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Title: The exon-junction complex proteins, Y14 and MAGOH regulate STAT3 activation

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Running title: Interactions between Y14, MAGOH and STAT3

Abstract

Signal transducer and activator of transcription 3 (STAT3), which is activated by cytokines and growth factors, mediates biological actions in many physiological processes. In a previous study, we found that Y14, a core component of the exon-junction complex (EJC) bound to STAT3 and upregulated the transcriptional activity of STAT3 by influencing its DNA-binding activity. In the present study, we demonstrate that STAT3 endogenously interacts with Y14. In addition, we found that MAGOH, a Y14 partner in the EJC, inhibits the STAT3-Y14 complex formation. Furthermore, small-interfering RNA-mediated reduction of MAGOH expression enhanced interleukin-6-induced gene expression. These results indicate that MAGOH regulates the transcriptional activation of STAT3 by interfering complex formation between STAT3 and Y14.

Key words: Cytokine, STAT3, Y14, MAGOH, exon-junction complex, gene expression, signal transduction

Introduction

STAT3 was originally cloned as an acute-phase response factor activated by interleukin (IL)-6 in the mouse liver, or as a homology to STAT1 [1,2]. Growth factors, such as epidermal growth factor, platelet-derived growth factor and colony-stimulating factor-1, can also stimulate STAT3 activation [1,2]. STAT3 plays crucial roles in early embryonic development as well as in other biological responses, including cell growth and apoptosis [1-3]. In recent years, constitutive or dysregulated expression of STAT3 has been identified in cancer cells and oncogene-transfected cells and found to be involved in a wide range of diseases including malignant and autoimmune diseases [4-7]. Studies in *Dictyostelium* and *Drosophila* have revealed that the Jak/STAT pathway is required for a broad set of developmental processes, including cell proliferation, cell fate determination, cell migration, planar polarity, convergent extension and immunity [8]. *Zebrafish* STAT3 has been shown to control cell movements during gastrulation [9]. Taken together, these findings imply that STAT3 activation must be tightly regulated.

Y14 is an RNA-binding protein that forms the exon-junction complex (EJC) with MAGOH. The complex binds preferentially to spliced mRNAs immediately upstream of exon-junctions, and remains bound to the mRNAs after nuclear export [11, 12]. In a previous study, we identified Y14 as a novel binding partner of STAT3 [10]. Y14 binds to STAT3 and affects IL-6-induced STAT3 transactivation by influencing the tyrosine-phosphorylation, nuclear accumulation and DNA-binding activity of STAT3.

Here, we confirm the enhancing effects of endogenous Y14 on STAT3 activation. Of

importance, MAGOH, which interacts with Y14, regulates the Y14 action via inhibiting the binding between Y14 and STAT3. Therefore, a pair of Y14 and MAGOH is a novel regulatory mechanism for cytokine-induced STAT3 activation.

Materials and Methods

Reagents and antibodies, Recombinant human IL-6 was a kind gift from Ajinomoto Co. (Tokyo, Japan). Recombinant human LIF was purchased from INTERGEN (Purchase, NY). Expression vectors, epipote-tagged STAT3 and its YF mutant were previously described [13, 14, 15]. Expression vectors, STAT3-LUC and STAT3-C were provided by Dr. T. Hirano (Osaka University, Osaka, Japan) and Dr. J. F. Bromberg (Rockefeller Univ., New York, NY), respectively [15, 16]. Epitope-tagged Y14 and MAGOH was generated by PCR and sequenced (primer sequences are available upon request) [10]. Anti-Myc, anti-HA, anti-STAT3, and Y14 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG antibody from Sigma-Aldrich (St. Louis, MO); anti-phospho-STAT3 Tyr705 (pSTAT3 Tyr705) from Cell Signaling Technologies (Beverly, MA); anti-actin antibody from Chemicon (Temecula, CA).

Cell culture, transfection, siRNA, RT-PCR and quantitative real-time PCR, Human embryonic kidney carcinoma cell line, 293T, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and transfected by the standard calcium precipitation protocol. The human hepatoma cell line Hep3B was cultured in DMEM, supplemented with 10% fetal bovine serum (FBS). Hep3B cells were treated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FCS as previously described [15]. siRNAs targeting human Y14 used in this study was follows: siY14, 5'-GGGGUAUACUCUAGUUGATT-3', siMAGOH,

5'-AGAGGCUUAUGUACAUAAATT-3'. Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in reverse transcription (RT)-PCR. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan) [16]. Primers used for RT-PCR were: Y14: 5'-AAGATTTCGCCATGGATGAG-3' (sense), 5'-ATCAAATCCTGGCCATTGAG-3' (antisense), MAGOH: 5'-AAAGCGTGATGGAGGAACTG-3' (sense), 5'-CCTTCTGGATCCTTGGATTG-3' (antisense). Quantitative real-time PCR analyses of SOCS3 and C/EBPδ as well as the control G3PDH mRNA transcripts were carried out using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system.

Immunoprecipitation and immunoblotting, Immunoprecipitation and Western blotting were performed as previously described [17]. The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 mg /ml each of aprotinin, pepstatin, and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to an Immobilon filter (Millipore; Bedford, MA), which was then immunoblotted with antibodies.

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In a previous study, we identified Y14 as a novel binding partner of STAT3 [10]. Y14 is an RNA-binding protein that forms the exon-junction complex (EJC) with MAGOH. The complex binds preferentially to spliced mRNAs immediately upstream of exon-exon junctions, and remains bound to the mRNAs after nuclear export [11, 12]. Y14 binds to STAT3 and affects IL-6-induced STAT3 transactivation by influencing the tyrosine-phosphorylation, nuclear accumulation and DNA-binding activity of STAT3. Here, we show that Y14 endogenously interacts with STAT3. We also demonstrate that

MAGOH releases STAT3 from the STAT3-Y14 complex. Furthermore, small-interfering RNA-mediated reduction of MAGOH expression enhances IL-6-induced gene expression. Therefore, MAGOH appears to act as a negative regulator of IL-6-induced STAT3 activation by displacing Y14 from STAT3.

Results and Discussion

STAT3 endogenously binds to Y14 in Hep3B cells

Previously, we demonstrated that Y14 binds to STAT3 and modulates its activation [10]. We also showed that Y14 interacts with STAT3 in unstimulated 293T cells. To further examine the molecular mechanisms of the interactions between STAT3 and Y14, we evaluated the time course analysis of their interactions after LIF stimulation in 293T cells. Following transfection of 293T cells with expression vectors for HA-tagged STAT3 and Myc-tagged Y14, the cells were treated with LIF for the indicated periods and lysed. The lysates were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were analyzed by immunoblotting with an anti-Myc antibody. The results of these co-immunoprecipitation experiments are shown in Fig. 1A. The binding potential of STAT3 with Y14 was markedly increased within 15 min after LIF stimulation, although significant amount of STAT3 bound to Y14 independently on LIF stimulation as described previously [10]. We also re-evaluated the involvement of the STAT3 activation state in the interactions between Y14 and STAT3. Specifically, we tested their interactions in 293T cells after 15 min of LIF stimulation using an inactive form (STAT3 YF) and a constitutively active form (STAT3-C) of STAT3. STAT3 YF was a STAT3 point mutant in which the tyrosine phosphorylation site (Tyr 705) was replaced by phenylalanine did not form protein-DNA complexes [15], whereas a constitutively active STAT3-C was created by introducing cysteines at residues 662 and 664 in a loop of the SH2 domain in STAT3 for dimer formation through inter-chain

disulfide bridges in the absence of tyrosine phosphorylation [16]. Expression vectors encoding Myc-tagged Y14 and HA-tagged STAT3 or STAT3 YF mutant were transiently transfected into 293T cells. The transfected cells were treated with LIF for 15 min and then lysed. The lysates were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were analyzed by immunoblotting with an anti-Myc antibody. As shown in Fig. 1B, STAT3 YF showed much less binding potential to Y14 than STAT3 WT, indicating that phosphorylated STAT3 prefers to bind to Y14. Similarly, we tested the interactions between Y14 and the STAT3-C mutant. Interestingly, STAT3-C showed a strong binding potential to Y14 in the absence of LIF stimulation, suggesting that the STAT3 activation state plays a critical role in the interaction between STAT3 and Y14.

To examine the physiological interactions between STAT3 and Y14 in more detail, a human hepatoma cell line Hep3B, which expresses endogenous Y14 and large amounts of STAT3, was employed. The cells were lysed and immunoprecipitated with a control antibody or an anti-STAT3 antibody. The immunoprecipitate with the anti-STAT3 antibody contained Y14. Conversely, the immunoprecipitate with the anti-Y14 antibody contained STAT3. These results indicate that STAT3 endogenously interacts with Y14 in Hep3B cells.

MAGOH interferes complex formation between STAT3 and Y14

Y14 has been shown to form the EJC complex with MAGOH [11, 12, 20]. Therefore, we tested whether MAGOH has an effect on the interactions between STAT3 and Y14.

293T cells were transfected with Myc-tagged Y14 or FLAG-tagged MAGOH together with HA-tagged STAT3. Western blot analysis of the associated proteins revealed that STAT3 interacted with Y14 but not with MAGOH (Fig. 2A). In contrast, when 293T cells were transfected with HA-tagged STAT3 or FLAG-tagged MAGOH together with Myc-tagged Y14, immunoprecipitation with an anti-Myc antibody revealed that Y14 interacted with both STAT3 and MAGOH (Fig. 2B). Importantly, STAT3 showed much less binding potential to Y14 in the presence of MAGOH (Fig. 2A), indicating that MAGOH inhibits the physical interactions between STAT3 and Y14. However, overexpression of STAT3 could not influence the amount of the Y14-MAGOH binding (Fig. 2B), suggesting Y14 may have high affinity to MAGOH than to STAT3. These results suggest that MAGOH decreases the formation of STAT3/Y14 complexes due to a tight complex formation between MAGOH and Y14.

MAGOH regulates STAT3-mediated transactivation

To assess the functional relevance of the interactions among STAT3, Y14 and MAGOH, we first examined whether reduction of Y14 and/or MAGOH expression affects IL-6-induced tyrosine-phosphorylation of STAT3. Specific siRNAs for Y14 and/or MAGOH or a control siRNA were transfected into Hep3B cells. Total cellular proteins or RNA extracted from the transfected cells was subjected to Western blot or RT-PCR analyses, which confirmed reductions in Y14 and/or MAGOH mRNA expression. As shown in Fig. 3A, tyrosine-phosphorylation of STAT3 was decreased following a reduction in Y14 expression in Hep3B cells as previously described [10]. On the

contrary, a reduction in MAGOH expression increased IL-6-induced tyrosine-phosphorylation of STAT3. Furthermore, reductions in both Y14 and MAGOH expressions decreased IL-6-induced tyrosine-phosphorylation of STAT3. These results suggest that MAGOH negatively regulates IL-6-induced tyrosine-phosphorylation of STAT3 by interfering with complex formation between Y14 and STAT3.

Finally, to clarify the physiological significance of the molecular interactions among STAT3, Y14 and MAGOH, we examined the effects of Y14 and/or MAGOH expression on STAT3 transactivation. We first tested whether ectopic expression of Y14 and/or MAGOH affected IL-6-mediated STAT3 activation by transient transfection experiments in Hep3B cells using STAT3-LUC, in which the α2-macroglobulin promoter drives expression of a luciferase (LUC) reporter gene. Expression vectors for Y14 and/or MAGOH together with STAT3-LUC were transfected into 293T cells. After 48 h, the cells were harvested and the STAT3-LUC activities were determined. As shown in Fig. 3B, Y14 markedly upregulated the IL-6-induced STAT3 activation. In contrast, the MAGOH transfectant failed to show enhancement of IL-6-induced STAT3 activation. Transfection of both Y14 and MAGOH caused slight upregulation of IL-6-induced STAT3 activation. These results were consistent with the influences of Y14 and/or MAGOH on IL-6-induced tyrosine-phosphorylation of STAT3 shown in Fig. 3A. To further test whether reductions in Y14 and/or MAGOH expression affect IL-6/STAT3-mediated gene expression, we used siRNAs to reduce the endogenous expression of Y14 and/or MAGOH in Hep3B cells. As shown in Fig. 4A and B, IL-6/STAT3-mediated SOCS3 and C/EBPδ mRNA expressions were remarkably

augmented in MAGOH siRNA-treated Hep3B cells, but reduced in Y14 and Y14/MAGOH siRNA-treated Hep3B cells. These results indicate that Y14 and MAGOH both play critical roles in the regulation of STAT3 activation *in vivo*.

Concluding remarks

We have demonstrated that Y14 is a binding partner for STAT3 and that a reduction in endogenous MAGOH increases STAT3-mediated transactivation by displacing Y14 from STAT3.

mRNAs produced by splicing are translated more efficiently than those produced from similar intronless precursor mRNAs. The EJC probably mediates this enhancement, but the specific links between the EJC and the translation machinery have not yet been identified. The EJC proteins Y14 and MAGOH play crucial roles by binding to spliced mRNAs after their export from the nucleus to the cytoplasm and are only removed when the mRNAs are translated. Y14 and MAGOH are both essential for cell viability, and mutants of Y14 and mago-nashi, the *Drosophila* homolog of MAGOH, show mislocalization of oskar mRNA in *Drosophila* oocytes. Y14 and mago-nashi form the *Drosophila* oskar mRNA localization complex and act as nuclear shuttling proteins for oskar mRNA cytoplasmic localization [21, 22]. Human Y14 has also been shown to associate with mRNAs upon splicing and shuttles to the cytoplasm where it interacts with MAGOH [20]. At the present time, there have been no previous reports of an mRNA shuttling protein involved in the regulation of transcriptional factors, such as the nuclear shuttling of STAT3.

Our data presented here clearly indicated that the binding of Y14 to STAT3 enhanced IL-6-induced STAT3 activation. The binding seemed to occur in the cytoplasm because Y14 showed high binding capacity to STAT3-C, which mainly located in the cytoplasm. The most important finding is that MAGOH negatively regulated IL-6-induced STAT3 activation via interfered the binding between Y14 and STAT3. Our immunoprecipitation experiments suggested the possible mechanism that Y14 has high affinity to MAGOH than to STAT3. Thus, we have identified a novel regulatory mechanism for cytokine-induced STAT3 activation by Y14 and MAGOH. Although we investigated whether IL-6 induces mRNA expression of Y14 and MAGOH in Hep3B cells, we did not observe any significant alterations in these mRNA levels after IL-6 stimulation (data not shown). STAT3 is known to play an essential role in the self-renewal of embryonic stem cells, and it is the only STAT family member whose knockout leads to embryonic lethality [23]. Therefore, it is possible that the expressions of Y14 and MAGOH are developmentally regulated and regulate STAT3 activation.

It is very important to identify signaling pathways in the STAT3-specific regulation of gene transcription, since STAT3 may be a key player not only in the pathogenesis of diverse human diseases, but also in the self-renewal and reprogramming of embryonic stem cells. Further understanding of the detailed molecular interactions among STAT3, Y14 and MAGOH may provide novel therapeutic strategies for human diseases or regenerative medicine.

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Figure legends

Fig. 1. STAT3 endogenously binds to Y14 in Hep3B cells

(A) 293T cells in a 6 cm dish were transfected with HA-STAT3 WT (WT) (3 μg) with Myc-Y14 (5 μg). At 48 h after transfection, cells were stimulated with LIF for the indicated periods. The cells were and lysed, immunoprecipitated with anti-HA, and blotted with anti-Myc or anti-HA antibody. An aliquot of total cell lysates (TCL) was was blotted with anti-Myc, anti-HA or anti-pSTAT3 (Tyr705) antibody. (B) 293T cells in a 6 cm dish were transfected with Myc-STAT3 WT (WT) or STAT3 YF (YF) (3 μg) with Myc-Y14 (5 μg). At 48 h after transfection, cells were stimulated or unstimulated with LIF for 15 min. The cells were and lysed, immunoprecipitated with anti-HA, and blotted with anti-Myc or anti-HA antibody. An aliquot of TCL was blotted with anti-Myc or anti-HA antibody. (C) 293T cells in a 6 cm dish were transfected with FLAG-STAT3 WT (WT) or STAT3-C (C) (3 μg) with Myc-Y14 (5 μg). At 48 h after transfection, cells were stimulated or unstimulated with LIF for 15 min. The cells were and lysed, immunoprecipitated with anti-FLAG, and blotted with anti-Myc or anti-FLAG antibody. An aliquot of TCL was blotted with anti-Myc or anti-HA antibody. (D) Hep3B cells ($3x10^7$ cells) were stimulated with IL-6 for 15 min. The cells were and lysed, immunoprecipitated with control IgG (Ig), anti-STAT3 Ig or anti-Y14 Ig, and blotted with anti-STAT3 or anti-Y14 antibody. An aliquot of TCL was blotted with anti-STAT3 or anti-Y14 antibody.

Fig. 2. MAGOH interferes complex formation between STAT3 and Y14

(A) 293T cells in a 6 cm dish were transfected with Myc-tagged Y14 (5 μg) and/or FLAG-tagged MAGOH (3 μg) together with HA-tagged STAT3 (3 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-HA antibody and immunoblotted with an anti-Myc, anti-FLAG or anti-HA antibody. An aliquot of total cell lysates (TCL) was immunoblotted with an anti-Myc, anti-FLAG or anti-HA antibody. (B) 293T cells in a 6 cm dish were transfected with HA-tagged STAT3 (3 μg) and/or FLAG-tagged MAGOH (3 μg) together with Myc-tagged Y14 (5 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-Myc antibody and immunoblotted with an anti-HA, anti-FLAG or anti-Myc antibody. An aliquot of TCL was immunoblotted with an anti-HA, anti-FLAG or anti-Myc antibody.

Fig. 3. Y14 and MAGOH are involved in IL-6-induced tyrosine-phosphorylation of STAT3

(A) Hep3B cells in a 24-well plate were treated with control, Y14 and/or MAGOH siRNA, and cells were stimulated with IL-6 (10 ng/ml) for the indicated periods. The cells were lysed, and an aliquot of total cell lysates (TCL) was blotted with anti-pSTAT3 (Tyr705), anti-STAT3, anti-Y14 or anti-actin antibody. Total RNA samples isolated from these cells were also subjected to RT-PCR analysis using MAGOH or G3PDH primers. (B) Densitometric quantification of the above results was also shown. Relative intensity of pSTAT3 (Tyr705) was normalized to the STAT3 protein of the same sample. (C) Hep3B cells in a 24-well plate were transfected with STAT3-LUC (200 ng) and/or the

increasing amount of FLAG-tagged MAGOH (10, 50, 250 ng) and Myc-tagged Y14 (10, 50, 250 ng). The cells in a 24-well plate were also transfected with STAT3-LUC (200 ng) and FLAG-tagged MAGOH (250 ng) and Myc-tagged Y14 (10, 50, 250 ng). At 48 h after transfection, the cells were treated with IL-6 (10 ng/ml) for 8 h. The cells were harvested and the luciferase activities were measured. At least three independent experiments were carried out for each assay.

Fig. 4. Y14 and MAGOH are involved in IL-6/STAT3-dependent gene expression

(A) Hep3B cells in a 6 cm dish were treated with control, Y14 and/or MAGOH siRNA, and cells were stimulated with IL-6 (10 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SOCS3, C/EBPδ, Y14, MAGOH or G3PDH primers. (B) SOCS3 and C/EBPδ expression levels were also quantified by reverse transcription and quantitative real-time PCR analysis using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems). Data represent the levels of SOCS3 and C/EBPδ mRNA normalized to that of a G3PDH internal control and are expressed relative to the value of control siRNA-treated samples without IL-6-stimulation. Results are representative of three independent experiments, and the error bars represent the S.D.

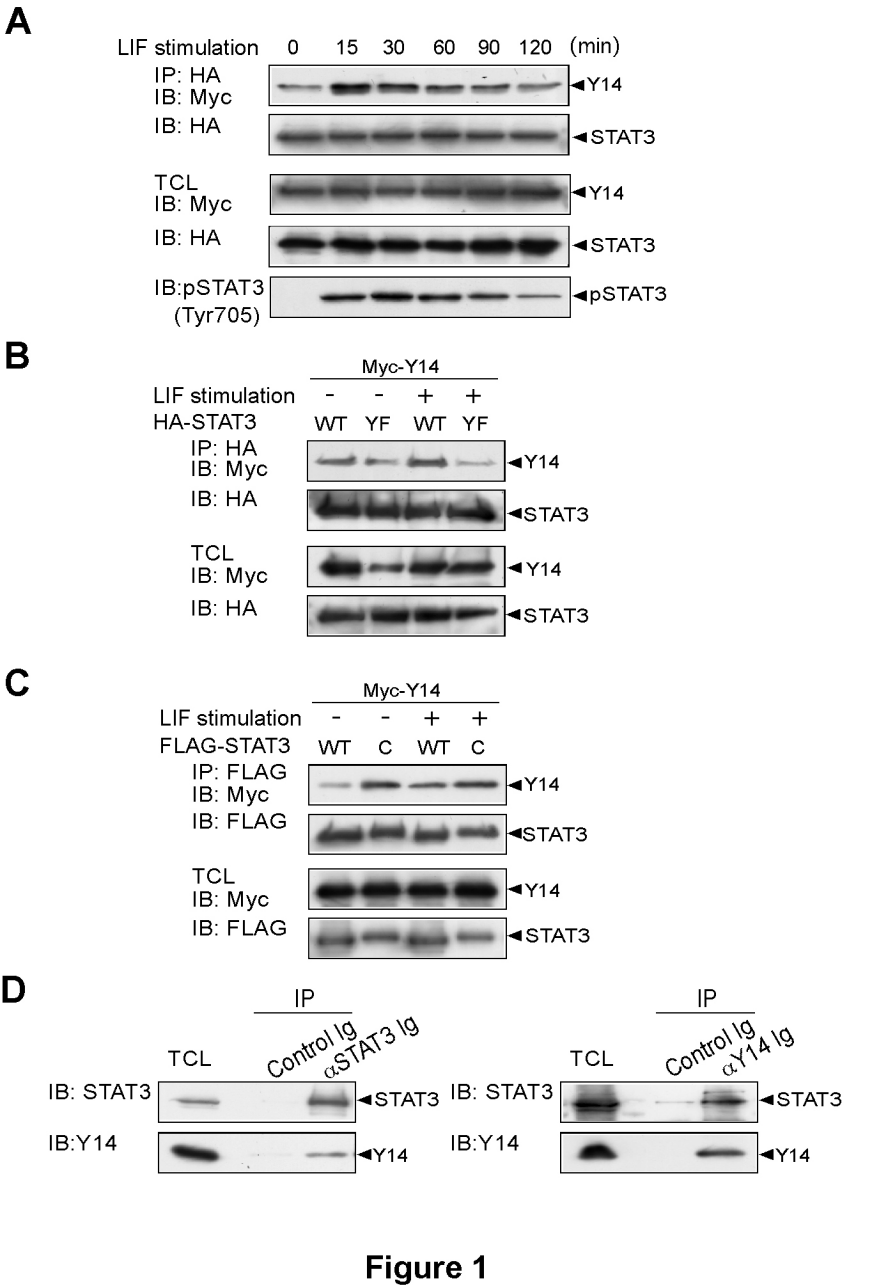
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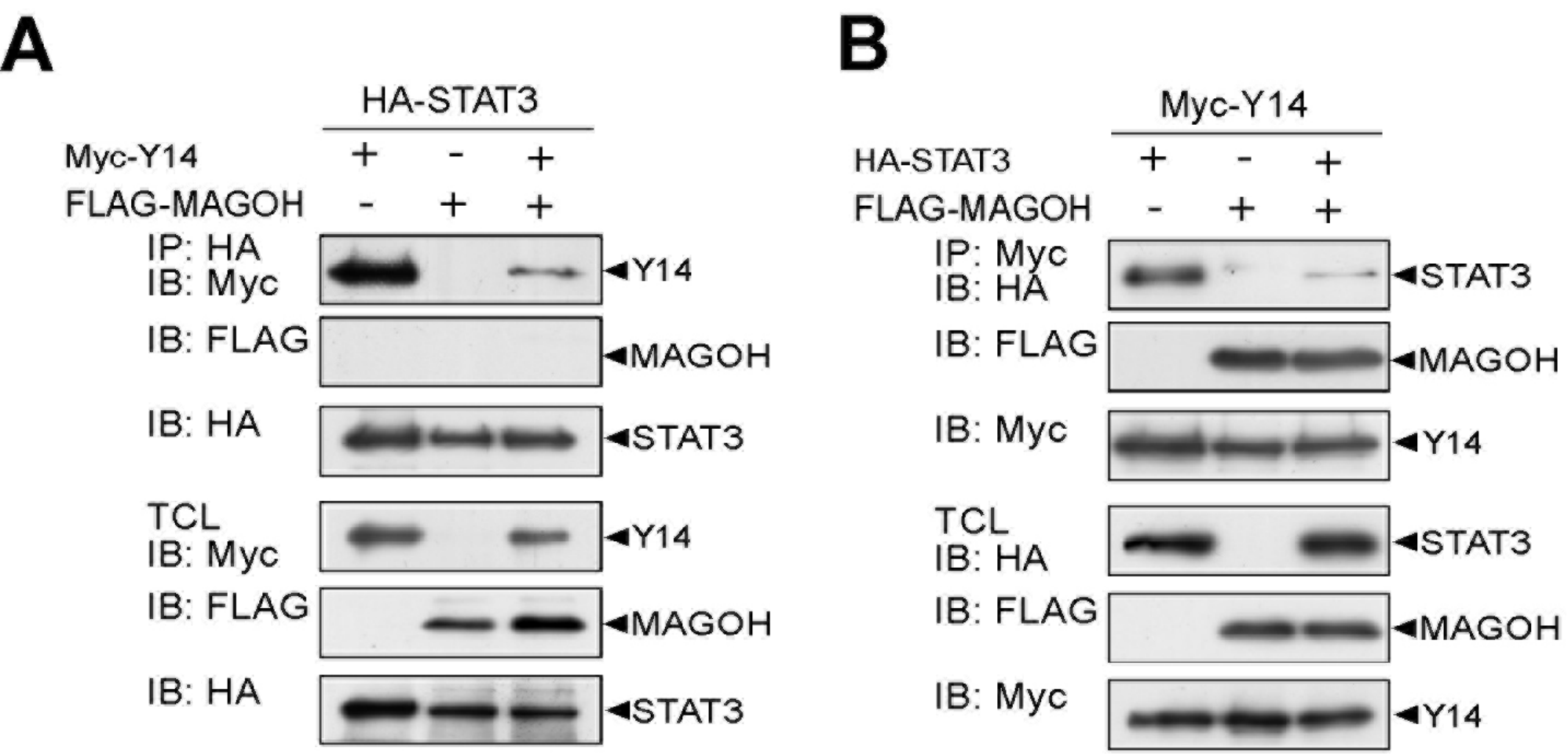
Figure 1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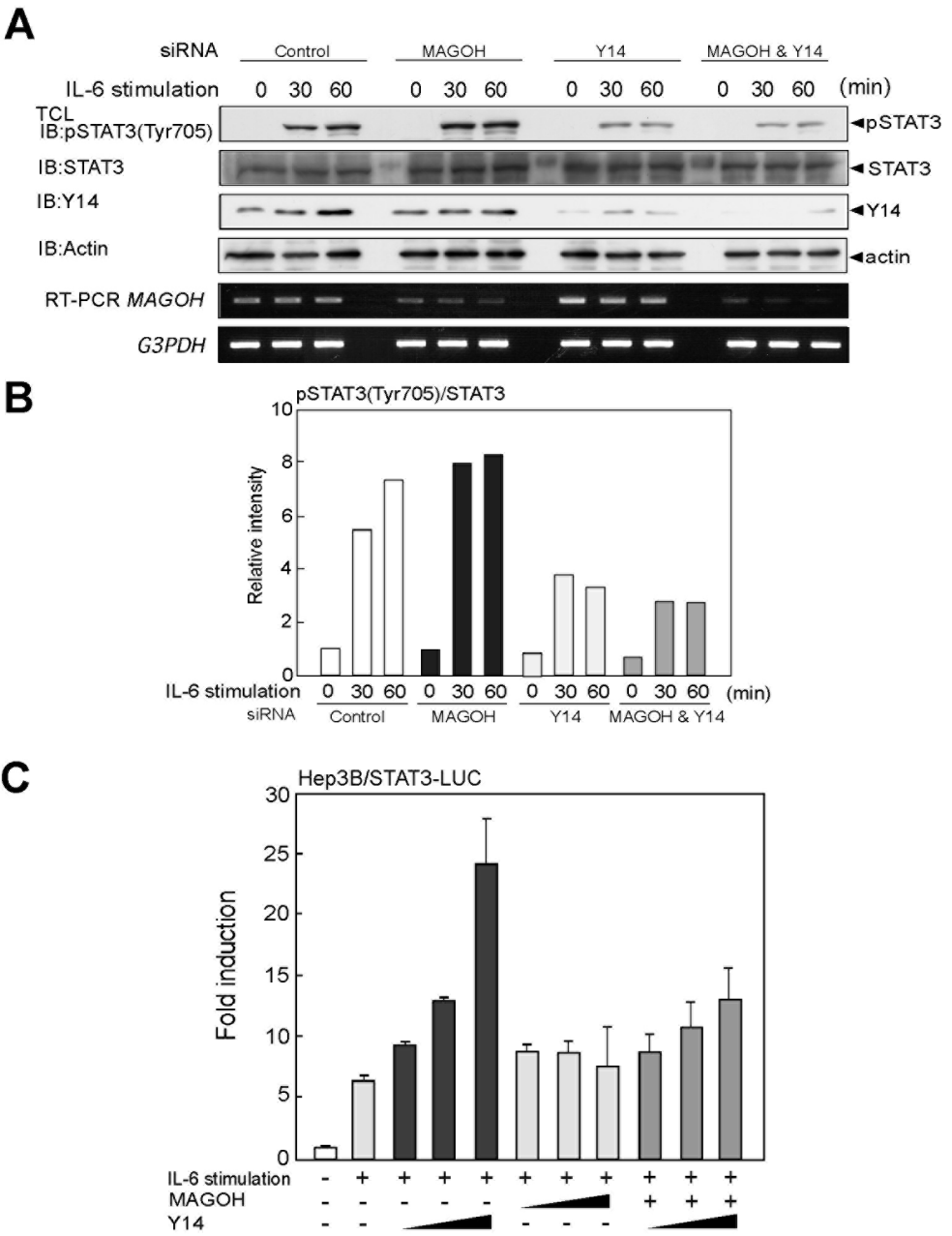
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MAGOH
Y14
MAGOH & Y14
IL-6 stimulation
0 30 60 0 30 60 0 30 60 0 30 60 (min)
TCL
IB:pSTAT3(Tyr705)
pSTAT3
IB:STAT3
STAT3
IB:Y14
Y14
IB:Actin
actin
RT-PCR MAGOH
G3PDH
B
pSTAT3(Tyr705)/STAT3
Relative intensity
10
8
6
4
2
0
IL-6 stimulation
0 30 60 0 30 60 0 30 60 0 30 60 (min)
siRNA
Control
MAGOH
Y14
MAGOH & Y14
C
Hep3B/STAT3-LUC
Fold induction
30
25
20
15
10
5
0
IL-6 stimulation - + + + + + + + + + +
MAGOH - - - - - + + +
Y14 - - - - - -

Figure 3

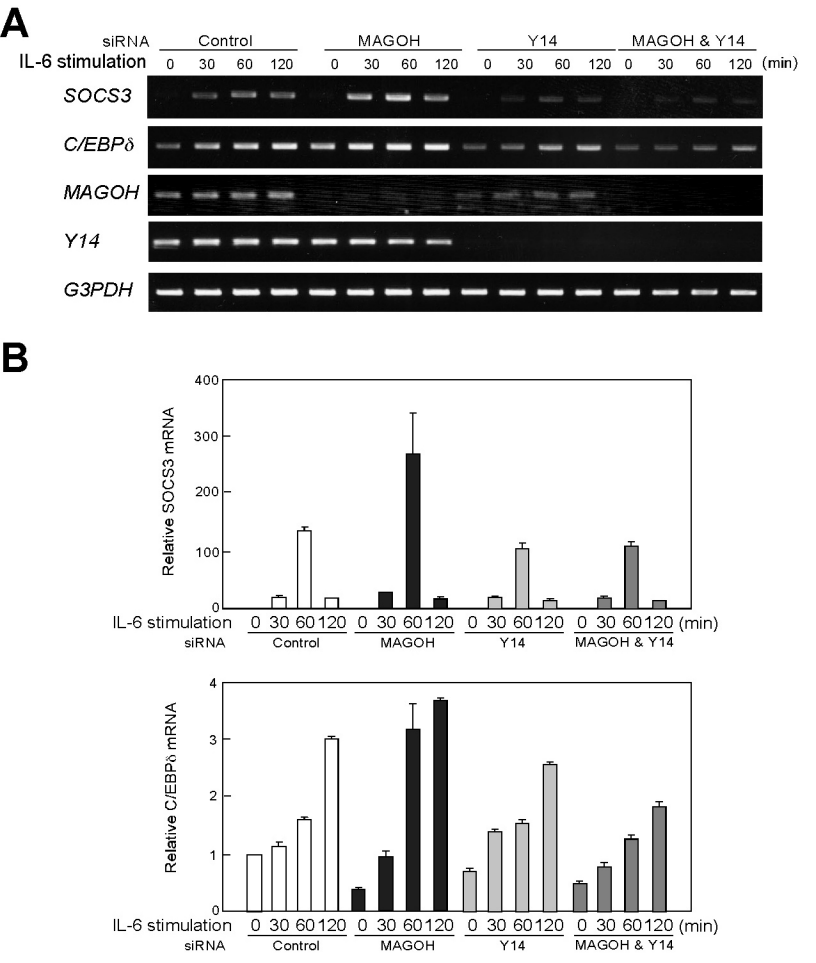
A
siRNA
Control
MAGOH
Y14
MAGOH & Y14
IL-6 stimulation
0 30 60 120 0 30 60 120 0 30 60 120 0 30 60 120 (min)
SOCS3
C/EBPδ
MAGOH
Y14
G3PDH
B
Relative SOCS3 mRNA
400
300
200
100
0
IL-6 stimulation 0 30 60 120 0 30 60 120 0 30 60 120 0 30 60 120 (min)
siRNA Control MAGOH Y14 MAGOH & Y14
Relative C/EBPδ mRNA
4
3
2
1
0
IL-6 stimulation 0 30 60 120 0 30 60 120 0 30 60 120 0 30 60 120 (min)
siRNA Control MAGOH Y14 MAGOH & Y14

Figure 4