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Mutations in the zinc finger domain of IKKγ block the activation of NF-κB and the induction of IL-2 in stimulated T lymphocytes

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Dedicated to the loving memory of Marshall S. Horwitz.

Abstract

Mutations in the zinc finger of IκB kinase gamma (IKKγ) are associated with hypohidrotic ectodermal dysplasia-immune deficiency (HED-ID) in which the major immune deficit is the inability to switch Ab heavy chain class. However, the pathophysiologic role of the mutations has not been fully delineated. Since help from activated Th cells is essential in Ab class switching, we sought to examine how these mutations affect T cell activation. Using a human T cell line that was null for IKKγ, we generated cells stably expressing two of the reported mutations, namely, D406V and C417R. Cells expressing either mutation failed to induce IL-2 following stimulation with PMA/ionomycin while the induction of IL-2 was restored in cells reconstituted with the wild type IKKγ. The lack of IL-2 upregulation correlated with the lack of NF-κB activation as evidenced by the inability to induce IκBα degradation, NF-κB binding to DNA and the expression of a reporter gene. However, both mutations did not prevent the incorporation of IKKγ into the IKK complex and, interestingly, the induced phosphorylation of IκBα at S32 and S36 and its subsequent ubiquitination were not affected. The suppression of IL-2 induction was solely due to the inhibition of NF-κB activation as the mutations did not impair the activation of AP-1 and NFAT. Our data indicated that the failure of T cells to undergo activation in response to TCR stimuli may play a role in the pathophysiology of HED-ID and also showed that IKKγ has a role in the post-ubiquitination processing of IκBα.

Keywords: T Cells; Immunodeficiency diseases; Cytokines; T cell receptors; Cell activation

1. Introduction

Three categories of mutations in IκB kinase gamma (IKKγ), also known as NF-κB essential modulator (NEMO), have been associated with human disease: hypomorphic mutations typically involving the zinc finger domain causing hypohidrotic ectodermal dysplasia-immune deficiency (HED-ID) in males; amorphic mutations causing incontinentia pigmenti in females and, generally, pre-natal death in males; and hypomorphic mutations at the stop codon causing anhidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis and lymphedema in males (Aradhya et al., 2001a; Doffinger et al., 2001; Jain et al., 2001; Zonana et al., 2000). The immune deficiency identified in individuals with HED-ID is usually characterized by high levels of IgM and low levels of IgG and IgA, a syndrome referred to as hyper IgM (HIGM) syndrome, but sometimes the immune abnormalities can be highly variable. Most cases of HIGM syndrome are X-linked and are caused by mutations in the CD154 (CD40 ligand) gene that results in defective expression of CD154 on activated helper T cells (Etzioni and Ochs, 2004; Fuleihan, 2001; Schneider, 2000). The remaining cases of HIGM syndrome are caused by mutations in CD40 (the receptor for CD154), activation-induced cytidine deaminase or uracil-DNA glycosylase, all of which are mainly expressed in B cells (Etzioni

Abbreviations: IKKγ, IκB kinase gamma; NEMO, NF-κB essential modulator; HED-ID, hypohidrotic ectodermal dysplasia-immune deficiency; HIGM, hyper IgM; JNK, Jun N-terminal kinase; p-JNK, phospho-JNK; TNFR, TNF receptor; qPCR, quantitative PCR; FC, flow cytometry; Th, helper T cell; Ab, antibody; mAb, monoclonal Ab; PMA, phorbol myristyl acetate; NFAT, nuclear factor of activated T lymphocytes; TCR, T cell receptor; aa, amino acid; FBS, fetal bovine serum; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence reagent; EMSA, electrophoretic mobility shift assay; NK cell, natural killer cell.

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and Ochs, 2004; Fuleihan, 2001; Schneider, 2000) or by mutations in IKKγ, which is expressed ubiquitously (Aradhya et al., 2001b).

IKKγ was identified as a non-catalytic component of the IKK complex which is essential for the full activity of the complex. The IKK complex consists of IKKα, IKKβ and IKKγ and is responsible for the activation of the NF-κB pathway by phosphorylating the IκB proteins (Li et al., 1999; Cohen et al., 1998; Yamaoka et al., 1998; Rothwarf et al., 1998). The human IKKγ gene is located on chromosome Xq28 (Jin and Jeang, 1999). The IKKγ protein consists of 419 aa residues with a zinc finger spanning residues 397 to 419 (Li et al., 1999). IKKγ is required for the activation of NF-κB by various stimuli, including TNFα, IL-1 and LPS in various cell types (Krappmann et al., 2000; Makris et al., 2002; Rudolph et al., 2000). However, the exact roles of IKKγ in the activation of the NF-κB pathway have not yet been fully characterized. IKKγ has been shown to be required for the normal development and for the activation of T cells (Nishikomori et al., 2004; Schmidt-Supprian et al., 2003). Jurkat cells deficient in IKKγ failed to secrete IL-2 following TCR stimulation due to the inability to induce IκBα degradation and NF-κB binding to DNA (He and Ting, 2003). In addition to its roles as a regulatory component of the IKK complex, IKKγ has been shown to interact with many other proteins, such as caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1) (Stilo et al., 2004), connection to IKK and SAPK/JNK (CIKS) (Leonardi et al., 2000), cylindromatosis (CYLD) protein (Kovalenko et al., 2003) and ubiquitinated receptor interacting protein (Kawadler and Yang, 2006). Thus, it is possible that mutations in the zinc finger domain of IKKγ could affect the ability of IKKγ to interact with those proteins and affect NF-κB activation via pathways that require the participation of IKKγ.

TCR engagement triggers several signaling cascades, which eventually lead to the activation of three transcription factors, NF-κB, AP-1 and NFAT (Huang and Wange, 2004). The activation of NF-κB requires the IKK-mediated phosphorylation of the IκB proteins, which, in the basal state, sequester the NF-κB subunits in the cytoplasm (Li and Stark, 2002; Rothwarf and Karin, 1999). Phosphorylated IκB proteins are ubiquitinated by the E3-SCF$^{\beta\text{-TrCP}}$ complex (Spencer et al., 1999) and subsequently degraded by the proteasomal pathway. This releases the NF-κB subunits which are then able to form dimers, translocate to the nucleus and function as transcription factors (Li and Stark, 2002; Perkins, 1997). The activation of AP-1 involves the phosphorylation of JNK; JNK in turn phosphorylates c-Jun, which then forms heterodimers with the other members of the AP-1 family of proteins resulting in the generation of dimers that function as transcription factors (Wisdom, 1999). The activation of NFAT involves the de-phosphorylation of NFAT by the calcium-calmodulin-regulated calcineurin, an event that allows NFAT to translocate to the nucleus and regulate gene expression (Huang and Wange, 2004). The combined activation of NF-κB, AP-1 and NFAT leads to the induction of IL-2 expression, which is a major marker of T cell activation (Gaffen and Liu, 2004). A deficiency in the activation of any one of those three transcription factors results in the impairment of IL-2 upregulation.

Some of the point mutations that are associated with HED-ID have been studied by other investigators in various cell types, including dendritic cells obtained from HED-ID patients (Temmerman et al., 2006), monocytes derived from HED-ID patients (Jain et al., 2001), human monocytes with an endogenous IKKγ expression (Yang et al., 2004), IKKγ-null human T cells (Tang et al., 2003), IKKγ-null mouse fibroblasts (Makris et al., 2002) and IKKγ-null mouse B cells (Huang et al., 2002). The results of those studies have been conflicting, with the effect on NF-κB activation being dependent on the stimulus and the cell type. However, the effects of the mutations on NF-κB activation via the TCR and on IL-2 induction remain unknown. Therefore, we examined two of the reported mutations of IKKγ in an IKKγ-null Jurkat cell line to understand the effects of the mutations on IL-2 induction and to characterize the roles of IKKγ in NF-κB activation. Here, we show that the IKKγ mutations D406V and C417R completely abolished the induction of IL-2 by blocking the activation of NF-κB. The mutations inhibited the degradation of IκBα but they did not prevent the serine phosphorylation of IκBα at its two key sites and its subsequent ubiquitination, indicating the existence of an additional role for IKKγ in the post-ubiquitination processing of IκBα.

2. Materials and methods

2.1. Cells

IKKγ-positive (SVT35) and IKKγ-null (SVT26) Jurkat cell lines were obtained from Dr. Shao-Cong Sun (Pennsylvania State University, Hershey, PA) (Harhaj et al., 2000). Jurkat cells were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 100 U/ml penicillin (Mediatech), 100 μg/ml streptomycin (Mediatech) and 50 μM 2-ME (Sigma). For cell stimulation, PMA was used at 50 ng/ml, ionomycin at 1 μM and TNFα at 20 ng/ml, unless otherwise indicated.

2.2. Plasmids

The plasmid pcDNA3-T7-IKKγ was described before (Li et al., 1999). Mutations were introduced using *Pfu* Turbo DNA polymerase (Stratagene). For the D406V (1217A > T) mutant IKKγ, overlapping fragments were generated by using the following two forward/reverse primer pairs: 5′-aacaggaggtgatcgataagct-3′/5′-ccataacaggggcctgatactgg-3′ and 5′-ggcccctgttatggacaccctgc-3′/5′-gtggttcgagcagacagaagg-3′. For the C417R (1249T > C) mutant IKKγ, overlapping fragments were generated using the following two forward/reverse primer pairs: 5′-aacaggaggtgatcgataagct-3′/5′-ctactcaatgcgctccatgac-3′ and 5′-ggagcgcattgagtagggc-3′/5′-gtggttcgagcagacagaagg-3′. The PCR conditions for the generation of the overlapping fragments were as follows: initial denaturation at 95 °C for 2 min; 35 cycles with denaturation at 95 °C for 30 s, annealing for 30 s at 54 °C for the D406V mutant and at 57 °C for the C417R mutant and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. Overlap extension was done using the forward/reverse primer pair 5′-aacaggaggtgatcgataagct-3′/5′-