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Research Paper

Calcipotriol Affects Keratinocyte Proliferation by Decreasing Expression of Early Growth Response-1 and Polo-like Kinase-2

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Purpose. Calcipotriol is a potent drug for topical treatment of psoriasis because it manages to inhibit keratinocyte proliferation. In the present study we investigated the effects of calcipotriol on gene expression in human keratinocytes in terms of mechanism of how calcipotriol decreases proliferation.
Materials and methods. Cell proliferation was analyzed by MTT assay. The differential display approach together with qPCR was used to assess the gene expression after treatment. In addition, Western immunoblotting revealed differences on the protein level. Finally, transfection of the KCs with specific small interfering RNA determined the genes necessary to inhibit proliferation.
Results. KCs proliferation was decreased in a concentration-dependent manner. Moreover, calcipotriol dowregulated the expression of two proliferation factors: early growth response-1 (EGR1) and polo-like kinase-2 (PLK2). The protein levels of EGR1 and PLK2 were also decreased. Specific siRNA against EGR1 and PLK2 in KCs resulted in marked reduction of EGR1 and PLK2 expression. In both cases, the reduction resolved in the decreased proliferation of KCs.
Conclusion. This study provides a new insight into how calcipotriol affects proliferation of keratinocytes by decreasing the expression of EGR1 and PLK2. Furthermore, the results offer groundwork for developing novel compounds for the treatment of hiperproliferative skin disorders like psoriasis.

KEY WORDS: calcipotriol; differential display; EGR1; PLK2; siRNA.

INTRODUCTION

Psoriasis is a chronic, genetically influenced, and immunologically based inflammatory disorder of the skin. It is characterized by the hyperproliferation and disrupted terminal differentiation of keratinocytes in the epidermis (1). Still, its complex pathogenesis is not completely clear. Abnormalities in signalling pathways involving kinases and in the expression or activation of different transcription factors in psoriatic keratinocytes have been hypothesized to play a role in the pathophysiology of psoriasis (2). Phosphorylase kinase activity was shown to correlate with psoriasis severity (3). AP1 and NF-κB have been two recently described transcription factors, whose binding activity is impaired in psoriatic skin (4,5). Furthermore, inducible epidermal deletion of JunB transcription factor and its functional companion c-Jun lead to a phenotype resembling psoriatic inflamed lesions (6).

Many agents are used to treat psoriatic skin lesions. These include topical glucocorticoids, systemic cyclosporine, methotrexate, retinoids, ultraviolet light irradiation, and new immunomodulatory antibodies. In addition, the synthetic vitamin D_3 analogue, calcipotriol (also known as MC-903), is also very potent in the topical treatment of psoriasis (7,8). Calcipotriol is much less potent in calcium metabolism than vitamin D_3, but retains similar effects on cell proliferation and differentiation in human lymphoma cell line U937, human keratinocyte, and squamous carcinoma cell line SCC13 in the 3-D raft culture (9–11). The inhibition of keratinocyte proliferation and the induction of differentiation are consistent with its anti-psoriatic activity. Calcipotriol exerts its effect by binding to an intranuclear vitamin D receptor (VDR), which is expressed in many cell types including keratinocytes (12,13). The VDR belongs to the superfamily of transcriptional factors and, through it, calcipotriol regulates the transcription of certain genes. However, the identity of the latter has not been established nor was any of the involved genes unequivocally linked to the proliferation. Recently, it was only proposed that calcipotriol might decrease cell proliferation by deactivating EGF receptor (11). Therefore, identification of genes modulated by calcipotriol would help to clarify the mechanism of action of the drug, and would reveal potential targets for more specific treatment of psoriasis. We have therefore looked for changes in gene expression in primary cultures of human keratinocytes following treatment with calcipotriol and found a mechanism of action for the inhibition of cell proliferation in normal human keratinocytes.

The investigation of how calcipotriol affects proliferation through the change in gene expression in primary

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cultures of human keratinocytes was performed by differential display experiments. Furthermore, the search for factors implicated in the reduction of proliferation after treatment with calcipotriol led to the identification of early growth response-1 (EGR1) and polo-like kinase-2 (PLK2) genes. We show that EGR1 and PLK2 are significantly downregulated at mRNA and protein levels after treatment with calcipotriol. Additionally, for the first time, the specific EGR1 and PLK2 small interfering RNA (siRNA) were used to inhibit EGR1 and PLK2 expression in cultured primary human keratinocytes. Transfection with specific siRNA resulted in similar decrease in EGR1 and PLK2 expression as after calcipotriol treatment and in evident inhibition of keratinocyte proliferation. In conclusion, we demonstrate that EGR1 and PLK2 gene downregulation is at least in part a mechanism by which calcipotriol inhibits cell proliferation, and therefore, EGR1 and PLK2 might serve as putative targets in the treatment of psoriasis.

MATERIALS AND METHODS

Isolation and Culture of Keratinocytes

With the approval of the National Bioethical Committee samples of normal human skin from two healthy donors were obtained during plastic surgery. Primary keratinocyte cultures were prepared according to the GibcoBRL (Life Technologies) protocol with minor modifications. Skin samples were incubated overnight in a defined keratinocyte serum free medium (defined keratinocyte-SFM), without bovine pituitary extract but supplemented with 2 μg/ml amphotericin B, 50 μg/ml gentamycin (all from Invitrogen, Paisley) and 100 U/ml penicillin (Sigma, Taufkirchen). Subsequently, most of the connective tissue layer was removed and the remaining skin cut into 5 mm^2 pieces. After 18 h digestion with 200 U/ml collagenase (Invitrogen, Paisley), the separated epidermis was immersed in 0.05% trypsin/0.02% EDTA (Sigma, Taufkirchen) solution for 10 min at 37°C. Trypsinization was terminated with 1 mg/ml final concentration soybean trypsin inhibitor (Sigma, Steinheim). The extracted keratinocytes were washed in Hanks balanced salt solution (HBSS) without Mg^{2+} and Ca^{2+} (Sigma, Steinheim), resuspended in defined keratinocyte-SFM medium supplemented with 2 μg/ml amphotericin B, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma, Taufkirchen), and left to grow to 50% confluence in collagen-coated culture plates. The cells were serially sub-cultured at a split ratio of 1:3 and the second passage was used for this study.

Treatment with Calcipotriol

Initially, keratinocytes intended for differential display experiments were incubated for 24 h in fresh culture medium containing the amount 2.85 nM calcipotriol (gift from Leo Pharma) dissolved in isopropanol to avoid uncontrolled cell response to the drug. In additional experiments, cells were treated with 0.285 μM calcipotriol for 8, 16 and 24 h to examine already identified genes at the most effective concentration, determined by previous studies. An equal volume of isopropanol was added to control cell cultures. After treatment, cells were washed, harvested and stored in liquid nitrogen for later use. To assess the viability and concentration of the cells, the samples were stained with Trypan blue solution (Invitrogen, Paisley) and counted in a haemocytometer.

MTT Assay

Non-radioactive cell proliferation assay using thiazolyl blue (Sigma, USA) was performed in the 24-well culture plates. Cells (2×10^4) were incubated with various concentrations of calcipotriol or solvent as control for 72 h at 37°C in the final volume of 0.5 ml of keratinocyte medium. Thereafter, 0.05 ml of MTT solution (5 mg/ml) was added to each well. After 2 h of incubation at 37°C, 0.5 ml solution of isopropanol and Triton X-100 (ratio 10:1) was added, and the culture plate left on the plate shaker for 15 min. Optical density was determined at 570 nm on a microplate reader (Tecan, Switzerland). Cells were seeded in four wells for each drug concentration. In addition, MTT assay was performed after electroporation of siRNA into the primary keratinocytes. Cells underwent the same procedure, only that they were seeded in six wells for each experiment condition.

Differential Display

Changes in gene expression were monitored using the method of Liang and Pardee (14). Total RNA was isolated using the RNeasy Mini Kit combined with RNase-Free DNase Set (from Qiagen, Maryland and Hilden, respectively) according to the manufacturer's protocol. The RNA was quantified by absorbance at 260 nm and its integrity determined using Agilent 2100 Bioanalyzer and Expert 2100 software. Approximately 250 ng of RNA was reverse transcribed at 37°C for 1 h with OmniScript reverse transcriptase (Qiagen, Hilden) using one of the anchored primers H-T_{11}C, H-T_{11}A, H-T_{11}G (5′-*Hin*dIII-T11A/C/G-3′) or β-actin (fw: 5′-CCA CGA AAC TAC CTT CAA CTC C-3′; rev: 5′-CCT GCT GCT TGC TGA TCC-3′) gene specific primer. One tenth of the cDNA obtained was used as a template in a 25-μl PCR reaction containing 0.4 μM arbitrary 13-mer primer, 0.4 μM appropriate anchored primer, 400 μM dNTP, 0.625 U Taq polymerase (Promega, Madison, WI), and 3 mM $MgCl_2$. PCR reactions were accomplished with combinations of 16 different random primers and all three anchored primers (all synthesized by Invitrogen). Kinetics of the PCR reactions were set to 30 s at 94°C, 2 min at 42°C, 30 s at 72°C, for 40 cycles, with a 5 min termination step at 72°C. RT-PCR reactions were all made in duplicate. PCR-products were separated on GeneGel Clean sequencing gel (Amersham Pharmacia, Uppsala) using a GenePhor electrophoresis unit (Amersham Pharmacia, Uppsala). Gels were stained with silver using a commercial kit (Amersham Pharmacia, Uppsala). cDNA bands that were stained differently for treated and control samples were isolated from the gel using a sterile needle and boiled in 20 μl Tris–EDTA buffer for 5 min. 2 μl of solution served as a template for PCR-reamplification and the subsequent cloning into a pGEM-T Easy vector (Promega, Madison, WI). Subsequently, the plasmid DNA was prepared and two independent clones for each original DNA band were submitted to non-radioactive cycle-sequencing (MWG-Biotech, Ebersberg) with the SP-6 reverse primer. The nucleotide sequence data obtained for the differentially expressed cDNA inserts were tested for homology to known sequences in the GenBank databases by BLAST search.

Quantitative Real Time PCR Analysis

mRNA was reverse transcribed from 80 ng total RNA using oligo-dT primer mix. qPCR was carried out on ABI PRISM 7000 apparatus (Applied Biosystems) in a total volume of 50 µl containing 10 µl of the RT mixture, 1X Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), 1 µl Rox reference dye, and 0.2 µM of each primer (EGR1 fw: 5′-GTC CCC GCT GCA GAT CTC T-3′; EGR1 rev: 5′-CTC CAG CTT AGG GTA GTT GTC CAT-3′; PLK2 fw: 5′-TGG ACA TGT GGT GGT ACG AAA-3′; PLK2 rev 5′-TGT ATG CCT TAG CCT GGT TCT G-3′; Involucrin: PrimerBank ID5031813a1). Concentration and time dependences of calcipotriol treatment were monitored using 25 µl reactions. The cycling program was always as follows: 2 min at 50°C, 2 min at 95°C, followed by 45 cycles (15 s at 95°C, 30 s at 60°C, 30 s at 72°C). We checked β-actin, B2M, HPRT, GAPD, YWHAZ and UBC (primer sequence of β-actin is given above, the rest of primers were found in Real Time PCR Primer and Probe Data Base; ID 1234, 1237, 55, 9 and 8, respectively) housekeeping genes for their stability and chose the three most stable genes to be our endogenous controls (β-actin, B2M, UBC) for qPCR analysis. A melting curve (60–95°C) and standard gel electrophoresis of PCR products were also performed to ensure the absence of artefacts.

Immunoblot Analysis

Keratinocytes were washed with HBSS (GibcoBRL) and collected for protein preparation 24 h after treatment with 0.1 nM or 100 nM of calcipotriol. Cell pellets were vortexed and sonicated in RIPA lysis buffer (50 mM Tris HCl, pH 8; 100 mM NaCl; 0.1% SDS; 1% NP-40; 0.5% sodium deoxycholate; 1 mM EDTA). Lysates were centrifuged at 16,000 g for 30 min, and protein concentration was determined using the Coomassie Plus—The better Bradford assay kit (Pierce). Equal amounts of protein were separated by 10 to 12.5% sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride Immobilon-P membrane (Millipore). The blots were first blocked for 1 h in Western blocking solution (1:10, Roche) in TBST at room temperature and probed by polyclonal antibodies against EGR1 (1:200 dilution, Santa Cruz Biotechnology), PLK2 (1:250 dilution, Abnova) and β-2-microglobulin (B2M, 1:200 dilution, Santa Cruz Biotechnology), in Western blocking solution (1:20, Roche) in TBST overnight at 4°C, then incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (1:5,000 dilution) for 1 hour at room temperature and developed using enhanced chemiluminescence reagents Lumi-LightPLUS Western Blotting Substrate (Roche) and Hyperfilm ECL (Amersham Bioscience). The developed films were scanned using analysis system G-Box-HR and adequate software SnapGene (Syngene). The band densities (heights) were quantified using GeneTools software (Syngene).

siRNA Transfection

Keratinocytes were grown to 70–80% confluency before transfection. All transfections were done by an Amaxa Nucleofector apparatus and corresponding kit (Amaxa Biosystems) according to the manufacturer's instructions. Cells were transfected with Nucleofector solution using Nucleofector program for high transfection efficiency (T-24) with 3 µg siRNA. Stealth EGR1 siRNA (HSS103118, Invitrogen) and stealth PLK2 siRNA (HSS116614, Invitrogen) were used to knockdown EGR1 and PLK2. Scrambled AllStars Negative Control siRNA (Qiagen) and electroporated cells without siRNA were used as controls. After electroporation, cells were split and seeded in the 24-well culture plate for MTT assay and in the 6-well culture plate for isolation of mRNA. mRNA was isolated 24 hours later and the level of knockdown determined by qPCR. In addition, transfection was performed using Lipofectamine 2000. Various siRNA/Lipofectaimne 2000 ratios (from 3/5 to 1/10) were used for the formation of transfection complexes. siRNA:Lipofectamine 2000 complexes were prepared in basal keratinocyte-SFM medium (GibcoBRL) or Opti-MEM medium (Invitrogen). Transfections were done with the final siRNA concentrations of 100 pmol–10 nmol. mRNA was isolated after 24 h of incubation at 37°C and 5% CO_2.

Statistics

Results were expressed as mean±standard deviation (SD). Statistical significance ($P<0.01$) was assessed by Student's t test.

RESULTS

Calcipotriol Inhibits Keratinocyte Proliferation

In order to determine the effect of calcipotriol on keratinocyte proliferation, we performed MTT assay. In defined keratinocyte-SFM medium, the doubling time of keratinocytes was approximately 22 h. Therefore as presented in Fig. 1, calcipotriol exhibits growth-inhibitory effect while it significantly decreases cell proliferation in a dose-dependent manner. Calcipotriol is already effective at concentration of 10^{-10} M. According to Takahashi *et al.* (15), a calcipotriol concentration of 10^{-7} M exerts the highest antiproliferative effect. The same concentration of calcipotriol is routinely used in topical ointments for treatment of psoriasis.

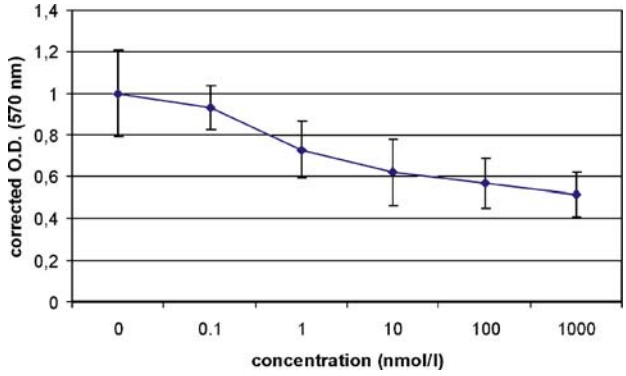


Fig. 1. Effect of calcipotriol on primary human keratinocyte cell proliferation. Cells were incubated with various concentrations of calcipotriol or solvent as control for 72 h. The doubling time of keratinocytes was approximately 22 h. MTT solution was then added to the cells and left for 2 h of incubation at 37°C. Formed formazan was resuspended with isopropanol and Triton X-100 with the aid of the plate shaker. Optical density (O.D.) was determined at 570 nm and corrected with O.D. at 620 nm. Results are the means±SD.

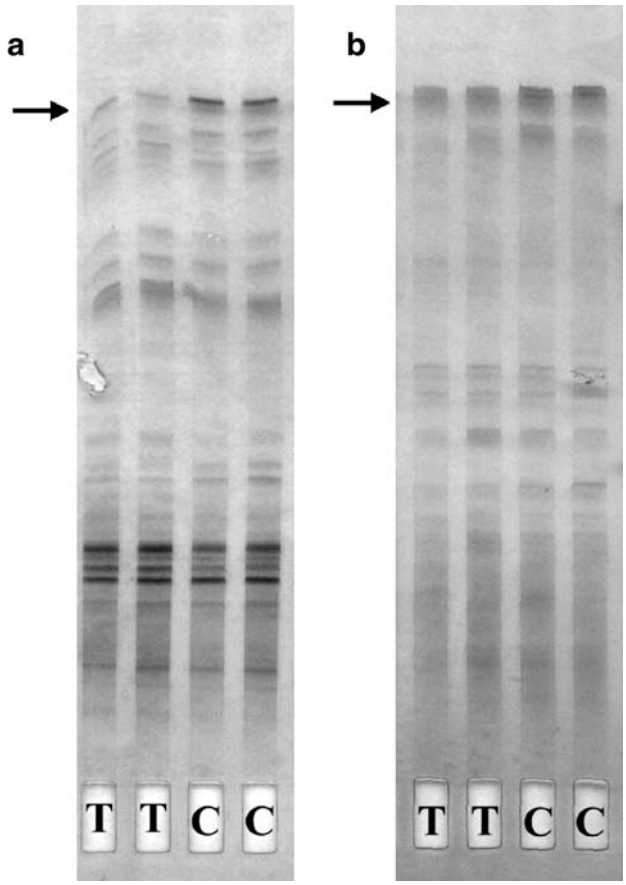


Fig. 2. Differential display (DD) PCR. mRNA was extracted from primary keratinocyte cultures treated with 2.85 nM calcipotriol. mRNA was reverse transcribed in duplicate and DDPCR was prepared from both reverse transcription reaction in parallel using different combinations of one base anchored oligo-dT and random arbitrary primers. Arrows indicate differentially expressed gene products. The fragment on gel (**a**) was later identified as being homologous to EGR1 mRNA, and from gel (**b**) to PLK2 mRNA. *T* cDNA from treated cells, *C* samples from control cells.

Calcipotriol Downregulates the Expression of EGR1 Gene

In this study, the differential display technique made use of 48 different combinations of arbitrary and one-base anchored primers to detect differentially expressed genes in primary keratinocytes on treatment with 2.85 nM calcipotriol after 24 h. Initial concentration of 2.85 nM was used to eliminate possible unspecific general cell response to the treatment. A total of 28 differentially stained cDNA bands were isolated from the gel, cloned, sequenced and tested for homology to known sequences in the GenBank databases. For one of the downregulated fragments (Fig. 2a) the BLAST search returned very high homology to EGR1 gene (Fig. 3). qPCR experiments were employed for further confirmation of the experimental results. The level of down-regulation in the preliminary experiment, normalized on β-actin and control cells, was twofold (Fig. 4a).

After identifying the candidate gene, we investigated the concentration and time dependence of calcipotriol treatment on its expression. Therefore we treated the cells with 0.285 μM calcipotriol, which is the most effective concentration, and observed the expression levels of EGR1 gene after 8, 16 and 24 h. Transcription of the EGR1 gene in treated cells obtained from two healthy donors was found to be significantly decreased after the treatment (Fig. 4b). The EGR1 gene was downregulated in the range of two- to fivefold, normalized on three housekeeping genes (β-actin, B2M, UBC) and control cells. Potent downregulation was detected after 8 h of treatment with calcipotriol and was sustained throughout the 24 h.

Calcipotriol Influences the Expression of PLK2 Gene

The second downregulated gene from the differential display study was identified as PLK2 (Fig. 2b). The cloned fragment sequence exhibited 100% homology to the middle region of PLK2 mRNA (Fig. 3). Even though fragments similar to the 3′-end of mRNA transcripts are expected, when performing differential display according to the protocol of Liang and Pardee (14), the observed homology to the middle part of the transcript can be explained by annealing of the oligo-dT primer to poly-A regions within the transcript. The observation was made with cells treated with 2.85 nM calcipotriol for 24 h. The difference was confirmed and quantified by qPCR. The expression level of PLK2 was downregulated 1.3-fold, normalized on β-actin and control cells (Fig. 4a).

Increasing the concentration of calcipotriol for treating cells to 0.285 μM resulted in a more marked decrease in PLK2 mRNA levels. The expression level of PLK2 in treated cells was downregulated by up to twofold, compared to control cells (Fig. 4c). The data were normalized to three endogenous controls (β-actin, B2M, UBC). Again, the

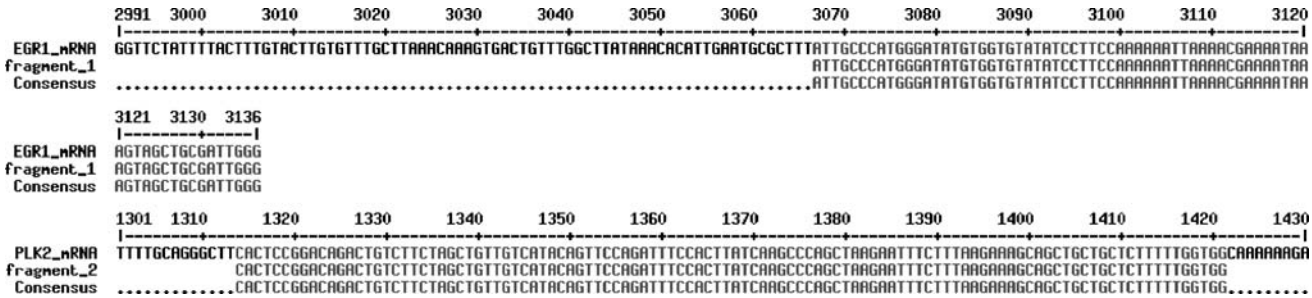
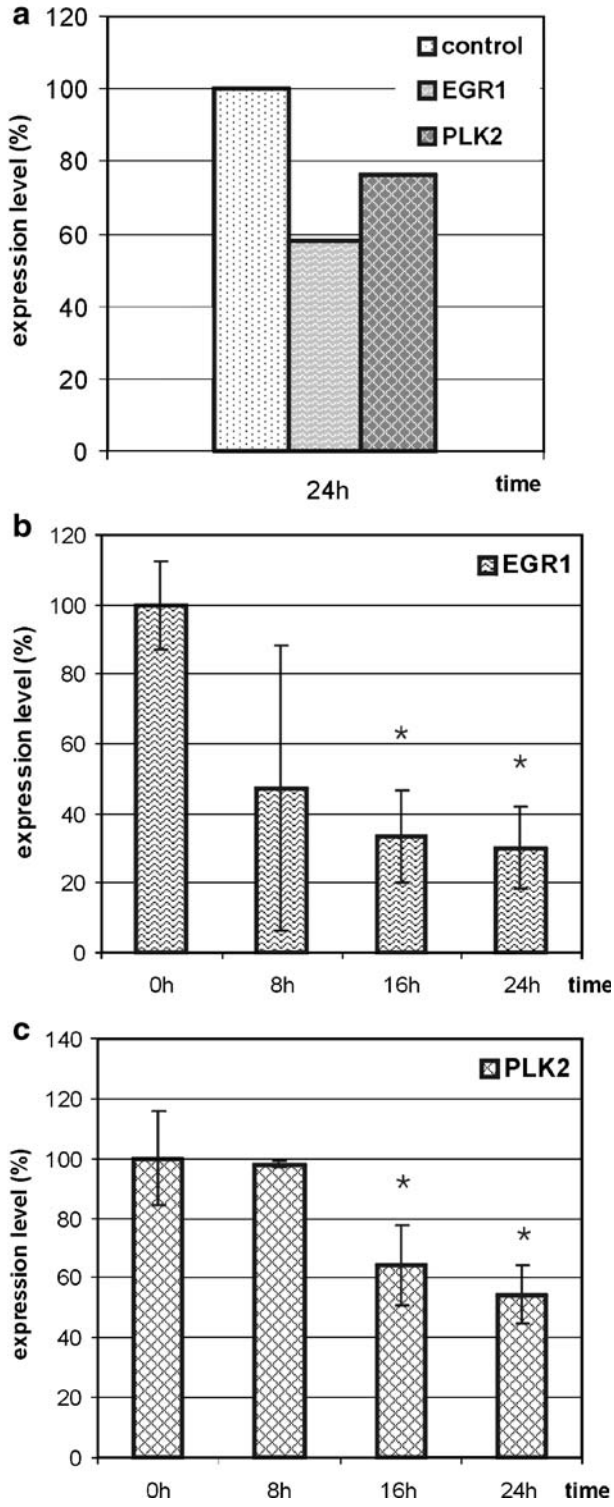


Fig. 3. Fragments identified as parts of EGR1 and PLK2 genes fitted against mRNA sequence. Differentially stained cDNA bands were isolated from the gel, cloned, sequenced and tested for homology to known sequences in the GenBank databases by BLAST search. Fragment 1 returned very high homology to the 3′-end of EGR1 mRNA. Fragment 2 sequence exhibited almost complete homology to the middle region of PLK2 mRNA. *EGR1* Early growth response-1, *PLK2* polo-like kinase-2.



◀**Fig. 4.** Real time PCR (qPCR) analysis determined downregulation of genes encoding EGR1 and PLK2. The differentially stained cDNA fragments were sequenced, leading to identification of the differentially expressed genes. The differential gene expression level in cells was confirmed by qPCR analysis using gene specific primers and SYBR Green I chemistry. **a** Expression level of EGR1 and PLK2 obtained by qPCR. Cells were treated with 2.85 nM calcipotriol for 24 h. **b**, **c** Expression level of EGR1 and PLK2 obtained by qPCR in primary keratinocytes, cultured in the presence of 0.285 μM calcipotriol, determined at 8, 16 and 24 h. The expression levels of EGR1 and PLK2 in keratinocytes at time 0 were designated as 100. *EGR1* Early growth response-1, *PLK2* polo-like kinase-2. Results are means±SD. **P*<0.05 compared to control.

observations were made after 8, 16 and 24 h of treatment with calcipotriol; levels of PLK2 mRNA at 8 h were unchanged and the most significant differences were observed at 24 h.

Additionally, we monitored as a positive control the effect of calcipotriol on the expression of involucrin, an early differentiation-associated marker in keratinocytes. Calcipotriol is well known to promote differentiation in keratinocytes (10). Calcipotriol stimulated the expression of involucrin mRNA by up to threefold, measured by qPCR (data not shown). The results were normalized on three housekeeping genes (β-actin, B2M, UBC) and control cells.

Calcipotriol Reduces EGR1 and PLK2 Protein Expression

Western immunoblotting was used to characterize the effects of calcipotriol on EGR1 and PLK2 protein expression in primary keratinocytes. Cells were incubated with 0.1 nM and 100 nM calcipotriol for 24 h and whole cell lysates were prepared. Both EGR1 and PLK2 protein levels were decreased after calcipotriol treatment (Fig. 5a). The decrease of EGR1 protein was more pronounced. The pattern completely corresponds to the results obtained by qPCR analysis. However, absolute differences in band densities obtained by software analysis of Western blots showed that the decrease at protein level is not as strongly expressed as it is at the mRNA level after calcipotriol treatment (Fig. 5b). The six housekeeping genes (β-actin, B2M, HPRT, GAPD, YWHAZ and UBC) were checked for their stability in qPCR analysis and the most stable gene was beta-2-microglobulin (B2M). Therefore, the antibody against B2M protein was used to assay equal loading in Western blot experiments.

Overall, these studies demonstrate that calcipotriol decreases expression of EGR1 and PLK2 on mRNA level and the change evidently reflects on the protein level.

EGR1 and PLK2 siRNA Transfection Reduces EGR1 and PLK2 mRNA Expression and Proliferation of Keratinocytes

To study the effect of suppression of the EGR1 and PLK2 gene expression in cultured human keratinocytes on cell proliferation, the cells were transfected with specific EGR1 or PLK2 siRNA. First, we tried to transfect keratinocytes with siRNA using Lipofectamine 2000. Even though we tested various amounts of Lipofectamine 2000, numerous ratios for detergent/siRNA complex building, and different transfection protocols, we failed to achieve any reduction in gene mRNA levels. Later, we succeeded to transfect cells with electroporation. Immediately after transferring siRNA into keratinocytes, part of them was seeded for evaluation of proliferation of the cells by MTT assay and the rest was used to determine the level of gene knockdown by qPCR. Analysis of mRNA levels was carried out 24 hours after transfection. The mean total EGR1 mRNA content in the keratinocytes was analyzed by qPCR and was found to be reduced by 89% (P<0.01) in the transfected keratinocytes

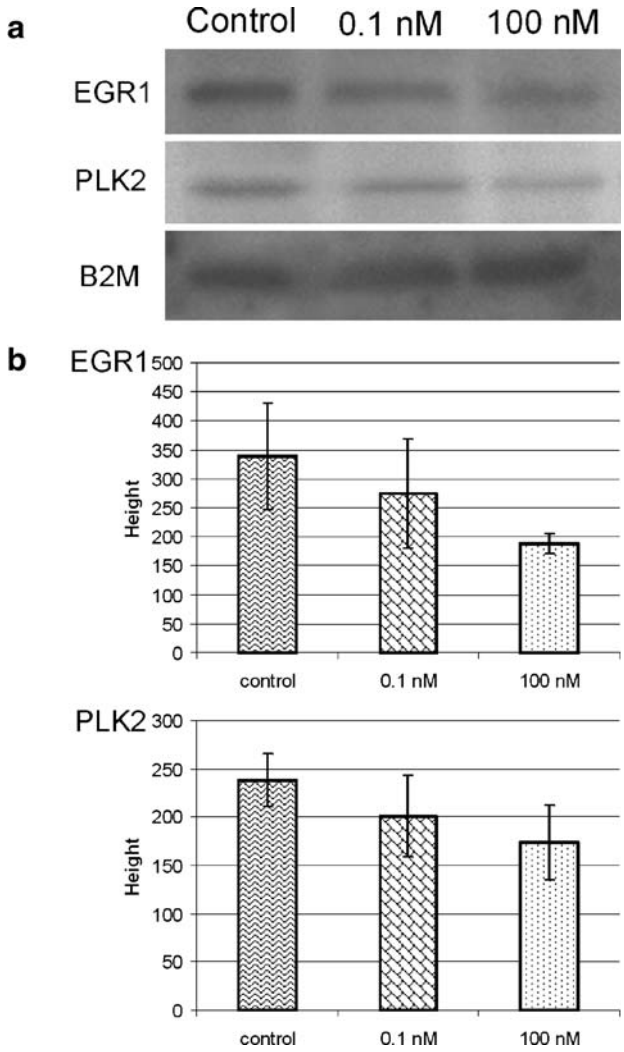


Fig. 5. The level of EGR1 and PLK2 proteins after treatment with calcipotriol determined by Immunoblot analyses. Primary keratinocytes were cultured without calcipotriol or in the presence of two concentrations of the drug (0.1 nM or 100 nM) for 24 h. The cells were harvested for the preparation of total cell lysates. **a** Western blot analysis was used as described in "MATERIALS AND METHODS" to confirm the expression levels of EGR1 and PLK2 determined by differential display. Equal loading was confirmed by assaying for β-2-microglobulin (*B2M*) levels. Similar results were obtained from two independent experiments. **b** Images were analysed using GeneTools software (Syngene) to quantify the band densities. Results are means±SD.

compared to control (Fig. 6a), always normalized on three housekeeping genes. Also the level of PLK2 mRNA was reduced (56%, $P<0.01$) compared to control (Fig. 6b), again, normalized on three endogenous controls. The drop in mRNA content after using siRNA is comparable to reduction after calcipotriol treatment. The second part of cells used in electroporation experiments were incubated for 72 h and examined in the MTT assay. We found in all experiments that the cells with either reduced EGR1 gene expression or reduced PLK2 gene expression showed a consistent inhibition in proliferation. Proliferation of primary human keratinocytes was reduced for approximately 40% ($P<0.01$) after EGR1 siRNA treatment and 20% ($P<0.01$) after PLK2 siRNA treatment (Fig. 7). The proliferation suppression was comparable to inhibition achieved by calcipotriol. However, the simultaneous transfection with siRNA against both EGR1 and PLK2 resulted in less pronounced inhibition of proliferation (14%, $P<0.01$; Fig. 7). Negative control scrambled siRNA did not influence proliferation (Fig. 7).

PLK2 Gene is Not Under EGR1 Control

EGR1 is a transcription factor and has various target genes, whose expression it regulates. We verified if PLK2 gene is a downstream target for EGR1 transcription factor and as such a secondary response gene to calcipotriol treatment. The siRNA experiments with EGR1 siRNA showed significant decrease in EGR1 gene. Also Western blot results showed marked reduction of EGR1 protein levels already after 24 h, meaning that 1 day is sufficient for EGR1 protein pool to diminish. However, there was no simultaneous reduction in PLK2 gene expression after the knockdown of EGR1 gene (Fig. 8).

DISCUSSION

Calcipotriol is widely used drug for the local treatment of psoriasis. Clinical treatment of psoriatic lesions with calcipotriol significantly improves their thickness, scaling and inflammation. Understanding the molecular basis of the action of the biologically active vitamin D analogue calcipotriol has implications on the treatment of psoriasis, as well as for various new potential targets capable of influencing the processes of proliferation, differentiation and apoptosis. Except abundant clinical trials so far, there has been only little data on calcipotriol molecular action on the keratino-

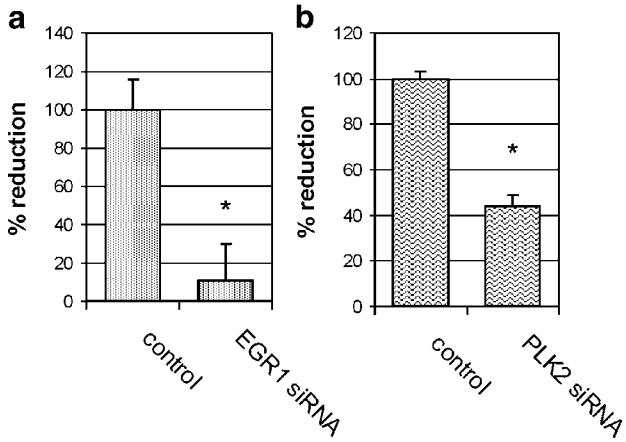


Fig. 6. Effect of specific siRNA transfection on total expression level of genes coding for EGR1 (**a**) and PLK2 (**b**). Primary keratinocytes were transfected with specific EGR1 and PLK2 siRNA or scrambled negative control siRNA using an Amaxa Nucleofector apparatus. Cells were transfected with Nucleofector solution using Nucleofector program for high transfection efficiency (T-24). After electroporation, one part of cells was seeded for evaluation of knockdown efficiency. Cells were grown in 6-well culture plates and after 24 h harvested for total RNA collection. The siRNA efficiency was determined using qPCR. The level of EGR1 and PLK2 mRNA was evaluated relative to corresponding mRNA in control cells and normalized on three housekeeping genes (β-actin, B2M, UBC). The expression levels of EGR1 and PLK2 mRNA in the control cells was designated as 100%. Results are the means±SD. *$P<0.01$ compared to control.

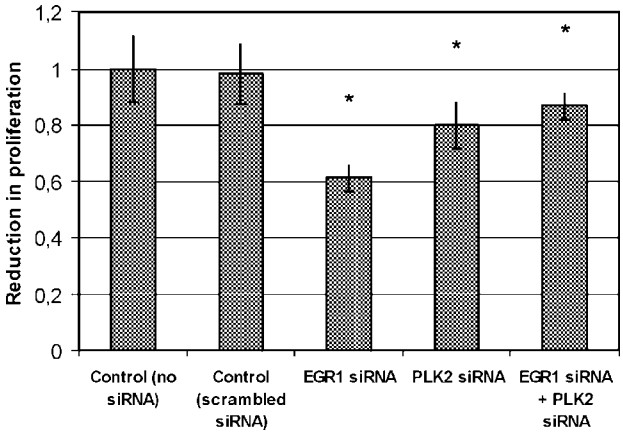


Fig. 7. Effect of specific siRNA transfection on keratinocyte proliferation. Normal human keratinocytes were transfected with specific siRNA designed to interfere with EGR1 and PLK2 mRNA. siRNA was transfected into cells with electroporation using Amaxa Nucleofector apparatus set to the program for high transfection efficiency (T-24). Experiments were controlled with scrambled negative control siRNA and electroporated cells without siRNA. We found in all experiments performed that the cells with either EGR1 siRNA or PLK2 siRNA had decreased gene expression (Fig. 6) and showed a consistent inhibition in proliferation. EGR1 siRNA reduced proliferation of primary human keratinocytes for approximately 40% (*P<0.01). PLK2 siRNA treatment reduced it for 20% (*P<0.01) and simultaneous transfection with siRNA against EGR1 and PLK2 resulted in 14% decrease in proliferation (*P<0.01). Results are the means±SD.

cytes. Johansen *et al.* reported that calcipotriol restores impaired activity of AP-1 and NF-κB transcription factors in the psoriasis (4,5). Experiments also revealed that calcipotriol reduces interleukin-19 (IL-19) and -20 (IL-20) after calcipotriol treatment (16). To our knowledge, the only attempt to elucidate the mechanism of how calcipotriol decreases proliferation of the keratinocytes was done by Lee *et al.* (11), who reported that calcipotriol inhibits autocrine phosphorylation of the EGF receptor causing its deactivation.

In this study, we searched for genes with differential expression in primary keratinocyte cultures following calcipotriol treatment. It has led us to the identification of two gene products, which were previously reported to be implicated in the proliferation. First gene, EGR1, codes for a zinc-finger transcription factor. Increasing the calcipotriol concentration and analysis of the time dependence provided more detailed results. EGR1 mRNA levels showed a marked decrease after 8 h of treatment and remained at constant low levels. In addition to mRNA, we found protein level of EGR1 also to be suppressed after calcipotriol treatment. The magnitude of protein reduction correlated well with the mRNA levels. Most elucidating and important are results from our experiments with siRNA. Transfection using specific EGR1 siRNA managed to significantly knockdown expression of EGR1. The decrease in EGR1 mRNA content obtained with siRNA was comparable to decrease after calcipotriol treatment. Interestingly, it resulted in marked inhibition of keratinocyte proliferation.

EGR1 (also known as NGFI-A, zif268, Krox24 and TIS8) is a transcriptional activator that is involved in growth and differentiation. It has been proposed to play a direct role in controlling proliferation in keratinocytes (17). A variety of growth factors induce EGR1 expression (18,19), indicating that EGR1 may couple extracellular signals to long-term responses by altering the expression pattern of its target genes. Kaufmann and Thiel explicitly showed that powerful mitogens for keratinocytes, like epidermal growth factor (EGF) and thrombin, upregulate EGR1 in their pathways. Furthermore, the search for EGR1 downstream target genes, using microarrays, revealed several genes coding for growth factors, including insulin-like growth factor-2 (IGF2), platelet-derived growth factor-A (PDGF-A), and transforming growth factor-β1 (TGF-β1) (20), suggesting that EGR1 is rapidly induced by a variety of extracellular stimuli and continues the mitogenic signalling cascade via the stimulation of growth factor synthesis. We propose that calcipotriol influences keratinocyte proliferation directly by tuning EGR1 expression levels and limiting growth factor synthesis. This mechanism of action is even more strongly supported in psoriasis, since EGR1 levels have been shown to increase in psoriatic plaques as reported by Chaturvedi *et al.* (21). Overexpressed EGR1 in psoriasis can stimulate keratinocyte proliferation. Calcipotriol, on the other hand, normalizes the unbalanced EGR1 levels and reduces proliferation. Furthermore, there is no evidence, how much curative effect of calcipotriol in psoriasis falls to inhibition of proliferation and how much to reduction of inflammation. Therefore it would be no surprise, if EGR1 were also to interfere in psoriasis in the inflammation pathways, since transcription of some key players in psoriasis, like intracellular adhesion molecule-1 (ICAM-1), macrophage colony-stimulating factor (M-CSF), interleukin-2 (IL-2) and TNF-α, are also orchestrated by EGR1 (reviewed in 22). Based on the available data, we suppose that calcipotriol elicits at least part of its effect in psoriasis by downregulating the transcription level of EGR1,

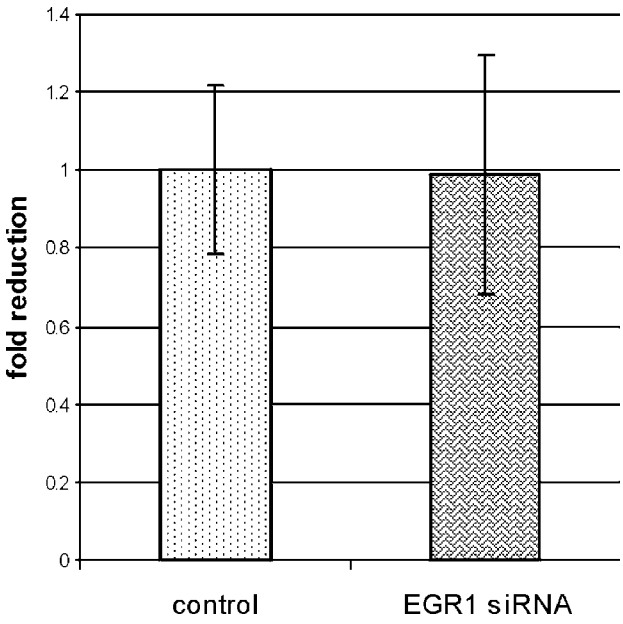


Fig. 8. Effect of EGR1 transcription factor on the PLK2 expression level. We verified if PLK2 gene is a downstream target for EGR1 transcription factor. The EGR1 siRNA was used to knockdown EGR1 expression level as described above. After decrease in EGR1 content, the PLK2 mRNA level was determined using qPCR. The levels were normalized on three housekeeping genes (β-actin, B2M, UBC). Results are the means±SD.

making keratinocytes less prone to proliferation and improving inflammation.

The second identified gene, which might have causal role in the proliferation of keratinocytes, was PLK2, a member of the polo-like kinase family. We could detect the decreased PLK2 levels only after 16 h of treatment. After that the level kept falling. In addition, the protein levels of PLK2 followed the same pattern of reduction, meaning that calcipotriol not only downregulates mRNA, but also affects protein levels. In recent years, PLKs have emerged as major regulators for cell cycle progression. Many key cell cycle regulators such as p53, Cdc25C, cyclin B, and mitotic motor proteins are directly targeted by PLKs. Although the exact mechanism of action of these protein kinases in vivo remains to be elucidated, PLKs are important mediators for various cell cycle checkpoints that monitor centrosome duplication, DNA replication, formation of the bipolar mitotic spindle, segregation of chromosomes, and mitotic exit (23). Early studies on PLK2 implied that the kinase is rapidly induced by mitogen stimulus (24). Ma *et al*. demonstrated that PLK2 functions primarily as a regulator of G1 progression in mammalian cells (25). More recently, PLK2 has been shown to be partly controlled by p53, and PLK2 expression is also induced when cell lines are treated with DNA-damaging agents (26). Both together imply that PLK2 is involved in the DNA damage checkpoint activation pathway. Furthermore, Warnke *et al*. connected PLK2 to centriol duplication (27). In summary, PLK2 protein may function to prevent mitotic catastrophe following spindle damage, but only by complementing other proteins involved in mitosis. The average level of downregulation of PLK2 in our study was twofold, normalized on three endogenous controls and control cells. This might appear only a minor change in the expression level. However, only minor changes in kinase activity may cause significant difference. Moreover, kinases work in reaction cascades, with each step amplifying the next one, meaning it could still have a significant effect on the highly complex processes of cell cycle regulation. Finally, the siRNA experiments show, that approximately 50%-reduction in PLK2 mRNA content, which is equal to twofold reduction, is sufficient to significantly reduce proliferation of keratinocytes. To sum up, these experiments undoubtedly outline PLK2 as effector gene for the influence that calcipotriol has on the keratinocytes.

PLK2 is not one of the EGR1 target genes, since its mRNA level does not change after EGR1 knockdown. On the other hand, EGR1 and PLK2 might act in a co-dependent manner, since co-transfection with siRNA against both of them reduces the effect on proliferation compared to the effect of each single siRNA. One possible junction of EGR1 and PLK2 pathways may represent p53. EGR1 has been previously shown to be a key regulator of p53 (reviewed in 28). It directly induces the transcription of p53. In addition, p53 might promote the expression of PLK2 (26). However, if this was the case, the downregulation of EGR1 caused by siRNA would only help to deflate the level of PLK2 in mixed siRNA experiments via decreased p53, but the EGR1 mRNA expression does not influence the level of PLK2. Therefore, there may be additional adaptor proteins involved in the process. Another pathway, which may influence the joint action of EGR1 and PLK2, leads to ERK and lies upstream of EGR1 and PLK2. Kaufmann *et al*. reported that the biosynthesis of EGR1 is strongly stimulated by activation of ERK (29). On the other hand, Zuber *et al*. came to a conclusion that PLK2 is significantly reduced in H-Ras-transformed cells with permanent ERK activation (30). Both results imply that EGR1 action might be influenced if there is any feed back control in ERK–PLK2 pathway. Activation of ERK pathway after reduction of PLK2 may compensate the knockdown of EGR1. However, the substrates of PLK2 are not known and this is highly speculative. More important, the summed effect of mixed siRNA experiments shows even less pronounced drop in proliferation as does the treatment with calcipotriol itself. With regard to the fact that calcipotriol reduces both of them, we can deduct that EGR1 and PLK2 are involved in the sequence of processes at different time points. We presented that calcipotriol causes the significant drop of PLK2 mRNA only 8 h after it influences the level of EGR1 mRNA. This enables calcipotriol to inhibit proliferation at its level even though it downregulates the expression of both EGR1 and PLK2 genes. Finally, when we compare the effects that calcipotriol has on EGR1 and PLK2, all the changes of EGR1 seem to be more vivid compared to those of PLK2. Therefore we might add that EGR1 gene is more important in the regulation of proliferation. However, fine tuning of PLK2 expression after treatment with calcipotriol must not be fully disregarded.

Taken together, our results contribute to the explanation of what an effect calcipotriol has at the molecular biology level—it modifies the expression of target genes. We prove that calcipotriol elicits its effect by changing the expression pattern to downregulating EGR1 and PLK2 genes. These changes of EGR1 and PLK2 expression in keratinocytes provide a new insight as to how calcipotriol modulates the process of proliferation and might serve for designing more specific and alternative therapies.

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