Phosphorylation of Ornithine Decarboxylase at Both Serine and Threonine Residues in the ODC-overproducing, Abelson Virus-transformed RAW264 Cell Line

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Abstract

Expression of ornithine decarboxylase (ODC), the initial enzyme in polyamine biosynthesis, is essential for cell growth. The Abelson virus-transformed, murine macrophage-derivative RAW264 cell line overexpresses ODC activity and enzyme protein at a level 100-1000-fold greater than in normal cells. Expression of ODC was completely dependent on extracellular stimulants and followed a temporally discrete pattern similar to that in normal cells. ODC was present in RAW264 cells as two major and one minor isoelectric forms. Analysis of ODC isolated from [32P]orthophosphate metabolically radiolabeled cells demonstrated that the basic isoelectric enzyme form was unphosphorylated, the two more acidic forms were phosphorylated, and both phosphoserine and phosphothreonine residues were present in the phosphorylated ODC. Therefore, in the RAW264 cell line, ODC is overexpressed and phosphorylated at multiple sites on the enzyme molecule.

Introduction

ODC (EC 4.1.1.17) is the initial, inducible enzyme in the polyamine biosynthetic pathway. The polynomials, small aliphatic nitrogenous bases, fulfill structural and regulatory roles in protein and nucleic acid biosynthesis and function (1). ODC has been shown to be phosphorylated in situ by immunoprecipitation of radiolabeled enzyme from [32P]orthophosphate metabolically radiolabeled cells. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The work was supported by Grant DK44331 from the NIH.

Materials and Methods

Cell Culture and ODC Purification. RAW264 cells (10) were maintained in culture in Dulbecco's modified Eagle's medium ( Gibco, Grand Island, NY) supplemented with 0.11 g/l sodium pyruvate, 3.7 g/l sodium bicarbonate, and 0.584 g/l l-glutamine. Cells were plated at 1 x 10^6 cells/ml of serum-free medium, incubated overnight, and stimulated by the addition of 10% FBS, 1 mM cyclic AMP, 1 mM IBMX, and 1 μg/ml LPS. Cellular ODC activity was assayed as described (11). ODC was purified to homogeneity by sequential DEAE-Sepharose CL-6B (Pharmacia/LKB, Uppsala, Sweden) and pyridoxamine 5-phosphate affinity chromatography (12).

Electrophoresis and Immunoblotting. SDS-PAGE was performed using 8% polyacrylamide gels, and two-dimensional gel electrophoresis was performed as described (13). Gels were stained with silver and visualized by autoradiography or transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) or to Immobion-P (Millipore, Bedford, MA). Experiments conducted with iodinated, purified ODC documented a uniform transfer of 85-90% of the enzyme protein from the gel to the membranes. For immunoblotting, the nitrocellulose was incubated for 2 h at room temperature in TBS (150 mM NaCl-50 mM Tris-HCl, pH 7.5) containing 5% bovine serum albumin and then for 16 h at 4°C with a 1:1000 dilution of rabbit anti-ODC antiseraum (14), washed with TBS, and then incubated with a 1:1000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) for 2 h at room temperature. The bound antibodies were visualized by incubation with 4-chloro-l-naphthol and 0.015% H2O2 in 20 mM Tris-HCl, pH 7.5, containing 0.5 mM NaCl. SDS-PAGE and immunoblotting of 0.5 to 20 ng of pure ODC protein generated a linear increase in laser densitometric-detected color. The amount of ODC protein present in whole cell extracts was quantitated by linear regression analysis of the density of the bands detected as compared to pure ODC standards. Apparent molecular weight values for proteins were determined by interpolation on a plot of log M versus the mobilities of methyl-3H-labeled standard proteins: phosphorylase B (M, 97,000), bovine serum albumin (M, 69,000), ovalbumin (M, 46,000), and carbonic anhydrase (M, 30,000; Amerham, Arlington Heights, IL).

Metabolic Radiolabeling of Cells and Immunoprecipitation. Thirty min prior to harvesting stimulated cells, the medium was replaced with 1 ml of 32P]orthophosphate-free supplemented Dulbecco's modified Eagle's medium containing 3 mM carrier-free [32P]orthophosphate (ICN, Irvine, CA). Cells were harvested into buffer A [1 mM dithiothreitol, 0.5% (v/v) Brij 35, 25 mM NaF, 1 mM EDTA, 1 mg/ml bovine serum albumin, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5]. Cell lysates were centrifuged at 10,000 X g for 5 min, and the supernatants were incubated with a 1:500 dilution of rabbit anti-ODC antiseraum for 1 h at 4°C. Pansorbitin (Calbiochem, LaJolla, CA) was added at a dilution of 20 μl/ml and incubated for 20 min at 4°C; the immune complexes were recovered by centrifugation at 10,000 X g for 5 min. Immunoprecipitates were washed three times at 4°C by resuspension in buffer A containing 2 mM NaCl and 5% (v/v) Brij 35 and centrifuged at 10,000 X g for 5 min; then they were washed three additional times in buffer A.

Analysis of Radiolabeled Amino Acids. ODC immunoprecipitated from RAW264 cells was separated by SDS-PAGE. Proteins were electrophoretically transferred to an Immobilon-P membrane, which was immunoblotted to localize ODC as referenced to samples of pure

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3 The abbreviations used are: ODC, ornithine decarboxylase; FBS, fetal bovine serum; IBMX, isobutylmethylxanthine; LPS, lipopolysaccharide; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.
ODC. The radiolabeled ODC band was excised, placed in 6 N HCl, and heated for 2 or 18 h at 110°C. One mg/ml of phosphoserine, phosphothreonine, and phosphotyrosine were added to the hydrolysates before lyophilization. Lyophilized samples were resuspended in 5 μl of 5.0% acetic acid-7.5% formic acid (pH 1.9) and spotted onto a thin-layer chromatography plate which was run in the same buffer at 1000 V for 1 h. The thin-layer chromatography plates were air dried, wetted with buffer containing 5.0% acetic acid-0.5% pyridine (pH 3.5), and run at 1100 V for 45 min. Plates were dried at 60°C for 10 min, sprayed with 0.25% ninhydrin/acetone, and baked at 60°C for 10 min to detect the phosphoamino acid standards and scanned using a Betascope to detect the radiolabeled phosphoamino acids.

Results and Discussion

Regulation of Overexpression of ODC in the RAW264 Cell Line. The RAW264 cell line was derived from murine macrophages by transformation with the Abelson virus (10). Each of the cell stimulants used, FBS, cAMP plus IBMX, or LPS, induced an increase in RAW264 cell ODC activity when added alone. When all of the stimulants were combined, a synergistic induction of ODC occurred. When these combined stimulants were added to serum-starved RAW264 cells, the cell line overexpressed ODC activity in a regulated manner, as shown in Fig. 1A. In serum-starved cells and after 1 h of stimulation, no ODC activity was detected. After 2 h, enzyme activity was detectable and continued to rise to a peak level of expression from 3–5 h. The enzyme activity then declined to 20% of the maximal level by 8 h. This temporal pattern of stimulated ODC activity expression is typical of that seen in normal tissues and cell lines. However, the peak activity of ODC achieved at 3 h, 4 nmol/min/mg cell protein, was 100–1000-fold greater than that achieved in stimulated normal tissues (1).

To determine whether the overexpression of ODC in RAW264 cells was the result of an overexpression of enzyme protein, whole cell extracts were harvested at hourly intervals after stimulation, separated by SDS-PAGE, and immunoblotted. As shown in Fig. 1B, the polyclonal anti-ODC antisera was monospecific; no protein was detected at zero-time in serum-starved cells, and only one protein was detected in stimulated cells which migrated coincident with authentic, purified ODC at M₆, 52,000. After cell stimulation, ODC protein was first detected at 2 h, reached a peak from 3 to 5 h, and then progressively declined in parallel to the changes in enzyme activity. At 3 h, the RAW264 cell ODC enzyme protein was 200 ng/mg cell protein, a level 100–1000-fold greater than that present in stimulated normal tissues in which ODC constitutes 1–10 ppm of the cellular protein (1). The ODC induced in the RAW264 cells at 3 h had a calculated specific activity of 20 μm/min/mg enzyme protein, consistent with the specific activity of ODC purified from these cells.

Multisite Phosphorylation of ODC in the RAW264 Cell Line. RAW264 cells were metabolically radiolabeled in the presence of [³²P]orthophosphate for 30 min prior to harvesting at different times after stimulation. Cell extracts were incubated with anti-ODC antiserum, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1C, no immunoprecipitable, radiolabeled ODC was present in RAW264 cells at zero-time when no enzyme activity was detectable. At 3 h, a radiolabeled band was detected which migrated coincident with ODC at M₆, 52,000. The amount of radiolabeled ODC remained elevated to 5 h and then declined. The incorporation of radiolabeled phosphate into ODC occurred throughout the time of ODC expression.

ODC was purified from stimulated RAW264 cells. The purified enzyme was separated by two-dimensional gel electrophoresis and detected by silver staining. As shown in Fig. 2A, the purified enzyme preparation contained two major and one minor isoelectric forms. To determine if all the isoelectric forms of ODC were reactive with the anti-ODC antiserum, a companion gel was electrophoretically transferred to nitrocellulose and immunoblotted, as shown in Fig. 2B. The anti-ODC antiserum reacted with all three isoelectric forms of the enzyme present in the purified preparation. To determine whether all of the isoelectric forms of ODC contained phosphate, RAW264 cells, which had been stimulated for 4 h, were metabolically radiolabeled for 30 min in the presence of [³²P]orthophosphate and immunoprecipitated and analyzed by two-dimensional electrophoresis. Fig. 2C, an immunoblot of that gel, shows that the three isoelectric forms of ODC that were present in the purified enzyme preparation were also present in and immunoprecipitable from RAW264 cell extracts. Fig. 2D is the autoradiograph of the blot shown in Fig. 2C, and the inset, 2E, is an alignment of the sections of the blot and autoradiograph where ODC was detected. No radioactivity was associated with the major basic isoelectric form of ODC, whereas the major and minor acidic isoelectric forms of the enzyme were radioactive. Multiple isoelectric forms of ODC have been observed previously by two dimensional electrophoresis, protein staining of enzyme preparations purified from kidney (15) and epidermal papillomas (16), high-resolution slab gel isoelectric focusing analysis, and immunoblotting of stimulated RAW264 whole cell extracts (17). This is the first direct demonstration that ODC can be present intracellularly in both unphosphorylated and phosphorylated states.
SERINE AND THREONINE PHOSPHORYLATION OF ODC

To determine whether the in situ incorporation of $^{32}$P into ODC was the result of phosphorylation of amino acids, cells were stimulated, metabolically radiolabeled with $^{32}$Porthophosphate, and immunoprecipitated with antibodies specific for ODC. The proteins were then subjected to acid hydrolysis, and the radiolabeled amino acids were separated by two-dimensional high voltage electrophoresis. Fig. 3A shows the separation achieved between the ninhydrin-stained internal standards of phosphoserine, phosphothreonine, and phosphotyrosine. Fig. 3B shows the Beta-Scan image of the radiolabeled phosphoamino acids present after 2 h of acid hydrolysis of the ODC. Phosphoserine was the major radiolabeled amino acid detected, and phosphothreonine was present but in lower amounts. Fig. 3C shows the Beta-Scan image of a similar plate on which the radiolabeled ODC phosphoamino acids were analyzed after 18 h of acid hydrolysis, a time optimal for the detection of phosphothreonine. Only radiolabeled phosphothreonine was detected, and it was present in a greater amount than after 2 h of acid hydrolysis. These results are consistent with the fact that the stability to acid hydrolysis of both the peptide bond and the phosphodiester bond is significantly greater for phosphothreonine versus phosphoserine.

Cell lines which overexpress ODC have been derived by a variety of strategies, the most common being mutagenesis followed by selection based on the presence of inhibitors of ODC activity. In these cases, elevated levels of ODC are often constitutively expressed, and growth factor addition stimulates a 5–10-fold increase in enzyme activity. In contrast, the RAW264 cell line was derived by oncogene transformation. This cell line overexpresses ODC at levels comparable to the cell lines described above, but enzyme expression is tightly regulated in that it is temporally discrete in a manner similar to normal cell lines and tissues. ODC is therefore an inducible enzyme that is absolutely dependent upon the induction of extracellular growth factors. Expression of an active c-Myc chimeric protein has been shown to induce ODC mRNA expression and elevate ODC activity in BALB/c 3T3 and Rat 1A fibroblasts; however, the induced ODC activity is not overproduced but increases only within the range of normal tissue expression. ODC expression in this system is also solely dependent on the activation of the c-Myc chimeric protein and does not require activation by extracellular regulatory stimulants, as does the overexpression of ODC in RAW264 cells.

ODC exists within the RAW264 cell in an unphosphorylated form and at least 2 phosphorylated forms. This report demonstrates for the first time that ODC can be phosphorylated on both serine and threonine residues in situ. It has recently been reported that ODC is phosphorylated intracellularly exclusively on serine residues by casein kinase II. There are several potential explanations for the different results reported here: (a) Abelson virus-transformed, murine macrophage-derived RAW264 cells were used in the present report, whereas murine myeloma 653-1 and EXOD-1 cells were used in the previous studies. Different cell lines and tissues may inherently display unique phosphorylation patterns of ODC; (b) cells may acquire unique ODC phosphorylation patterns after transformation with different oncogenes; (c) in the previous reports, the cells were grown in the presence of FBS alone, whereas in this report, cells were also stimulated with cAMP, IBMX, and LPS. These stimulants may lead to activation of phosphorylation pathways for which ODC is a substrate which are not activated by FBS; and (d) in the previous reports, acid hydrolysis of metabolically radiolabeled ODC was conducted for just a single time, 2 h. Thus, the selection of only a single, relatively short hydrolysis time could obscure the detection of radiolabeled phosphothreonine.

Fig. 3. Two-dimensional high voltage electrophoretic analysis of RAW264 cell ODC radiolabeled amino acids. ODC was immunoprecipitated from stimulated RAW264 cells metabolically radiolabeled by incubation in the presence of $^{32}$Porthophosphate. Amino acid analysis was accomplished as described in “Materials and Methods”. A, ninhydrin-stained amino acid standards. ODC-radiolabeled phosphoamino acids after 2 h (B) or 18 h (C) of acid hydrolysis.
Since casein kinase II phosphorylates only serine residues on purified RAW264 cell ODC in vitro, as also reported for bacterial expression vector pET-8C-expressed ODC (4), it can be inferred from the present results that a different protein kinase must be phosphorylating the ODC threonine residue(s) in intact RAW264 cells. The phosphorylation of ODC throughout the entire time of enzyme expression studied indicates that the protein kinases responsible belong to signal transduction pathways activated throughout the time of cell proliferation, such as the cyclin regulated cdc-2 family of protein kinases (20). Current investigation is directed towards identifying the protein kinases which phosphorylate ODC and elucidating the functional significance of the multisite in situ phosphorylation of the enzyme.

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