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Glyceraldehyde-3-phosphate dehydrogenase in the extracellular space inhibits cell spreading

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Abstract

The occurrence and the novel function of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the extracellular space were studied. The extracellular GAPDH with the same molecular mass as the intracellular GAPDH was detected in the conditioned medium of mammalian cultured cell lines such as COS-7, HEK293, MCF-7, HepG2, PC-12, and neuro-2a cells. Western blot analysis represented the occurrence of GAPDH, but not α-tubulin (an intracellular marker protein), in the conditioned medium of COS-7 cells. Furthermore, GAPDH was found in rat serum. These results indicate that GAPDH was secreted outside of the cells. Addition of GAPDH to the cultured medium of COS-7, HEK293 and HepG2 cells allowed cells to undergo morphological changes. In COS-7 cells, the extracellular GAPDH inhibited cell spreading without influencing the cell growth. Western blot and immunofluorescent microscopy analyses revealed that the extracellular GAPDH bound to COS-7 cells in time- and dose-dependent manners. However, a mutant substituting Ser for Cys at position 151 of GAPDH resulted in no binding to the cells, no decreased cell-spreading efficiency and no cell morphological changes. These results indicate that the Cys151 was involved in the binding of GAPDH to cells and the GAPDH-inhibited cell spreading.

Keywords: Glyceraldehyde-3-phosphate dehydrogenase; Extracellular space; Cell spreading; Cell morphology; Active center

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is ubiquitously expressed in mammals and composed of a homotetramer assembled four identical 36 kDa subunits. The protein reversibly catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate as a glycolytic enzyme in the cytosol. The reaction proceeds through the thiol of a cysteine residue (Cys151) at each of the four active centers of the tetramer. The highly reactive cysteine in the active center undergoes oxidation and nitrosylation, resulting in the loss of activity [1–3].

Recently, GAPDH has been demonstrated to occur in the nuclear and particulate fractions in addition to the cytosolic fraction and has been identified to be involved in a number of diverse nonglycolytic cellular processes such as apoptosis, neuronal disorders, viral pathogenesis, endocytosis, microtubule bundling, phosphotransferase/kinase reactions, the translational regulation of gene expression, nuclear RNA export, DNA replication and DNA repair [4]. GAPDH has been thought to be a house-keeping gene and is used to standardize Northern blots. The GAPDH mRNA level, however, is up-regulated in hypoxic endothelial cells [5] and alveolar epithelial cells [6] in highly metastatic rat adenocarcinoma cell lines [7], in lung cancer [8] and in apoptosis-induced cells [9,10]. Dissociation of GAPDH from the tetrameric form to the monomeric one seems to be responsible for diminution of the dehydrogenase activity [11]. In contrast, the monomeric form of GAPDH exhibits uracil DNA glycosylase activity as a different function in the nucleus [12]. The cytosine β-D-arabinoside-induced neuronal apoptosis results in the overexpression and nuclear accumulation of GAPDH, whereas such nuclear GAPDH

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contributes to neither the dehydrogenase activity nor the uracil DNA glycosylase activity [13]. Furthermore, in the hypoxic endothelial cells, the GAPDH expression is induced not only in the cytosolic fraction, but also in the nuclear and particulate fractions [5]. However, even in the cytosolic fraction, the GAPDH activity insufficiently increases in proportion to the increase in the GAPDH protein. Thus, the subcellular expression of GAPDH is independent of its classical glycolytic function even in the cytosolic fraction, and a number of diverse biological properties of GAPDH might be exerted depending on the oligomeric state and/or the different locations in the cells.

The immunoglobulin production stimulating factor (IPSF)-IIa purified from the cell lysate of human Burkitt's lymphoma, Namalwa cell, is composed of a 40-kDa polypeptide chain and two homogeneous 36 kDa polypeptide chains, and the 36-kDa subunit with IPSF activity is identified as GAPDH [14]. In fact, rabbit muscle GAPDH is demonstrated to enhance the IgM production of human–human and mouse–mouse hybridomas. However, there is an inconsistency that intracellular GAPDH acts as an IPSF outside of the cells. In the present study, we report that GAPDH occurs in the conditioned medium of several mammalian cultured cell lines and in rat serum and that extracellular GAPDH inhibits cell spreading. Furthermore, we demonstrate the involvement of the Cys151 of an active center in the extracellular GAPDH-inhibited cell spreading.

2. Materials and methods

2.1. Cell culture

HEK293 cells and COS-7 cells were maintained in Dulbecco's modified Eagle's medium-low glucose (5.6 mM glucose) supplemented with 25 mM HEPES, 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). HepG2 cells were grown on a collagen (type I)-coated dish in the above medium. Neuro-2a cells were cultured in Dulbecco's modified Eagle's medium-high glucose (33 mM glucose) supplemented with 25 mM HEPES, 10% fetal bovine serum and the above antibiotics. MCF-7 cells were grown in Dulbecco's modified Eagle's medium-high glucose (25 mM glucose) supplemented with 25 mM HEPES, 10% fetal bovine serum and the above antibiotics. PC-12 cells were plated on a collagen (type I)-coated dish in RPMI1640 medium supplemented with 25 mM HEPES, 10% fetal bovine serum, 5% horse serum and the above antibiotics. Cells were grown at 37 °C in humidified 5% CO_2.

2.2. Subcellular fractionation

COS-7 cells were washed twice with ice-cold phosphate buffered saline (PBS), pH 7.4, resuspended in 20 mM HEPES–NaOH, pH 7.5, containing 250 mM sucrose, 1 mM EDTA, 2 mM dithiotreitol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin and 1 μg/ml aprotinin, and homogenized as previously described [5]. Briefly, the homogenates were centrifuged at 270×*g* for 2 min and the supernatants were further centrifuged at 750×*g* for 10 min to precipitate the nuclei. The post-nuclear fraction was centrifuged at 105,000×*g* for 1 h, and the supernatant and precipitate are referred to as the cytosolic fraction and particulate fraction, respectively. The proteins (10 μg) in each fraction were subjected to SDS-PAGE in 12.5% gel and transferred to a poly(vinylidene difluoride) membrane. The membrane was incubated with polyclonal anti-GAPDH [5], polyclonal anti-triosephosphate isomerase (TPI) (a cytosolic protein marker) [5], or monoclonal anti-lamin B_1 (a nuclear protein marker) (Clone No. L-5: Zymed Laboratories Inc., South San Francisco, CA, USA) antibodies. The anti-GAPDH or anti-TPI antibodies were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG, and the anti-lamin B_1 antibody was incubated with the horseradish peroxidase-conjugated goat anti-mouse IgG. The immunoreactive proteins were detected using the Super Signal Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA). Digitized images were obtained with luminescent image analyzer (LAS-1000 plus: Fuji Film, Tokyo, Japan).

2.3. Construction of plasmids

A two-sequential nested PCR was performed to obtain the human GAPDH cDNA. The primer sequences correspond to the human GAPDH cDNA sequence (GenBank accession number M33197). The first PCR was performed using a sense primer 1 (5′-GTTCGACAGTCAGCCG-CATCTTCT-3′; positions 1–24), an antisense primer 1 (5′-AACTGTGAGGAGGGGAGATTCAGT-3′; positions 1184–1161) and human brain (cerebral cortex) Marathon-ready cDNA (BD Biosciences Clontech, Palo Alto, CA, USA) as a template. The second PCR was performed using a sense primer 2 (5′-GGgaattcATGGGGAAGGT-GAAGGTCGGAG-3; positions 61–82), an antisense primer 2 (5′-GGggatccTTACTCCTTGGAGGCCATGTGG-3′; positions 1068–1047) and the first PCR products as a template. The lower case letters in the sequences were designed for *Eco*RI and *Bam*HI restriction sites, respectively. The PCR products were cloned into the pCR2.1TOPO-TA vector, termed pCR2.1-hGAPDH. To construct the expression vector for a wild type of recombinant human GAPDH (hGAPDH), the PCR was performed using a sense primer 3 (5′-gacgacgacaagATG-GGGAAGGTGAAGGTCGGAG-3′; positions 61–82), an antisense primer 3 (5′-gaggagaagcccggtTTACTCCTTG-GAGGCCATGTGG-3′; positions 1068–1047) and the above first PCR products as a template. The lower case letters in the sequences were designed for ligation-independent cloning sequences of pET30 Ek/LIC vector. The PCR products were cloned into the pET-30 Ek/LIC vector