Localization of Enolase in the Subfractions of a Breast Cancer Cell Line

Ewa Seweryn*, Jadwiga Pietkiewicz, Iwona S. Bednarz-Misa, Ireneusz Ceremuga, Jolanta Saczko, Julita Kulbacka, and Andrzej Gamian

Department of Medical Biochemistry, University of Medicine in Wroclaw, ul. Chalubinskiego 10, 50–368 Wroclaw, Poland. E-mail: eseweryn@bioch.am.wroc.pl

* Author for correspondence and reprint requests

Z. Naturforsch. **64c**, 754–758 (2009); received May 11/June 17, 2009

Enolase detected on the cell surface may be a receptor for certain ligands, especially for plasminogen. It is important for the pathogen invasiveness and in the development of a tumour. Therefore, we sought to preliminarily determine the enolase location and catalytic activity in the subfractions of MCF-7 cells. The latter was done on intact cells and in subfractions of MCF-7 cells. We identified enolase by immunoblotting. The binding of human plasminogen to enolase was performed by immunoblotting using monoclonal antibodies against plasminogen. The intact MCF-7 cells demonstrated activity of enolase. Enolase in postnuclear and perinuclear fractions is catalyticly active too. We identified the enolase protein in immunoblots of these fractions, except for the nuclear subfraction. These results provide evidence that enolase is present on the intact surface of MCF-7 cells and in post- and perinuclear fractions. The surface protein maintained catalytic activity, which suggests that its location in the plasma membrane didn't change the active centre of the enzyme.

Key words: α-Enolase, MCF-7 Breast Cancer Cells, Plasminogen

Introduction

The glycolytic enzyme enolase (EC 4.2.1.11) catalyzes the reversible dehydration of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) and is composed of two subunits with a molecular weight of 47 kDa each. For stabilizing the catalytic centre two magnesium ions are required. As far as humans and mammals are concerned, there are three independent genetic loci, α, β and γ, that encode three types of subunits for homo- or heterodimeric enzymes (Pancholi, 2001). Enolases from several mammalian species have been purified and their gene structures were determined (Tanaka *et al*., 1998). Despite the differences found in species, α-enolase is highly conserved. There is above 90% homology among the α-enolases of mammals (Pancholi, 2001). Enolase exists as a multienzyme complex with other glycolytic enzymes, such as pyruvate kinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate mutase, in the cytoplasm (Liliom *et al*., 1999).

Recent studies have provided interesting and important findings about several noncatalytic functions of enolase in various types of mammalian cells. These new functions include: enolase as a myc-binding protein (MBP-1), as a heat-shock protein, as a plasminogen (Plg)-binding receptor, as a component of an eye lens, and as a hypoxic stress protein (Seweryn *et al*., 2007). Its multiple activities may have distinct subcellular locations and control mechanisms. For these reasons, we have begun to examine the interrelationship between the subcellular distribution and activities of enolase and its possible interaction with plasminogen in the MCF-7 cell line.

Material and Methods

Cell culture

The MCF-7 (human breast carcinoma cells) cell line was cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The medium was replaced every 72 h and the cells were trypsinized for passaging when confluent. Cells analysis was performed when cultures were visibly confluent.

Cell fractionation

Cells were washed twice in cold PBS and then resuspended in hypotonic buffer containing 10 mM

imidazole, 1.5 mM KCl, 2 mM $MgSO_4$, 1 mM PMSF (added immediately before use), and 10 μg/ml aprotinin, at pH 6.8, followed by 10 min of incubation. After that, cells were sonicated (UP 200 s sonicator, Dr. Hilscher GmbH, Berlin, Germany) for 30 s at 60 W and 4 ºC. The subcellular fractions were obtained according to the method of Mazzola and Sirover (2003). The suspension was centrifuged at 380 × *g* for 10 min at 4 ºC. The supernatant was recovered and used as the postnuclear fraction. The pellet was resuspended in hypotonic buffer and centrifuged at 380 × *g* at 4 ºC. The supernatant was removed and termed the perinuclear fraction. The pellet was resuspended in hypotonic buffer and comprised nuclear fraction.

Determination of enolase activity

The reaction mixture for surface enolase activity determination was composed of 10 mM $MgSO_4$, 10 mM KCl, and 1 mM 2-PGA in 50 mM imidazole buffer, pH 6.8. The level of enolase activity was monitored at 25 ºC as an increase of ΔA_{240}/min adequate to the formation of PEP from 2-PGA under these conditions. The specific activity of enolase from cell subfractions was measured in 2 ml of test sample containing 50 mM imidazole buffer, pH 6.8, 1 mM 2-PGA as a substrate, 3 mM $MgSO_4$, and 0.4 M KCl. The reaction sustained for 1 min at 25 ºC after addition of the enzyme. One unit of enolase activity is defined as the amount (in U) of protein, which catalyzes the synthesis of 1 μmol of PEP for 1 min under these conditions.

The enolase concentration (in mg/ml) was determined spectrophotometrically at 280 nm using the absorption coefficient $A^{01\%} = 0.89$ determined for 1 mg of rabbit muscle enolase per ml of solution.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Each fraction (150 μg) was resolved by electrophoresis according to Laemmli (1970) using 10% acrylamide gels, an electrode buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS for 45 min at 200 V. SDS-PAGE was performed using a mini gel apparatus (Biometra, Goettingen, Germany). Electrophoresis was conducted at 150 V and 4 ºC. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R 250 in 10% acetic acid and 40% methanol and destained using a solution of 5% methanol and 7.5% acetic acid in water. Whole fractions were transferred to an immobilon P membrane (Millipore, Bedford, MA, USA) using transfer buffer (10 mM Tris, 150 mM glycine, 20% methanol) at 4 ºC for 1 h and 100 V. Transferred proteins were stained using 0.005% Ponceau S in 3% TCA. The membrane with transferred proteins was incubated at 37 ºC for 1 h with rabbit antihuman α-enolase antibodies diluted 1:1000 [polyclonal antibodies were produced in our laboratory according to Witkowska *et al.* (2005)] in 1% BSA in TBS-T [Tris-buffered saline-Tween-20 (20 mM Tris-HCl, pH 7.0, 50 mM NaCl, 0.05% Tween-20)]. The membrane was stepwise incubated at 37 ºC for 1 h with horseradish peroxidase-labeled goat antirabbit IgG (ICN Pharmaceutical, Inc., Costa Mesa, USA) diluted 1:500 in TBS-T. The immunoblots were developed with substrate for peroxidase 4-chloro-1-α-naphthol (Sigma, St. Louis, MO, USA) and 0.1% H_2O_2 in PBS.

Interaction with human plasminogen

The immobilon P membrane with transferred fractions (and purified enolase as a control) was incubated with 40 μg of human plasminogen (R & D Systems, Minneapolis, USA) in PBS for 2 h at 37 ºC and the whole night at 4 ºC. The immunoreactivity of human α-enolase with human Plg was determined using monoclonal rabbit antihuman Plg antibodies (Santa Cruz Biotechnology, Inc., USA) diluted 1:200 in TBS-T with 1% BSA for 2 h at 37 ºC. The secondary antibodies were horseradish peroxidase-labeled goat antirabbit IgG diluted 1:500 in TBS-T. The immunoblots were developed with substrate for peroxidase 4-chloro-1-α-naphthol and 0.1% H_2O_2 in PBS.

Statistical analysis

Experiments were performed in triplicate. Activity data of surface enolase were analyzed by Student's t-test. A p value < 0.05 was considered statistically significant.

Results and Discussion

The well-known glycolytic enzyme is overexpressed in some human tumour cells (Altenberg and Greulich, 2004; Altenberg *et al.*, 2006). The results presented in this study indicated that α-enolase exists not only in the cytoplasm of

MCF-7 cells. We verified that surface enolase of intact MCF-7 cells maintains catalytic activity, because it has the ability to form PEP from the glycolytic substrate 2-PGA under nonlysis conditions. These results suggest, that surface exposition of a molecule did not disturb the catalytic centre. Similar observations were reported by Witkowska *et al*. (2005) for a enolase-like protein of the surface bacterial cells of *Klebsiella pneumonia*. This phenomenon was observed in experiments with intact cell lines of rat sarcoma and normal cardiomyocytes (Seweryn *et al*., 2008).

The enolase specific activity in cell subfractions obtained from MCF-7 cells was measured. The active enzyme was detected only in the postnuclear and perinuclear fractions. The postnuclear subfraction of MCF-7 cells demonstrated higher specific activity (54.6 mU/mg) compared to the perinuclear one (10.9 mU/mg). This agrees with a main function of enolase in the cell cytoplasm. Perinuclear α-enolase may be involved in several nonglycolytic functions. For example, α-enolase binds to F-actin and tubulin and has been localized in the centrosome in HeLa cells (Walsh *et al*., 1989; Johnstone *et al.*, 1992). We did not observe α-enolase in the nuclear fraction of MCF-7 cells; nevertheless that was recognized to be a negative transcriptional regulator of *c-myc* and has been identified as a C-terminal fragment of α-enolase in a cell line cultivated at high level of glucose (Sedoris *et al*., 2007). Probably, nuclear C-terminal analogues of α-enolase in MCF-7 cells do not have epitopes recognized by polyclonal antibodies used in our experiments. Reports of Feo *et al*. (2000) demonstrated that α-enolase mRNA gives rise to an alternative translation product, which negatively regulates *c-myc* transcription by binding to the P_2 promoter. In contrast to native α-enolase, MBP-1 is preferentially localized in the cell nucleus. α-Enolase constitutes a clear example of proteins for which alternative functions have been identified, also named as "moonlighting proteins" (Malorni *et al*., 2006). Surface enolase is recognized as receptor of some ligands, especially for plasminogen. Although the mechanisms of α-enolase expression in the cell membrane are not clear, its role as a receptor of plasminogen in myogenesis, metastasis, pathogen invasiveness and activation of host defence system cells in the inflammatory state was shown (Pancholi, 2001). It may play an important role in the initiation of disease processes by modulating the pericellular and intravascular fibrinolytic system (Liu and Shih, 2007).

To determine whether or not α-enolase was present in the fractionated MCF-7 cells, immu-

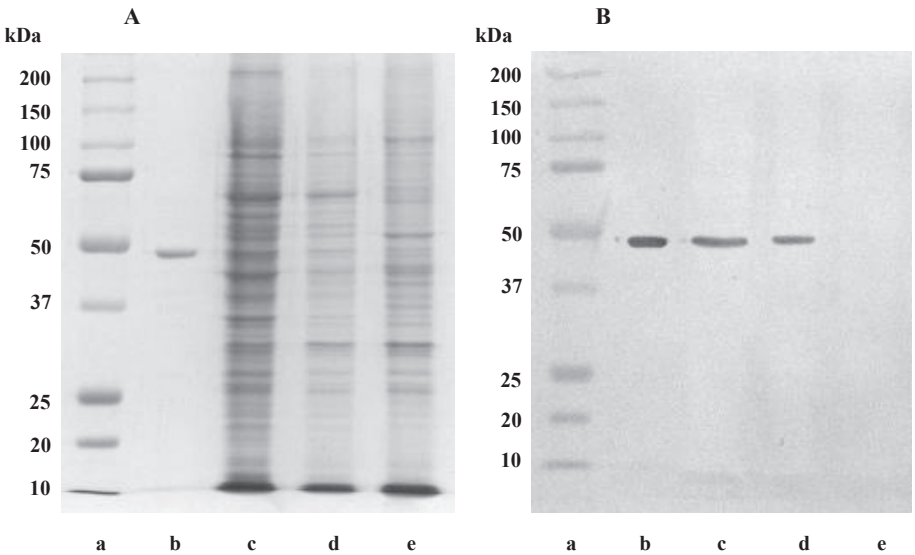

Fig. 1. (A) The 10% SDS-PAGE analysis of each fraction and (B) immunoblotting. Lane a, the molecular mass protein standards; lane b, the human α-enolase; lane c, the postnuclear fraction; lane d, the perinuclear fraction; lane e, the nuclear fraction.

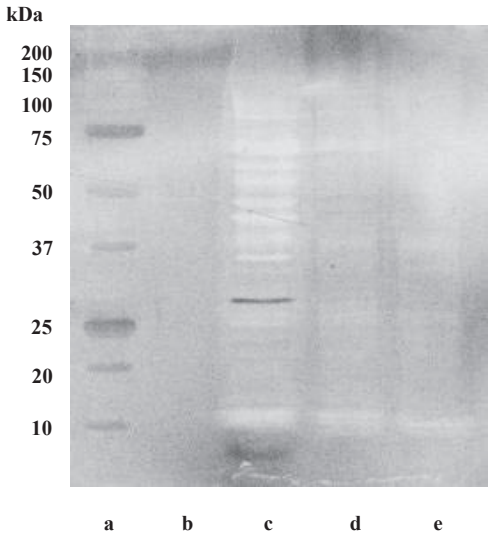

Fig. 2. The immunoblotting of each fraction with human plasminogen antibodies. Lane a, the molecular mass protein standards; lane b, the human α-enolase; lane c, the postnuclear fraction; lane d, the perinuclear fraction; lane e, the nuclear fraction.

noblotting with polyclonal rabbit antihuman α-enolase antibodies was performed. Subcellular expression of α-enolase is shown in Fig. 1. A single 47-kDa band was detected in the postnuclear and perinuclear fraction corresponding to the enolase monomer. As expected, the significant enolase protein was identified in the postnuclear fraction in accordance with its known glycolytic function (Fig. 1, lane c). Further, α-enolase was not detected in the nucleus (Fig. 1, lane e). We also determined the enolase in the perinuclear fraction (Fig. 1, lane d).

For detection of the α-enolase-Plg interaction, we used human unlabeled Plg. There was no reaction with monoclonal antihuman plasminogen antibodies in the Western blotting experiment which could indicate lack of binding of Plg with α-enolase from subfractions and with purified human α-enolase (Fig. 2).

In our experiments we demonstrated the presence of α-enolase in perinuclear and postnuclear subfractions, but Western blotting method was not succesfull for the detection of Plg binding to α-enolase in MCF-7 subfraction. The lack of any interaction between α-enolase and plasminogen does not prove that there is no such enzyme on the cell surface, because the intact cells show enzyme activity of enolase. Eukaryotic α-enolases may bind Plg by presenting two C-terminal lysine residues (binding site 1 or BS1) (Redlitz *et al.*, 1995). Recently, however, an additional Plg binding site (binding site 2 or BS2) on pneumococcal α-enolase has been identified (Bergmann *et al.*, 2003). The BS2 regions contain positively charged lysine and arginine residues and negatively charged glutamate and/or aspartate residues near the centre of the surface loop, flanked by hydrophobic residues (Ehinger *et al.*, 2004). Only human, rat and parasitic *Pneumocystis carinii* α-enolases lack negatively charged residues within the region BS2 (Ehinger *et al.*, 2004). Therefore, it seems possible that the surroundings of BS2 is essential in the interaction with Plg. On the other hand, α-enolase from parasitic *Pneumocystis carinii* binds the human Plg in the study by Fox *et al.* (2001). However, interaction of Plg with human α-enolase is dependent on the C-terminal lysine residue (Andronicos *et al.*, 1997). Removal of the C-terminal lysine from the enzyme with carboxypeptidase B significantly reduces its Plg-binding capacity. Furthermore, it was also reported that the monoclonal antibody anti-α-enolase abrogates 85% cell-dependent Plg activation (Lopez-Alemany *et al.*, 2003).

Acknowledgements

This work was financially supported by Wroclaw Medical University (grant number 1602).

Altenberg B. and Greulich K. O. (2004), Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. Genomics **84**, 1014–1020.

Altenberg B., Gemuend C., and Greulich K. O. (2006), Ubiquitous cancer genes: multipurpose molecules for protein micro-arrays. Proteomics **6**, 67–71.

Andronicos N. M., Ranson M., Bognacki J., and Baker M. S. (1997), The human ENO1 gene product (recombinant human alpha-enolase) displays characteristics required for a plasminogen binding protein. Biochim. Biophys. Acta **1337**, 27–39.

Bergmann S., Wild D., Diekmann O., Frank R., Bracht D., Chatwal G. S., and Hammerschmidt S. (2003), Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. Mol. Microbiol. **49**, 411–423.

Ehinger S., Schubert W. D., Bergmann S., Hammerschmidt S., and Heinz D. (2004), Plasmin(ogen)-binding α-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. J. Mol. Biol. **343**, 997–1005.

Feo S., Arcuri D., Piddini E., Passantino R., and Giallongo A. (2000), ENO 1 gene product binds to the c-myc promoter and acts as a transcriptional repressor: Relationship with Myc promoter-binding protein 1 (MBP-1). FEBS Lett. **473**, 47–52.

Fox D. and Smulian A. G. (2001), Plasminogen-binding activity of enolase in the opportunistic pathogen *Pneumocystis carinii*. Med. Mycol. **39**, 495–507.

Johnstone S. A., Waisman D. M., and Rattner J. B. (1992), Enolase is present at the centrosome of HeLa cells. Exp. Cell Res. **202**, 458–463.

Laemmli K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685.

Liliom K., Wagner G., Kovacs J., Comin B., Cascante M., Orosz F., and Ovadi J. (1999), Combined enhancement of microtubule assembly and glucose metabolism in neuronal systems *in vitro*: decreased sensitivity to copper toxicity. Biochem. Biophys. Res. Commun. **264**, 605–610.

Liu K. J. and Shih N. Y. (2007), The role of enolase in tissue invasion and metastasis of pathogens and tumor cells. J. Cancer Mol. **3**, 45–48.

Lopez-Alemany R., Longstaff C., Hawley S., Mirshahi M., Fabregas P., Jardi M., Merton E., Miles L. A., and Felez J. (2003), Inhibition of cell surface mediated plasminogen activation by a monoclonal antibody against α-enolase. Am. J. Hematol. **72**, 234–242.

Malorni L., Cacace G., Cuccurullo M., Pocsfalvi G., Chambery A., Farina A., Di Marco A., Parente A., and Malorni A. (2006), Proteomic analysis of MCF-7 breast cancer cell line exposed to mitogenic concentration of 17β-estradiol. Proteomics **6**, 5973–5982.

Mazzola J. L. and Sirover M. A. (2003), Subcellular localization of human glyceraldehydes-3-phosphate dehydrogenase is independent of its glycolytic function. Biochim. Biophys. Acta **1622**, 50–56.

Pancholi V. (2001), Multifunctional α-enolase: its role in diseases. Cell. Mol. Life Sci. **58**, 902–920.

Redlitz A., Fowler B. J., Plow E. F., and Miles L. A. (1995), The role of an enolase-related molecule in plasmin(ogen)-binding to cells. Eur. J. Biochem. **227**, 407–415.

Sedoris K. C., Thomas S. D., and Miller D. M. (2007), c-Myc promoter binding protein regulates the cellular response to an altered glucose concentration. Biochemistry **46**, 8659–8668.

Seweryn E., Pietkiewicz J., Szamborska A., and Gamian A. (2007), Enolase on the surface of prokaryotic and eukaryotic cells is a receptor for human plasminogen. Postepy Hig. Med. Dosw. **61**, 672–682 (online).

Seweryn E., Bednarz-Misa I. S., Danielewicz R., Saczko J., Kulbacka J., Dawiskiba T., and Pietkiewicz J. (2008), Distribution of β-enolase in normal and tumor rat cells. Folia Histochem. Cytobiol. **46**, 519–524.

Tanaka M., Taniguchi T., Ohkubo T., and Nakashima K. (1998), c DNA cloning and characterization of neuron-specific enolase from chicken. Biochim. Biophys. Acta **1395**, 28–33.

Walsh J. K., Keith T. J., and Knull H. R. (1989), Glycolytic enzyme interactions with tubulin and microtubules. Biochim. Biophys. Acta **999**, 64–70.

Witkowska D., Pietkiewicz J., Szostko B., Danielewicz R., Maslowski L., and Gamian A. (2005), Antibodies against human muscle enolase recognize a 45-kDa bacterial cell wall outer membrane enolase-like protein. FEMS Immunol. Med. Microbiol. **45**, 53–62.