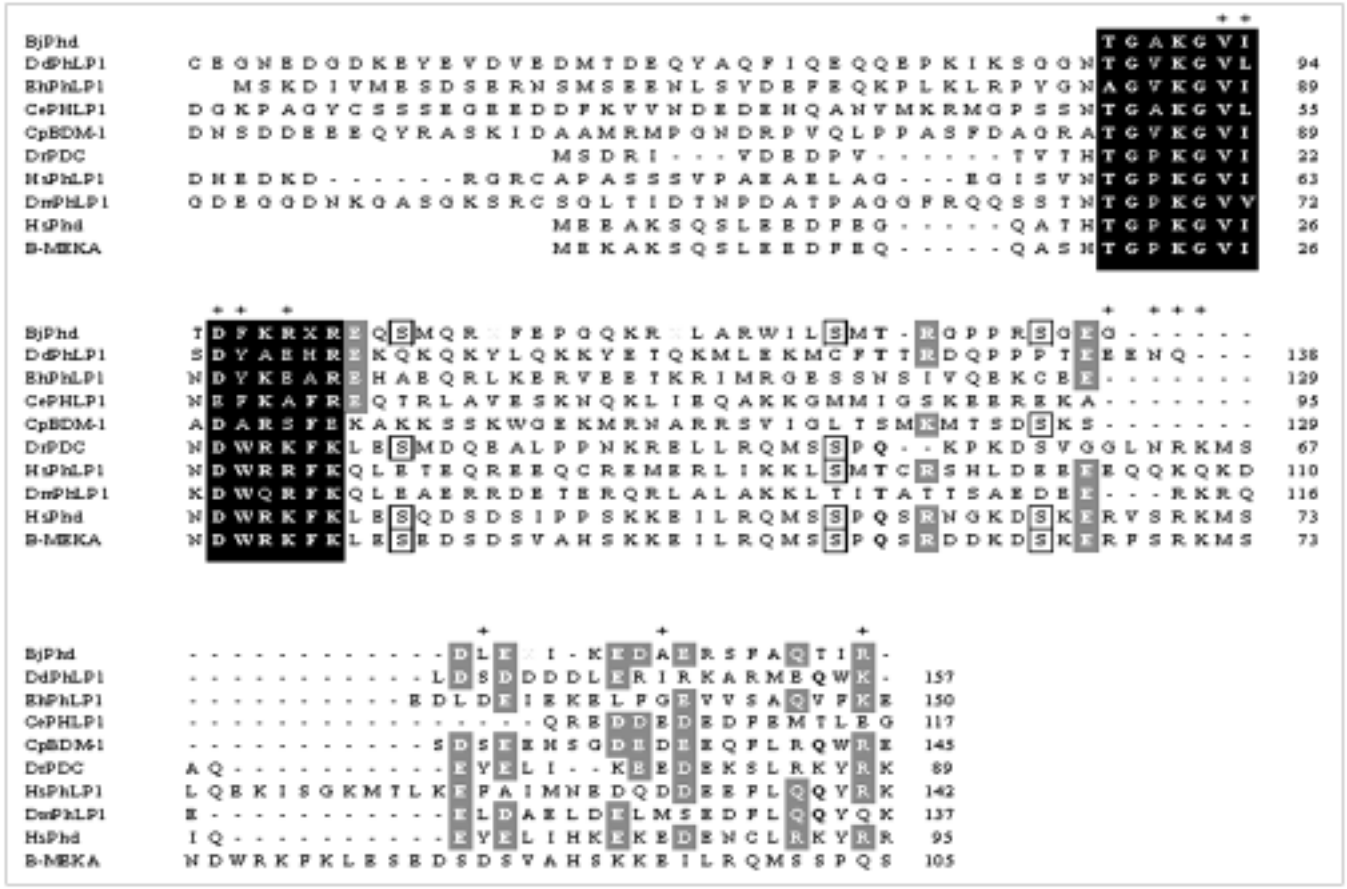

Fig. 4. Alignment of the derived amino acid sequences of *Blepharisma japonicum* phosducin and members of the subgroup I of phosducin protein family. Residues shaded in black are conserved in 70 - 100% of all sequences; residues shaded in grey are conserved in 60-70% of all sequences; residues bold characters are conserved in 75 -100% of all sequences of this subgroup; serine residues localized in the same place in *Blepharisma japonicum* and other organisms are in frames. The Gβ-containing residues of human phosducin (HsPh) are denoted by + above the aligned sequences. The abbreviations used are: Phd - phosducin; PhLP - phosducin like protein; BjPhd - putative fragment of Phd from *Blepharisma japonicum*; DdPhLP1 - PhLP from *Dictyostelium discoideum*, gi 333331889; EhPhLP1 - PhLP from *Entamoeba histolytica*, gi 56467495; CePhLP1- PhLP from *Ceanorhabditis elegans*, gi 17543862; CpBDM-1 - PhLP from *Cryphonectria parasitica*, gi 6714950; DrPDC - PhLP from *Danio rerio*, gi 56207464; HsPhLP1 - PhLP from *Homo sapiens*, gi 13642199; DmPhLP1 - PhLP from *Drosophila melanogaster*, gi 23093421; HsPhd - Phd from *Homo sapiens*, gi 187517; B-MEKA - Phd from *Bos taurus*, gi130133

phosducin was found to be ubiquitous cytosolic G-protein regulator, since in its dephosphorylated form can bind several different G-proteins, such as Gs, Go, or Gi and prevents reassociation of α-subunit of G-proteins with Gβγ, resulting in lower signal amplification at the G-protein level (Bauer *et al.* 1992). It appears that phosducin immunoanalogue found in the photosensitive *Blepharisma japonicum* might also play distinct physiological roles in the ciliate photobehavior. The obtained data may also have important evolutionary significance, since they suggest that the sensory transduction cascade in this ciliate is under the control of phosducin phosphorylation changes processes similar to those operating in evolutionarily distant photoreceptor like the cells of vertebrate retina (Thulin *et al.* 1999, Lee *et al*. 2004).

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