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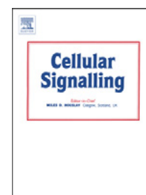

Disease-associated mutations in IRF6 and RIPK4 dysregulate their signalling functions

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ABSTRACT

IRF6 and RIPK4 are critical regulators of keratinocyte differentiation and their mutation cause the developmental syndromes Van der Woude syndrome (VWS) and Bartsocas-Papas syndrome (BPS), respectively. RIPK4 promotes keratinocyte differentiation, in part, by inducing IRF6 transactivator function through the phosphorylation of its C-terminal domain at Ser413 and Ser424. Although more than 200 IRF6 mutations have been identified in VWS, a p.Arg412X nonsense mutation is particularly prevalent. A RIPK4 p.Ser376X nonsense mutation in BPS was also recently identified. Here, we demonstrated for the first time that the truncation of IRF6 at Arg412 causes its rapid proteasome-dependent degradation. The truncation of IRF6 also prevented the induction of its transactivator function by RIPK4. Similarly, the p.Ser376X mutation in RIPK4 impaired its induction of IRF6 transactivator function. The mutation also inhibited the stabilisation of β-catenin by RIPK4, and thus may additionally impair Wnt signalling. Collectively, our findings provide important mechanistic insight into how the p.Arg412X and p.Ser376X mutations may cause VWS and BPS, respectively.

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1. Introduction

The formation of the skin as well as many associated structures (e.g. lips, oral cavity, digits, and genitalia) relies upon a balance between keratinocyte proliferation and differentiation. This requires the correct spatial and temporal expression of interconnected regulatory gene networks [1]. The dysregulation of these networks in utero can manifest in various abnormalities, such as cleft lip/palate. Van der Woude syndrome (VWS) is the most common syndromic form of cleft lip/palate [2]. Clefting of the lip/palate also occurs in popliteal pterygium syndrome (PPS), which is characterised further by additional abnormalities, including skin webbing, syndactyly, and genital malformations [3]. Bartsocas-Papas syndrome (BPS) is a more severe form of popliteal pterygium and typically results in death early in life [4].

VWS and PPS are caused by mutations in interferon regulatory factor 6 (IRF6) [5], while BPS is caused by mutations in receptor-interacting protein kinase 4 (RIPK4) [6,7]. IRF6 is a key transcriptional regulator of keratinocyte differentiation [8–10]. In the mouse, Irf6-deficiency results in a greatly expanded epidermal spinous layer and the absence of the granular and cornified layers; it also causes intra-oral adhesions [8]. Ripk4-deficient mice similarly have a greatly expanded and dysregulated epidermis, resulting in all external orifices being fused [11]. We recently demonstrated that IRF6 operates downstream of RIPK4 to promote keratinocyte differentiation [10]. In this context, RIPK4 induces IRF6 transactivator function by phosphorylating regulatory residues (Ser413 and Ser424) in the C-terminal domain (CTD) of IRF6 [10]. RIPK4 can also activate other regulators of keratinocyte differentiation, including NF-κB and JNK [12,13].

More than 200 different IRF6 mutations have been identified in VWS [14]. However, a p.Arg412X nonsense mutation predicted to cause the truncation of IRF6 at Arg412 was the most common mutation identified in two geographically separate cohorts of >300 families with VWS [15]. The same mutation was also identified in other studies [5,16–21], suggesting that it represents a mutational hotspot in VWS. A small number of RIPK4 mutations in BPS have been identified [6,7]. In addition to missense mutations, including those that inhibit its kinase activity [6,10], a nonsense mutation (p.Ser376X) was also identified [7]. RIPK4 is characterised by a serine/threonine kinase domain, an intermediate domain, and a C-terminal ankyrin repeat domain [12,22]. Ser376 is located in the intermediate domain. Interestingly, caspase-dependent cleavage in the intermediate domain (after Asp340 and/or Asp378) may serve to repress RIPK4 activity by allowing the ankyrin repeat domain to inhibit the kinase domain in *trans* [12].

Given the clinical significance of the p.Arg412X and p.Ser376X mutations, we sought to establish their impact on the signalling functions of

Abbreviations: ARD, ankyrin repeat domain; BPS, Bartsocas-Papas syndrome; CTD, C-terminal domain; DBD, DNA-binding domain; IAD, IRF-association domain; IRF, interferon regulatory factor; PPS, popliteal pterygium syndrome; RIPK, receptor-interacting protein kinase; VWS, Van der Woude syndrome.

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IRF6 and RIPK4. Our data indicate that the truncation of IRF6 at Arg412 not only makes it far more susceptible to proteolytic degradation, but also prevents its activation by RIPK4. The p.Ser376X mutation in RIPK4 impairs IRF6 activation as well as inhibits its stabilisation of the Wnt signalling protein β-catenin. This study therefore provides molecular explanations for how these mutations may cause VWS and BPS.

2. Materials and methods

2.1. Material

Cell culture medium, foetal calf serum (FCS), Penicillin–Streptomycin, GlutaMax-1, precast 10% Nu-PAGE gels, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG antibodies, and ProLong Gold Antifade reagent were from Life Technologies. The anti-IRF6 antibody, anti-phospho JNK and anti-JNK antibodies, and HRP-conjugated anti-HA antibody were from Cell Signaling. The anti-β-catenin and anti-HSP90 antibodies were from BD Biosciences. Cycloheximide, MG132, anti-β-tubulin antibody, and agarose-conjugated, HRP-conjugated, and unconjugated anti-FLAG antibodies were from Sigma. Concanavalin A-Sepharose beads were from GE Healthcare. Restriction enzymes were from New England Biolabs, and *Pfu* DNA polymerase was from Promega. PCR primers were synthesised by GeneWorks, Complete protease inhibitors were from Roche, and [γ-^{32}P] ATP (3000 Ci/mmol) was from PerkinElmer Life Sciences.

2.2. Expression and gene promoter reporter plasmids

The IRF6 expression plasmids, pEF-HA-IRF6 and pEF-V5-IRF6 (express HA- and V5-tagged human IRF6, respectively), and the GST-IRF6 fusion protein expression plasmid, pGEX-IRF6 CTD (expresses the IRF6 C-terminal domain fused to GST), are as described previously [10,23]. The IRF6 expression plasmid, pEF-HA-IRF6 R412X (lacks Arg412 to Gln467), was created by PCR using the primer pair 5′-GCCCTCCACCCCCGCAGAGTCCGGCTAAAG-3′ and 5′-TCATGTGAAATCACCAGAAAACATCTCGTA-3′. The Ripk4 expression plasmid, pCMV-FLAG-Ripk4 (expresses FLAG-tagged mouse Ripk4), was a generous gift from Dr Shiv Pillai (Harvard Medical School, USA) [22]. The Ripk4 S378X expression plasmid (Ser378 is equivalent to Ser376 in human RIPK4) was created using the mutagenic primer pair 5′-CTCGGGGGTGTCCTAAGTGGACTCAGC-3′ and 5′-GCTGAGTCCACTTAGGACACCCCCGAG-3′ and a QuikChange II site-directed mutagenesis kit (Agilent Technologies). The human JNK expression plasmid, pXJ41-HA-JNK, was a generous gift from Dr Marie Bogoyevitch (The University of Melbourne) [24]. The E-selectin and interferon-β (IFNβ) gene promoter reporter plasmids are as described previously [10]. The control *Renilla* luciferase reporter plasmid pRL-TK was from Promega.

2.3. Expression and purification of GST-IRF6 CTD

The expression and purification of a GST fusion protein containing the IRF6 CTD was as recently described [10]. Briefly, BL21(DE3) *Escherichia coli* transformed with the pGEX-IRF6 CTD expression plasmid were induced with IPTG (0.4 mM) for 2–3 h at 25 °C, followed by lysis via sonication in ice-cold phosphate-buffered saline (PBS) supplemented with 1.0% Triton X-100, DNase I (30 μg/ml) and protease inhibitors. The lysate was clarified by centrifugation (27,000 × *g* for 40 min at 4 °C) and the GST-IRF6 CTD fusion protein purified by affinity chromatography using a GSTrap FF column (GE Healthcare).

2.4. Cell culture and transient transfection

HEK293T cells were cultured in Dulbecco's-modified Eagle's medium supplemented with 10% FCS, 100 units/ml Penicillin, 100 μg/ml Streptomycin, and 2 mM GlutaMax-1. OKF6/TERT-2 cells [25] were cultured in Keratinocyte serum-free medium supplemented with 25 μg/ml bovine pituitary extract, 0.2 ng/ml EGF, 0.4 mM $CaCl_2$, 100 units/ml Penicillin, 100 μg/ml Streptomycin, and 2 mM GlutaMax-1. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. HEK293T cells were transfected using FuGENE 6 (Promega), while OKF6/TERT-2 cells were transfected using Lipofectamine 2000 (Life Technologies).

2.5. Immunofluorescent staining and confocal microscopy

HEK293T cells (which were seeded on glass coverslips prior to transfection) were fixed with 4% paraformaldehyde (30 min), solubilised with 0.1% Triton X-100 (5 min), and blocked in 5% goat serum (60 min). The cells were subsequently stained overnight at 4 °C with a mouse anti-FLAG antibody or rabbit anti-IRF6 antibody. Following washing with PBS, the cells were incubated at room temperature with an Alexa Fluor 594-conjugated goat anti-mouse IgG antibody or an Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody for 60 min. The cells were washed with PBS, mounted on glass microscope slides using ProLong Gold Antifade reagent containing DAPI, and allowed to cure for 24 h in the dark. Images were acquired on an Olympus FV1000 scanning confocal microscope. No anti-FLAG or anti-IRF6 staining was apparent in HEK293T cells transfected with empty vector only.

2.6. Cell lysis and Western blotting

Cells were washed twice with ice-cold PBS and then lysed (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 10 mM NaF, 10 mM β-glycerol phosphate, and protease inhibitors) on ice for 60 min. The lysates were clarified by centrifugation (13,000 × *g* for 10 min at 4 °C) and protein concentrations measured using a protein assay kit (Bio-Rad). Cell lysates were subjected to SDS-PAGE, followed by Western blotting according to standard protocols. Immunoreactive bands were visualised using ECL reagents (Millipore) and a LAS-3000 Imager (Fujifilm) or by exposure to x-ray film (Fujifilm), which was scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad).

2.7. In vitro dephosphorylation

Transfected HEK293T cells were lysed as above, except that phosphatase inhibitors were omitted. Protein dephosphorylation was carried out in 50 μl reactions consisting of 50 μg of cell protein and 10 units of calf intestinal phosphatase (CIP). The reactions were incubated at 37 °C for 30 min, followed by SDS-PAGE and Western blotting.

2.8. Immunoprecipitation

FLAG-tagged Ripk4 proteins were immunoprecipitated from cell lysates by incubating 1 mg of cell lysate with 10 μl of anti-FLAG agarose beads for 4 h at 4 °C with constant mixing. The beads were washed four times with lysis buffer, and then subjected to Western blotting and/or used for in vitro kinase assays.

2.9. In vitro kinase assay

Anti-FLAG (RIPK4) immunoprecipitates were incubated for 30 min at 30 °C in kinase assay buffer (20 mM HEPES (pH 7.4), 25 mM $MgCl_2$, 3 mM $MnCl_2$, 10 mM β-glycerol phosphate, 10 μM ATP, 5 μCi [γ-^{32}P] ATP) containing 1 μg of GST-IRF6 CTD or GST. The reactions were terminated by the addition of SDS-PAGE sample buffer and heating at 95 °C for 5 min, after which they were subjected to SDS-PAGE. The gel was stained with Coomassie G-250, dried and ^{32}P incorporation detected with a Typhoon Trio Phosphorimager (GE Healthcare).

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