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The *ASAP2* gene is a primary target of 1,25-dihydroxyvitamin D_3 in human monocytes and macrophages

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ABSTRACT

A genome-wide data set on vitamin D receptor (VDR) binding sites in human THP-1 cells (monocytes) led us to the genomic region around the *ASAP2* (Arf-GAP with SH3 domain, ankyrin repeat and PH domain 2) gene, whose product is involved in the regulation of vesicular transport, cellular migration and autophagy. Using ENCODE data, we demonstrated that the *ASAP2* gene is flanked by conserved binding sites of the insulating transcription factor CTCF. These sites define different chromosomal domains containing the *ASAP2* gene, up to six additional genes and three VDR binding sites. In human monocytes (THP-1 cells) the *ASAP2* gene is more weakly expressed but more and faster inducible by the biologically active form of vitamin D, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), than in M2-type macrophages (phorbol ester-differentiated THP-1 cells). Within the investigated genomic region, the basal mRNA expressions of the neighboring genes are comparably high in both monocytes and macrophages, but the *ASAP2* gene is the only primary $1,25(OH)_2D_3$ target. The three VDR binding sites located 54, 436 and 574 kb downstream of the *ASAP2* transcription start site each carry a sequence formed by a direct repeat with three intervening nucleotides (DR3). Ligand-inducible VDR binding was confirmed to all three genomic sites in monocytes and macrophages. Taken together, the region around the *ASAP2* gene is genome-wide highlighted as a special attraction point for the VDR, but the presently sole known functional consequence of the binding of VDR to three sites within this chromosomal region is that *ASAP2* is a primary $1,25(OH)_2D_3$ target gene in monocytes and macrophages.

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1. Introduction

The nuclear hormone $1,25(OH)_2D_3$ is the active compound of the vitamin D endocrine system [1]. It binds directly to the transcription factor vitamin D receptor (VDR) [2], which is a member of the nuclear receptor superfamily [3]. $1,25(OH)_2D_3$ achieves direct modulation of transcription by stimulating the specific association of VDR with its genomic binding sites, which via DNA looping get into contact with the transcriptional start sites (TSSs) of primary $1,25(OH)_2D_3$ target genes. According to the traditional understanding VDR binding sites are formed by a direct repeat of two hexameric binding motifs spaced by three nucleotides (DR3) [4,5]. Within the last three years the genome-wide binding of VDR has been determined by chromatin immunoprecipitation (ChIP) coupled with massive parallel sequencing (ChIP-seq) in human lymphoblastoids (treated for 36 h with $1,25(OH)_2D_3$ [6]), in human monocytes (THP-1, stimulated for 40 min [7]), in human colorectal cells (LS180, exposed for 180 min with ligand [8]) and in human hepatic stellate cells (LX2, incubated for 16 h with the $1,25(OH)_2D_3$ analog MC903 [9]). These four studies revealed 1600–6200 specific VDR binding sites, but only a low percentage of them are identical in all investigated cellular models [10]. Moreover, only approximately

Abbreviations: $1,25(OH)_2D_3$ or 1,25D, 1α,25-dihydroxyvitamin D_3; ADAM17, ADAM metallopeptidase domain 17; Arf, ADP-ribosylation factor; ASAP2, Arf-GAP with SH3 domain, ankyrin repeat and PH domain 2; B2M, beta-2-microglobulin; ChIA-PET, chromatin interaction analysis with paired-end tag sequencing; ChIP, chromatin immunoprecipitation; CPSF3, cleavage and polyadenylation specific factor 3, 73 kDa; CTCF, CCCTC-binding factor; DDEF2, development- and differentiation-enhancing factor-2; DR3, direct repeat with 3 intervening nucleotides; FCS, fetal calf serum; GAP, GTPase-activating protein; GAPDH, gylcerinaldehyd-3-phosphate-dehydrogenase; H3K4me, histone 3 lysine 4 methylation; HPRTI, hypoxanthine phosphoribosyltransferase 1; IAH1, isoamyl acetate-hydrolyzing esterase 1 homolog; ITGB1BP1, integrin beta 1 binding protein 1; IGV, Integrative Genomics Viewer; MB, myoglobin; PMA, phorbol 12-myristate 13-acetate; qPCR, real-time quantitative polymerase chain reaction; SH3, Src homology 3; TAF1B, TATA box binding protein (TBP)-associated factor, RNA polymerase I, B, 63 kDa; TSS, transcription start site; VDR, vitamin D receptor; YWHAQ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide.

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30% of these VDR binding sites carry a DR3-type sequence that has a high similarity score with the consensus sequence. This suggests that there are additional modes of VDR binding to its genomic targets [11].

On the level of transcriptome analysis rather short incubations (2–4 h) with $1,25(OH)_2D_3$ are essential, in order to identify primary VDR target genes, while for the overall physiological or consequential effects of $1,25(OH)_2D_3$ far longer treatment times (24–72 h) are used. In earlier studies [7,12–14] we have demonstrated that THP-1 cells are a well responding and physiologically meaningful model system for the investigation of $1,25(OH)_2D_3$ signaling in the context of innate immunity and cancer. We found in these cells already 4 h after stimulation with $1,25(OH)_2D_3$ 408 genes statistically significantly up-regulated and after 24 h even 1651 genes are induced [7]. In this context we found that the *ASAP2* gene is one of the most up-regulated $1,25(OH)_2D_3$ target genes in monocytes showing a 3.7- and 5.3-fold induction after 4 and 24 h stimulation, respectively. The *ASAP2* gene, which is also called development- and differentiation-enhancing factor-2 (*DDEF2*) [15], encodes for a 1006-amino acid multi-domain protein composed of an N-terminal α-helical region with a coiled-coil motif, followed by a pleckstrin homology domain, an ADP-ribosylation factor (Arf)-GTPase-activating protein (GAP) domain, an ankyrin homology region, a proline-rich region and a C-terminal Src homology 3 (SH3) domain [16]. The Asap2 protein is localized in the Golgi apparatus and at the plasma membrane, where it co-localizes with the protein tyrosine kinase 2-beta (Pyk2) [16]. In this way, the Asap2 protein functions as an Arf-GAP that controls Arf-mediated vesicle budding when recruited to Golgi membranes modulating constitutive secretion [17]. Moreover, the Asap2 protein modulates phagocytosis (via the Fcγ receptor and Arf6) [18] and cell migration (via the organization of focal contacts) [19].

In this study, we made intensive use of publically available data sets from the ENCODE project [20], in order to monitor how the *ASAP2* gene is surrounded by conserved binding sites of the transcription factor CCCTC-binding factor (CTCF). CTCF acts as a chromatin organizer that is able to link chromosomal loci [21]. These CTCF sites define different sizes of chromosomal domains containing the *ASAP2* gene, up to six additional genes and three VDR binding sites. We found that the genomic region around the *ASAP2* gene is genome-wide highlighted as a special attraction point for the VDR by containing in close vicinity three DR3-type sequence-carrying binding sites. However, the only presently known functional consequence of the favored binding of VDR to this chromosomal region is that in both monocytes and macrophages *ASAP2* is a primary $1,25(OH)_2D_3$ target gene.

2. Materials and methods

2.1. Cell culture

The human acute monocytic leukemia cell line THP-1 [22] was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin and the cells were kept at 37 °C in a humidified 95% air/5% CO_2 incubator. Prior to mRNA or chromatin extraction of monocytes THP-1 cells were grown overnight in a density of 500000–650000 cells/ml in phenol red-free RPMI 1640 medium supplemented with 5% charcoal-stripped FCS. For the differentiation into M2-type macrophages, THP-1 cells were grown for 72 h in normal culture medium supplemented with 20 nM PMA (Sigma–Aldrich). Monocytes were treated with 100 nM $1,25(OH)_2D_3$ (Sigma–Aldrich) or solvent (0.1% ethanol), while macrophages were exposed to 10 nM $1,25(OH)_2D_3$ or solvent (0.001% ethanol).

2.2. RNA extraction, cDNA synthesis and qPCR

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche) or the Quick RNA Miniprep Kit (Zymo Research). For cDNA synthesis the Transcriptor First Strand cDNA Synthesis Kit (Roche) was applied, where total RNA and oligo$(dT)_{18}$ primers were denaturated at 65 °C and reverse transcription was carried out for 30 min at 55 °C. qPCR reactions were performed using 250 nM of reverse and forward primers and the LightCycler 480 SYBRGreen I Master (Roche). The hotstart Taq polymerase was activated for 10 min at 95 °C, followed by 42 amplification cycles of 20 s denaturation at 95 °C, 15 s annealing at primer-specific temperatures (Table S1) and 15 s elongation at 72 °C and a final elongation for 10 min at 72 °C. PCR product specificity was monitored using post-PCR melt curve analysis. Relative expression levels were determined with the comparative delta threshold cycle (ΔCt) method. Relative expression levels of the target genes were normalized to the internal reference genes *B2M*, *GAPDH* and *HPRT1* as determined by the geNorm algorithm [23]. Briefly, the arithmetic mean of replicated Ct values for each gene is transformed to a relative quantity (setting the sample with the highest expression as calibrator to 1), using the ΔCt formula $Q = E^{\Delta Ct} = E^{(calibratorCt - sampleCt)}$ (Q = quantity sample relative to calibrator sample; E = amplification efficiency). For normalization, the relative quantities were divided by the normalization factor being the geometric mean of the three reference genes.

2.3. ChIP

After treatment of cells, nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% and incubating at room temperature for 5–10 min on a rocking platform. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating at room temperature for 5 min on a rocking platform. The cells were collected, washed with ice-cold PBS and resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris–HCl, pH 8.1) and the lysates were sonicated with a Bioruptor Plus (Diagenode, Liege, Belgium) to result in DNA fragments of 200–400 bp. Cellular debris was removed by centrifugation. For output samples, aliquots of the lysate were diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, protease inhibitors, 250 μg/ml BSA, 16.7 mM Tris–HCl, pH 8.1). Anti-VDR antibody (sc-1008X, Santa Cruz) or non-specific IgG (12–370, Millipore) were bound overnight to Magna ChIP™ Protein A Magnetic Beads (Millipore) or protein A agarose beads (Millipore). The pre-formed bead-antibody complexes were then washed three times with ChIP dilution buffer and added to the output aliquots. The samples were incubated for overnight at 4 °C on a rotating platform to form and collect the immuno-complexes. The beads were washed sequentially for 4 min with the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris–HCl, pH 8.1) and LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1). Finally, the beads were washed twice with TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8.0) and the immune complexes were eluted twice using elution buffer (1% SDS, 100 mM $NaHCO_3$) at room temperature for 15 min with rotation. Both output and input samples were reverse cross-linked overnight at 65 °C in the presence of proteinase K (Roche). The DNA was isolated with the ChIP DNA Clean & Concentrator Kit (Zymo Research). Selected genomic regions containing VDR peaks were analyzed by qPCR using equal DNA amounts of chromatin fragments, 250 nM of reverse and forward primers and the LightCycler 480 SYBRGreen I master mix. The qPCR reactions were performed using the following profile: 10 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 15 s annealing at

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