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Reduced viability of neuronal cells after overexpression of protein histidine phosphatase

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ABSTRACT

Protein histidine phosphatase (PHP) has just recently been discovered in eukaryotes and ATP-citrate lyase (ACL) was shown to be one of its substrates. Since ACL is crucial for cellular energy and fat metabolism we made an attempt to study the influence of PHP on cell viability. Using an adenoviral vector PHP was overexpressed in SN56 cholinergic murine neuroblastoma cells and in primary cultures of hippocampal neurons obtained from embryonic rats (E18). Overexpression of PHP in these cells caused a decrease in ACL activity and consequently impaired viability. To be sure that the reduced cellular viability was achieved by overexpression of PHP we also downregulated ACL in SN56 cells using RNAi-technology. Downregulation of ACL was harmful to the cells similar to what was observed upon overexpression of PHP. Taken together, it is concluded that overexpression of PHP results in increased dephosphorylation with concomitant inactivation of ACL, thus finally leading to cell damage.

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1. Introduction

The biological significance of reversible protein phosphorylation has been described already in the 1960s by Fischer et al. (1959). Meanwhile we know that almost all aspects of cell life are regulated by protein kinases and phosphatases. Previously, it has been assumed that vertebrate proteins are phosphorylated exclusively on serine, threonine and tyrosine residues. However, proteins have been discovered in higher organisms with phosphates covalently bound to histidine, like histone H4 (Fujitaki et al., 1981), annexin I (Muimo et al., 2000), P-selectin (Crovello et al., 1995) and the β subunit of heterotrimeric G-proteins (Kowluru et al., 1995; Wieland et al., 1993).

Studying *N*-phosphorylation is hampered by the fact that antibodies against those sites are not available. Inhibitors that specifically interfere with the enzymes involved in reversible *N*-phosphorylation are also not existing. Over the years, however, and despite analytical difficulties, a variety of proteins could be identified carrying one or more phosphates reversibly bound to histidine residues (Klumpp and Krieglstein, 2005). Evidence gradually accumulates that reversible phosphorylation of histidine residues in vertebrate proteins might be as important as the well-established reversible phosphorylation of serines, threonines and tyrosines.

A protein histidine phosphatase (PHP) from vertebrates was identified and cloned just recently (Hermesmeier and Klumpp, 1999; Klumpp et al., 2002; Ek et al., 2002). This protein was shown to dephosphorylate ACL (Klumpp et al., 2003) and Gβ (Mäurer et al., 2005), and even more substrates might be disclosed in the future. So far, nothing is known about the regulation of PHP activity or potential posttranslational modifications. The primary structure of PHP is different from all kinases and phosphatases described.

The 14 kDa PHP protein is found in virtually all vertebrate species and in almost any vertebrate tissue. In *C. elegans*, however, PHP was exclusively located in neurons (Klumpp et al., 2002). Accordingly, we overexpressed PHP in different cultured neuronal cells to shed some light on the functional role of this enzyme.

2. Experimental procedures

2.1. Materials

Cell culture media, amino acids and antibiotics were obtained from Gibco (Eggenstein, Germany). Hoechst 33258, polyethylenimine, trypsin inhibitor, staurosporine and paraformaldehyde were from Sigma–Aldrich (Taufkirchen,

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Abbreviations: PHP, protein histidine phosphatase; ACL, ATP-citrate lyase; siRNA, small interfering RNA.

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Germany). Fetal calf serum (FCS) was purchased from PAA (Marburg, Germany). A polyclonal antibody against the amino acid sequence 57–70 of ACL was generated in rabbits and affinity purified. Polyclonal antibodies against PHP were prepared and used as described (Klumpp et al., 2002). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs were from GE Healthcare (Freiburg, Germany). siRNA oligonucleotides were purchased from Ambion (Austin, TX, USA).

2.2. Cell cultures and cell extracts

All cell types were grown at 37 °C in humified atmosphere containing 5% CO_2. SN56.B5.G4 cholinergic murine neuroblastoma cells were kindly provided by C. Culmsee (Marburg, Germany). These cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) plus gentamycin. HEK293 cells were cultured in EarlesMEM containing 10% FCS plus penicillin/streptomycin. Primary cultures of hippocampal neurons were generated from brain hippocampal pieces from E18 fetuses of Sprague–Dawley rats (Charles-River, Sulzfeld, Germany) and treated with trypsin. Cells were dissociated by gentle trituration, seeded on PEI-coated dishes and cultured in Neurobasal™ medium supplemented with 1% B27 and 2 mM L-glutamine essentially as described by Mattson and Kater (1988).

Cell extracts for the determination of ACL activity were prepared by washing cells with ice-cold phosphate-buffered saline (PBS) and resuspending them in homogenisation buffer containing 10% glycerol, 130 mM Tris–HCl pH 6.8, 1 mM phenylmethylsulfonyl fluoride (PMSF), 7 μg/ml trypsin inhibitor and 1 μM calpain inhibitor. After brief sonication, cells were centrifuged (13,000 × g, 10 min) and the supernatant was frozen at −80 °C. Protein concentration was determined by using the bicinchoninic acid assay and bovine serum albumin as a standard.

2.3. Nuclear staining with Hoechst 33258

Cells were washed with ice-cold PBS, fixed for 30 min in 4% paraformaldehyde, washed and incubated for 30 min at 37 °C with the DNA fluorochrome Hoechst 33258 (10 μg/ml). Nuclear morphology was analysed using a fluorescence microscope.

2.4. Western blotting and immunocytochemistry

Cell extracts for Western blotting were prepared as described above with the exception that 3% SDS was added to the homogenisation buffer. 20–40 μg of those extracts were applied to SDS-PAGE (7.5% gels with special emphasis for ACL resolution, 15% gels for PHP). Proteins were transferred electrophoretically to nitrocellulose membranes and immunodetection was performed essentially as described (Klumpp et al., 2002).

Cells were grown on coverslips, fixed with 4% paraformaldehyde for 20 min, permeabilised with 0.2% Triton X-100 for 5 min, treated with 5% horse or goat serum for 30 min, then incubated with the primary antibody over night at 4 °C (anti-ACL 1:100, or anti-PHP 1:100), followed by incubation with the secondary Cy3-labeled anti-rabbit antibody for 1 h at room temperature (1:200), and analysed using a confocal laser scanning microscope.

2.5. Determination of ACL activity

The enzyme was assayed using the malate dehydrogenase coupled procedure and monitoring the oxidation of NADH by the change in absorbance at 340 nm as described (Linn and Srere, 1979). The assay mixture (150 μl) contained 100 mM Tris–HCl (pH 7.4), 16 mM potassium citrate, 1 mM dithiothreitol, 10 mM $MgCl_2$, 2.5 units malate dehydrogenase, 0.2 mM CoA, 0.14 mM NADH, 1.5 mM ATP, and cell extracts containing ACL. Assays were performed at 30 °C.

2.6. Autophosphorylation of ACL at his-760 and dephosphorylation by PHP

Phosphorylation of proteins in SN56 cell extracts (15 μg protein/10 μl assay) was carried out at 37 °C for 30 min in the presence of 1 μM ATP including 3 μCi [γ-^{32}P]ATP (1000 Ci/mmol), 5 mM EDTA and 25 mM Tris–HCl (pH 7.5). Reactions were either immediately used for dephosphorylation by PHP or stopped on ice with 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 0.06% bromphenol blue), run on 15% SDS-PAGE minigels, dried and autoradiographed using Kodak X-Omat R films. Dephosphorylation reactions (15 μl, 37 °C, 15 min) were performed after phosphorylation by adding PHP (1 μg/assay) instead of sample buffer and proceeded as described above. Exposure time was overnight without intensifying screens.

2.7. Downregulation of ACL by siRNA

To induce knockdown of ACL by RNA interference, SN56 cells were transfected with chemically synthesised small interfering RNA (siRNA) oligonucleotides directed against nucleotides 2093–2111 of mRNA encoding ACL (5′-GGC AUG UCU AAU GAA CUC-3′).

SN56 cells were washed with OptiMEM containing 10% FCS and adjusted to 4×10^6 cells/375 μl in the same medium. siRNA was added (25 μl of a 20 μM solution) and electroporation performed by using a Genpulser Xcell (Bio-Rad, Munich). Thereafter, the cells were seeded in OptiMEM with 10% FCS and kept in culture until harvested.

2.8. Recombinant PHP adenovirus

A 474 bp cDNA encoding human PHP (378 bp) was excised from pVL1392-PHP-wt by NotI/HindIII and ligated into the shuttle vector pAd-Track-CMV carrying an additional green fluorescent protein (GFP) expression cassette. Recombinant adenovirus (type 5) encoding for PHP was generated after linearisation of pAd-Track-CMV-PHP and cotransformation with the adenoviral plasmid pAd-easy-1 in *E. coli* BJ5183. Kanamycin was used for selection. Recombinant plasmids were

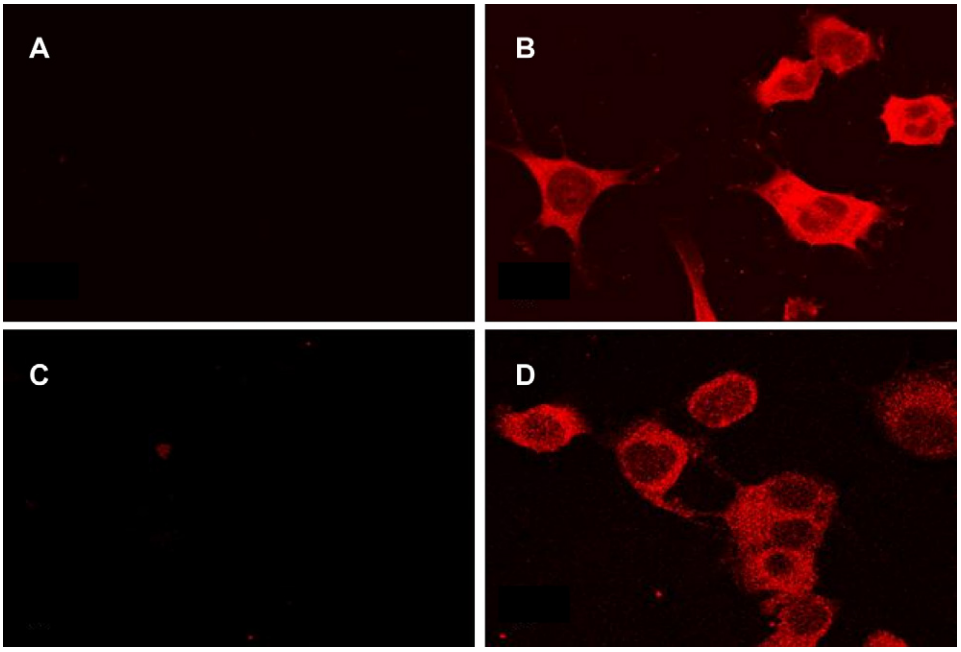

Fig. 1. Fluorescence laser scanning photographs showing subcellular localization of PHP and ACL in SN56 cells. Controls omitting primary antibodies against PHP (A) or against ACL (C). Incubation with antibodies against PHP (B), or antibodies directed against ACL (D). Both enzymes are mainly localized in the cytosol.

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