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TRAF6 is required for the GM-CSF-induced JNK, p38 and Akt activation

Yiwu Wang[a,e,1], Chenchen Zhou[a,1], Jiang Huo[c,1], Yanli Ni[b], Pengfei Zhang[a], Cheng Lu[a], Bin Jing[a], Fengjun Xiao[d], Wenxia Chen[a], Wei Li[a], Peng Zhang[a,*], Luo Zhang[a,b,**]

[a] Department of Biomedical Engineering, Chinese PLA 307 Hospital, Beijing 100071, China
[b] 307-Ivy Translational Medicine Center, Laboratory of Oncology, Chinese PLA 307 Hospital, Beijing 100071, China
[c] Department of Anesthesiology, Chinese PLA 307 Hospital, Beijing 100071, China
[d] Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing 100850, China
[e] Department of Infectious Diseases, Chinese PLA 532 Hospital, Anhui 242700, China

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ABSTRACT

JNK, p38 and Akt signalings have been shown to be activated by granulocyte-macrophage colony-stimulating factor (GM-CSF) and are pivotal for GM-CSF-mediated survival, proliferation and differentiation of macrophages and their progenitors. However, the detailed mechanism of how these signalings is activated by GM-CSF is not fully elucidated. We report here that E3 ligase TRAF6 is required for the GM-CSF-induced activation of JNK, p38 and Akt. GM-CSF triggers autoubiquitination of TRAF6 and TRAF6 knocked down results in impaired activation of JNK and p38 signaling. TRAF6 is also required for GM-CSF-induced ubiquitination and activation of Akt. These findings reveal novel roles of TRAF6 in GM-CSF signaling.

1. Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF, also known as Csf-2) is a hematopoietic growth factor that controls the differentiation of the myeloid lineage (Metcalf, 2008). In addition to its effects on hematopoietic progenitors, GM-CSF can also induce the production of granulocytes and macrophages from myeloid progenitor cells, and is a potent activator of neutrophils, macrophages, and dendritic cells, promoting their differentiation, survival, and function (Hamilton and Anderson, 2004).

GM-CSF binds to a tetrameric type I cytokine receptor composed of two α and two β subunits. The α subunit (GM-CSFRα [GMRα]) confers binding specificity, whereas the shared β subunit (β common receptor or GM-CSFR β [βc]) initiates signaling. Type I receptors all share signal-transducing subunits with other receptors within their receptor subfamily. For instance, GM-CSF shares its βc receptor subunit with IL-3 and IL-5 (Martinez-Moczygemba and Huston, 2003). Activation of the GM-CSF receptor is known to stimulate at least three pathways: the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, the phosphoinositide 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway (de Groot et al., 1998; Guthridge et al., 1998). The MAPK pathways include the extracellular signal regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 signaling cascades. These pathways are vital mediators of a number of cellular responses, including growth, proliferation, and survival (Guthridge et al., 1998; Liu et al., 1997; Smith et al., 1997).

The JNK and p38 MAP kinases are at the tertiary layer of a kinase cascade, in which they are activated by the MAP kinase kinases (MKKs), specifically the MKK4 or MKK3/6, respectively. Further upstream, MKKs are activated by the MAP kinase kinase kinases (MAP3Ks); in the case of MKK4 or MKK3/6, the TGF-β-activated kinase 1 (TAK1) is one of the activating MAP3Ks (Chang and Karin, 2001). However, whether GM-CSF initiates this MAP3K/MKK/MAP cascade has not been demonstrated.

GM-CSF also rapidly activates PI3K in a tyrosine phosphorylation-dependent manner, βc phosphorylation event becomes a recruitment site for the adaptor protein 14-3-3, which in turn recruits the p85 and p110 subunits of PI3K to the receptor complex (Klein et al., 2000; McLeish et al., 1998; Stomski et al., 1999). PI3K leads to the generation of PIP3, resulting in the recruitment and binding of the serine/threonine

* Corresponding author.
** Corresponding author at: Department of Biomedical Engineering, Chinese PLA 307 Hospital, Beijing 100071, China. Tel.: +86 010 66947835; fax: +86 10 66947241.
E-mail addresses: yxgck307@126.com (P. Zhang), marbleluo@126.com (L. Zhang).
[1] These authors contributed equally to this work.

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kinase Akt/PKB, whose activation is pivotal for cell survival and proliferation (Cantley, 2002). However, how these cascades initiate, especially how TAK1 is activated and Akt is recruited to plasma membrane, after GM-CSF stimulation are still not fully understood.

Recent evidence suggested that GM-CSF might also induce NF-κB signaling through TNFR-associated factor 6 (TRAF6), a RING domain ubiquitin ligase that catalyze the synthesis of polyubiquitin chains linked via a lysine at position 63 (K63) (Meads et al., 2010). TRAF6-binding motifs locating in the cytoplasmic tail of βc mediate the interaction between TRAF6 and βc. Previous work also showed that for activation of JNK by GM-CSF, a membrane proximal region of βc including box1 and a more C-terminal region between amino acids 544–589 are required (Liu et al., 1997). Given that the TRAF6-binding region and the JNK-activating region are coincide partially, we hypothesized that the βc subunit of the GM-CSFR initiates JNK as well as p38 and Akt signalings by recruitment of TRAF6. Our investigation demonstrated that GM-CSF induces K63 polyubiquitination of TRAF6, which is required for activation of the downstream JNK, p38 and Akt.

2. Materials and methods

2.1. Cells and reagents

RAW264.7 cells were cultured in RPMI 1640 medium with standard formulations. 32D cells were maintained in RPMI 1640 containing 5% WEHI3-conditioned medium. GM-CSF was purchased from PeproTech.

2.2. Immunoprecipitation, immunoblot and ubiquitination assays

Immunoprecipitation (IP), immunoblot (IB) and ubiquitination assays were carried out as previously described. Briefly, RAW264.7 cell in the TNE lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and a cocktail of protease and phosphatase inhibitors. To remove debris, lysates were centrifuged at 10,000 × *g* for 10 min. Then a two-step IP protocol with 3-h primary antibody incubation and subsequent overnight incubation with Protein A/G Sepharose beads. Beads were then washed with TNE buffer 3 times. The following antibodies were used for IP and IB: anti-TRAF6 antibody (IP: 1:200; IB: 1:1000, Santa Cruz), anti-ubiquitin antibody (IB: 1:1000, Santa Cruz), anti-ubiquitin K48 antibody (IB: 1:500, Cell Signaling), anti-ubiquitin K63 antibody (IB: 1:500, Cell Signaling), anti-GAPDH antibody (IB: 1:1000, Santa Cruz), anti-Akt antibody (IP: 1:200; IB: 1:1000, Santa Cruz), anti-phospho (S473)-Akt antibody (IB: 1:2000, Cell Signaling), anti-phospho (T308)-Akt (IB: 1:1000, Cell Signaling), anti-phospho-JNK antibody (IB: 1:1000, Cell Signaling), anti-JNK antibody (IB: 1:1000, Cell Signaling), anti-phospho-p38 antibody (IB: 1:1000, Cell Signaling), anti-p38 antibody (IB: 1:2000, Cell Signaling), anti-phospho-TAK antibody (IB: 1:500, Cell Signaling), anti-phospho-MEKK4 antibody (IB: 1:1000, Cell Signaling), anti-phospho-ATF2 antibody (IB: 1:1000, Cell Signaling), anti-phospho-MSK antibody (IB: 1:1000, Cell Signaling), anti-phospho-c-Jun antibody (IB: 1:1000, Cell Signaling).

2.3. Viral infection

TRAF6 shRNA were inserted to U6-Puro-GFP vector for knock down assay. TRAF6-lentivivral shRNA#1 (5′-GCC-CAGGCTGTT-CATAATGTT-3′), shRNA#2 (5′-CCTGTGAATTTCAGAG-GCT-3′), control shRNA (5′-TTCTCCGAACGTGTCACGT-3′) were transfected with packing plasmids into 293FT cells for 2 days, and virus particles containing TRAF6 or control shRNAs were used to infect RAW264.7 and 32D cells. All the infected cells were cultured in medium with 5 μg/ml puromycin for 2 days.

2.4. Cell proliferation and survival assay

GM-CSF-stimulated cells were labeled for 1 h with BrdU (5-bromodeoxyuridine; 10 μM) and incorporation was measured by flow cytometry with a BrdU Flow kit (BD Pharmingen) according to the manufacturer's instructions. When cell count and viability were assayed, cells were stained with trypan blue and an automated BioRad TC20 cell counter was used.

3. Result

3.1. GM-CSF promotes K63-linked polyubiquitin chain modification of TRAF6

It has previously shown that TRAF6 binds to GM-CSF-R β chain to initiate NF-κB signaling. However, it is unclear whether its

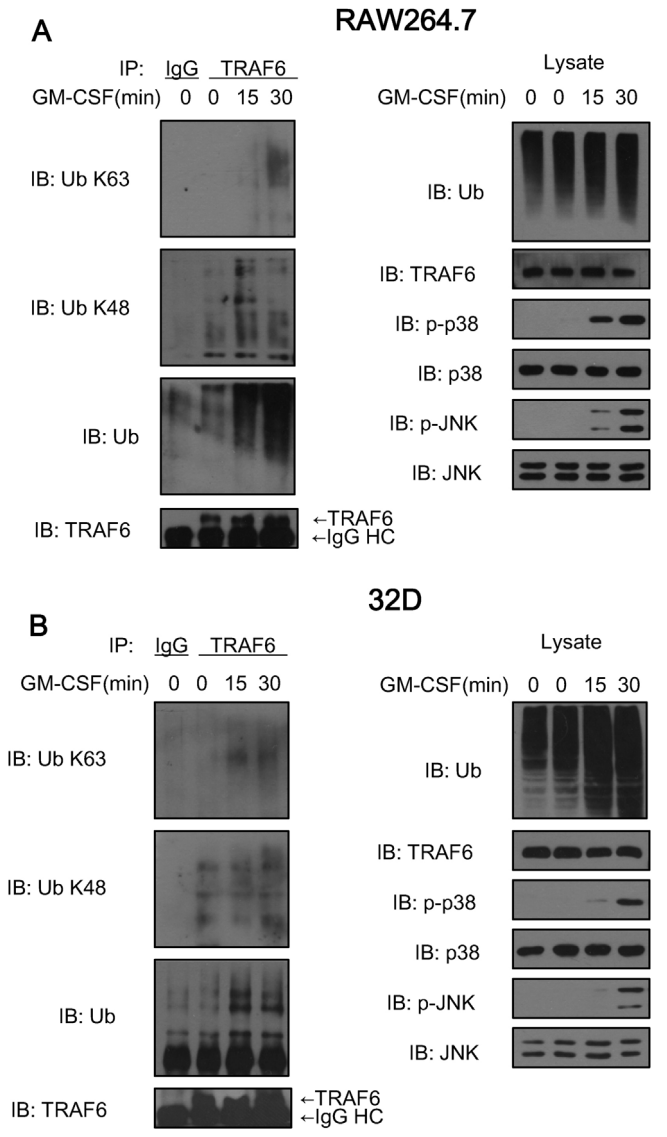

Fig. 1. GM-CSF promotes K63-linked polyubiquitin chain modification of TRAF6. (A) RAW264.7 cells were serum-starved for 1 day, treated with GM-CSF (50 ng/ml) for various times; WCE were collected IP with TRAF6 antibody for in vivo ubiquitination assay. (B) 32D cells were serum-starved for 4 h, treated with GM-CSF (50 ng/ml) for various times; WCE were collected IP with TRAF6 antibody for in vivo ubiquitination assay. Data is representative of at least three separate experiments.