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Nuclear proteome analysis reveals a role of Vav1 in modulating RNA processing during maturation of tumoral promyelocytes

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ABSTRACT

Vav1 is a key molecule in the ATRA-induced acquisition of a mature phenotype by tumoral myeloid precursors. Since ATRA acts throughout events that require extensive changes of nuclear architecture and activity and considering that Vav1 accumulates inside the nuclear compartment of differentiating APL-derived cells, the possible role of this protein in modulating the nuclear proteome was investigated. Membrane-depleted nuclei purified from NB4 cells induced to differentiate with ATRA in the presence of forcedly down-modulated Vav1 were subjected to 2D-DIGE followed by mass spectra analysis. The obtained data demonstrated that, in NB4 cells treated with ATRA, Vav1 is involved in determining the nuclear amount of proteins involved in molecular complexes with DNA and may participate to RNA processing by carrying in the nucleus molecules involved in modulating mRNA production and stability, like hnRNPs and SR proteins. Our results provide the first evidence that, at least in maturation of tumoral myeloid precursors, Vav1 is part of interconnected networks of functionally related proteins ended to regulate different aspects of gene expression. Since defects in mRNA processing are common in tumor development, our data suggest that Vav1 is a potential target molecule for developing new anti-cancer strategies.

1. Introduction

Vav1, whose physiological expression is restricted to the hematopoietic system, is a critical signaling mediator downstream of several surface receptors which plays functional roles associated to agonist-induced activation of both lymphoid and myeloid cells during immune response [1–3]. In addition to promote the acquisition of a mature phenotype by normal hematopoietic cells [4], Vav1 is a crucial molecule in the completion of the differentiation program to neutrophils of cells derived from Acute Promyelocytic Leukemia (APL) induced by All-*trans* retinoic acid (ATRA) [5]. In particular, the down-modulation of Vav1 prevents, and the Vav1 over-expression potentiates, the effects of ATRA [5], that is known to activate a complex network of events including the degradation of the PML/RARα fusion protein, the activation of RARα-mediated gene transcription [6] and the regulation of posttranscriptional events, such as mRNA processing and export [7].

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In parallel with the best know function as a guanosine exchange factor (GEF) for small G proteins [8], other roles have been demonstrated for Vav1 [9]. Indeed, the presence inside Vav1 of diverse functional domains and of several tyrosine residues enables this protein to act as an adaptor/regulator molecule in both cytoplasm and nuclear compartment [8,10]. Inside the nucleus of lymphoid and myeloid cells, Vav1 seems to play its most intriguing role by regulating gene transcription as part of transcriptionally active complexes and facilitating NFAT and NFkB-like transcriptional activity [11–14].

Vav1 accumulates inside the nuclear compartment of APL-derived cells as a consequence of ATRA treatment [5,15], suggesting that it may be involved in the changes of nuclear architecture and activity required for the ATRA-dependent regulation of both transcription and posttranscriptional events. Consistent with a role of Vav1 in modulating the transcriptional machinery of differentiating tumoral promyelocytes is the decrease in the number of ATRA-induced genes that we have demonstrated to take place in HL-60 cells in which Vav1 was forcedly reduced during ATRA treatment [5]. A direct interaction of Vav1 with transcription factors known to drive the ATRA-induced maturation of myeloid cells was also demonstrated. In particular, our recent data show that, during the ATRA-induced differentiation of NB4 cells, both PU.1 and Vav1 are recruited *in vivo* to a DNA sequence known to drive the expression of the surface antigen CD11b [16]. We have also shown, by proteomic approaches on both HL-60 and NB4 cells, a more general role of Vav1 in modulating the expression level of the protein tool by means of which ATRA accomplishes the maturation program of tumoral promyelocytes [17,18].

Aim of this study was to establish whether Vav1 may have a role in modulating the nuclear protein tools by means of which ATRA completes the maturation program of tumoral promyelocytes. For this purpose, both proteomic and genomic technologies were used. Proteomic investigation was performed with the high-resolution 2D differential in gel electrophoresis system (2D-DIGE) and mass spectrometry of tryptic digests of nuclear proteins from NB4 cells in which the expression of Vav1 was forcedly down-modulated. To establish whether Vav1 has a role in regulating the levels of mRNA for the identified proteins, TaqMan® assay-based real-time PCR (RT-PCR) was performed. We have found that, during ATRA treatment of NB4 cells, Vav1 plays a role in determining the nuclear amount of several proteins mainly involved in the processing of RNA. The involvement of Vav1 in the ATRA-dependent regulation of mRNA levels for proteins variously involved in nuclear processes was also demonstrated, indicating that Vav1 contributes to modulate the complex nuclear network underlying protein expression during implementation of the differentiation program of tumoral promyelocytes.

2. Materials and methods

2.1. Cell culture and differentiation

All the used reagents were from Sigma Chemicals Co. (St. Louis, MO) if not otherwise indicated. The APL-derived NB4 cell line was obtained from the "German Collection of Microorganisms and Cell Cultures" (Braunschweig, Germany) and was cultured in RPMI 1640 (Gibco Laboratories, Grand Islands, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco Laboratories) in a 94/6% (v/v) air/CO_2 atmosphere. Cell density was maintained between 5×10^5/mL and 1.5×10^6/mL.

To induce neutrophil-like maturation, cells were treated with 1 μM ATRA for 4 days and the degree of granulocytic differentiation was evaluated by measuring the expression level of the CD11b myeloid surface antigen by direct staining with a PE-conjugated anti-CD11b-antibody (Immunotech, Coulter Company, Marseille, France), as previously reported [18]. After staining, samples were analyzed by flow cytometry (FACScan, Becton-Dickinson, San Jose´, CA) with Lysis II software (Becton-Dickinson). Data collected from 10000 cells are presented as mean fluorescence intensity values.

2.2. Down-modulation of Vav1 expression (RNA interference assays)

Exponentially growing NB4 cells were transfected with a mixture of Vav1 siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA) by using the electroporation procedure, as previously described [17]. For experiments in which mRNA levels for identified proteins were measured, a further Vav1 siRNAs mixture (#Vav1 siRNAs, Qiagen, Inc., Germantown, MD) was used. As a control of transfection efficiency, which resulted always higher than 60%, a non-silencing fluorescein-labeled duplex RNA (Qiagen) was used.

Following electroporation, cells were recovered in 600 μl of RPMI culture medium with 20% FBS and, after 5 h, centrifuged, suspended in RPMI plus 10% FBS at a density of 5×10^5 cells/mL and then treated with ATRA. After 96 h of treatment, transfected cells were subjected to evaluation of their differentiation level, to Western blot analysis and to 2D-DIGE.

2.3. Isolation of membrane-deprived nuclei and immunochemical analysis

Purification of nuclei deprived of nuclear membrane was performed essentially as previously reported [5]. The absence of the outer nuclear membrane as well as of cytoplasmic contaminations was assessed by ultrastructural analysis and marker enzyme assays, as previously reported [15].

The nuclear pellet was suspended in 10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 10 mM NaCl (TM5) and protease (0.5 mM PMSF, 1 μg/mL Aprotinin, 1 μg/mL Leupeptin) and phosphatase (1 mM Na_3VO_4) inhibitors, all from Calbiochem (La Jolla, CA), then subjected to Western blot analysis or to 2D electrophoresis.

For Western blot analysis, total lysates (50 μg protein) from cells (1×10^6) and membrane-depleted nuclei (10×10^6) were separated on 7.5% polyacrylamide denaturing gels and blotted to nitrocellulose membranes (GE Healthcare Life Science, Little Chalfont, United Kingdom).

For analysis of Vav1, membranes were saturated for 1 h in TBS containing 0.05% Tween-20 and 5% milk and incubated with a specific anti-Vav1 polyclonal antibody (Santa Cruz Biotechnology), as previously described [5].

For the analysis of β-tubulin, membranes were saturated and reacted with the specific monoclonal antibody, as previously reported [17].