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The β-subunit of G proteins is a substrate of protein histidine phosphatase ☆

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Abstract

Increasing evidence suggests that reversible phosphorylation of *histidine* residues in proteins is important for signaling cascades in eukaryotic cells. Recently, the first eukaryotic protein histidine phosphatase (PHP) was identified. The β_1-subunit of heterotrimeric G proteins (Gβ) undergoes phosphorylation on His^{266} which is apparently involved in receptor-independent G protein activation. We studied whether phosphorylated Gβ-subunits are substrates of PHP. Phosphorylated Gβγ dimers of the retinal G protein transducin and Gβ in membrane preparations of H10 cells (neonatal rat cardiomyocytes) were dephosphorylated by PHP. Overexpression of PHP in H10 cells showed that PHP and Gβ also interfere within cells. In membranes of cells overexpressing PHP, the amount of phosphorylated Gβ was largely reduced. Both our in vitro and cell studies indicate that phosphorylated Gβ-subunits of heterotrimeric G proteins are substrates of PHP. Therefore, PHP might play a role in the regulation of signal transduction via heterotrimeric G proteins.

Keywords: G protein; Histidine; Phosphatase; Phosphorylation; ATP-citrate lyase; Nucleoside diphosphate kinases; β-subunit; Signal transduction; Guanine nucleotides; Transducin

The reversible phosphorylation of proteins regulates almost all aspects of cell life. In bacteria, phosphorylation is often observed on histidine and aspartate residues, whereas in eukaryotes it is mostly found on serine, threonine or tyrosine residues. Nevertheless, an increasing number of covalent protein histidine phosphates has been discovered in higher organisms over the past few years. Histone H4, a nuclear protein, was the first vertebrate protein identified containing phosphorylated histidine residues [1]. Annexin I, a 37 kDa member of a family of Ca^{2+}-dependent phospholipid-binding proteins, undergoes histidine phosphorylation in airway epithelia [2]. P-selectin, a leukocyte adhesion protein, is phosphorylated on histidine following platelet activation with thrombin or collagen [3]. Recently, it has been demonstrated that the β-subunit of heterotrimeric G proteins can be phosphorylated on histidine residue 266 [4–7]. Surprisingly, nucleoside diphosphate kinase B (NDPK B), an enzyme that primarily catalyzes the transfer of terminal phosphate groups from 5′-triphosphate to 5′-diphosphate nucleotides, has been

☆ *Abbreviations:* ACL, ATP-citrate lyase; (P)-Gβ, (phosphorylated form of) β-subunit of heterotrimeric G protein, (P)-NDPK, (phosphorylated form of) nucleoside diphosphate kinase; PHP, protein histidine phosphatase.

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identified to serve as histidine kinase in that particular reaction. The His^{266}-phosphate in Gβ is of high energy and can be transferred onto GDP leading to the formation of GTP. Recent evidence suggested that NDPK B and Gβγ form a complex which specifically catalyzes the transfer of a high energetic phosphate onto GDP derived from G protein α-subunits (Gα) of the G_s- [7] as well as the G_i-family [8,9] of heterotrimeric G proteins. Apparently, this phosphotransfer reaction is involved in the basal, receptor-independent activation of heterotrimeric G proteins.

In the meantime, already 6% of all protein phosphorylation taking place in eukaryotic cells are supposed to be histidine phosphorylation [10]. As protein phosphorylation is regulated by kinases on the one hand and phosphatases on the other, it was not surprising that recently the first eukaryotic histidine phosphatase from vertebrate tissues was purified, characterized, and sequenced [11,12]. This protein histidine phosphatase is a soluble, low molecular mass protein (14 kDa), which is ubiquitously expressed. Orthologues of this enzyme are present in vertebrates and also in *Caenorhabditis elegans*. It is, however, not found in yeast and prokaryotes. The function of this novel phosphatase is still unknown. Yet, ATP-citrate lyase (ACL) has been identified as a substrate of PHP [13]. As ACL catalyzes the reaction from citrate and citryl-CoA to acetyl-CoA and oxaloacetate, PHP might be involved in the regulation of energy metabolism as well as in neurotransmitter production.

In search for further substrates of PHP, we studied whether P-His^{266} on Gβ might be recognized as substrate. Herein, we provide evidence that purified recombinant PHP dephosphorylates P-His in Gβ subunits of the retinal G protein transducin as well as phosphorylated Gβ in membranes of H10 cells, a cell line derived from neonatal rat cardiomyocytes. In contrast, the intermediately occurring histidine phosphate on NDPK B which is present during the above described phosphate transfer reaction is not affected by PHP. Furthermore, stable overexpression of PHP in H10 cells led to a strong reduction of phosphate incorporated into Gβ but not into NDPK in these cells. It is, therefore, concluded that phosphorylated Gβ subunits are substrates for PHP.

Materials and methods

Chemicals and proteins. ECL Western blotting detection reagent, Hybond ECL nitrocellulose membranes, horseradish peroxidase linked secondary anti-rabbit antibody, and [γ-^{32}P]GTP (5000 Ci/mmol), were from Amersham Bioscience. [γ-^{32}P]GTP (0.1 Ci/mmol) and [γ-^{3}H]GDP (0.1 Ci/mmol) were from NEN-DuPont. The prestained molecular weight standard was from preQlab, alkaline phosphatase and puromycin from Sigma, Superfect transfection reagent from Quiagen. pIRESpuro and pIRESpuro-EGFP were obtained from Clontech and polyethyleneimine cellulose F thin layer chromatography plates from Merck. Antibodies against Gβ (T-20) sc-378 were purchased from Santa Cruz Biotechnology and antibodies against the N-terminus of PHP were prepared as described [11]. PHP was expressed and purified according to standard procedures [11,13]. The retinal G protein transducin was prepared as described [6,8].

Tissue culture, preparation of cell homogenates and membranes. Neonatal rat heart myocytes, immortalized with a temperature-sensitive SV40 T antigen (H10 cells) were cultured at 33 °C in DMEM, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 10 μg/ml gentamycin. For cell lysis, cells were washed twice with ice-cold phosphate-buffered saline, scraped off in buffer A (10 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), and homogenized with two 10 s bursts applied by a Polytron (Kinematica) at a setting of 20,000 rpm. Cell lysates were then centrifuged at 100,000*g* for 30 min. Pellets were resuspended and centrifuged three more times in buffer A to obtain the membrane fraction.

Subcloning of PHP, stable transfection of H10 cells. The cDNA encoding human PHP was subcloned into pIRESpuro linearized with *Eco*RV and *Not*I. The construct was confirmed by restriction digest analysis. The day before transfection, H10 cells or H10 cells stably transfected with NDPK B (C3 cells) [7] were seeded at a density of 7×10^5 cells per 60-mm dish in 5 ml DMEM with 10% fetal calf serum. For transfection, 8 μg DNA of pIRES-EGFP encoding enhanced green fluorescent protein or the pIRESpuro-PHP construct and 50 μl Superfect transfection reagent were used according to the manufacturer's protocol. Forty-eight hours later, cells were diluted 1:15 and placed in a selective medium containing 2 μg/ml puromycin. Stably transfected cell clones were isolated after two weeks, and PHP expression was screened by Western blot analysis.

Tissue preparation. Heart, brain, lung, kidney, liver, and spleen, as well as cortex, cerebellum, striatum, and hippocampus, were isolated from adult, female Fischer-rats. Organs and brain tissues were homogenized at 4 °C, using a Dounce homogenizer (heart, brain, lung, kidney, liver, and spleen) or an Eppendorf micropestle (cortex, cerebellum, striatum, and hippocampus). The homogenization buffer contained 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM phenymethylsulfonyl fluoride, 1 mM benzamidine, and 0.1% (v/v) 2-mercaptoethanol. Four milliliters thereof were added per gram tissue. Homogenates were stored frozen at −80 °C. Protein was determined by the method of Lowry using bovine serum albumin as a standard.

Western blotting. Proteins were separated by SDS–PAGE on 15% polyacrylamide minigels ($9 \times 9.5 \times 0.05$ cm), transferred to membranes by semidry-blotting (1 h, 10 V) and stained with Ponceau S. Blots were blocked in TBST (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween) containing 5% non-fat milk powder for 1 h at room temperature. Membranes were cut horizontally at 25 kDa. The upper part was used for detection of Gβ (36 kDa), and the lower part was used to monitor PHP (14 kDa) content. Membranes were incubated overnight at 4 °C with polyclonal anti-PHP antibodies (1:400) in TBST containing 0.1% BSA or with anti-Gβ antibodies (1:2000) in TBST. This was followed by incubation with horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:2500) in TBST for 1 h at room temperature. Between blocking and the different antibody incubations the membranes were washed with TBST for 25 min (buffer was changed twice). Finally, the blots were developed with enhanced chemiluminescence reagent.

Phosphorylation and dephosphorylation. Phosphorylation of transducin preparations (10 μg) and C3 cell membranes (7 μg) was carried out in a volume of 10 μl at 37 °C in 25 mM Tris–HCl (pH 7.5), 1 mM Mg^{2+}, and 1 μM GTP including 2 μCi [γ-^{32}P]GTP (Amersham Bioscience) at 37 °C and for the indicated periods of time. Thereafter, the phosphorylation was stopped by addition of 5 mM EDTA. Dephosphorylation reactions (15 μl) followed immediately at 37 °C for 30 min with PHP (2.5 μg) in the presence of 25 mM Tris–HCl (pH 7.5), and 5 mM EDTA. Dephosphorylation with alkaline phosphatase (0.3 μg) was performed in the presence of 25 mM Tris–HCl (pH 7.9). Reactions were stopped by adding 5 μl sample buffer (15 mM Tris–HCl (pH 6.8), 4% SDS, 2% 2-mercaptoethanol, 8 M urea, 10% sucrose, 10 mM EDTA, and 0.01% bromphenol blue). Membranes of H10 cells stably