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Two-dimensional electrophoresis database of fluorescence-labeled proteins of colon cancer cells

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Abstract

We constructed a novel database of the proteome of DLD-1 colon cancer cells by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of fluorescence-labeled proteins followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) analysis. The database consists of 258 functionally categorized proteins corresponding to 314 protein spots. The majority of the proteins are oxidoreductases, cytoskeletal proteins and nucleic acid binding proteins. Phosphatase treatment showed that 28% of the protein spots on the gel are phosphorylated, and mass spectrometric analysis identified 21 of them. Proteins of DLD-1 cells and of laser-microdissected colon cancer tissues showed similar distribution on 2D gels, suggesting the utility of our database for clinical proteomics.
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Keywords: Proteome; Two-dimensional gel electrophoresis; 2D database; 2D-DIGE; colon cancer

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is an established method of protein separation [1,2], and has been used in many types of biological studies to monitor protein expression in a global manner [3,4]. However, analysis by 2D-PAGE is hampered by low reproducibility due to electrophoretic artifacts and by the limited dynamic range of spot intensity. Recently, novel fluorescent dyes have been developed and used for fluorescence two-dimensional difference gel electrophoresis (2D-DIGE) (Amersham Biosciences, Buckinghamshire, UK) with the aim of solving the problems inherent to 2D-PAGE. In 2D-DIGE, multiple samples are differentially labeled with different fluorescent dyes, mixed together, and co-separated in the same gel. Multiple 2D images are obtained by scanning the gel with appropriate wavelengths specific for each fluorescent dye [5–8]. Because samples are run concomitantly in the same gel, artifactual differences produced by electrophoresis can be distinguished from biological alterations, and spot matching can be performed in a less labor-intensive manner. 2D-DIGE also allows more accurate quantitative analysis of protein expression, since, by their nature, fluorescent dyes have a broader and more quantitative dynamic range than do colorimetric-based staining systems. In addition, the replacement of labor-intensive staining procedures by a simple laser scan enables high-throughput spot detection. With these advantages, 2D-DIGE has been applied to the study of breast cancer [9], esophageal cancer [10], and colon cancer [11]. However, global identification of the proteins corresponding to protein spots has been performed only on 2D images visualized by colorimetric methods, including

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 2D-DIGE, fluorescence two-dimensional difference gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CIAP, calf intestine alkaline phosphatase; IPG, immobilized pH gradient; DHB, dihydroxybenzoic acid; DTT, dithiothreitol; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone

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silver staining [12], SYPRO Ruby [13] and Coomassie Brilliant Blue [14]. Because 2D databases have been created using only these images, the potential of 2D-DIGE for differential expression analysis has not been fully explored.

In this study, we studied the 2D image of fluorescence-labeled proteins. We identified by mass spectrometry (MS) 258 proteins corresponding to 314 fluorescence-labeled protein spots and functionally grouped the identified proteins according to the Panther Category classification in the Celera Discovery System. We discuss the characteristics of the proteins observed by 2D-DIGE, and the possible application of this technology for cancer research.

2. Materials and methods

2.1. Materials

1-(5-Carboxypentyl)-1′-propylindocarbocyanine halide *N*-hydroxy-succinimidyl ester (Cy3 fluorescent dye), 1-(5-carboxypentyl)-1′-methylindodi-carbocyanine halide *N*-hydroxysuccinimidyl ester (Cy5 fluorescent dye), and Decyder software Version 4.0 were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK).

2.2. Cell culture, clinical material, and protein extraction

The human colon cancer cell line DLD-1 was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in a humidified CO_2 incubator with RPMI-1640 medium supplemented with 10% FBS. DLD-1 cell protein samples were prepared as follows. After washing the cells with PBS and incubating them with 10% trichloroacetic acid (TCA) for 30 min on ice, they were scraped off and collected by brief centrifugation. The cell pellet was then suspended with lysis buffer (6 M urea, 2 M thiourea, 3% CHAPS and 1% Triton X-100) for 30 min on ice. The protein sample was centrifuged at 15,000 rpm for 30 min at 4 °C, and the supernatant was recovered.

Colon cancer tissue was obtained from a surgical specimen from a patient with colon cancer at Tokyo Medical University after obtaining the patient's informed consent. Laser microdissection was performed as described previously [15]. Briefly, the surgically resected colon cancer tissue was snap-frozen in liquid nitrogen immediately after resection and embedded in optimal cutting temperature (O.C.T.) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). The O.C.T. embedded tissue blocks were cut into 10 μm thick sections with a Leica cryostat CM 3050 S (Leica, Milton Keynes, UK). The sectioned tissues were placed on a membrane-coated slide glass (Leica) pretreated with tissue adhesive solution, 0.1% poly-L-lysine (Sigma, St. Louis, MO). The sectioned tissues were fixed with 95% ethanol for 30 s [DD1] and washed in water. After being soaked in Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan) for 1 min, they were washed twice in 95% ethanol and once in water, each for 10 s. The neighboring section was occasionally stained with standard hematoxyline and eosine to confirm the diagnosis. All staining procedures were carried out on ice. Under microscopic observation, colon cancer cells were isolated with the Leica Microdissection System (Leica). The isolated tumor cells were treated with lysis buffer as described above.

Protein concentration was measured with a Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA) and adjusted to 1 mg/ml with lysis buffer. The pH of the samples was adjusted to 8.5 with 30 mM Tris–HCl, a 50 μg protein sample was labeled with Cy3 or Cy5 fluorescent dye for 30 min, and the labeling reaction was terminated by adding 0.2 mM lysine for 10 min. The labeled sample was then incubated for 15 min with an equal volume of lysis buffer containing 130 mM DTT and 2.0% ampholine (Amersham Biosciences), and the total volume of the sample was adjusted to 420 μl with lysis buffer containing 65 mM DTT and 1.0% ampholine. All procedures after the start of the labeling reaction were carried out on ice in the dark.

2.3. 2D-PAGE

2D-PAGE was performed as described previously [1]. Briefly, IPG strip gels (24 cm long, p*I* range 3.0 and 10.0; Amersham Biosciences) were allowed to rehydrate with a labeled protein sample for 12 h at 20 °C. Isoelectric focusing was carried out with an IPGphor (Amersham Biosciences) for a total of 80 kVh at 20 °C. The IPG gel was equilibrated with equilibration buffer containing 3 M urea, 50 mM Tris–HCl (pH 8.8), 30% glycerol, 1.0% SDS, and 16 mM DTT for 15 min with gentle agitation. Another incubation was then carried out with the equilibration buffer in which DTT was replaced by 122 mM iodoacetamide. A 9–15% gradient polyacrylamide gel between low-fluorescence glass plates measuring 200 mm × 240 mm × 1 mm was used in the second separation. The equilibrated IPG gel was placed on the top of the second-dimension gel with embedding agarose (Amersham Biosciences), and the second-dimension electrophoresis was performed at 20 °C for 15 h at 18 W per gel with the EttanDalt II system (Amersham Biosciences). For preparative purposes, we applied 550 μg of protein sample. Although several hundreds of protein samples were required for preparative purpose, it is very costly to label whole proteins with flup
rescent dyes. Because labeled and unlabeled proteins had almost identical electrophoretic mobility and 50 μg protein was enough for spot detection, we mixed 500 μg of unlabeled protein sample with 50 μg of labeled protein and separated them together in the same gel for preparative purpose. The second-dimension separation was performed as described above, except that the concentration of DTT was increased to 162 mM for the first equilibration, and iodoacetamide was replaced by 0.05% acrylamide for the second equilibration. The second-dimension gel was bonded to the inner plate

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