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A multidimensional approach to an in-depth proteomics analysis of transcriptional regulators in neuroblastoma cells

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HIGHLIGHTS

- An optimised neuroproteomic method for the analysis of transcriptional regulators.
- This method detected more than 1800 nuclear proteins, which constitutes one of the largest datasets reported for a neuronal cell.
- This method will allow in-depth analysis of transcriptional regulators for the study of neurological diseases.

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ABSTRACT

The dynamic regulation of transcriptional events is fundamental to many aspects of neuronal cell functions. However, proteomics methods have not been routinely used in global neuroproteomics analyses of transcriptional regulators because they are much less abundant than the "house-keeping" proteins in cells and tissues. Recent improvements in both biochemical preparations of nuclear proteins and detection sensitivities of proteomics technologies have made the global analysis of nuclear transcriptional regulators possible. We report here an optimised neuroproteomic method for the analysis of transcriptional regulators in the nuclear extracts of SHSY-5Y neuroblastoma cells by combining an improved nuclear protein extraction procedure with multidimensional peptide separation approaches. We found that rigorous removal of cytoplasmic proteins and solubilisation of DNA-associated proteins improved the number of nuclear proteins identified. Furthermore, we discovered that multidimensional peptide separations by either strong cation exchange (SCX) chromatography or electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) analysis detected more than 1800 nuclear proteins, which constitutes one of the largest datasets of nuclear proteins reported for a neuronal cell. Thus, in-depth analysis of transcriptional regulators for studying neurological diseases are increasingly feasible.

1. Introduction

Proteomics approaches have been successfully used for the large-scale analysis of protein expression patterns, post-translational modifications and protein–protein interactions (Cahill, 2001; Pandey and Mann, 2000). The rapid evolution of quantitative proteomics technologies has enabled routine analysis of global proteomic changes among diverse tissues and cells (Gauss et al., 1999; Shevchenko et al., 1996; Yan et al., 2001). More recently, specialised proteomic studies such as those on neuroproteomics have become increasingly useful for understanding the dynamic regulatory protein networks that underlie neuronal development and neurological diseases (Lin et al., 2009; Liu et al., 2006; Tyler et al., 2011). In addition, neuroproteomics has branched into more in-depth studies of the sub-proteomes, including synaptoproteomics and neural plasma membrane proteomics (Zhang, 2010). However, compared with other high-throughput tools for the system-wide analyses of genes and proteins, the sensitivities of proteomics technologies for the characterisation of less abundant signalling molecules and transcriptional regulators have remained low for routine biochemical studies.

The eukaryotic nucleus is an important organelle for regulating gene expression and other diverse functions (Trinkle-Mulcahy

Abbreviations: 2DE, 2D gel electrophoresis; ACN, acetonitrile; ATN1, atrophin-1; C/EBP, CCAAT enhancer binding protein; CID, collision induced dissociation; DMEM, Dulbecco's modified eagle medium; DRPLA, dentatorubral-pallidoluysian atrophy; ERLIC, electrostatic repulsion-hydrophilic interaction chromatographic; FDR, false discovery rate; HD, Huntington's disease; ICAT, isotope-coded affinity tag; IPA, ingenuity pathway analysis; SCX, strong cation exchange; TEAB, triethylammonium bicarbonate; TF, transcription factor.

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and Lamond, 2008). Within the nuclear proteomes, many cellular signals, including signals for the stress response, growth and differentiation, ultimately target specific gene promoters to induce alterations in gene expression or DNA replication. The ability to comprehensively identify and quantify transcriptional regulators is important for understanding their functions under different physiological and diseased conditions. Unfortunately, transcription factors are often underrepresented in global proteomic studies due to their relatively low abundance in comparison with the "house-keeping" proteins, such as metabolic enzymes, cytoskeletal proteins and heat shock proteins. To address this limitation, subcellular fractionation approaches for organelle-specific proteomic analyses have been attempted for more sensitive examinations of low-abundance proteins (Andersen et al., 2002; Boisvert et al., 2010; Dreger et al., 2001; Trinkle-Mulcahy and Lamond, 2008). For example, several groups have investigated the nuclear or chromatin proteomes, using a variety of biochemical approaches for the enrichment of nuclear proteins from diverse cell lines and primary cells (reviewed by Albrethsen et al., 2009). In one study, a 2D gel electrophoresis (2DE) reference map of total nuclear proteins isolated from human liver was established (Jung et al., 2000); however, both heat shock proteins and cytoskeletal proteins were still abundantly represented. Additional subnuclear fractionation can further improve the depth of the proteome coverage. For example, the nuclear proteome of human HeLa cells was extensively analysed by Andersen et al., which resulted in the identification of 271 nucleolar proteins (Andersen et al., 2002). Similarly, Tchapyjnikov et al. (2010) used a nanospray LC/MS/MS-based approach to analyse cell nuclei extracted with a commercially available nuclear extraction kit and identified 154 transcription factors and many other transcriptional co-regulators, kinases and phosphatases. Shakib et al. (2005) analysed the nuclear proteins from NRK49F rat kidney fibroblasts after prolonged hypoxia by 2DE. Among the 791 proteins identified, 17 transcription factors or cofactors were found to be possibly regulated by hypoxia. In addition to 2DE, LC-based shotgun proteomics methods have also been used effectively for the analysis of nuclear proteomes. Shiio and Eisenman used the isotope-coded affinity tag (ICAT) approach to identify Myc-induced changes in the nuclear proteome (Shiio et al., 2003). After chromatin enrichment, they applied ICAT in combination with LC/MS/MS and identified 282 proteins, including 64 known nuclear proteins. Among the 18 transcription factors identified, ATF-3 reduction and NIFK induction were found to be Myc-modulated. Recently, several advanced mass spectrometry-based studies have made notable progress in characterising human chromatin. Garcia's group used three different chromatin extraction methods and identified over 1900 proteins, 40% of which were classified as nuclear proteins by independent bioinformatics analyses (Torrente et al., 2011). Overall, it appears that a balance must be reached between nuclear protein specificity and the depths of the nuclear proteome coverage.

Nuclear proteomics analyses of neuronal cells have not been widely reported, in part because of the difficulties associated with the unusual morphologies and processes of cells of the central and peripheral nervous systems. In this study, we have developed a comprehensive approach for the characterisation of the nuclear proteome from a SHSY-5Y neuroblastoma cell line. We found that, by both rigorous removal of cytoplasmic proteins and extensive extraction of chromatin-associated proteins, we could dramatically improve the nuclear proteome coverage in this cell line. Furthermore, by adopting multidimensional chromatographic approaches, including ERLIC and SCX fractionations, to further expand the nuclear proteome coverage, we could achieve one of the most in-depth identifications of transcription regulators in SHSY-5Y cells.

2. Materials and methods

2.1. Materials

HPLC-grade solvents and water were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Triethylammonium bicarbonate (TEAB), protease inhibitors cocktail and phosphatase inhibitors cocktail were purchased from Sigma (St. Louis, MO). Sequencing-grade modified trypsin was purchased from Promega Corp. (Madison, WI). PepClean C_{18} spin columns were purchased from Pierce (Rockford, IL). Western blot reagents were obtained from BioRad (Redmond, WA). The antibody against actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the antibody against histone H1 (Clone AE-4) was purchased from Millipore (Billerica, MA).

2.2. Cell lines and cell culture

Human neuroblastoma cell line SHSY-5Y was obtained from ATCC (Manassas, VA). Cells were propagated as monolayers in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and F12 medium supplemented with 0.1 mmol/L nonessential amino acids, 1% penicillin/streptomycin and 10% foetal bovine serum at 37 °C in 5% CO_2. Exponentially growing and nearly confluent (90%) cells were harvested after several passages and were washed twice with PBS.

2.3. Nuclear protein extraction and analysis

2.3.1. Basic extraction method (Method 1)

Nuclear extracts were prepared from the SHSY-5Y cells using a cell lysis and salt extraction procedure described by Dignam et al. (1983). Briefly, the PBS-washed cell pellets were gently resuspended in a hypotonic lysis buffer comprising 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, protease inhibitors and phosphatase inhibitors. After incubation of the re-suspended cells on ice for 15 min, 0.5% NP-40 was added, and the extracts were vigorously vortexed for 10 s to disrupt the cell membranes. The cellular extracts were then centrifuged at 800 × *g* for 10 min at 4 °C to separate the cytoplasmic components (supernatants) from the nuclei-enriched fractions (pellets). The cytoplasmic fractions (supernatants) were stored at −80 °C until subsequent analyses. The nuclear pellets were resuspended in a hypertonic buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% (v/v) glycerol, 0.5 mM DTT, 0.2 mM EDTA and cocktails of protease and phosphatase inhibitors). Nuclear proteins were extracted via vigorous agitation for 15 min on ice. The solutions were further sonicated 3 times at 10 s intervals on ice. The resulting solutions were centrifuged at 16,000 × *g* and 4 °C for 15 min. The supernatants containing solubilised nuclear proteins were stored at −80 °C until further analyses. Protein extracts were further concentrated by the addition of 5 volumes of ethanol for precipitation. After centrifugation at 16,000 × *g* for 15 min, the protein pellets were re-suspended in 500 μL of a buffer containing 8 M urea, 50 mM TEAB (pH 8.0) and protease and phosphatase inhibitor cocktails. Protein concentrations were determined using the Bradford assay according to the manufacturer's instructions (Bio-Rad). The proteins were reduced with 10 mM DTT at RT for 1 h and alkylated with 50 mM iodoacetamide for 30 min in the dark. To maintain, trypsin digestion efficiency, the urea concentration was diluted to 1 M with the addition of 50 mM TEAB. For in-solution proteolytic digestion, trypsin was added into the protein solutions at a ratio of 1:25 (trypsin/protein by weight), and the solutions were incubated overnight at 37 °C. The resulting tryptic peptides were desalted using C_{18} spin columns (Pierce) and stored at −80 °C until LC/MS/MS analysis.