Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 114–116

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Heat shock protein 90 interacts with vitamin D receptor in human leukemia cells☆

Ewa Marcinkowska*, Elżbieta Gocek

Faculty of Biotechnology, University of Wrocław, Tamka 2, 50-137 Wrocław, Poland

ARTICLE INFO

Article history:
Received 19 October 2009
Accepted 30 January 2010

Keywords:
1α,25-Dihydroxyvitamin D_3
Vitamin D receptor
Heat shock protein 90
Acute myeloid leukemia
HL60
THP-1
Co-immunoprecipitation
Nucleus

ABSTRACT

The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25D), has a broad range of effects which are mediated by nuclear vitamin D receptor (VDR). Many experiments that investigate the role of VDR can be done in human acute myeloid leukemia (AML) cells, since these cells are responsive to 1,25D and express VDR in a 1,25D-regulated manner. In this paper we show that in HL60 and in THP-1 cells VDR protein interacts with heat shock protein 90 (Hsp90) and that Hsp90 is important for differentiation of AML cells. Geldanamycin (GA), an Hsp90 inhibitor, is able to suppress 1,25-induced differentiation of HL60 cells.

1. Introduction

1α,25-Dihydroxyvitamin D_3 (1,25D) is a seco-steroid hormone which exerts broad biological activities by interaction with a vitamin D receptor (VDR) [1]. VDR belongs to the superfamily of steroid-thyroid-retinoid acid receptor proteins, which act as ligand-dependent transcription factors [2]. These receptors are divided into two major subgroups: class I receptors for estrogens, progesterone, androgens, glucocorticoids and mineralocorticoids, and class II receptors for 1,25D, retinoids, thyroid hormone and peroxisome proliferators. Class I receptors in order to be active must form homodimers, while class II receptors usually heterodimerize with retinoid X receptor (RXR) protein [2]. It is well documented that most class I receptors create multi-protein chaperone complexes containing heat shock proteins (Hsps) [3], but the roles played by these proteins are much more complicated than just help in folding [4]. For example, it has been shown that Hsp90 is required for folding, maturation, nuclear translocation and degradation of glucocorticoid receptor [5,6]. The situation is not so clear in case of class II receptors. An interaction of VDR with Hsp90 has not been documented yet, but it was shown that bacterially expressed VDR binds to DnaK and DnaJ, which are bacterial homologues of Hsps [7]. Moreover, a recent publication has presented that in human intestinal Caco-2 cells, Hsp90β is necessary for transcriptional activity of VDR [8].

HL60 cells (AML subtype M2) when exposed to 1,25D undergo a concentration- and time-dependent differentiation towards macrophage-like cells [9]. Expression of VDR protein in untreated HL60 cells is low, and it increases significantly within minutes from treatment with 1,25D, mostly in nuclear compartment of the cells [10]. Similar effect can be observed also in other acute myeloid leukemia (AML) cell lines, such as THP-1 (AML subtype M5) and in leukemia cells from AML patients [10,11]. This rapid increase of VDR expression is caused by its reduced degradation, when receptor is liganded with 1,25D. Therefore HL60 and other AML cells constitute an appropriate model for studies of possible interaction between VDR and Hsp90 proteins.

2. Materials and methods

2.1. Cell lines

HL60 and THP-1 cells were obtained from the European Collection of Cell Cultures. These cells were propagated as suspension cultures at standard cell culture conditions [11].

2.2. Chemicals and antibodies

1,25D was obtained from the Pharmaceutical Research Institute (Warsaw, Poland). Chemiluminescence Blotting Substrate

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

* Corresponding author. Tel.: +48 71 375 29 29; fax: +48 71 375 26 08.
E-mail address: ewa@protein.pl (E. Marcinkowska).

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doi:10.1016/j.jsbmb.2010.01.013

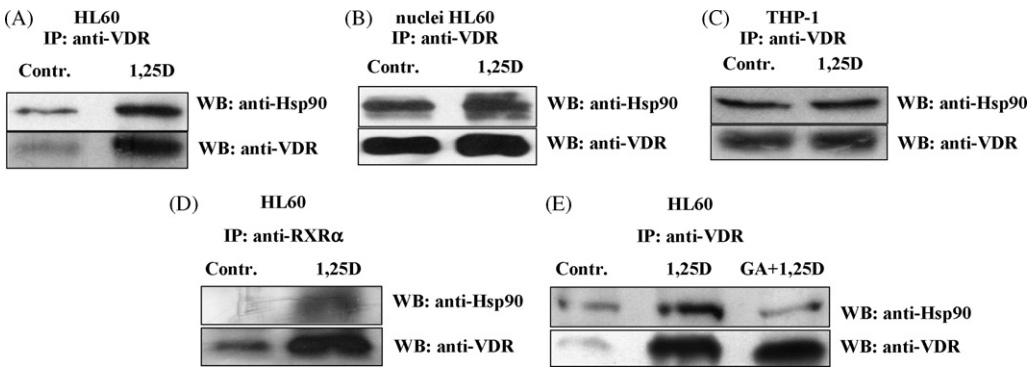

Fig. 1. Interaction of VDR with Hsp90 in acute myeloid leukemia cells. VDR interacts with Hsp90 in lysates from whole (A) and from nuclei (B) of HL60 cells, as well as in lysates from THP-1 cells (C) and this interaction is weakened by Hsp90 inhibitor (E). RXRα co-immunoprecipitates with VDR and Hsp90 in HL60 cells (D). The cells treated with 10 nM 1,25D for 3 h were lysed and either VDR or RXRα was immunoprecipitated from the lysates derived from equal numbers of the cells. In experiments with an Hsp90 inhibitor, 1 μM GA was added to the cells at 1 h before treatment with 10 nM 1,25D. All co-precipitated proteins were separated in SDS-PAGE and blotted to the membranes. The membranes were probed with anti-Hsp90, stripped and blotted again with anti-VDR.

was from Roche Diagnostics (Mannheim, Germany). Mouse monoclonal and rabbit polyclonal anti-VDR, mouse monoclonal anti-Hsp90α/β, anti-RXRα, anti-Lamin A/C antibodies and ExactaCruz™ IP/Western Blot Reagent were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Geldanamycin was from LC Laboratories (Woburn, MA). Other reagents were from Sigma (St. Louis, MO).

2.3. Immunoprecipitation and western blotting

After treatment the cells (1.5×10^7 cells/sample) were washed and lysed for 20 min on ice in 0.5 ml of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100; pH 7.5) containing protease and phosphatase inhibitors cocktails. Then the lysates were sonicated for 10 s and centrifuged. To prepare nuclear extracts, 2×10^7 cells were lysed without sonication, and then nuclei were separated from cytosol by centrifugation. Nuclei remaining in pellets were washed and suspended in 0.5 ml of lysis buffer containing protease and phosphatase inhibitors, sonicated for 10 s and centrifuged. The desired proteins were immunoprecipitated from supernatants overnight at 4 °C. Antibodies used for immunoprecipitation were immobilized using ExactaCruz precipitation Matrix according to manufacturer's instructions and washed three times with PBS before adding to cell lysates. After overnight precipitation ExactaCruz beads were washed in lysis buffer, suspended in SDS-PAGE sample buffer and boiled for 10 min. Immunoprecipitated proteins were separated in SDS-PAGE and transferred to PVDF membranes. The membranes were blotted with primary antibody, washed and then blotted for 1 h with ExactaCruz Detection Reagent (which does not recognize immunoglobulin bands). The protein bands were visualized with a chemiluminescence assay system. Then the membranes were stripped, dried again and probed with subsequent antibodies.

2.4. Determination of cell differentiation by flow cytometry

The cells were washed and incubated with PE-conjugated anti-CD14 (ImmunoTools GmbH; Friesoythe; Germany) antibody on ice for 45 min. The cell fluorescence was analyzed using FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The acquisition parameters were set for an isotype control. Data analysis was performed with use of WinMDI 2.8 software (freeware by Joseph Trotter). In order to analyze statistical significance of the results, Student's *t*-test for independent samples was used and *p*-value < 0.05 was considered to be a significant difference.

3. Results

3.1. VDR interacts with Hsp90 in HL60 and THP-1 cells

VDR protein is not abundantly expressed in AML cells, as compared to other cell components. However, upon treatment of AML cells with 1,25D, the amount of VDR protein increases significantly [11]. Moreover HL60 cells grow very fast and it is relatively easy to obtain a large number of these cells and this is why these cells were used in this study. In order to examine possible interactions between VDR and Hsp90, equal numbers of HL60 cells were treated for 3 h with either 10 nM 1,25D or with an equivalent amount of ethanol (solvent for 1,25D). The lysates were prepared from the cells and were used for immunoprecipitation using mouse monoclonal anti-VDR antibodies. All precipitated and co-precipitated proteins were separated in SDS-PAGE, transferred to PVDF membrane and then blotted with either mouse or rabbit anti-VDR and with mouse anti-Hsp90α/β antibodies. To exclude unspecific binding of the proteins negative control was performed: irrelevant mouse monoclonal antibody was used for separate immunoprecipitations (anti-Lamin A/C) and neither of the proteins of interest bound to this bait.

There appeared that in lysates from whole HL60 cells VDR immunoprecipitated using anti-VDR antibody interacted with Hsp90, which could be detected in western blots (Fig. 1A). These proteins interacted also in the cell nuclei, since when lysates containing only nuclear fractions of HL60 cells were used for immunoprecipitation using anti-VDR, Hsp90 could be detected in western blots as well (Fig. 1B). To confirm that interaction of VDR and Hsp90 is not limited to HL60 cell line, another AML cell line, THP-1 was used, and the results were similar to these obtained in HL60 cells (Fig. 1C). When VDR heterodimerization partner, RXRα, has been used as a bait in immunoprecipitation, there appeared that co-immunoprecipitated complexes contained both VDR and Hsp90 (Fig. 1D).

3.2. Interaction of VDR with Hsp90 and 1,25D-induced differentiation of HL60 cells are inhibited by geldanamycin

It has been shown recently that geldanamycin (GA), a chemical inhibitor of Hsp90 activity [12] can reduce 1,25D-induced transcriptional activity in Caco-2 cells [8]. In a next series of experiments HL60 cells were pretreated with 1 μM GA for 1 h before addition of 10 nM 1,25D for the next 3 h, and these cells were compared with untreated ones and with the cells treated with 10 nM 1,25D for 3 h alone. Fig. 1E shows that GA has partly abolished

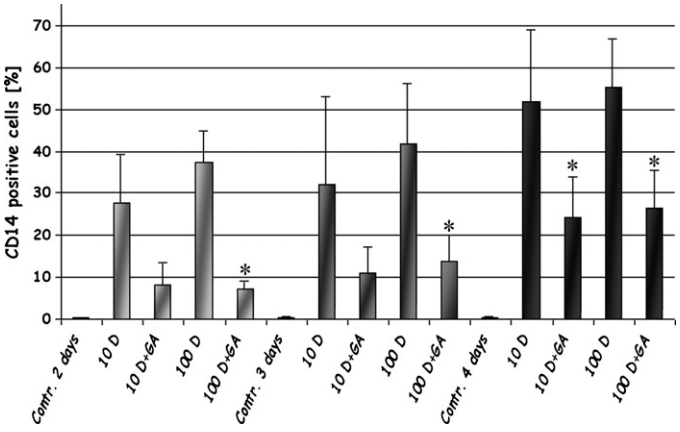

Fig. 2. Effect of GA on 1,25D-induced expression of CD14 in HL60 cells. The cells were treated with 10 nM 1,25D (10 D) or 100 nM 1,25D (100 D) ± 1 μM GA for 2 (light grey), 3 (medium grey) or 4 (dark grey) days and then the expression of CD14 cell surface marker was determined by flow cytometry. Mean values (±SEM) of percentage of positive cells are presented ($n = 5$). Results that differ significantly ($p \leq 0.05$) from results of respective 1,25D concentration are marked by asterisks.

the interaction between VDR and Hsp90 in 1,25D-treated HL60 cells.

It is well known and documented that 1,25D can induce differentiation of HL60 cells [9]. The extent of cell differentiation can be measured by many different parameters, but expression of CD14 cell surface marker seems to be the most accurate [10]. In order to evaluate whether inhibition of Hsp90 affects 1,25D-induced cell differentiation HL60 cells were treated simultaneously with 1 μM GA and either 10 nM or 100 nM 1,25D for 2, 3 or 4 days. Fig. 2 shows that in all these treatment procedures HL60 cells differentiation was inhibited by GA.

4. Discussion

It is widely accepted that Hsp90 proteins chaperone many class I nuclear receptors. Initially it was believed that Hsps are necessary to stabilize them in cytosol in the absence of their ligands, but further research revealed much more complicated role of these chaperones in regulation of the activities of nuclear receptors [4]. Interactions of class II nuclear receptors with Hsps have not been studied as extensively as that of class I receptors, but recently a report showing that Hsp90β is necessary for transcriptional activity of VDR has been published [8]. Data presented here indicate that in human AML cells VDR interacts with Hsp90, and that such interaction is not restricted to cytosolic VDR. Despite Hsp90 in HL60 cells is located predominantly in cytosol, its substantial portion resides in the nuclei (not shown) and interaction between VDR and Hsp90 occurs also in the cell nuclei. The data presented here do not predict if interaction of VDR and Hsp90 proteins is direct. One should consider a possibility that there is a third partner which interacts with both proteins studied and causes their co-immunoprecipitation. However, an inhibitory action of GA towards 1,25D-induced cell differentiation suggests that Hsp90 is an important mediator of VDR's activity.

Acknowledgments

The authors want to thank Igor Fijałkowski and Paweł Póda, Bachelorate Degree students, for their help in performing immunoprecipitation experiments. This work was supported by Polish Ministry of Science and Higher Education (Grant No. 2622/P01/2006/31).

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