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Acetylation regulates the MKK4-JNK pathway in T cell receptor signaling

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ABSTRACT

T cell functions are regulated by multiple signaling cascades, including the MKK4-JNK (c-Jun NH_2 terminal kinase) pathway. However, the mechanism regulating the MKK4-JNK axis in T cells remains unclear. Herein, we demonstrated that protein acetylation modulates JNK activity induced by T cell receptor (TCR) activation. The acetyltransferase, CREB-binding protein (CBP), is transported from the nucleus to the cytoplasm in response to TCR cross-linking. To investigate the role of CBP in TCR signaling, we overexpressed CBP in the cytoplasm of Jurkat cells, a human T lymphocyte line. Enforced expression of cytoplasmic CBP led to MKK4 acetylation and interfered with MKK4-mediated JNK phosphorylation. Insufficient JNK activity decreased the activity of the transcription factor, AP-1. In contrast, other transcription factors, NF-κB and NFAT, stimulated with anti-CD3 and anti-CD28 antibodies were activated normally in the presence of cytoplasmic-CBP. These results provide valuable insights into the role of acetylation in MKK4-JNK signaling in T cells.

1. Introduction

Lymphocyte activation is a key step in the adaptive immune response. Activated T cells undergo clonal expansion and acquire the capacity to kill target cells infected with pathogens or produce cytokines essential for regulating the immune response. T cell activation and proliferation are initiated by the interaction of the T cell receptor (TCR) with antigen peptides presented in the context of a major histocompatibility complex (MHC) molecule by antigen presenting cells (APCs). In addition to the antigen signal, a co-stimulatory signal is generated through the interaction between the B7/B7-2 molecule on APCs and CD28 on T cells [1]. TCR engagement activates several signaling pathways, resulting in the transcriptional activation of numerous genes. Within minutes of antigenic stimulation, a complex network of signal transducers enhances the transient transcription of early activating genes, which in turn regulates nuclear events essential for T cell survival, proliferation, differentiation, effector function, and cytokine release [2,3]. Among these early genes, c-Jun [4–6], a component of the transcription factor complex, activator protein 1 (AP-1), plays a critical role in diverse physiological processes in T cells, such as regulating IL-2 expression [7,8], stabilizing the interaction of nuclear factor of activation T cells (NFAT) with DNA [9], and regulating the induction of anergy [10,11]. In particular, the binding of Jun and Fos proteins to the AP-1 site in promoters is essential for the transcription of several cytokines and other gene products that regulate the immune response [12].

In response to extracellular stimulators such as growth factors, cytokines, or stress signals, c-jun is phosphorylated by c-Jun NH_2-terminal kinase (JNK) [13,14]. MKK4 (also termed SEK1 or JNKK) is the first kinase to specifically and directly phosphorylate JNK, resulting in JNK activation [15]. A second JNK activator, MKK7, has been identified in mammalian cells [16–18] and is considered to be redundant with MKK4. MKK4 and MKK7 share similar molecular characteristics, as well as sharing several upstream activators and scaffold proteins. While both MKKs synergistically phosphorylate JNK, MKK4-deficient cells are defective in both JNK activation and transcriptional induction of the AP-1 complex [19,20]. MKK4 is known to be involved in a variety of physiological processes. For example, in mice *mkk4* mRNA is exclusively expressed in the central nervous system up until embryonic day 10 (E10) [21] and mice lacking *mkk4* (*mkk4*$^{-/-}$) die between E11.5 and E13.5, as a result of anemia and abnormal hepatogenesis [19,20]. MKK4 is also important in the immune system, since it has been shown that *mkk4*$^{-/-}$ mice have smaller thymi, along with decreased numbers of peripheral T cells [22].

The function of the JNK pathway in T cells has been studied extensively. These studies have demonstrated that MKK4 plays an

Abbreviations: AP-1, activation protein 1; CBP, CREB-binding protein; CREB, camp response element binding; ER, estrogen receptor; ERK, extracellular-signlaing-regulated kinase; JNK, c-Jun HN_2 terminal kinase; NFAT, nuclear factor of activated T cell; NF-κB, nuclear factor κB; ZAP70, ζassociated protein-70

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important role in T cell development in the thymus [22,23]. In mature T cells, the MKK4-JNK pathway has been shown to play a role in the signal integration that occurs following stimulation by antigen and co-stimulatory receptors, leading to the activation of AP-1-directed transcription [24,25]. In addition, MKK4 is required for maintaining T cell homeostasis [26]. Thus, MKK4 plays an important role in both the development of T cells in the thymus, as well as in the function of mature T cells in the periphery. In addition to T cell activation, activation of the MKK4-JNK pathway can also lead to apoptotic cell death, a process which is necessary to avoid excessive T-cell activity and tissue damage which can occur upon prolonged JNK activation [27]. It is known that the ubiquitin ligase, ITCH, plays an important role in regulating MKK4 signaling [28]. Following MKK4 activation, ITCH binds to MKK4 and ubiquitinates it at specific lysine residues. This ubiquitination promotes MKK4 degradation, and thus prevent long-term MKK4 activation.

The molecular mechanisms responsible for modulating the MKK4-JNK axis and down-regulating AP-1 activity, remain a critical missing link in understanding the control of T cell function. IL-2-activated Stat5 undergoes dynamic post-translational modifications, including both phosphorylation and acetylation. Recently, we showed that CREB-binding protein (CBP)-mediated acetylation can regulate Stat5 activity in T cells [29]. These results suggest that CBP might function as a cytoplasmic regulator of T cell signaling. It is therefore possible that CBP is also an important regulator of TCR-mediated MKK4 activity. Based on this, we hypothesized that CBP recruitment to the cytoplasm modifies the JNK signaling cascade after TCR activation. In this study, we found that CBP was transported to the cytoplasm following TCR activation, and that ectopic expression of CBP in the cytoplasm attenuated JNK activity and led to the acetylation of MKK4. Cytoplasmic CBP-expressing Jurkat cells exhibited a defect in AP-1 activity, suggesting that CBP recruitment to the cytoplasm may be therefore be important in regulating T cell function.

2. Materials and methods

2.1. Cell culture

Human leukemia Jurkat cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin G, and 200 μg/mL streptomycin at 37 °C under a humidified atmosphere of 5% (v/v) CO_2 as previously described [30]. For transient gene expression, 3.0×10^6 cells were added to electroporation cuvettes (0.4 cm gap, Bio-Rad, Hercules, CA, USA) together with 10 μg of reporter plasmids encoding either NFAT-luc, NF-κB-luc, or AP-1-Luc [31]. The plasmid/cell mixture was incubated for 5 min on ice and the Jurkat cells were then electroporated at 300 mV and 950 μF using a Gene Pulser electroporation system (Bio-Rad). Cells were incubated for 5 min on ice prior to resuspension in 2 mL of RPMI-1640 medium supplemented with 10% FCS, transferred to 6-well culture plates, and cultured at 37 °C and 5% CO_2 for 48 h.

2.2. Plasmids

The cDNA encoding CBP was cloned into the expression plasmid pcDNA3. A modified estrogen receptor (ER), prepared from pMXs by PCR, was constructed with a FLAG tag and fused to the amino terminus of CBP (ERCBP). Site-directed mutagenesis (QuikChange, Stratagene, San Diego, CA, USA) was used to generate a catalytically dead CBP mutant [32] and a CBP lacking the putative nuclear localization signal [33]. MKK4 and JNK1, amplified from Jurkat cells by RT-PCR, were cloned into the expression vectors pCMV tag 3B and pCMV tag 2B, respectively. These plasmids were also electroporated into Jurkat cells using a Gene Pulser. All experiments were performed according to the guidelines approved by the Toho University Safety Committee for Recombinant DNA Experiments (16-51-307).

2.3. Immunoprecipitation and immunoblot analysis

Jurkat cells were lysed in Nonidet P-40 cell extraction buffer (1% Nonidet P-40, 25 mM Tris-HCl pH 7.5, 140 mM NaCl, 2 mM EDTA 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, 10 mM NaF, and 1 mM Na_3VO_4). Following removal of nuclei and other cellular debris by centrifugation (12,000*g* for 30 min at 4 °C), the lysates were precleared with control IgG and protein G-Sepharose (Sigma, St Louis, MO, USA) (30 μL/sample of a 1:1 slurry). After pre-clearing, the lysates were incubated overnight with either an anti-MKK4 antibody, an anti-c-Jun antibody, or an anti-acetyl lysine antibody. Specific immunoprecipitates were recovered by the addition of protein G-Sepharose beads for 2 h, and were washed three times in lysis buffer.

Immunoblot analysis was performed as described previously [34]. Briefly, cell extracts were generated from cultured cells using extraction buffer (50 mM Tris-HCl (pH7.4), 1% Triton X-100, 450 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 1 mM dithiothreitol) and proteinase inhibitors. The lysates were centrifuged at 12,000*g* for 10 min. Protein concentrations in the supernatants were determined using the BCA protein assay (ThermoFisher Scientific, Waltham, MA, USA). Samples were suspended in 2x sample buffer (75 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.05% bromophenol blue, and 2.5% beta-mercaptoethanol). Immunoprecipitates and SDS-PAGE samples were then separated by standard SDS-polyacrylamide electrophoresis gel, and proteins transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). After blocking with 1% bovine serum albumin in Tris buffered saline containing 0.05% Tween 20 at room temperature for 2 h, the membranes were incubated with the indicated antibodies, followed by incubation with anti-mouse IgG or anti-rabbit IgG coupled with horseradish peroxidase and visualized using an enhanced chemiluminescence detection system (GE Healthcare, Chicago, IL, USA).

2.4. Reporter assays

Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Jurkat T cells were transfected with a pcDNA3-CBP expression plasmid using a Gene Pulser. Following electroporation, cells were further cultured in fresh medium for 48 h.

Jurkat cells expressing reporter constructs were deprived of serum for 6–8 h. Subsequently, the cells were stimulated with anti-CD3 and anti-CD28 antibodies, harvested, and assayed for luciferase activity using the Dual Luciferase Assay System according to the manufacturer's protocol (Promega, Madison, WI, USA). The reporter luciferase activity was normalized to that of *Renilla* luciferase.

2.5. ELISA assays

Human IL-2 and IL-8 ELISA assays were purchased from BioLegend (San Diego, CA, USA) and used according to the manufacturer's protocol. Data were statistically analyzed with the two-tailed unpaired *t*-test with Welch's correction (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

2.6. Cell imaging

For confocal imaging, ERCBP-expressing Jurkat cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were then incubated with 50 mM NH_4Cl in PBS for 10 min and then permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min. After blocking for 30 min with 1% (w/v) BSA in PBS, the cells were labeled with an anti-FLAG antibody (Sigma) followed by a FITC-conjugated rat anti-mouse IgG (TAGO Inc., Camarillo, CA, USA). The cells were observed using a confocal laser scanning microscope (Carl Zeiss LSM510, Jena, Germany). Fluorescence images