Biochemical and Biophysical Research Communications 413 (2011) 270–276

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Protein tyrosine nitration in the cell cycle

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ARTICLE INFO

Article history:
Received 16 August 2011
Available online 27 August 2011

Keywords:
Tyrosine nitration
Cell cycle
Two-dimensional gel electrophoresis
Proteomics

ABSTRACT

Nitration of tyrosine residues in proteins is associated with cell response to oxidative/nitrosative stress. Tyrosine nitration is relatively low abundant post-translational modification that may affect protein functions. Little is known about the extent of protein tyrosine nitration in cells during progression through the cell cycle. Here we report identification of proteins enriched for tyrosine nitration in cells synchronized in G0/G1, S or G2/M phases of the cell cycle. We identified 27 proteins in cells synchronized in G0/G1 phase, 37 proteins in S phase synchronized cells, and 12 proteins related to G2/M phase. Nineteen of the identified proteins were previously described as regulators of cell proliferation. Thus, our data indicate which tyrosine nitrated proteins may affect regulation of the cell cycle.

1. Introduction

Protein tyrosine nitration is a low abundant post-translational modification. It is a potential marker of oxidative/nitrosative stress [1,2], and associated with many physiological and pathological processes such as neurodegenerative diseases, cancer and inflammatory diseases [2]. Protein tyrosine nitration results mainly from the in vivo peroxynitrite pathway, and also from metalloperoxidase reaction pathways [3]. The main source of nitration is nitric oxide (NO). The interaction between NO and superoxide leads to the formation of peroxynitrite (ONOO_), a reactive molecule with strong nitrating activity [4]. The nitration (addition of a $-NO_2$ group) of a tyrosine residue in a protein decreases the electron density of the phenolic ring of tyrosine [1,2], affects the chemical properties of the tyrosine residue, and may interfere with the phosphorylation of this tyrosine residue. Therefore, tyrosine nitration can regulate a protein's function. Nitration involves the redox signaling system, occurs under physiological conditions, is affected under pathological conditions, and can be reversed by enzymatic or nonenzymatic mechanisms [5].

The cell cycle is controlled by many mechanisms, including the cyclins, CDKs and CDK inhibitors, a number of signaling processes, and Redox and Reactive nitrogen species (RNS) (nitric oxide (NO) and peroxynitrite (ONOO_)) [6]. Kong et al. [7] have shown in a cell-free system that tyrosine nitration of cdc2 fragment completely inhibits the ability of the fragment to become phosphorylated by a lymphocyte-specific tyrosine kinase (LCK). Since phosphorylation of cdc2 is required for cell cycle progression through mitosis, nitration of cdc2 in vivo should cause an accumulation of cells with 4n DNA copy number. Redox-dependent regulation of cyclin D1 has been proposed as the primary target of oxidants upon induction of the cell growth [8]. Several groups have reported S-phase arrest upon exposure to ·NO-generating compounds and other ·NO-derived reactive species (RNS), occasionally with coincident checkpoint activation [9]. The impacts of Redox/RNS during cell cycle progression are not well-understood, as the extent of tyrosine nitration in the cell cycle has not been explored. In this study, we report identification of 27 nitrated proteins in human breast epithelial cells in G0/G1 phase, 37 proteins in cells in S phase, and 12 nitroproteins in cells in G2/M phase.

2. Materials and methods

2.1. Cell culture, treatment, and synchronization

MCF-7 human breast carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

MCF-7 cells were synchronized in G0/G1 phase by starving cultured cells for 48 h in DMEM medium supplemented with 0.1% FBS. Synchronization of the MCF-7 cells in the S phase was obtained by hydroxyurea block [10]. In brief, culturing medium was replaced with 10% FBS-supplemented medium containing 2 mM hydroxyurea (H8627, Sigma–Aldrich, Steinheim, Germany) for 16 h. The medium was removed and the cells were washed three times with

Abbreviations: NO, nitric oxide; ONOO_, peroxynitrite; NT, nitrotyrosine; 2DE, two dimensional gel electrophoresis; IEF, isoelectrofocusing; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; ILK, integrin-linked kinase; FASTKD2, FAST kinase domains 2; ESR2, estrogen receptor 2.

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doi:10.1016/j.bbrc.2011.08.084

PBS solution and incubated in 10% FBS-supplemented DMEM for 13 h. Subsequently, hydroxyurea was added from a 500 mM stock solution to yield a final concentration of 2 mM hydroxyurea, and the cells were incubated for another 13 h. Replacement of the medium with one without hydroxyurea permitted the cells to progress along the cell cycle in a synchronized fashion. Cell synchronization in G2/M phase were obtained by incubating exponentially growing cells in complete DMEM medium containing 0.4 μg/ml nocodazole (M1404, Sigma–Aldrich, Steinheim, Germany) for 16 h. Release from nocodazole was obtained by removing the medium, washing with PBS solution and incubating the cells with 10% FBS-supplemented medium.

2.2. Flow cytometry

Flow cytometry was used to monitor distribution of synchronized cells in the cell cycle phases. Cells were collected by trypsinization and fixed for at least 30 min in 70% ethanol at −20 °C. Then, ethanol was removed, and cells were stained with staining solution (PBS supplemented with 0.1% (v/v) sodium citrate, pH 7.4, 0.2 mg/ml RNase A, 20 μg/ml propidium iodide (PI)). Cell cycle was analyzed in a FACscan flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.3. Immunoprecipitation (IP), Coomassie brilliant blue R250 staining and immunoblotting

Protein concentration was adjusted to 3 mg/ml and the solution was subjected to IP with 40 μl of agarose-bound monoclonal anti-3-nitrotyrosine (NT) antibody (Cat# 389549, Cayman Chemicals, Ann Arbor, MI, USA) overnight at 4 °C. The protein-bound beads were collected by centrifugation at 12,000 rpm for 1 min and washed thoroughly (3 times) with the lysis buffer. The bounded proteins were eluted with 10 mM nitrityrosine (Cat# 89540, Cayman Chemicals, Ann Arbor, MI, USA) two times, followed by another two time elution with a buffer containing 100 mM DTT, 1% SDS, 50 mM Tris/HCl, pH 6.8 for 5 min at 95 °C.

Collected protein fractions were equally divided into two parts (one for immunoblotting, and one for Coomassie blue staining), and resolved on SDS polyacrylamide gels, with nitrated tyrosine containing proteins as positive control (Cat# 12-354, Upstate, Lake Placid, NY, USA). The proteins on the gels were transferred onto Hybond P membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked with 5% (v/v) BSA and then incubated with a primary antibody as recommended by the manufacturer, and followed by an HRP-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden). The proteins were visualized using western blotting luminol reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The following primary antibodies were used: anti-nitrotyrosine antibodies (clone 1A6, Upstate, Lake Placid, NY, USA), anti-ILK antibody (06-1113, Millipore, Bedford, MA, USA), anti-FASTKD2 antibody (ab70127, Abcam, Cambridge, UK) and anti-ESR2 antibody (ab16813, Abcam, Cambridge, UK). For Coomassie brilliant blue staining, gels were fixed in 50% methanol and 10% acetic acid for 1 h. Then gels were stained for 30 min in a solution of 0.15% Coomassie brilliant blue R-250 (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 20% methanol, 10% acetic acid and water. The gels were destained in a mixture of 50% methanol, 10% acetic acid and water. Quantization of protein band intensity was performed by using ImageJ tool (http://rsbweb.nih.gov/ij/).

2.4. Two-dimensional gel electrophoresis (2DE) and gel image analysis

Proteins eluted from anti-3NT antibody conjugated beads were dissolved and diluted in 2DE buffer (8 M urea, 4% CHAPS, 0.5% DTT, IPG buffer, pH 3–10). Eluted proteins were subjected to isoelectrofocusing (IEF) using 18-cm linear IPG dry strips with a pH range of 3–10 (GE Healthcare, Uppsala, Sweden). IEF was performed in an IPGphor (GE Healthcare, Uppsala, Sweden) using the following protocol: rehydration, 10 h; 50 V, 3 h; 90 1000 V, 1 h; 8000 V, 10 h. After IEF, strips were equilibrated in 50 mM Tris–HCl, pH 8.8, 6 M urea, 2.0% SDS, 30% glycerol with 1% DTT for 10 min, and then for 10 min in the same buffer containing 4% iodoacetamide instead of DTT. Equilibrated strips were placed on top of 10% polyacrylamide gels and were fixed with 0.5% agarose in 62.5 mM Tris–HCl, pH 6.8, 0.1% SDS. SDS–PAGE was performed in a DALTsix following the manufacturer's recommendations (constant power 50 W, for 6–8 h; GE Healthcare, Uppsala, Sweden). Gels were fixed in 10% acetic acid and 20% methanols for 10–12 h. Proteins were detected by silver staining. We generated three 2DE gels for each condition.

Silver-stained gels were scanned in an ImageScanner with the MagicScan32 software, and analyzed with calculation of volumes of spots by the ImageMaster 2-D Platinum software (GE Healthcare, Uppsala, Sweden).

2.5. Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Protein spots were excised from 2DE gels, destained and subjected to in-gel digestion with trypsin (modified sequence-grade porcine; Promega, Madison, WI, USA). Tryptic peptides were concentrated and desalted on a microC18 ZipTip (Millipore Billerica, MA, USA). Peptides were eluted with 60% acetonitrile containing α-cyano-4-hydroxycinnamic acid as the matrix, applied directly onto the metal target and analyzed on M@LDI-R TOF mass spectrometer (Micromass-Waters, Manchester, UK). Peptide spectra were internally calibrated using trypsin autolytic peptides (842.51, 1045.56, 2211.10). To identify proteins, we performed a search of the NCBInr database by using the ProFound search engine (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). One miscut and partial oxidation of methionine were allowed. Tolerance of searches was set on less than 0.1 Da. The probability of identification was evaluated according to the probability, *Z* values, number of peptides used for identification and sequence coverage. Experimental and theoretical pI and Mr value of proteins were also considered.

2.6. Systemic analysis

Systemic analysis of obtained data was performed using GoMiner (http://discover.nci.nih.gov/gominer/index.jsp) and Funcoup (http://funcoup.sbc.su.se/). GoMiner allows classification of identified proteins into biologically coherent categories and assesses these categories. FunCoup is a statistical framework of data integration for finding functional coupling between proteins.

3. Results and discussion

3.1. Enrichment for nitrotyrosine-containing proteins

To enrich for tyrosine nitrated proteins, we used immunoprecipitation with anti-3-NT antibodies. We optimized binding, washes, and elution of nitrated proteins from antibody beads. Enriched proteins were then analyzed using 1DE, followed by Coomassie brilliant blue staining or immunoblotting with antibodies specific for nitrotyrosine (Fig. 1A and B). Coomassie brilliant blue-stained gels showed that the protein fraction eluted with buffer containing 1% SDS has high abundance of proteins (Fig. 1A). Our trials with elution with nitrotyrosine showed that significant quantities of nitrated proteins were not released from the beads