Journal of Chromatography A, 1218 (2011) 7003–7015

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Transcription factor proteomics: Identification by a novel gel mobility shift-three-dimensional electrophoresis method coupled with southwestern blot and high-performance liquid chromatography–electrospray-mass spectrometry analysis

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ARTICLE INFO

Article history:
Received 31 May 2011
Received in revised form 5 August 2011
Accepted 9 August 2011
Available online 16 August 2011

Keywords:
Electrophoresis
Transcription factor
Proteomics
AP1
C/EBP

ABSTRACT

Transcription factor (TF) purification and identification is an important step in elucidating gene regulatory mechanisms. In this study, we present two new electrophoretic mobility shift assay (EMSA)-based multi-dimensional electrophoresis approaches to isolate and characterize TFs, using detection with either southwestern or western blotting and HPLC–nanoESI-MS/MS analysis for identification. These new techniques involve several major steps. First, EMSA is performed with agents that diminish non-specific DNA-binding and the DNA–protein complex is separated by native PAGE gel. The gel is then electro-transferred to PVDF membrane and visualized by autoradiography. Next, the DNA–protein complex, which has been transferred onto the blot, is extracted using a detergent-containing elution buffer. Following detergent removal, concentrated extract is separated by SDS-PAGE (EMSA-2DE), followed by in-gel trypsin digestion and HPLC–nanoESI-MS/MS analysis, or the concentrated extract is separated by two-dimensional gel electrophoresis (EMSA-3DE), followed by southwestern or western blot analysis to localize DNA binding proteins on blot which are further identified by on-blot trypsin digestion and HPLC–nanoESI-MS/MS analysis. Finally, the identified DNA binding proteins are further validated by EMSA-immunoblotting or EMSA antibody supershift assay. This approach is used to purify and identify GFP-C/EBP fusion protein from bacterial crude extract, as well as purifying AP1 and CEBP DNA binding proteins from a human embryonic kidney cell line (HEK293) nuclear extract. AP1 components, c-Jun, Jun-D, c-Fos, CREB, ATF1 and ATF2 were successfully identified from 1.5 mg of nuclear extract (equivalent to 3×10^7 HEK293 cells) with AP1 binding activity of 750 fmol. In conclusion, this new strategy of combining EMSA with additional dimensions of electrophoresis and using southwestern blotting for detection proves to be a valuable approach in the identification of transcriptional complexes by proteomic methods.

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1. Introduction

There is considerable interest in transcription factors (TFs) and their role in gene regulation. Gene expression is important to all fundamental biological processes and is regulated by sequence-specific DNA binding proteins. Prior to transcription initiation, a transcription complex forms at the promoter that contains TFs, DNA and co-regulators, of which the TFs are the core members. The individual transcription factors bind to discrete, specific DNA-sequences, called response elements, in the promoter region. The formed DNA–protein complex functions either to activate or repress the expression of a target gene by integrating signaling cues through protein–protein interaction and translating these cues into transcriptional regulation [1].

In humans, TFs compose the second largest group of proteins after the metabolic enzymes. They play a central role in many

Abbreviations: TF, transcription factor; EMSA, electrophoretic mobility shift assay; RE, response elements; TRE, TPA response element; CRE, cyclic AMP responsive elements; C/EBP, CCAAT enhancer binding protein; hnRNP, heterogeneous nuclear ribonucleoprotein; PVDF, polyvinylidene fluoride; NC, nitrocellulose membrane; SOT, systematically optimized oligonucleotide trapping; PT, promoter trapping; IEF, isoelectric focusing; 2-DE, two-dimensional gel electrophoresis; 2D-SW, two-dimensional southwestern blot; WB, western blotting; EMSA-3DE, EMSA followed by IEF and SDS-PAGE; EMSA-3DE-SW, EMSA-3DE using southwestern blot detection; HPLC–ESI-MS/MS, high performance liquid chromatography–electrospray ionization-tandem mass spectrometry; FT-ICR, Fourier transform ion cyclotron resonance mass spectrometer.

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doi:10.1016/j.chroma.2011.08.023

biological processes including cell cycle regulation [2], maintenance of the intracellular environment, cellular differentiation and development [3,4]. Abnormal TF activity leads to numerous diseases and developmental disorders [5,6]. Surprisingly little is known about TF proteins due to their difficulty of study. For example, how many transcription factors does the human genome encode? Based on annotations of DNA-binding domains of TF, the initial analyses of the human genome sequence estimated that there are 2000–3000 sequence-specific TFs [7]. The DBD database (http://dbd.mrc-lmb.cam.ac.uk/DBD/index.cgi?Home) predicts 1508 human loci as potential TFs in human [8]. According to the characteristic DNA-binding domains, TFs are classified into different families by InterPro, the C2H2 zinc-finger (675 TFs), homeodomain (257 TFs), helix–loop–helix (87 TFs) and basic leucine zipper (bZIP, about 50 TFs) account for over 80% of the total TF repertoires. Among the 20 most highly cited transcription factors (TFs) in PubMed, the transcription factors Jun and Fos, which are the most common components of activator protein 1 (AP1) complex, rank third and fifth, respectively, of studies performed in humans and all other organisms [9]. c-Jun and c-Fos function to regulate various cellular behaviors, from the cell cycle, proliferation and development, the stress response and apoptosis. AP1 represents diverse homodimeric or heterodimeric combinations of members of the Jun family (JUN, JUNB and JUND), Fos (FOS, FOSB, FRA1 and FRA2), the closely related activating transcription factor (ATF and CREB) subfamily, the Maf subfamily and other bZIP TFs [10]. The dimeric combinations of AP1 mainly depends on the cell or tissue-specific expression patterns of individual proteins and their post-translational modifications in response to extracellular stimulation. The individual proteins dimerize with other partners and bind DNA via the bZIP domain [11]. For example, Jun proteins can form stable homodimers or form heterodimers with Fos that bind to the TPA response element (TRE, 5′-TGAC/GTCA-3′) based on their ability to mediate transcriptional induction in response to the phorbol ester TPA. Conversely, Jun and ATF proteins form heterodimers that preferentially bind the cyclic AMP responsive elements (CRE, 5′-TGACGTCA-3′) [12,13]. The different AP1 dimers bind to DNA with different affinities, resulting in different transactivation activity, protein stability and localization, and ultimately influencing the transcriptional repertoire of these proteins [14]. So identifying the protein composition of TF complexes and their post-translational modification becomes important to elucidate gene regulatory mechanism.

Currently, less than 5% of TFs have been experimentally characterized [15]. TF purification is the very first step for not only biochemical and structure study, but also for TF functional investigation in gene regulation. TF purification and characterization is complicated by the large numbers of TFs and their relatively low abundance in human cells, which is always less than 0.1% of the total nuclear protein [16]. Assuming 1 pmol of pure TF (50 ng for a 50 kDa. protein) is required for successful MS identification, it is estimated that 2×10^9–10^{11} cells are needed for a TF (10^3–10^5 molecules per cell) purification, supposing the overall yield will be 50%. So purification of TFs often requires large amounts of starting material. Successful characterization requires sensitive analytical methods such as mass spectrometry (MS). The significant increase in mass accuracy and resolving power now allows for the identification of low abundant proteins as their spectral peaks become increasingly distinguishable from background noise in complex mixtures, especially when the MS is equipped with advanced mass analyzer such as the Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR) or the Orbitrap [1].

Since TFs have an affinity several orders of magnitude greater for their consensus binding site (response element) than for nonspecific DNA, DNA affinity chromatography was developed to isolate sequence-specific-binding TFs. In this technique, double stranded oligonucleotides containing a consensus binding site for a TF is either absorbed or linked covalently to a chromatographic support, and TFs are purified by exploiting the inherent capacity of the TF to bind to DNA. Single-step DNA affinity chromatography results in high amounts of nonspecific co-purifying proteins that interfere or mask the identification of low-abundant TF [9]. Currently, TF purification often involves four or five different chromatographic steps that may include ion exchange, gel filtration, nonspecific and sequence-specific DNA affinity columns [15]. In some studies, the consensus site-mutated DNA was used to pre-clean prior to specific DNA affinity chromatography [17]. Recently, Mann et al. combined one-step DNA affinity chromatography with stable isotope labeling with amino acid in cell culture (SILAC) quantitative proteomics to identify specific TFs that bind to the methyl-CpG site in a promoter. This strategy eliminated a large excess of copurifying background proteins by calculating the peptide isotope ratios between wild type and the methyl-CpG bait, and only the significantly expressed proteins were taken as methyl-CpG binding candidates [18]. Despite the success of this approach, it is laborious, and since it requires homogeneous tissue cultured cells, is not feasible for all research. Our research group has been endeavoring to develop simple and rapid techniques to improve TF purification for over a decade, developing systematically optimized oligonucleotide trapping (SOT) [19], promoter trapping (PT) [20] and two-dimensional southwestern blot (2D-SW) [21] to purify and identify c-Jun promoter binding proteins [20], CCAAT enhancer binding protein (C/EBP) [19] and MafA in cell nuclear extract [22]. However, in oligonucleotide or promoter trapping, the single stranded $(GT)_5$ tail at 3′-end of double stranded oligonucleotide or promoter DNA causes unspecific binding of heterogeneous nuclear ribonucleoprotein (hnRNP) family members and several other abundant nuclear proteins. To avoid interference of the single stranded tail, in this study, we explored the electrophoretic mobility shift assay (EMSA)-based purification using DNA without a tail and combining this with additional dimensions of electrophoresis.

Developed in 1981, EMSA is extensively used to monitor the ability of a protein binding to a segment of DNA and to quantify DNA binding activity [23,24]. The DNA used in EMSA is typically a double-stranded oligonucleotide of 20–25 bp containing a response element (RE) and it can be radio-, fluoro- or hapten-labeled. An image of the gel reveals the positions of the free DNA and TF–DNA complex. The formed TF–DNA complex migrates more slowly than the free DNA when subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE). Previous studies have combined EMSA with proteomic techniques for TF identification. TFs were separated by two-dimensional gel electrophoresis (2-DE), which combines isoelectric focusing (IEF) in a first dimension with SDS-PAGE in a second dimension. Spots were cut from the gel for MS identification according to their p*I* and molecular mass and the proteins were also eluted and renatured for EMSA assay [16]. This method requires that the proteins be efficiently renatured from a denaturing (SDS-PAGE) gel for DNA binding activity analysis by EMSA. Only monomeric or homodimeric proteins binding with DNA can be characterized since heterodimeric proteins are dissociated during the denaturing gel separation. Another technique called 2D-EMSA [25] has also been developed. A TF is separated by non-denaturing PAGE in either the presence or absence of DNA. The protein bands from the two conditions are then applied to a second dimension of SDS-PAGE. The protein showing a DNA-dependent electrophoretic shift was extracted from the corresponding SDS-PAGE gel for MS identification. This approach successfully identified recombinant AtrA in a bacterial lysate [26], but for low abundance TFs in a complicated cell nuclear extract, this technique requires improvement. Our group has also applied bands cut from EMSA gels for western blotting analysis, which helped to verify the identity of a TF [27].

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