

Cyclin G associated kinase interacts with interleukin 12 receptor β 2 and suppresses interleukin 12 induced IFN- γ production

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Abstract Interleukin 12 receptor β 1 (IL-12R β 1) and β 2 (IL-12R β 2) 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-12R β 2-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-12R β 2 and may play a role in regulating IL-12 signaling.

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Keywords: Cyclin G associated kinase; Interleukin 12 receptor β 2; 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-12R β 1 and interleukin 12 receptor β 2 (IL-12R β 2) [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-12R β 2 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-12R β 2 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-12R β 2, in order to seek key proteins interacting with IL-12R β 2, 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-12R β 2 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),

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

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-12R β 2 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-12R β 2 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-12R β 2 was cloned in-frame into pGBKT7 to generate bait plasmid, pGBKT7-IL-12R β 2cyto. 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 μ g/ml streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine at 37 °C in 5% CO₂. 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 μ g/ml OVA and 50 U/ml human IL-2 for 24 h and then infected with the retroviral supernatant in the presence of 8 μ g/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 Sall/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 μ Ci [³H] thymidine for 8 h before harvesting cells.

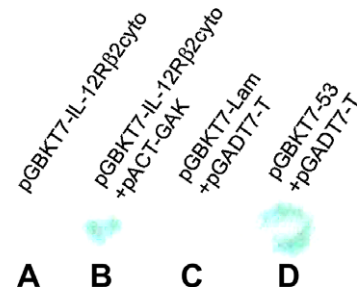


Fig. 1. Identification of interaction between GAK and the cytoplasmic domain of IL-12R β 2 in the yeast two-hybrid system by assaying β -galactosidase activities. (A) No auto-activity was found in pGBKT7-IL-12R β 2cyto. (B) Interaction of GAK with the cytoplasmic domain of IL-12R β 2. (C) Negative control, human lamin C did not interact with SV40 large T-antigen. (D) Positive control, murine P53 interacted with SV40 large T-antigen.

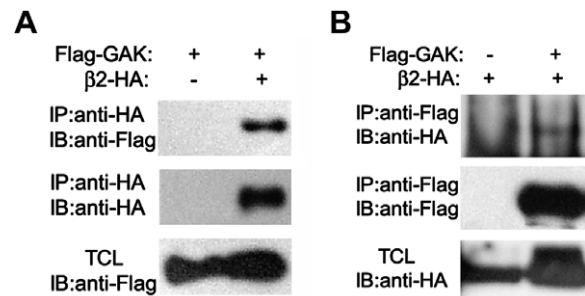


Fig. 2. GAK interacts with IL-12R β 2 in mammalian cells. (A) HEK293T cells were cotransfected with pBudCE4.1-IL-12R β 2-HA or the empty vector along with pcDNA3.1-N-Flag-rGAK. After 24 h, the cell lysates were subjected to immunoprecipitation with anti-HA antibody and detected by immunoblotting with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

2.10. Statistical analysis

Experiments were routinely repeated usually three or more times. Statistical analysis was performed using Student's *t*-test. A value of $P < 0.05$ was statistically significant.

3. Results

3.1. GAK interacts with IL-12R β 2 in yeast and in mammalian cells

To identify proteins that interact with IL-12R β 2, we performed a yeast two-hybrid screening of mouse T cell lymphoma cDNA library using the cytoplasmic region of mouse IL-12R β 2 cDNA as bait. About 7×10^5 colonies were screened and 16 positive clones were obtained on yeast synthetic medium lacking tryptophan, leucine, histidine and adenine. All the clones were further shown to be positive when analyzing for β -galactosidase activity using the colony shift assay. Positive clones were sequenced and blast analysis revealed that one of the 16 positive clones harbored a partial sequence of mouse GAK, which corresponded to amino acid residue 900–1200. When pGBKT7-IL-12R β 2cyto itself was transformed into yeast strain AH109, the lacZ gene was not activated indicated by β -galactosidase assay (Fig. 1). As we expected, cotransfection of pGBKT7-IL-12R β 2cyto and GAK significantly activated lacZ gene, which demonstrated the potential interaction between IL-12R β 2cyto and GAK. To further confirm this interaction, HEK293T cells were cotransfected with HA-tagged IL-12R β 2 or a control plasmid and Flag-tagged GAK. Cell lysates were immunoprecipitated

with anti-HA and the immunoprecipitates obtained were analyzed by Western blotting with an anti-Flag antibody to detect the presence of GAK. As shown in Fig. 2A, GAK was present in immunoprecipitates from cotransfection of IL-12R β 2 and GAK, but not from the control groups. The interaction was also confirmed in a similar experiment using anti-Flag antibody for immunoprecipitation and anti-HA antibody for immunoblotting (Fig. 2B). These results indicated that GAK associated with IL-12R β 2 in mammalian cells.

3.2. Mapping of the binding domain in GAK to the IL-12R β 2

GAK is composed of an N-terminal Ser/Thr kinase domain (K), a central tensin domain (T), a clathrin-binding domain (C) and a C-terminal J domain (J). We generated a series of Flag-tagged GAK deletions (as indicated in Fig. 3A) and tested their binding ability to HA-tagged IL-12R β 2 in HEK293T cells using coimmunoprecipitation assays. These truncated mutants and the full length of GAK were transfected into HEK293T cells along with HA-tagged IL-12R β 2, and then whole cell lysates were immunoprecipitated with anti-Flag antibody. The immunoprecipitates were immunoblotted with anti-HA antibody. As shown in Fig. 3B, the truncated mutants (K, KT and C) and full length of GAK retained a higher binding capacity with IL-12R β 2 and mutant (CJ) had the weak binding capacity. The semiquantitative analysis was also used to show the binding activity (Fig. 3C). These data suggested that multiple binding sites exist between IL-12R β 2 and GAK, at least the kinase domain and the clathrin-binding domain interacted with IL-12R β 2.

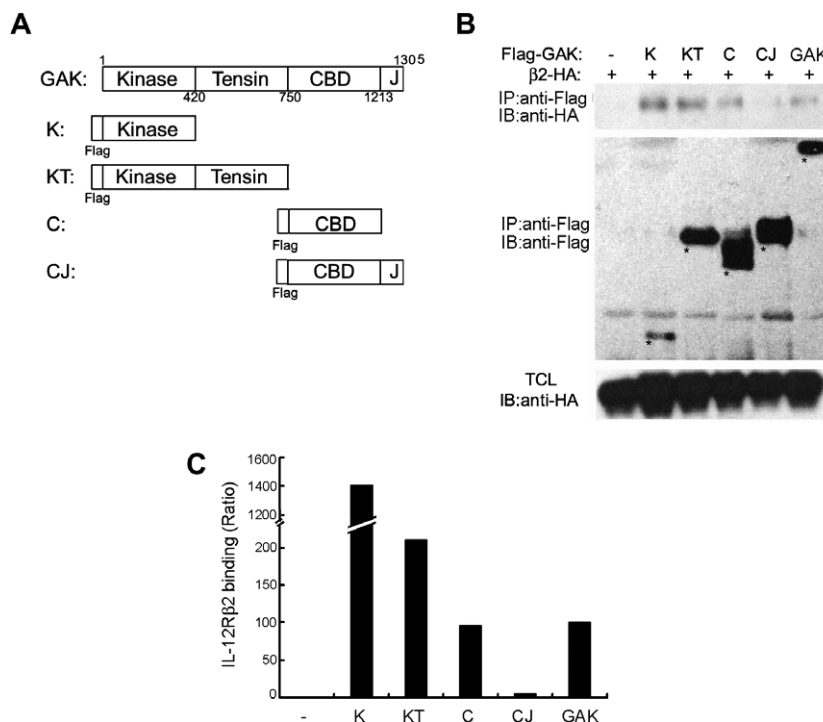


Fig. 3. Mapping of the binding domain in GAK to the IL-12R β 2 cytoplasmic region. (A) Schematic representation of GAK deletion mutants used for determining IL-12R β 2-binding domains. Amino acid residues corresponding to the four domains were indicated. Flag tag at the N-terminus of GAK deletion mutants were represented by small boxes. (B) Lysates of HEK293T cells cotransfected with the indicated plasmids were subjected to immunoprecipitation with anti-Flag antibody and detected by immunoblotting with anti-HA or anti-Flag antibody. (C) The band density was analyzed by Furi SmartView software. The IL-12R β 2 binding indicated ratio of the amount of IL-12R β 2 over GAK mutants in precipitates.

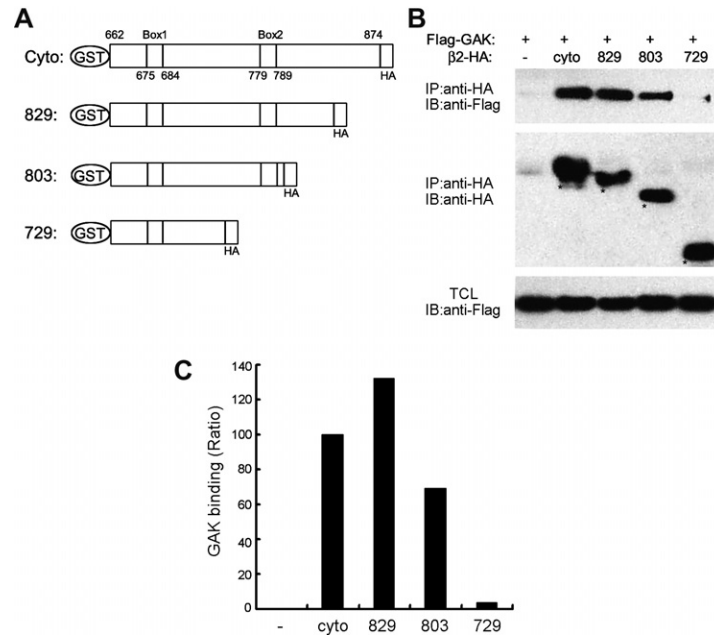


Fig. 4. Mapping of the binding domain in the IL-12R β 2 cytoplasmic region to GAK. (A) Schematic representation of the cytoplasmic domain of IL-12R β 2 truncated mutants. Amino acid positions corresponding to the box 1 and box 2 motif were indicated. HA tag at the C-terminus of IL-12R β 2 constructs were represented by small boxes. The truncated constructs were named according to C-terminal amino acid number of the cytoplasmic region. GST tag at the N-terminal domain was fused with IL-12R β 2 cytoplasmic domain. (B) Lysates of HEK293T cells cotransfected with the indicated plasmids were subjected to immunoprecipitation with anti-HA antibody and detected by immunoblotting with anti-Flag or anti-HA antibody. (C) The band density was analyzed by Furi SmartView software. The GAK binding indicated ratio of the amount of GAK over IL-12R β 2 mutants in precipitates.

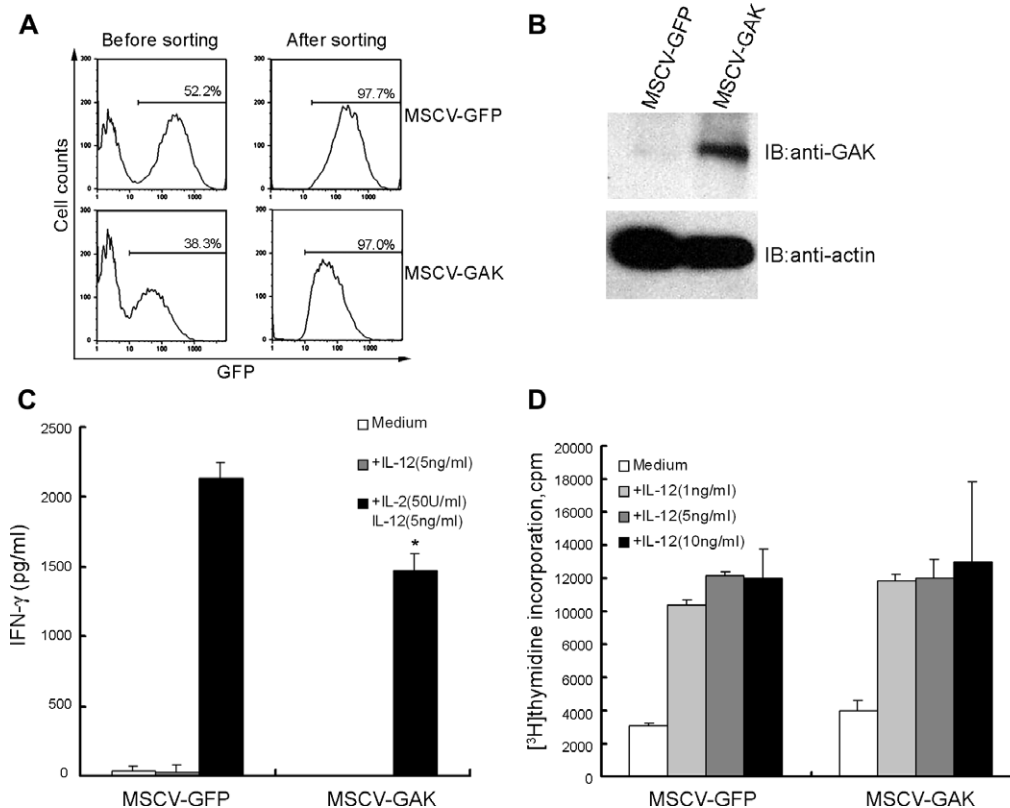


Fig. 5. Suppression of IL-12-induced IFN- γ production by retroviral expression of GAK in activated primary mice T cells. DO11.10 TCR-Tg mice spleen cells were activated by OVA and IL-2 and were infected with retroviruses expressing GAK/GFP or GFP alone. (A) Infected cells were purified by sorting for expression of GFP. (B) GAK expression was confirmed in sorted cells by Western blotting. (C) IFN- γ production in response to rIL-12 or rIL-12/IL-2 was measured. (D) T cell proliferation in response to rIL-12 was determined. Data are shown as the means \pm S.D. Similar results were obtained in three independent experiments (* P < 0.05).

3.3. Mapping of the binding domain in the IL-12R β 2 cytoplasmic region to GAK

To further determine which region of IL-12R β 2 was important for this interaction, four truncated mutants were generated according to different domains of IL-12R β 2 (designated as cyto, 829, 803 and 729) as shown in Fig. 4A. All these mutants were expressed as GST fusion proteins. The data showed that the cytoplasmic domain of IL-12R β 2 (cyto) and mutant (829) kept the interaction with GAK. The mutant (803) still bind to GAK, but with weaker binding activity. In contrast, mutant (729) significantly reduced this binding activity (Fig. 4B). The semiquantitative analysis was also used to show the binding activity (Fig. 4C). Taken together, these data suggested that the cytoplasmic domain (730aa–829aa) of IL-12R β 2 was involved in the interaction with GAK.

3.4. Overexpression of GAK suppressed IL-12-induced IFN- γ production in primary T cells

Since IL-12R β 2 interacts with GAK, it was reasonable to ask whether GAK affects IL-12 signaling. The retrovirus system was employed to introduce GAK into activated T cells. After administering the recombinant retrovirus supernatant, about 30–50% cells were infected. The infected cells were purified by sorting GFP-positive cells and the expression of GAK was also confirmed by Western blotting in the cells (Fig. 5A and B). Then responsiveness of the cells to rIL-12 was analyzed. After stimulating cells by cocktail of rIL-12 (5 ng/ml) and IL-2 (50 U/ml), it was found that the activated T cells expressing ectopic GAK produced less IFN- γ than those

expressing GFP alone (Fig. 5C). In contrast, IL-12-induced proliferation was not significantly impaired by retroviral expression of GAK as compared with the control (Fig. 5D). These results suggested that GAK may play a role in modulating IL-12 induced IFN- γ production in T cells.

3.5. Knockdown of GAK enhanced IL-12-induced IFN- γ production in primary T cells

To further investigate the possible role of GAK in IL-12 signaling, the endogenous GAK was silenced in activated T cells by RNAi. We prepared the retrovirus expression plasmid of the GAK/siRNA and infected the activated T cells using the retrovirus supernatants or control virus. GFP positive cells were sorted. The Western blotting demonstrated a significant down-regulation of GAK expression in the T cells infected with pMSCV-U6/GAK/siRNA (Fig. 6A). As expected, inhibition of GAK increased the production of IFN- γ in response to IL-2 and IL-12 (Fig. 6B), while the cell proliferation was not affected (Fig. 6C).

4. Discussion

In order to further understand the regulation and the downstream signaling of IL-12R β 2, we searched for IL-12R β 2-interacting proteins by yeast two-hybrid screening and identified GAK as a novel binding partner of IL-12R β 2. We demonstrated that overexpression or silence of GAK in T cells led to a change of IFN- γ production, but not in cell proliferation.

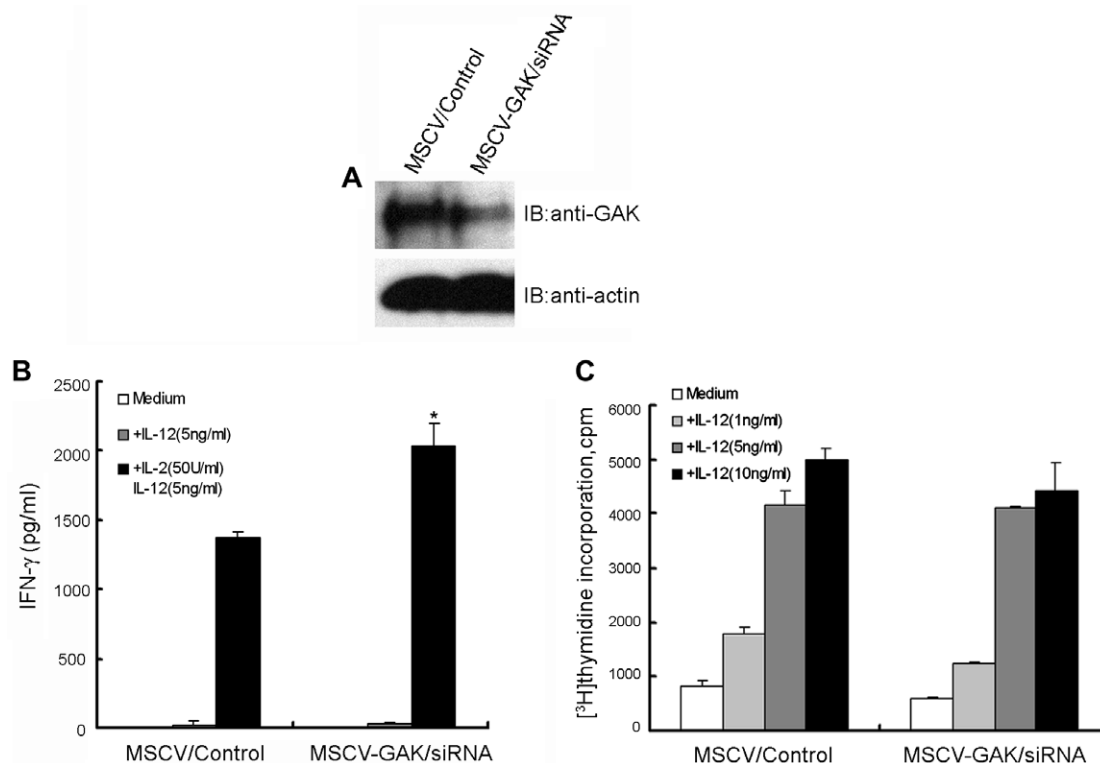


Fig. 6. Enhancement of IFN- γ production by knockdown of GAK in activated primary mice T cells. DO11.10 TCR-Tg mice spleen cells were activated by OVA and IL-2 and were infected with retroviruses expressing GAK/siRNA or control virus. (A) The expression of GAK was determined by Western blot using anti-GAK antibody. (B) GFP positive cells were sorted and production of IFN- γ in response to rIL-12 or rIL-12/IL-2 was measured. (C) T cell proliferation in response to rIL-12 was determined. Data are shown as the means \pm S.D. Similar results were obtained in three independent experiments (* P < 0.05).

GAK belongs to the Ser/Thr protein kinase family and has been shown to associate with cyclin G and cyclin-dependent kinase 5 (CDK5). It differs from auxilin by its N terminal kinase domain. Unlike auxilin, which expresses only in neurons, GAK is ubiquitously expressed. It has been reported to act as an auxilin homolog involved in the uncoating of clathrin-coated vesicles by constitutive heat shock protein 70 (Hsc70). GAK is one of the two active kinases presenting in clathrin-coated vesicles. Its Ser/Thr kinase activity was directed toward the $\mu 2$ component of CCVs [16].

Coimmunoprecipitation assays demonstrated that multiple domains of GAK were able to interact with IL-12R β 2. The kinase domain interacts strongly with IL-12R β 2, which suggested that GAK might be involved in the phosphorylation on the cytoplasmic Ser/Thr of IL-12R β 2. Meanwhile, we found several potential Ser/Thr phosphorylation sites on the cytoplasmic domain of IL-12R β 2 using NetPhos 2.0 Server prediction software. However, this needs further investigation.

As the signaling subunit of IL-12 receptor, IL-12R β 2 was reported to interact directly with several important molecules, such as STAT4, JAK2, and suppressor of cytokine signaling 3 (SOCS3) [17–19]. It was reported that membrane-proximal region in the cytoplasmic domain of IL-12R β 2 was necessary for the association between IL-12R β 2 and JAK2, while box 2 motif was not required for the association. In our truncated experiments, it was demonstrated that the fragment covering region from amino acid residues 730–829 (containing box 2 domain) was involved in the interaction between IL-12R β 2 and GAK. The function behind this interaction may be different from available information and need to be further investigated. And IL-12R β 1 was found to interact with sphingosine kinase 2 (SPHK2) [20], which have a potential role in positive regulation of IL-12 signaling.

Signaling receptors often undergo receptor-mediated endocytosis. IL-6 signal transducer gp130 undergoes constitutive internalization independent of the presence of a ligand [21]. In human microglial cells, IL-12 binds to the IL-12 receptor and the IL-12/IL-12 receptor complex is internalized and accumulates in early endosomes [22]. Since GAK has been reported to play an important role in clathrin mediated endocytosis and receptor trafficking, it is tempting to speculate that GAK might regulate the IL-12R β 2 trafficking and then led to the changes of IFN- γ production.

It has been demonstrated that serine phosphorylation of STAT4 by MKK6/p38 MAPK are critical for the IFN- γ production. Coimmunoprecipitation assay demonstrated that GAK did not interact with STAT4 in HEK293T cells (data not shown). How GAK is linked with p38/MAPK pathway is an important issue that will need to be resolved. Since IFN- γ production is critical for Th1 differentiation, GAK maybe also involved in Th1 cell differentiation. In this study, it should be emphasized that we can only demonstrate the interaction between IL-12R β 2 and GAK and GAK has potential to modulate IFN- γ production. We could not exclude other functions of this interaction due to limited reagents and techniques.

In summary, we have identified GAK as a novel binding partner for IL-12R β 2 via yeast two-hybrid screening. We have also mapped the molecular domains required for this interaction. Functional studies using overexpression or RNAi knockdown of GAK suggest that GAK potentially

plays an important role in regulating IL-12 signaling. The molecular mechanism of this regulation needs to be further elucidated.

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