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Original Article

pVHL mediates K63-linked ubiquitination of IKKβ, leading to IKKβ inactivation

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ABSTRACT

Nuclear factor (NF)-κB is a transcription factor that plays an important role in many biological functions. Regulation of NF-κB activity is complicated, and ubiquitination is essential for NF-κB activation. Hypoxia can activate NF-κB. However, the underlying mechanism remains unclear. pVHL is a tumour suppressor and functions as an adaptor of E3-ligase. In this study, we demonstrated that pVHL inhibits NF-κB by mediating K63-ubiquitination of IKKβ, which is dependent on oxygen. We found that pVHL mediates K63-linked ubiquitination of IKKβ, which is an upstream regulator of NF-κB. The pVHL-mediated K63-ubiquitination of IKKβ prevents TAK1 binding, which leads to the inhibition of IKKβ phosphorylation and NF-κB activation. pVHL-mediated K63-ubiquitination of IKKβ is inhibited under hypoxia. DMOG, which is a specific inhibitor of prolyl hydroxylases, also suppresses K63-ubiquitination of IKKβ. Prolyl hydroxylase (PHD) 1 enhances K63-ubiquitination of IKKβ and inhibits IKKβ phosphorylation. These results suggest a novel function for pVHL in mediating K63-linked ubiquitination of IKKβ, which plays a role in the regulation of IKK/NF-κB signalling. The results also provide new insight into the mechanism of NF-κB activation through hypoxia.

Introduction

Nuclear factor (NF)-κB is a transcription factor and is associated with diverse biological and pathological processes [1,2]. In most cells, NF-κB proteins are normally inactive because they are sequestered in the cytoplasm by the IκB family of inhibitory proteins. Extracellular stimuli, such as TNFα and IL-1β, lead to activation of the IκB kinase (IKK) complex. Activated IKK phosphorylates IκB, which results in its release from NF-κB [3,4]. This release allows for translocation of NF-κB from the cytoplasm to the nucleus, where it regulates gene expression. IKK contains two catalytic subunits, IKKα and IKKβ, and one regulatory scaffold protein called NEMO (also known as IKKγ). Activation of IKK requires phosphorylation of the T loop serines of IKKα and IKKβ [5].

The protein pVHL, which is a product of the von Hippel-Lindau (*VHL*) tumour suppressor gene, acts as a multipurpose adaptor protein that controls a diverse array of gene expression programs as well as extracellular matrix assembly and microtubule-based processes [6]. It functions as an adaptor of ubiquitin ligase that targets the alpha subunit of the hypoxia-inducible factor (HIFα) for ubiquitination and degradation by the 26S proteasome [7,8]. In addition to HIFα, pVHL has been found to have other targets and functions, and it has been implicated in diverse cellular processes, including cell division, apoptosis, differentiation, and control of extracellular matrix formation [7,8]. We recently demonstrated that pVHL mediated lysine (K)63-linked ubiquitination of nuclear clusterin, which led to its nuclear translocation [9].

Ubiquitination is a post-translational modification that has a pivotal role in numerous biological functions. Although ubiquitination is thought to achieve these functions by targeting proteins for degradation, many studies suggest that it also has non-proteolytic functions, such as protein trafficking, kinase and phosphatase activation [10]. Seven lysine (K) residues (K6, K11, K27, K29,

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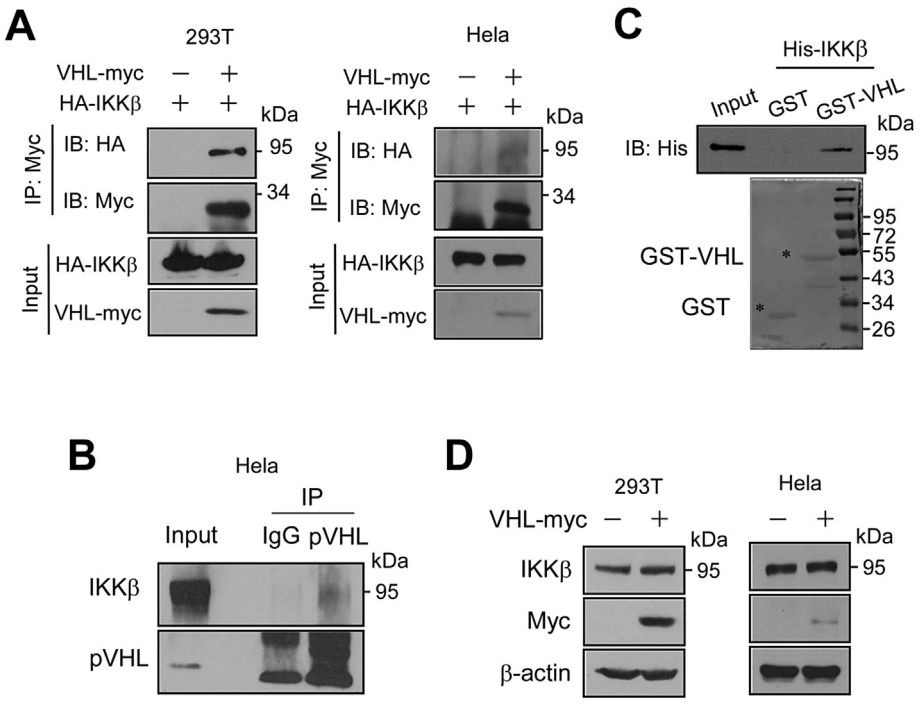

Fig. 1. pVHL bound IKKβ. (A) 293T and Hela cells were transfected with VHL-myc and HA-IKKβ. In 24 h, the cells were harvested and immunoprecipitation was performed using a Myc antibody. The data are representative of three independent experiments. (B) Cellular extracts from cells were used in immunoprecipitation experiments with an anti-pVHL antibody. Following fractionation of the immunoprecipitates by SDS-PAGE, immunoblotting was performed using an anti-IKKβ antibody. The data are representative of three independent experiments. (C) GST-pVHL and His-IKKβ proteins were produced in *E. coli*, and His-IKKβ was purified with a nickel column. The bacterial lysates containing GST-pVHL were incubated with glutathione-Sepharose 4B beads at 4 °C for 2 h. Then, the beads were washed and incubated with His-IKKβ purified protein at 4 °C overnight. The beads were washed and boiled in SDS-PAGE loading buffer for 5 min. The supernatants were collected and resolved on SDS-PAGE. (D) Overexpression of pVHL did not affect the total protein level of IKKβ. 293T and Hela cells were transfected with Myc-VHL. After 24 h, the cells were harvested, and the protein levels of IKKβ were determined with a Western blot.

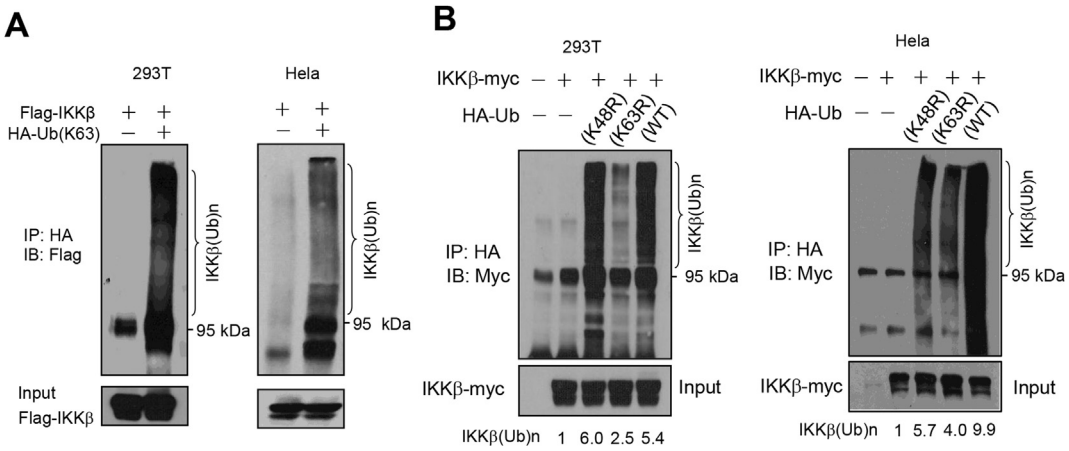

Fig. 2. IKKβ had K63-linked ubiquitination. (A) Overexpression of Ub(K63) enhanced ubiquitination of IKKβ. The cells were transfected with Flag-IKKβ or Flag-IKKβ plus HA-Ub(K63) as indicated. In 24 h, the cells were harvested and lysed in RIPA buffer. The cellular proteins were prepared and used for immunoprecipitation and immunoblotting. The data are representative of three independent experiments (B) 293T and Hela cells were transfected with Myc-IKKβ and various Ub plasmids as indicated. In 24 h, the cells were harvested and lysed in RIPA buffer as described in the Methods section. Cellular proteins were prepared for immunoprecipitation with an HA antibody and immunoblotting with a Myc antibody. The data are representative of three independent experiments. WT, wild-type; IKKβ(Ub)n, polyubiquitinated IKKβ.

K33, K48 and K63) were found in ubiquitin (Ub), and polyubiquitin chains involving those sites have been identified. The K48-linked ubiquitination is recognized by the 26S proteasome and triggers protein degradation. K63-linked ubiquitination does not trigger protein degradation. Instead, it has non-proteolytic functions [11–13].

Ubiquitination plays an essential role in the regulation of NF-κB activity. At least three steps in NF-κB activation directly involve ubiquitination: proteasomal degradation of IκB, processing of NF-κB precursors, and activation of transforming growth factor (TGF)-β-activated kinase 1 (TAK1) and IKK complexes [14–16]. K48-linked ubiquitination of IκBα leads to IκBα degradation and NF-κB activation. The majority of the key proteins, including receptor interaction protein (RIP), TAB2 and NEMO, in the IKK activation modules are modified by the K63-linked polyubiquitin chain, which is essential for IKK activation [16–19]. IKKβ has K48-linked ubiquitination at K555, which leads to its proteasomal degradation [20]. IKKβ was also found to have mono-ubiquitination, which inhibits