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KAP1 regulates type I interferon/STAT1-mediated IRF-1 gene expression

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ABSTRACT

Signal transducers and activators of transcription (STATs) mediate cell proliferation, differentiation, and survival in immune responses, hematopoiesis, neurogenesis, and other biological processes. Recently, we showed that KAP1 is a novel STAT-binding partner that regulates STAT3-mediated transactivation. KAP1 is a universal co-repressor protein for the KRAB zinc finger protein superfamily of transcriptional repressors. In this study, we found KAP1-dependent repression of interferon (IFN)/STAT1-mediated signaling. We also demonstrated that endogenous KAP1 associates with endogenous STAT1 *in vivo*. Importantly, a small-interfering RNA-mediated reduction in KAP1 expression enhanced IFN-induced STAT1-dependent IRF-1 gene expression. These results indicate that KAP1 may act as an endogenous regulator of the IFN/STAT1 signaling pathway.

The intracellular processing of cytokine signals entails the activation and nuclear translocation of STAT family transcription factors [1–3]. STATs are unusual among transcription factors in that they have characteristics of cytoplasmic signaling molecules, such as a Src-homology 2 (SH2) domain and tyrosine phosphorylation sites. Upon tyrosine phosphorylation, STATs dimerize through their phosphorylated SH2 domains and translocate to the nucleus. In recent years, constitutive or dysregulated expression of STATs has been found in cancer cells and oncogene-transfected cells and shown to be involved in a wide range of diseases. Therefore, STAT activation is tightly regulated by a variety of mechanisms.

Type I interferons (IFNs) are pleiotropic cytokines that possess several biological activities and play a central role in basic and applied research as mediators of antiviral and antigrowth responses, modulators of the immune system, and therapeutic agents against viral diseases and cancer [4,5]. Treatment of cells with IFN induces the activation of Jak-STAT molecules including Jak1 and Tyk2 as well as STAT1 and STAT2. Previous experiments using knock out mice have revealed that the Jak1-STAT1, STAT2 signals are major pathways for the IFN-mediated functions [6–8]. IFN-activated Jak/STAT signaling results in the transcriptional induction of many target genes, which include the genes for dsRNA-activated serine/threonine protein kinase, 2′,5′-oligoadenylate synthetase and the interferon-regulatory factor (IRF) family proteins [9–11].

In attempt to identify novel STAT-binding partners, we screened a mouse embryo cDNA library with a yeast two-hybrid system using the C-terminal domain of STAT4 as bait. We recently identified KAP1 [12], a co-repressor of the Kruppel-associated box (KRAB)-domain-containing zinc finger proteins [13–15] as a protein that interacts specifically with STAT1, STAT3, STAT4, and STAT6. KAP1 can recruit and coordinate many components involved in gene silencing. KAP1-mediated gene silencing involves the recruitment of the histone deacetylase (HDAC) complex [16–18], and binding to a histone methyltransferase [19]. Therefore, KAP1 orchestrates the function of these co-repressor complexes to inhibit the transcription of its target genes.

In the present study, we show a reduction in the level of endogenous KAP1 expression in HeLa cells resulting in enhanced IFN/STAT1-mediated transcriptional activation and gene expression of IRF-1. We also demonstrate that endogenous KAP1 interacts with STAT1 *in vivo*. Furthermore, a combination of KAP1 and HDAC1 knock-down demonstrated that HDAC1 cooperatively acts with KAP1 in repression of IFN/STAT1-induced IRF-1-LUC activation. Therefore, these results indicate that KAP1 is an endogenous transcriptional regulator of IFN/STAT1-mediated signaling.

Materials and methods

Reagents and antibodies. Recombinant human IFN-α was kindly provided from Dainippon Sumitomo Pharma Company (Osaka, Japan). Expression vectors for STAT1, KAP, and IRF-1-LUC were kindly provided by J.N. Ihle (St. Jude CRH, Memphis, TN), H. Ariga (Hokkaido University, Sapporo, Japan) and T. Tanaka (RCAI, RIKEN, Yokohama, Japan) [18,20]. Anti-STAT1, anti-KAP1, anti-HDAC1, anti-HDAC2, and anti-HDAC3 antibodies were obtained from Santa Cruz Biotechnology (Santa

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Cruz, CA); anti-HA antibody from Sigma–Aldrich (St. Louis, MO); anti-pSTAT1 (Tyr705) and anti-pSTAT1(Ser727) antibodies from Cell Signaling Technologies (Beverly, MA); anti-Actin antibody from Chemicon International (Temecula, CA).

Cell culture, transfection, small-interfering RNA (siRNA), and luciferase assays. Human cervix carcinoma cell line HeLa and human embryonic kidney carcinoma cell line 293T were maintained in DMEM containing 10% FCS. HeLa cells were transfected using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instruction. 293 T cells were transfected with the standard calcium precipitation protocol [21]. siRNAs targeting KAP1 and control siRNA used in this study was described previously [22]. siRNAs targeting human HDAC1, HDAC2, and HDAC3 used in this study were as follows: HDAC1, 5′-GCUUCAAUCUAACUAUCAATT-3′; HDAC2, 5′-CAGUGAUGAGUAUAUC AAATT-3′; HDAC3, 5′-GCCGGUUAUCAACCAGGUATT-3′. HeLa cells were plated on a 24-well plate at 2×10^4 cells/well, and then incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FCS. The cells were then transfected with IRF-1-LUC using jetPEI. Twenty-four hours after transfection, the cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Three or more independent experiments were carried out for each assay.

Immunoprecipitation and immunoblotting. The immunoprecipitation and Western blotting assays were performed as described previously [21]. The immunoprecipitates from cell lysates were resolved on SDS–PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA).

RNA isolation and quantitative real-time PCR. Cells were harvested and total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high-Plus-Kit (TOYOBO, Tokyo, Japan). Quantitative real-time PCR analyses of IRF-1, Mx1 as well as the control G3PDH mRNA transcripts were carried out using the assay-on-demand™ gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems) [22].

Statistical analysis. The significance of differences between group means was determined by Student's *t*-test.

Results and discussion

KAP1 regulates IFN-induced gene expression and transcriptional activation

In our previous work, we have shown that KAP1 negatively regulates IFN-induced transcription by a transient reporter assay using the IFN-stimulated responsive element (ISRE)-LUC [12]. We further confirmed that KAP1 affects IFN-induced transcriptional activation and gene expression using siRNA to reduce the endogenous expression of KAP1 in HeLa cells. A specific siRNA for KAP1 or a control siRNA was transfected into HeLa cells and aliquots of cell lysates and total RNAs were analyzed by Western blotting and RT-PCR, respectively, to confirm reduced expression

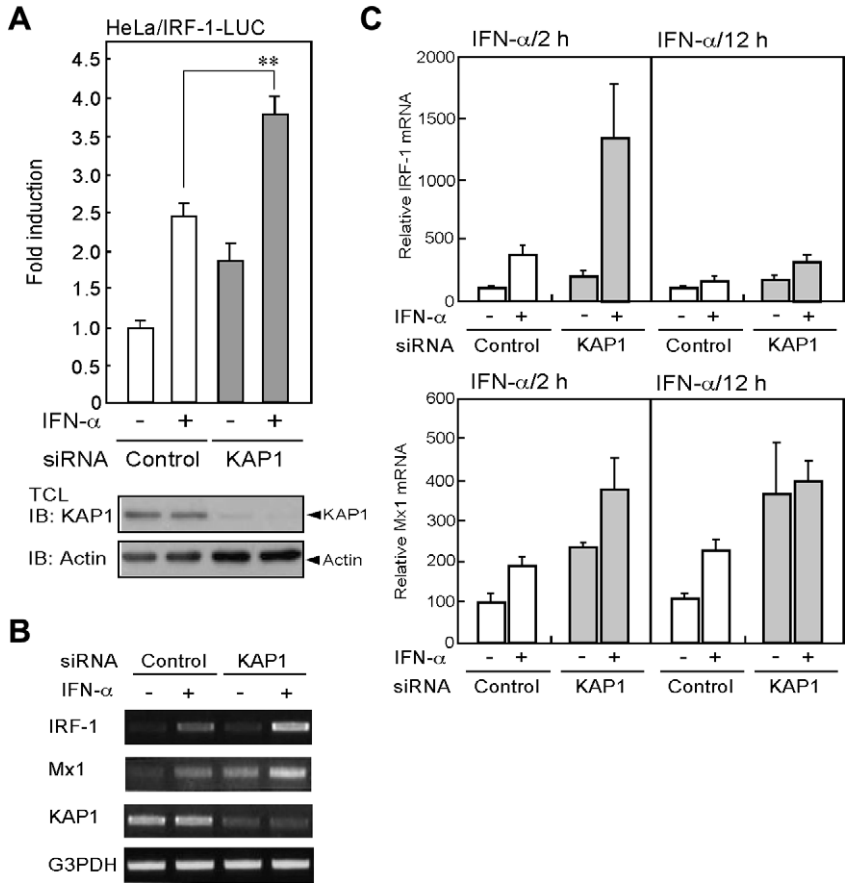

Fig. 1. KAP1 regulates IFN-induced gene expression and transcriptional activation. (A) HeLa cells in a 24-well plate were transfected with control siRNA or siRNA targeting human KAP1 using Lipofectamine2000. The cells were then transfected with IRF-1-LUC using jetPEI. At 36 h after transfection, cells were treated with IFN-α (10,000 U/ml) for an additional 8 h. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the SD. $^{*}p < 0.01$. An aliquot of total cell lysates (TCL) was analyzed by immunoblotting using anti-KAP1 or anti-Actin antibody. (B) HeLa cells in a 24-well plate were treated with control or KAP1 siRNA, and cells were stimulated with IFN-α (1000 U/ml) for an additional 2 h. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using IRF-1, Mx1, KAP1, or G3PDH primers. (C) HeLa cells in a 24-well plate were treated with control or KAP1 siRNA, and cells were stimulated with IFN-α (1000 U/ml) for an additional 2 or 12 h. IRF-1 and Mx1 expression levels were also quantified by reverse transcription and quantitative real-time PCR analysis using the assay-on-demand™ gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system. Data represent the levels of IRF-1 and Mx1 mRNA normalized to that of a G3PDH internal control and are expressed relative to the value of control siRNA-treated samples. Results are representative of three independent experiments, and the error bars represent the SD.