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Threonine 454 phosphorylation in Grainyhead-like 3 is important for its function and regulation by the p38 MAPK pathway

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ABSTRACT

The mammalian Grainyhead-like 3 (GRHL3) transcription factor is essential for epithelial development and plays a protective role against squamous cell carcinoma of the skin and of the oral cavity. A single nucleotide polymorphism (SNP) in *GRHL3*, rs141193530 (p.P455A), is associated with non-melanoma skin cancer in human patients. Moreover, it is known that this SNP, as well as another variant, rs41268753 (p.T454M), are associated with nonsyndromic cleft palate and that rs41268753 negatively affects GRHL3 transcriptional activity. These SNPs are located in adjacent codons of the *GRHL3* gene, and the occurrence of either SNP abolishes a putative threonine-proline phosphorylation motif at T454 in the encoded protein. The role of phosphorylation in regulating mammalian GRHL function is currently unknown. In this work we show that GRHL3 is phosphorylated at several residues in a human keratinocyte cell line, among them at T454. This site is essential for the full transcriptional activity of GRHL3. The T454 residue is phosphorylated by p38 MAPK *in vitro* and activation of p38 signaling in cells causes an increase in GRHL3 activity. The regulation of GRHL3 function by this pathway is dependent on T454, as the substitution of T454 with methionine inhibits the activation of GRHL3. Taken together, our results show that T454 is one of the phosphorylated residues in GRHL3 in keratinocytes and this residue is important for the upregulation of GRHL3 transcriptional activity by the p38 pathway.

1. Introduction

The Grainyhead-like (GRHL) proteins constitute a family of transcription factors whose first member, Grainyhead (GRH), was discovered in the fruit fly *Drosophila melanogaster* [1]. In mammals there are three members of this family, currently termed GRHL1–3. In the present study, we focus on the GRHL3 transcription factor, which is involved in a wide variety of biological processes. These include cancer development (recently reviewed in [2]), skin barrier formation and maintenance [3], epidermal wound healing [4], neural tube closure (recently reviewed in [5]), craniofacial development (recently reviewed in [6]) and controlling locomotor activity [7].

Phosphorylation is one of the key mechanisms allowing for control of transcription factor activity. It can directly regulate distinct aspects of transcription factor function, including cellular localization, protein-protein interactions and DNA binding [8]. At present, the data on the role of phosphorylation in regulating mammalian GRHL1–3 function is scarce. Thus far, phosphorylation of proteins from the GRHL family was investigated primarily in *D. melanogaster*, where the earliest studies determined that the GRH protein can be phosphorylated *in vitro* by

Abbreviations: ATF-2, activating transcription factor 2; BLNK, B-cell linker; CaMK, calcium/calmodulin-dependent protein kinase; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; CK, casein kinase; DNAPK, DNA-dependent protein kinase; EPHX3, epoxide hydrolase 3; ERK, extracellular signal-regulated kinase; GRHL, Grainyhead-like; GRK, G protein receptor kinase; GSK, glycogen synthase kinase; IVL, involucrin; JNK, c-Jun N-terminal kinase; LC, liquid chromatography; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MS, mass spectrometry; NHEK, normal human epidermal keratinocytes; NMSC, non-melanoma skin cancer; PKA, protein kinase A; PKC, protein kinase C; RSK, ribosomal S6 kinase; SBSN, suprabasin; SCC, squamous cell carcinoma; SNP, single nucleotide polymorphism; WT, wild type

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extracellular signal-regulated kinase 2 (ERK2), also known as mitogen-activated protein kinase 1 (MAPK1) [9]. Further indication of post-translational regulation of this factor was provided by the studies of GRH function in the development of trachea; however, the nature of the postulated modification was not determined [10]. Subsequently it was established that ERK can regulate GRH activity during epidermal wound healing in the fly [11]. Receptor tyrosine kinase Stitcher (STIT) may trigger GRH phosphorylation by ERK in the epidermis, as STIT induces ERK phosphorylation along with GRH-dependent expression of cuticle repair genes at the wound sites [12]. Serine 91 is the principal residue in GRH that is phosphorylated by ERK, but ERK can also phosphorylate GRH at serine 88 and some threonines [13]. These phosphorylation sites are essential for epidermal barrier repair; however, they are not conserved in the mammalian orthologs of GRH.

There is evidence that all the GRHL1–3 transcription factors undergo phosphorylation and other post-translational modifications in human cells. Relevant information is provided by various resources, such as PhosphoSitePlus [14]. However, all the reported phosphorylation sites in GRHL1–3 proteins were assigned using only large-scale proteomic discovery-mode mass spectrometry approaches, which provide minimal sequence coverage of individual proteins [15]. Furthermore, these findings were not followed by more detailed analyses. Thus the post-translational modifications of mammalian GRHL1–3 transcription factors have never been investigated in detail, and their biological significance remains unknown.

Recently it was found that two single nucleotide polymorphisms (SNP), rs41268753 and rs141193530, are associated with non-syndromic cleft palate [16,17]. These two polymorphisms are missense variants located in adjacent codons of the *GRHL3* gene. The occurrence of either SNP abolishes a putative threonine-proline (TP) phosphorylation motif at T454, which could be targeted by proline-directed kinases. Furthermore, in our latest research we observed increased frequency of SNP rs141193530 in human patients with non-melanoma skin cancer (NMSC), which further supports some effect of this change on *GRHL3* functioning and increased risk of disease [18]. However, the molecular mechanism responsible for these effects remains unknown.

In this study we present the results of a comprehensive analysis of phosphorylation of the GRHL3 protein in a human keratinocyte cell line. In particular detail we investigated the phosphorylation of the T454 residue in this protein using site-specific methods. We show that T454 is phosphorylated in cells and is important for upregulation of GRHL3 transcriptional activity by the p38 MAPK pathway.

2. Materials and methods

2.1. Cell culture

Human immortalized keratinocyte HaCaT cells were cultured in DMEM GlutaMAX medium supplemented with 10% fetal bovine serum and 100 IU/mL penicillin-streptomycin at 37 °C in a humidified incubator under 5% CO_2. Cell culture reagents were purchased from Thermo Fisher Scientific. Primary normal human epidermal keratinocytes (NHEK), neonatal, were purchased from Cell Systems (Cat. No. FC-0007), and were cultured using DermaLife K Medium, Complete Kit (Cell Systems, Cat. No. LL-0007), at 37 °C in a humidified incubator under 5% CO_2.

2.2. Plasmids

The pEZ-M12 plasmid with GRHL3 cDNA (termed from now on M12-3xFLAG-GRHL3) and the control plasmid were purchased from GeneCopoeia. M12-3xFLAG-GRHL3 plasmid was used as a template for generating GRHL3^{T454M}, GRHL3^{T454A}, GRHL3^{T454E} and GRHL3^{P455A} mutants using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies), with specific primers. pGEX-GRHL3, -GRHL3^{T454A} and -GRHL3^{P455A} were generated by amplification of GRHL3 cDNA from appropriate M12 plasmids using specific primers and insertion into the pGEX-4T-1 vector (GE Healthcare). pcDNA-MEK6ca (coding for constitutively active MEK6) and pcDNA-MEK6dn (coding for dominant negative MEK6) were constructed by PCR amplification of cDNA sequences from plasmids pcDNA3-Flag-MEK6(Glu) [19], kindly provided by Dr. Li-Jin Chew and Dr. Roger Davis, or pCMV5-FLAG-MKK6-K82M, a gift from John Kyriakis (Addgene plasmid #21583, [20]), respectively, and insertion into the pcDNA3.1(+) vector (Invitrogen). GMFFluc [16], containing a multimerized GRHL3 binding site in the firefly luciferase vector, was a kind gift from Dr. Robert Cornell, pRL-CMV was purchased from Promega. Primers used are listed in Table S1.

2.3. Identification of phosphorylated residues in GRHL3

HaCaT cells cultured in 10 cm plates were transfected with 20 μg of M12-3xFLAG-GRHL3, using Lipofectamine LTX (Thermo Fischer Scientific) according to the protocol recommended by the manufacturer. After 24 h cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 25 mM β-glycerophosphate, 50 mM NaF, 2× Complete Protease Inhibitor Cocktail and 2× PhosSTOP from Roche) and lysates were precleared with agarose beads. Immunoprecipitation was performed from 25 mg of total protein using Pierce Co-Immunoprecipitation Kit (Thermo Fischer Scientific), with mouse monoclonal anti-FLAG M2 antibodies cross-linked to agarose beads (Cat. No. F2426, Sigma), according to the protocol recommended by the manufacturer. Proteins bound to the beads were resolved by SDS-PAGE. The band corresponding to FLAG-GRHL3 was excised from the gel and sent to the Taplin Mass Spectrometry Facility, Harvard Medical School, for identification of phosphorylated residues. Further procedures were performed as described in [21], with the exception that apart from in-gel trypsin digestion, part of the sample was also digested with chymotrypsin.

2.4. In silico prediction of kinases that may phosphorylate residues in the GRHL3 protein

We took advantage of the following publicly available online tools to investigate which kinases are likely to phosphorylate amino acid residues identified in the GRHL3 protein: KinasePhos [22], PhosphoMotif Finder [23] and NetPhos [24].

2.5. Immunofluorescent staining

HaCaT cells cultured on glass slides in 24-well plates were transfected with 1 μg of M12-3xFLAG-GRHL3 plasmid or its mutated versions as described above. After 24 h cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and non-specific binding sites were blocked with 4% BSA. Cells were incubated with primary mouse anti-FLAG M2 antibody (1:400) (Cat. No. F1804, Sigma) and then with Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody (Cat. No. R37115, 1:200) (Thermo Fisher Scientific). Nuclei were counterstained by DAPI in mounting medium (Vectashield, DAKO) and the images were obtained using Zeiss AxioObserver Z.1 microscope equipped with appropriate fluorescence filters and monochromatic camera.

2.6. RT-qPCR

HaCaT cells cultured in 6-well plates were transfected with 2.4 μg of M12-3xFLAG-GRHL3 plasmid, or its mutated versions, using Lipofectamine 3000 (Thermo Fischer Scientific) according to the protocol recommended by the manufacturer. After 24 h total RNA was extracted using the GeneMATRIX Universal RNA Purification Kit (EURx). Next, 1 μg of total RNA was reverse-transcribed with the ReadyScript cDNA Synthesis Mix (Sigma). Levels of gene expression were assayed using Applied Biosystems chemistry (Thermo Fisher