FEBS Letters 581 (2007) 5151–5157

Cyclin G associated kinase interacts with interleukin 12 receptor β2 and suppresses interleukin 12 induced IFN-γ production

Ying Lin[a,c], Yu Jie Tang[a,c], Hong Liang Zong[b], Jian Xin Gu[b], Wei Wen Deng[a,c], Chen Wang[a,*], Bing Sun[a,d,e,*]

[a] *Laboratory of Molecular Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*
[b] *Gene Research Center, Shanghai Medical College, Fudan University, Shanghai 200032, China*
[c] *Graduate School of the Chinese Academy of Sciences, China*
[d] *Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200025, China*
[e] *E-institutes of Shanghai Universities Immunology Division, China*

Received 13 February 2007; revised 13 August 2007; accepted 7 September 2007

Available online 4 October 2007

Edited by Beat Imhof

Abstract Interleukin 12 receptor β1 (IL-12Rβ1) and β2 (IL-12Rβ2) constitute the functional and high-affinity receptor complex for interleukin 12 (IL-12) and mediate important functions in activated T cells. In this study, we identified cyclin G associated kinase (GAK) as a new IL-12Rβ2-interacting protein using yeast two-hybrid system and confirmed it by coimmunoprecipitation assays. Overexpression of GAK in activated T cells suppresses IL-12 induced IFN-γ production but has no detectable effects on its proliferation, whereas knockdown of GAK by RNA interference (RNAi) increases IFN-γ production. These results suggest that GAK associates with IL-12Rβ2 and may play a role in regulating IL-12 signaling.

Keywords: Cyclin G associated kinase; Interleukin 12 receptor β2; T cell activation; IFN-γ induction

1. Introduction

Interleukin 12 (IL-12), a pleiotropic cytokine that is produced mainly by macrophages and dendritic cells, plays a central role in the initiation and control of cell-mediated immune responses. IL-12 enhances the cytotoxic activity of NK cells, induces cytokine production (primarily of IFN-γ from NK and T cells) and promotes differentiation of naive T cells into Th1 cells [1].

IL-12 exerts its function through specific, high affinity receptor which is composed of two subunits termed IL-12Rβ1 and interleukin 12 receptor β2 (IL-12Rβ2) [2,3]. Upon binding of IL-12, both chains of the IL-12 receptor heterodimerize and activate the associated Janus kinases (JAKs) including TYK2 and JAK2 [4]. IL-12Rβ2 is subsequently tyrosine phosphorylated and recruits signal transducer and activator of transcription 4 (STAT4) to a specific docking site. This in turn results in phosphorylation, dimerization and nuclear translocation of STAT4, and the activation of IL-12-responsive genes [5,6]. In addition to JAK-STAT pathway, IL-12 also activates other signaling pathways such as p38 MAPK, PI3K/Akt pathways. MKK6/p38 MAPK pathway is essential for STAT4 serine phosphorylation on serine 721 and IL-12-induced IFN-γ secretion, but not T cell proliferation [7,8], while the PI3K/Akt pathway is critical for IL-12-induced T cell proliferation and the inhibition of apoptosis [9]. The mechanism on how IL-12Rβ2 is coupled with the upstream signaling molecules of these signaling pathways is still unclear. We performed the yeast two-hybrid assay using the cytoplasmic domain of IL-12Rβ2, in order to seek key proteins interacting with IL-12Rβ2, and may play critical roles in IL-12 signaling.

Cyclin G associated kinase (GAK) is a Ser/Thr kinase that has multiple functional domains [10]. Although GAK was initially reported to be associated with cyclin G, subsequent studies suggest that it has an important role in uncoating clathrin-coated vesicles (CCVs) in non-neuron cells [11]. Down-regulation of GAK by small hairpin RNA dramatically changed the epidermal growth factor receptor expression in cells as well as the downstream signaling from the receptor [12]. In addition, GAK was implicated to interact with androgen receptor and act as a transcription coactivator [13]. In the current study, we demonstrate that GAK interacts specifically with IL-12Rβ2 and suppresses IL-12 induced production of IFN-γ.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female DO11.10 TCR-Tg mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were kept in a specific pathogen-free facility at the Chinese Academy of Sciences. Animal care and use were in compliance with institutional guidelines.

2.2. Reagents

MATCHMAKER GAL4 two-hybrid system and mouse lymphoma cDNA library were products of CLONTECH (Mountain View, CA). Anti-Flag M2 and anti-HA monoclonal antibodies were purchased from Sigma–Aldrich (St. Louis, MO) and Bibco (Berkeley, CA),

*Corresponding authors. Address: Laboratory of Molecular Immunology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China. Fax: +86 21 54921011 (B.Sun).
E-mail addresses: cwang01@sibs.ac.cn (C. Wang), bsun@sibs.ac.cn (B. Sun).

Abbreviations: IL-12, interleukin 12; GAK, cyclin G associated kinase; IL-12Rβ2, interleukin 12 receptor β2; STAT4, signal transducer and activator of transcription 4; IL-2, interleukin 2; GFP, green fluorescence protein

0014-5793/$32.00
doi:10.1016/j.febslet.2007.09.056

respectively. Protein A/G PLUS-Agarose and anti-GAK (SC-7864) polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The LipofectAMINE™ reagents were bought from Invitrogen (Carlsbad, CA). Mouse IFN-γ ELISA Set and mouse rIL-12 were purchased from BD Biosciences (San Jose, CA). Recombinant human interleukin 2 (IL-2) was from Shanghai Hua Xin High Biotechnology Incorporation.

2.3. Plasmid construction

The mouse IL-12Rβ2 cDNA was obtained by RT-PCR using mRNA prepared from ConA-activated spleen cells and was cloned into the pBudCE4.1 vector with HA tag at the C-terminal sequence. Mutants of cytoplasmic domain of IL-12Rβ2 containing C-terminal HA tag were cloned into pcDNA3-GST vector. pcDNA3.1-N-Flag was described previously [14] and contained a Flag-tag at the N-terminal sequence. pcDNA3.1-N-Flag-rGAK was constructed by subcloning full length GAK cDNA from pGEX3-rGAK (kindly provided by Dr. Lois E. Greene, Laboratory of Cell Biology, NHLBI, National Institutes of Health) into pcDNA3.1-N-Flag vector. For truncated mutants of GAK, the corresponding cDNAs were created by PCR and were cloned into pcDNA3.1-N-Flag vector. All constructs were confirmed by sequencing.

2.4. Yeast two-hybrid screening

The cytoplasmic domain of mouse IL-12Rβ2 was cloned in-frame into pGBKT7 to generate bait plasmid, pGBKT7-IL-12Rβ2cyto. The bait plasmid was transformed into the yeast strain AH109, and this strain was subsequently transformed with a mouse T lymphoma cDNA library in pACT and selected on yeast synthetic medium lacking tryptophan, leucine, histidine and adenine. Colonies surviving after 4–6 d at 30 °C were tested for β-galactosidase activity, and plasmid DNA was prepared from positive colonies and sequenced for identification of cDNA clones.

2.5. Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine at 37 °C in 5% CO_2. Cells were plated at 80% confluence in 60 mm tissue culture dishes and the following day were transfected with relevant plasmids using LipofectAMINE™ reagents according to the manufacturer's recommendations. After 24 h, the cells were harvested for further analysis.

2.6. Immunoprecipitation and Western blotting

Twenty-four hours after transfection, the HEK293T cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with the lysis buffer containing 20 mM Tris–HCl, pH 8.0, 135 mM sodium chloride, 1% Nonidet P-40, 10% glycerol, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM pyrophosphoric acid and Complete™ protease inhibitors (Roche Biochemical). Whole cell lysates were incubated with mouse monoclonal anti-Flag M2 antibody or anti-HA antibody and protein A/G PLUS-agarose beads at 4 °C for 3 h. The immunocomplexes were pelleted and washed three times with cold lysis buffer in the absence of protease and phosphatase inhibitors. For Western-blot analysis, the bound proteins were eluted by boiling in SDS sample buffer, resolved on SDS–PAGE gel and transferred onto a nitrocellulose membrane (Pall Incorporation) and immunoblotted with corresponding antibodies.

2.7. Retroviral infection

The full length GAK cDNA was subcloned into a bicistronic retroviral vector pMSCV-internal ribosome entry site (IRES)/green fluorescence protein (GFP) (kindly provided by Dr. Xingxing Zang, Department of Molecular and Cell Biology, University of California, Berkeley). HEK293T cells were transfected with pMSCV-GAK/IRES/GFP or the empty vector, Eco-Envelope plasmid, gag-pol plasmid using LipofectAMINE™ reagents and cultured to generate retroviral supernatants. Primary T cells (3×10^6 cells/ml) of DO11.10 TCR-Tg mouse depleted of erythrocytes were activated with 3 μg/ml OVA and 50 U/ml human IL-2 for 24 h and then infected with the retroviral supernatant in the presence of 8 μg/ml polybrene by centrifugation at 1800 rpm for 90 min. After incubating with 50 U/ml IL-2 for 2 d, GFP-positive cells were purified by sorting using a FACSCalibur (BD Biosciences).

2.8. RNA interference

The sequence of nucleotides 820–840 of GAK gene (GenBank™ accession number: NM_153569) was used to produce a siRNA directed to GAK mRNA. Selected sequences were submitted to BLAST searches to ensure only GAK mRNA is targeted. Annealed complementary oligonucleotides synthesized were cloned into the ApaI/EcoRI sites of the pBS/U6 vector to generate pBS/U6-GAK/siRNA as described [15]. The U6 promoter and the hairpin cDNA insert were subcloned into the transition vector pGBKT7 and then subcloned into the SalI/EcoRI sites of the pMSCV-IRES/GFP vector to generate pMSCV-U6/GAK/siRNA.

2.9. IFN-γ production and proliferation assays

For IFN-γ production assay, GFP-positive cells (2×10^5/well) were stimulated with mouse rIL-12 and human IL-2, and culture supernatants were harvested after 24 h and assayed for IFN-γ concentrations by ELISA. For proliferation assay, GFP-positive cells (2×10^5/well) were stimulated with rIL-12 for 24 h and pulsed with 0.5 μCi [^{3}H] thymidine for 8 h before harvesting cells.

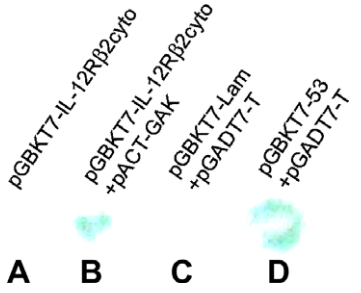

Fig. 1. Identification of interaction between GAK and the cytoplasmic domain of IL-12Rβ2 in the yeast two-hybrid system by assaying β-galactosidase activities. (A) No auto-activity was found in pGBKT7-IL-12Rβ2cyto. (B) Interaction of GAK with the cytoplasmic domain of IL-12Rβ2. (C) Negative control, human lamin C did not interact with SV40 large T-antigen. (D) Positive control, murine P53 interacted with SV40 large T-antigen.

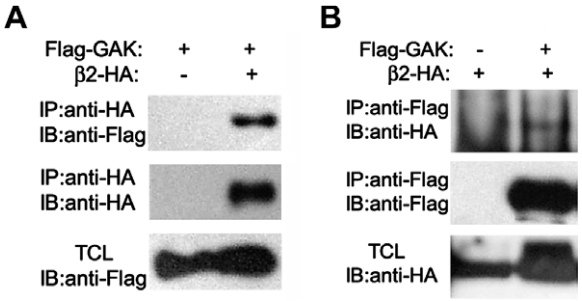

Fig. 2. GAK interacts with IL-12Rβ2 in mammalian cells. (A) HEK293T cells were cotransfected with pBudCE4.1-IL-12Rβ2-HA or the empty vector along with pcDNA3.1-N-Flag-rGAK. After 24 h, the cell lysates were subjected to immunoprecipitation with anti-HA antibody and detected by immunoblotting with anti-Flag or anti-HA antibody. (B) HEK293T cells were cotransfected with pcDNA3.1-N-Flag-rGAK or the empty vector along with pBudCE4.1-IL-12Rβ2-HA. After 24 h, the cell lysates were subjected to immunoprecipitation with anti-Flag antibody and detected by immunoblotting with anti-HA or anti-Flag antibody. The protein levels of GAK and IL-12Rβ2 in the total cell lysates (TCL) are presented (lower panel). IP: immunoprecipitation. IB: immunoblotting.