J. Biochem. **143,** 695–709 (2008)
doi:10.1093/jb/mvn022

Physical and Functional Interactions between STAT5 and Runx Transcription Factors

Shinya Ogawa[1,2], Masanobu Satake[3] and Koichi Ikuta[1,*]

[1]Laboratory of Biological Protection, Department of Biological Responses, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507; [2]Graduate School of Biostudies, Kyoto University, Yoshida-Honmachi, Sakyo-ku, Kyoto 606-8501; and [3]Department of Molecular Immunology, Institute of Development, Aging and Cancer, Tohoku University, Aoba-ku, Sendai 980-8575, Japan

Received January 31, 2008; accepted February 11, 2008; published online February 22, 2008

The signal transducers and activators of transcription (STAT) and the Runt-related (Runx) are two of major transcription factor families that play essential roles in lymphocyte development. Although the interaction of Runx2 with STAT1 and STAT3 has been reported before, the interaction between STAT5 and Runx family proteins has not been characterized. In this study, we first showed that STAT5 physically interacts with Runx1, Runx2 and Runx3 by co-immunoprecipitation experiments. The Runt domain of Runx proteins and the DNA-binding domain and α-helix loop structure of STAT5 are responsible for the interaction. When expressed in CHO cells, STAT5 inhibits the nuclear localization of Runx proteins and retains them in the cytoplasm. In addition, we showed by reporter assay that the interaction between STAT5 and Runx proteins mutually inhibits their transcriptional activity. Furthermore, Runx proteins inhibit the DNA-binding activity of STAT5. Finally, we found that Runx proteins suppress the transcription of an endogenous STAT5 target gene, cytokine-inducible SH2 protein-1, in an interleukin-3-dependent pro-B cell line, Ba/F3. These results collectively suggested that STAT5 and Runx proteins physically and functionally interact to mutually inhibit their transcriptional activity. Thus, this study implies a potential role of the STAT5–Runx interaction in lymphocyte development.

Key words: interleukin-7, Runx, signal transduction, STAT5, transcription factor.

Abbreviations: CA, constitutively active; ChIP, chromatin immunoprecipitation; CIS1, cytokine-inducible SH2 protein-1; DAPI, 4, 6-dia-midino-2-phenylindole; EMSA, electrophoretic mobility shift assay; hnRNP, heterogeneous nuclear ribonucleoprotein; IL, interleukin; PABP, poly(A)-binding protein; Runx, Runt-related; SH2, Src homology 2; STAT, signal transducers and activators of transcription; WT, wild type.

Interaction of transcription factors plays an important role in the regulation of gene expression. Transcription factors bind to specific DNA sequences in the promoters, enhancers and silencers of target genes. Transcription factors then control the transcription by changing histone modifications and chromatin structures through recruiting transcriptional co-activators, co-repressors and chromatin-remodelling factors. Transcription factors sometimes form a complex called enhanceosome that simultaneously interacts with the promoter and enhancer. Many transcription factors synergize or repress each other by direct and indirect interactions. For example, haematopoietic master transcription factors GATA-1 and PU.1 interact and inhibit each other (*1–3*). This antagonism is important for determining erythroid *versus* myeloid lineage commitment during haematopoiesis. In another case, glucocorticoid receptor and NF-κB mutually repress transactivation through direct interaction (*4–6*). This negative cross talk partly explains anti-inflammatory action of glucocorticoids.

*To whom correspondence should be addressed. Tel: +81-75-751-4012, Fax: +81-75-751-4810, E-mail: ikuta@virus.kyoto-u.ac.jp

The signal transducers and activators of transcription (STAT) family proteins play essential roles in signal transduction of various cytokines and growth factors (*7*). In mammals, the STAT family is comprised of six members, STAT1–6. As for STAT5, two highly conserved genes, *STAT5a* and *STAT5b*, are present in human and mice. STAT5 is crucial for the development of lymphocyte and mammary gland. With regard to lymphocyte development, STAT5 deficiency results in a severe combined immunodeficiency phenotype similar to mice lacking interleukin (IL)-7 receptor α chain, JAK3 or common γ chain (*8*). STAT5 consists of N-terminal, coiled-coil, DNA-binding, Src homology 2 (SH2) and transactivation domains. Cytokine and growth factor signalling activates tyrosine kinases, Jak and Tyk. Upon tyrosine phosphorylation by the kinases, STAT5 forms a homodimer *via* phosphotyrosine interaction of the SH2 domain and translocates into the nucleus (*7*). STAT5 binds the consensus DNA sequence motif (TTCNNNGAA) and activates the promoters of various target genes by recruiting transcriptional co-activators, CBP and p300 (*9*), and a chromatin-remodelling factor, Brg1 (*10*). STAT5 is known to interact with other transcription factors. For example, GR interacts with STAT5 and enhances the activity of STAT5 (*11*, *12*).

Additionally, STAT5 interacts with Oct-1 and induces the transcription of cyclin D1 (*13*).

The Runt-related (Runx) transcription factors comprise a family of transcriptional regulators important for haematopoiesis and osteogenesis (*14*). The Runx family consists of Runx1/AML1/PEBP2αB, Runx2/AML3/PEBP2αA and Runx3/AML2/PEBP2αC. Runx1 is essential for definitive haematopoiesis, and regulates the expression of haematopoietic cell-specific genes (*15*). Runx2 is critical for the generation and maturation of osteoblasts by regulating the expression of bone-specific genes (*16*). Runx3 is required for the development of $CD8^+$ cytotoxic T cells (*17–19*). Runx transcription factors consist of Runt homology, transcription activation and transcription inhibition domains. The Runt homology domain is the 128-amino acid region that is highly homologous to the *Drosophila* segmentation gene *runt*. Runx transcription factors bind the consensus DNA sequence motif (PuACCPuCA, where Pu stands for a purine residue) *via* the Runt domain, and their binding affinity is increased by heterodimerization through the Runt domain with a ubiquitously expressed β subunit, CBFβ/PEBP2β (*20–22*). Runx transcription factors interact with transcriptional co-activators, CBP and p300, and a co-repressor, mSin3A and function as activator and suppressor of the target gene, respectively (*23*, *24*). Moreover, other transcriptional factors such as Ets-1, Pax5, AP-1, PU.1, CEBP/α, ALY, MOZ and Smads also interact with Runx, and regulate transcriptional activation of specific target genes (*14*).

Interaction between STAT and Runx transcription factors has been partially characterized. It was reported that STAT1 associates with Runx2, and inhibits the transcriptional activity of Runx2 by retaining in the cytoplasm (*25*). In addition, STAT3 physically interacts with Runx2 by growth hormone stimulation, and impairs its transcriptional activity without affecting DNA-binding capacity (*26*). On the other hand, the interaction of STAT5 with Runx family proteins is still to be elucidated. As STAT5, Runx1 and Runx3 play critical roles in T-lymphocyte differentiation, we characterized the interaction between STAT5 and Runx family proteins. We found that the Runt domain of Runx proteins and the DNA-binding domain and α-helix loop structure of STAT5 are responsible for the interaction. We further demonstrated that STAT5 retains Runx proteins in the cytoplasm and that the interaction between STAT5 and Runx proteins mutually inhibits their transcriptional activity. Finally, we found that Runx proteins suppress the expression of an endogenous STAT5 target gene. These results collectively suggested that STAT5 and Runx proteins physically and functionally interact to mutually inhibit their transcriptional activity.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293T, CHO (a kind gift from Dr T. Sudo at Pharmaceutical Research Laboratories, Toray Industries, Inc.), and Plat-E (*27*) (a kind gift from Dr T. Kitamura at University of Tokyo) were maintained in DMEM supplemented with 10% fetal bovine serum. A mouse pre-T cell line, Scid.adh-TAC:CD3ε (*28*) (a kind gift from Dr D. L. Wiest at Fox Chase Cancer Center), was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate and 1× non-essential amino acids. An IL-3-dependent pro-B cell line, Ba/F3, was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol and 0.2% conditioned medium of an X63 transfectant of a mouse IL-3 expression vector, BMGNeo-mIL-3 (*29*), as a source of IL-3.

Plasmid Constructs—cDNA fragments for mouse wild type (WT)- and constitutively active (CA)-STAT5a (*30*) (kind gifts from Dr T. Kitamura at University of Tokyo), mouse Runx1, Runx2 and Runx3, and a series of their deletion mutants were subcloned by PCR into pcDNAFlag, pcDNAHA and pcDNAMyc vectors. The *Eco*RI fragments containing Runx1 or Runx3 cDNA with HA epitope tagged at the N-terminus were cut from pMX-IG-HA.Runx1 and pMX-IG-HA.Runx3, and then subcloned in the *Eco*RI site of pMXs-IB (a pMXs vector with internal ribosome entry site and blasticidin-resistant gene) (*31*). Runx2 cDNA with HA epitope tagged at the N-terminus were subcloned by PCR into pMXs-IB.

Immunoprecipitation and Immunoblotting—HEK293T cells were transiently transfected by lipofection (FuGENE 6; Roche Diagnostics) with pcDNAMyc-WT-STAT5a, pcDNAFlag-Runx1, -Runx2, or -Runx3 and/or their deletion mutants. After 36–48 h, the cells were lysed for 30 min in 1 ml of ice-cold lysis buffer [1% NP-40, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.02% sodium azide, 100 μM phenylmethanesulphonyl fluoride, 1 μM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml α_1-anti-trypsin and 10 μg/ml soybean trypsin inhibitor]. The lysate was then centrifuged at 4°C for 15 min, and supernatant was collected. Protein concentration was measured by BCA Protein Assay Kit (Pierce) standardized with bovine serum albumin. An equal amount of protein (200 μg) was pre-cleared with protein G-Sepharose beads (GE Healthcare Bio-Science) at 4°C for 1.5 h, and then subjected to immunoprecipitation with the following antibodies: anti-STAT5a antibody (PA-ST5A, R&D Systems), anti-STAT5b antibody (PA-ST5B, R&D Systems), polyclonal anti-Flag antibody (Sigma), rabbit anti-pan-Runx antibody (*32*), and normal rabbit IgG (Upstate Biotechnology). The immunoprecipitates were washed five times with lysis buffer, and eluted in 2× SDS loading buffer by boiling. The samples were separated by 8–12% SDS–PAGE and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore). The blot was incubated with the following antibodies: anti-STAT5a antibody, anti-STAT5b antibody, anti-Myc antibody (purified from culture supernatant of 9E10 hybridoma), anti-HA 3F10 antibody (Roche), anti-Flag M2 antibody (Sigma) or biotin-anti-pan-Runx [anti-pan-Runx antibody was biotinylated with Biotin Labeling Kit (Roche)], and visualized with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), horseradish peroxidase-goat anti-rabbit IgG (Cappel), horseradish peroxidase-conjugated goat anti-rat IgG

(Jackson ImmunoResearch Laboratories) or horseradish peroxidase-streptavidin (ZYMED) by ECL or ECL plus detection system (GE Healthcare Bio-Science). Immunoblots were analysed and quantitated with a Lumino-image Analyzer (LAS-3000mini; Fuji Film) and Image Gauge software (version 4.23, Fuji Film).

Immunofluorescence Staining—CHO cells were grown on glass cover slips in a well of 6-well plate, and transiently transfected by lipofection (GeneJuice transfection reagent, Novagen) with 0.5 μg of pcDNAMyc-STAT5a and/or 1.0 μg of pcDNAFlag-Runx1, -Runx2 or -Runx3. The total amount of DNA (1.5 μg) was adjusted with the pcDNA vector. After 24 h, the cells were fixed and permealized in 3% paraformaldehyde and 0.5% Triton X-100 for 15 min, and incubated in phosphate-buffered saline containing 5% fetal bovine serum and 5% bovine serum albumin for 30 min. The cells were incubated with anti-STAT5a and anti-Flag M2 antibodies for 1 h, followed by staining with Alexa Fluor 568-goat anti-rabbit IgG and Alexa Fluor 488-goat anti-mouse IgG for 1 h. The nucleus was detected by 4, 6-dia-midino-2-phenylindole (DAPI) staining. The cells were viewed with a laser scanning spectral confocal microscope (TCS SP2, Leica Microsystems).

Sub-cellular Fractionation—Nuclear and cytosolic fractions were isolated as previously described (*33*). Briefly, CHO cells in a ϕ60-mm dish were transiently transfected with 1.0 μg of pcDNAMyc-STAT5a and/or 1.0 μg of pcDNAFlag-Runx1, -Runx2 or -Runx3 using GeneJuice transfection reagent. Total amount of DNA (2 μg) was adjusted with pcDNA vector. After 24 h, the cells were collected, lysed in 500 μl of RSB-100 buffer [10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 2.5 mM $MgCl_2$] containing 0.5% Triton X-100 by passing through 26-gauge needle and then incubated for 5 min on ice. The soluble supernatant was recovered as cytosolic fraction after centrifugation at 2,500*g* for 1 s. The nuclear pellets were re-suspended in 170 μl of the RSB-100 buffer, and sonicated by Bioruptor (COSMO BIO) for 10 cycles of on (30 s) and off (60 s) at high power. The sonicate was layered on 30% sucrose cushion in the RSB-100 buffer, and centrifuged at 5,000*g* for 15 min. The supernatant was collected as nuclear fraction. Each fraction corresponding to 2.5×10^4 cells was immunoblotted with anti-heterogeneous nuclear ribonucleoprotein (hnRNP)-C1/C2 and anti-poly(A)-binding protein (PABP) antibodies (ImmuQuest) as nuclear and cytosolic markers, respectively. The remainder was immunoprecipitated with anti-STAT5a and polyclonal anti-Flag antibodies, followed by immunoblotting with anti-STAT5a and anti-Flag M2 antibodies.

Luciferase Reporter Assay—HEK293T cells in a well of 24-well plate were transiently transfected by lipofection (FuGENE 6; Roche Diagnostics) with 200 ng of the luciferase reporter plasmids driven by the 400-bp Jγ1 promoter (pGL4-Jγ1) (*34*) or the T-cell receptor β-chain enhancer-TK promoter (Tww-tk-Luc) (*35*), 50 ng of pcDNAMyc-CA-STAT5a or pcDNAFlag-Runx1 and the *Renilla* luciferase control vector, EF1-Renilla (0.1 ng) or pGL4.74(hRluc/TK) (Promega) (5 ng), as well as a various amount of pcDNAFlag-Runx1, -Runx2 and -Runx3, pcDNAFlag-Runx1 deletion mutants, or pcDNAMyc-STAT5a. The total amount of DNA (400 ng) was kept constant with the pcDNA vector. Reporter gene analysis was performed 36 h after transfection. Cell lysates were subjected to Dual-Luciferase Reporter Assay System (Promega), and luciferase activity was measured with a luminometer (Lumat LB9507; Berthold). Firefly luciferase activity was normalized by Renilla luciferase activity. In each experiment, samples were analysed in triplicate, and each experiment was repeated at least twice.

Electrophoretic Mobility Shift Assay (EMSA)—HEK293T cells in a well of 6-well plate were transfected by lipofection (GeneJuice) with 0.5 μg of pcDNAMyc-CA-STAT5a and/or 0.5, 1.5 and 1.0 μg of pcDNAFlag-Runx1, -Runx2 or -Runx3, respectively. Total amount of DNA (2 μg) was adjusted with the pcDNA vector. After 24 h, the cells were recovered, and incubated in 400 μl of sucrose I buffer [10 mM Tris–HCl (pH 8.0), 0.32 M sucrose, 3 mM $CaCl_2$, 0.2% NP-40, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol] on ice for 5 min. The lysate was mixed with 400 μl of sucrose II buffer [10 mM Tris–HCl (pH 8.0), 2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol]. The mixture was layered on a cushion of 440 μl of the sucrose II buffer, and centrifuged at 20,000*g* for 15 min. The nuclear pellet was re-suspended in 30 μl of low salt buffer [20mM HEPES–KOH (pH 7.9), 25% glycerol, 20 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1.5 mM $MgCl_2$]. The nuclear suspension was mixed with 30 μl of high salt buffer [20 mM HEPES–KOH (pH 7.9), 25% glycerol, 300 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1.5 mM $MgCl_2$], incubated on ice for 30 min and centrifuged at 20,000*g* for 30 min. The supernatant was recovered as nuclear extract.

The nuclear extract (10 μg) was incubated on ice for 20 min in binding buffer [10 mM HEPES–KOH (pH 7.9), 0.1 mM EDTA, 50 mM NaCl, 5% glycerol and 0.1% NP-40) containing 10 μg/μl poly(dI–dC). The binding reaction was added with 3′-biotinylated STAT5 oligonucleotide (mouse β-casein element: 5′-AGATTTCTAGGAA TTCAATCC-3′, STAT consensus motif is underlined), and 1 μg of normal rabbit IgG (Upstate Biotechnology) or mouse anti-STAT5 antibody (Santa Cruz Biotechnology), and incubated on ice for 30 min. The binding reactions were electrophoresed through 5% gel (19:1 acrylamide/bis acrylamide) in 0.5× TBE buffer at 4°C. DNA–protein complex was transferred to Zeta-Probe blotting membrane (Bio-Rad Laboratories) and visualized by LightShift Chemiluminescent EMSA Kit (PIERCE). Luminescence intensity was quantitated by lumino-image analyser (LAS-3000 mini; Fuji Film).

Virus Infection—Ba/F3 cells were stably transfected with pMXs-IB vectors with Runx1, Runx2 or Runx3 cDNA by retrovirus-mediated gene transfer using Plat-E packaging cells (*27*).

RNA Isolation, cDNA Synthesis and Real-Time RT–PCR—Total RNA was prepared form cells using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from total RNA with random primer and ReverTra Ace (TOYOBO). Random primed-cDNA was amplified in triplicate using *Taq*Man Ribosomal RNA control reagents VIC probe (Applied Biosystems) or

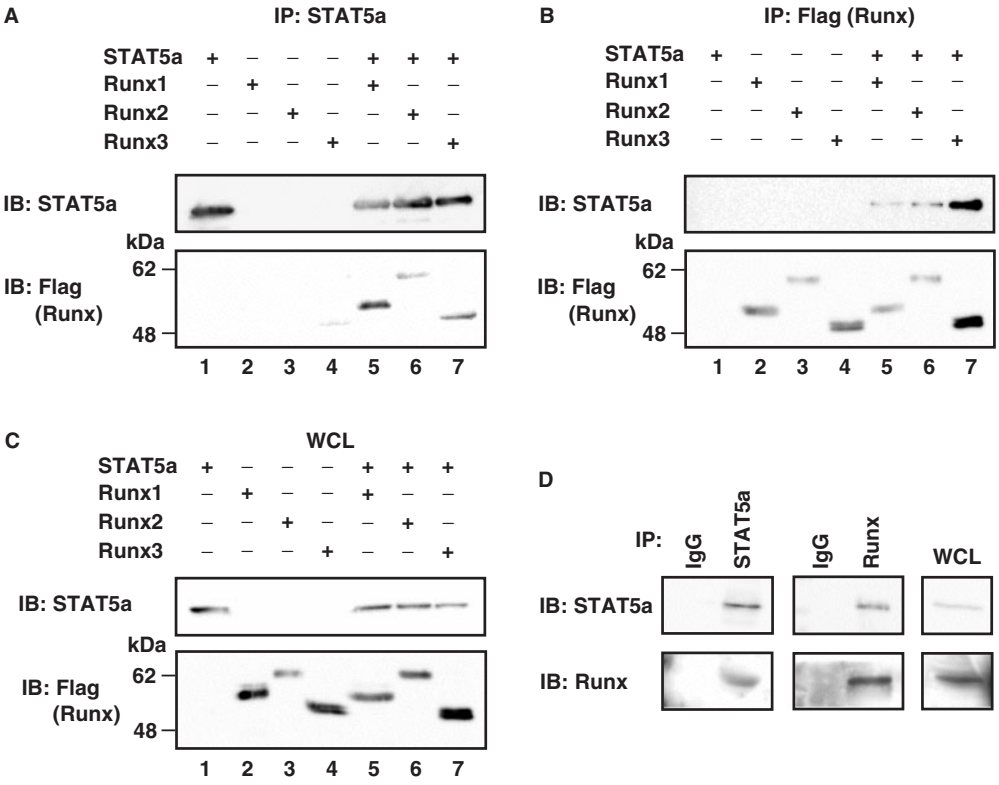

Fig. 1. **Runx proteins interact with STAT5.** (A) HEK293T cells were transfected with the expression vectors encoding Flag-Runx1, -Runx2 and -Runx3, and Myc-STAT5a. The cells lysate was immunoprecipitated with anti-STAT5a antibody, and immunoblotted with monoclonal anti-Flag antibody, followed by re-probing with anti-STAT5a antibody. Protein size marker is shown at left. (B) The same lysate was immunoprecipitated with polyclonal anti-Flag antibody, and immunoblotted with anti-STAT5a antibody, followed by re-probing with monoclonal anti-Flag antibody. (C) The whole-cell lysate (WCL) (2.5% input) was immunoblotted with anti-STAT5a antibody, followed by re-probing with monoclonal anti-Flag antibody. (D) The cell lysate of Scid.adh cells was immunoprecipitated with anti-STAT5a antibody, anti-pan-Runx antibody or control IgG, and immunoblotted with anti-STAT5a antibody or biotinylated pan-Runx antibody (left and middle panels). The whole-cell lysate (WCL) (2.5% input) was blotted with anti-STAT5a antibody or biotinylated pan-Runx antibody (right panels).

QuantiTect SYBR Green PCR Kit (QIAGEN) with primers for 40 cycles at 95°C for 15 s and 60°C for 1 min (rRNA), or at 94°C for 15 s, 60°C for 30 s and 72°C for 40 s [cytokine-inducible SH2 protein-1 (CIS1)] by ABI 7500 Sequence Detector (Applied Biosystems). The results were analysed using the Sequence Detection System 1.3.1 software (Applied Biosystems). Serial dilution of Ba/F3 cDNA was used as standard control. The levels of CIS1 mRNA were normalized with that of 18S rRNA. Sequences of the primers are as follows: CIS1, 5′-CATGGTCCTTTGCGTACAGG-3′ (sense) and 5′-TCAT TCTCTGCCTGGACAGG-3′ (anti-sense). Dissociation curve analysis and agarose gel electrophoresis were done to verify specific amplification of PCR.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed as previously described (*9*). Briefly, BaF3/pim-1 cells (5×10^6) were fixed with 1% formaldehyde for 5 min at room temperature and for 45 min at 4°C. Soluble chromatin containing DNA of 200- to 1,000-bp length was immunoprecipitated with 1.5 µl each of mouse anti-STAT5a and anti-STAT5b antibodies (R&D Systems) or 3 µl normal rabbit IgG (Upstate Biotechnology) for overnight at 4°C. Purified ChIP DNA was measured by real-time PCR. After amplification, melting curve analysis was performed to verify the specificity of the reaction. Serial dilution of sonicated genomic DNA of Ba/F3 cells was used as control for calibration. The level of ChIP DNA was normalized with that of input DNA. In each experiment, samples were analysed in triplicate. The sequences of PCR primers for the CIS-1 promoter were as follows: CIS-1 promoter sense, 5′-GGCACGTCAGT TCAGGGTC-3′ and anti-sense, 5′-GGCTCGAGAGTCGG AGTTC-3′.

RESULTS

Interaction of STAT5 and Runx Family—Previously, STAT1 and STAT3 were shown to interact with a member of Runx family, Runx2 (*25*, *26*). To test whether STAT5 interacts with Runx family proteins *in vivo*, we carried out co-immunoprecipitation experiment. HEK293T cells were transfected with STAT5a and Runx1, Runx2 or Runx3 expression vectors, and cell lysate was immunoprecipitated with anti-STAT5 antibody, followed by immunoblotting with anti-Flag (Runx) antibody. All members of Runx family were co-immunoprecipitated with STAT5a (Fig. 1A, lanes 5–7). In the absence of STAT5a, Runx proteins were not

immunoprecipitated (Fig. 1A, lanes 2–4). The association of STAT5a with Runx proteins was further examined in a reciprocal experiment in which the cell lysate was immunoprecipitated with anti-Flag (Runx) antibody, followed by immunoblotting with anti-STAT5a antibody. Certain amount of STAT5a was co-immunoprecipitated with Runx1, Runx2 and Runx3 (Fig. 1B, lanes 5–7). In the absence of Runx proteins, STAT5a was not immunoprecipitated (Fig. 1B, lane 1). Expression of STAT5a and Runx proteins was confirmed by immunoblotting of whole-cell lysate (Fig. 1C). We observed similar results with STAT5b (Supplementary Fig. 1). These results suggested that STAT5 specifically interacts with Runx proteins in forced expression system.

To test whether endogenous STAT5 and Runx proteins interact with each other, we next carried out immunoprecipitation experiment with the mouse pre-T cell line, Scid.adh. The Scid.adh cells only expressed Runx1 among the Runx family proteins (data not shown). First, cell lysate of Scid.adh cells was immunoprecipitated with anti-STAT5a antibody, followed by immunoblotting with anti-Runx antibody. Endogenous Runx1 was co-immunoprecipitated with STAT5a (Fig. 1D, left panels). The association of STAT5 with Runx1 was further examined in a reciprocal experiment in which the cell lysate was immunoprecipitated with anti-Runx antibody, followed by immunoblotting with anti-STAT5 antibody. Certain amount of STAT5a was co-immunoprecipitated with Runx1 (Fig. 1D, middle panels). These results suggested that endogenous STAT5 and Runx1 specifically interact with each other *in vivo*.

Mapping of the Interaction Domains of STAT5 and Runx Family—Since all members of Runx family were associated with STAT5, we assumed that a homologous region within Runx proteins was responsible for interaction with STAT5. Indeed, the Runt domain interacted with various transcriptional factors including CBFβ (*14*). To determine which domain of Runx proteins is responsible for interaction with STAT5, we constructed a series of Runx deletion mutants (Fig. 2A), and carried out co-immunoprecipitation experiment. HEK293T cells were transfected with the expression vectors for full-length STAT5a and Flag-tagged Runx1 deletion mutants, and cell lysate was immunoprecipitated with anti-STAT5a antibody, followed by immunoblotting with anti-Flag (Runx) antibody. The N-terminal (amino acid 1–51) and C-terminal (amino acid 178–451) regions of Runx1 were not necessary for the interaction with STAT5a (Fig. 2B, left panel, lanes 2 and 3). In contrast, the deletion of the N-terminal region and the Runt domain resulted in loss of association with STAT5a (lane 4). In addition, the Runt domain alone also interacted with STAT5a (lane 5). These results suggested that the Runt domain of Runx1 was necessary and sufficient for the interaction with STAT5. We obtained similar results with Runx3 deletion mutants (Fig. 2C and D) and the Runt domain of Runx2 (Supplementary Fig. 2), suggesting that the Runt domain of Runx family transcription factors is responsible for the interaction with STAT5.

Next, we dissected the domain of STAT5 interacting with Runx proteins. As shown in Fig. 3A, STAT5 consists of the N-terminal (amino acid 1–143), coiled-coil (amino acid 144–329), DNA-binding (amino acid 330–496) and transactivation (amino acid 497–793) domains. To determine which domain of STAT5 is responsible for interaction with Runx proteins, we constructed a series of STAT5a deletion mutants (Fig. 3A). As the protein levels of the STAT5a deletion mutants were highly variable after transient expression in HEK293T cells (data not shown), we carried out a co-immunoprecipitation experiment after *in vitro* association with Runx proteins. HEK293T cells were transfected with the expression vectors for Flag-tagged full-length Runx1 or Runx3, or Myc-tagged STAT5a deletion mutants. The cell lysate was first checked for expression of each protein by immunoblotting. The lysates containing the equal amount of STAT5a deletion mutants were mixed with the lysate containing Runx1 *in vitro*, and incubated at 4°C for 1 h. The mixtures were then immunoprecipitated with anti-Flag (Runx) antibody, followed by immunoblotting with anti-Myc (STAT5) antibody. The N-terminal, coiled-coil, and transactivation domains were dispensable for interaction with Runx1 (Fig. 3B, left panel, lanes 1, 2 and 4). In contrast, the DNA-binding domain of STAT5 showed weak interaction with Runx1 (lane 3). However, the STAT5 mutant with the DNA-binding domain and α-helix loop structure strongly interacted with Runx1 (lane 5). These results showed that the DNA-binding domain of STAT5 was necessary but not sufficient for full interaction with Runx1. Furthermore, we obtained similar results with Runx3 (Fig. 3C), suggesting that the DNA-binding domain and the α-helix loop structure of STAT5 are responsible for the interaction with Runx proteins *in vitro*.

Co-localization of STAT5 with Runx Proteins—It has been reported that STAT5 and Runx are mainly localized in the cytoplasm and the nucleus, respectively (*30*, *36*). We next analysed whether the interaction of STAT5 and Runx proteins takes place in the cytoplasm or in the nucleus. CHO cells were transfected with the expression vectors for WT-STAT5a and Flag-tagged Runx proteins, and stained with anti-STAT5a and anti-Flag antibodies, followed by staining with Alexa Fluor 568- and Alexa Fluor 488-labeled secondary antibodies. Immunofluorescence microscopy revealed that, in accordance with the previous reports, STAT5a and Runx1 were present in the cytoplasm and the nucleus, respectively, when expressed alone (Fig. 4, rows A and B). In contrast, when STAT5a and Runx1 were expressed together, Runx1 was retained in the cytoplasm (row C). We obtained similar results with Runx3 (rows F and G) and less prominent results with Runx2 (rows D and E). These results suggested that Runx proteins are retained in the cytoplasm by the interaction with STAT5.

Next, to confirm the localization of STAT5 and Runx proteins in biochemical way, we separated nuclear and cytosolic fractions of transfected CHO cells. Each fraction was immunoprecipitated with anti-STAT5a and anti-Flag (Runx) antibodies, followed by immunoblotting with anti-STAT5a and anti-Flag (Runx) antibodies. The separation was monitored with a cytosolic marker, PABP, and a nuclear marker, hnRNP C1/C2. When expressed alone,

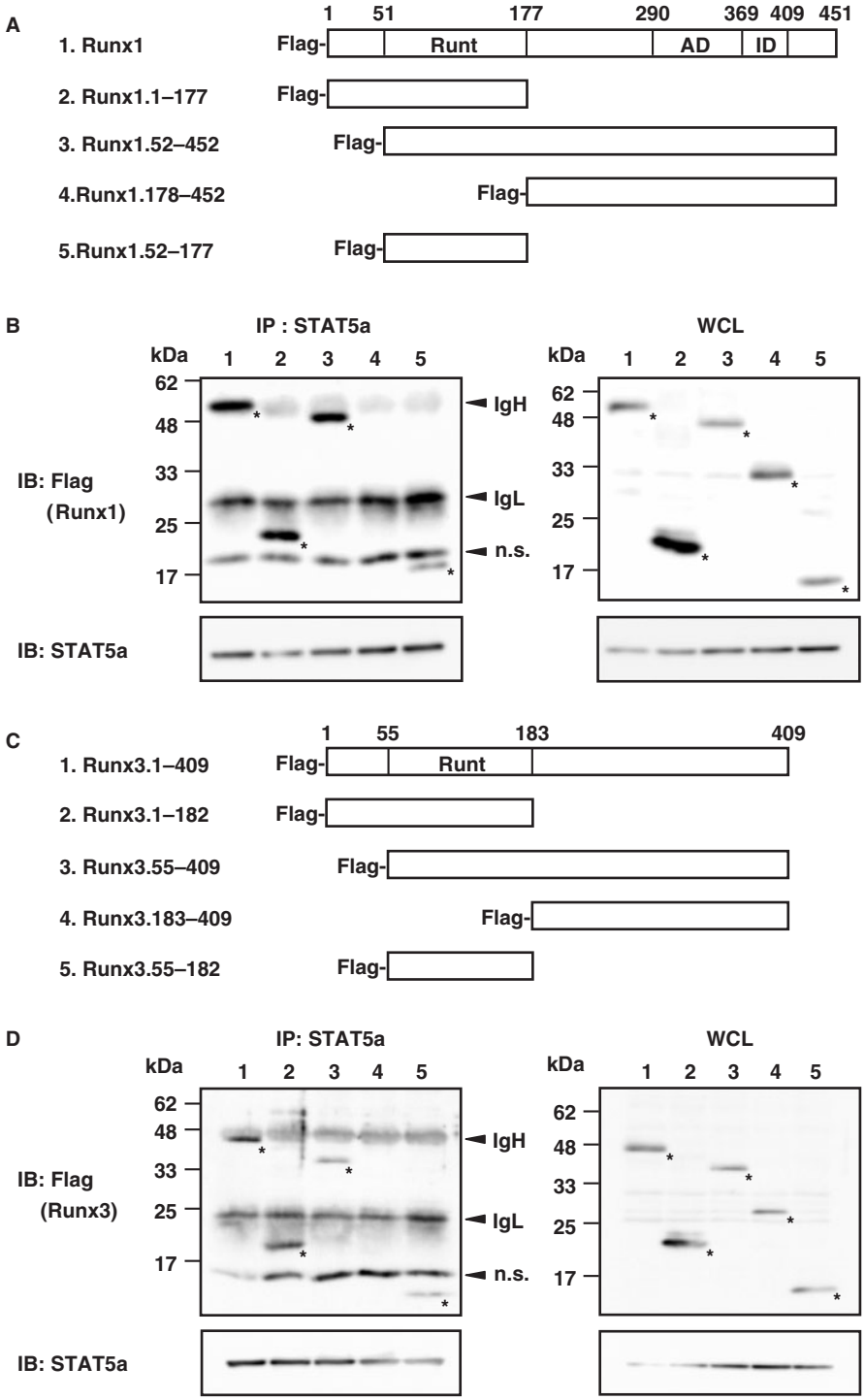

Fig. 2. **Mapping of the interaction domain of Runx proteins.** (A) Schematic presentation of Runx1 deletion mutants. Runt, Runt homology domain; AD, transcription activation domain; ID, transcription inhibition domain. Numbers denote the position of amino acids. (B) HEK293T cells were transfected with the expression vectors encoding Flag-Runx1 deletion mutants and Myc-STAT5a. The cell lysate was immunoprecipitated with anti-STAT5a antibody, and immunoblotted with monoclonal anti-Flag antibody, followed by re-probing with anti-STAT5a antibody (left panels). The whole-cell lysate (WCL) (2.5% input) was immunoblotted with monoclonal anti-Flag antibody, followed by re-probing with anti-STAT5a antibody (right panels). Lane numbers correspond to those of Runx1 deletion mutants in (A). Protein size marker is shown at left. The asterisks (*) indicate Runx deletion mutants. 'n.s.' indicates non-specific proteins. (C) Schematic presentation of Runx3 deletion mutants. (D) HEK293T cells were transfected with the expression vectors encoding Flag-Runx3 deletion mutants and Myc-STAT5a. Immunoprecipitation and immunoblotting were carried out as in (B).

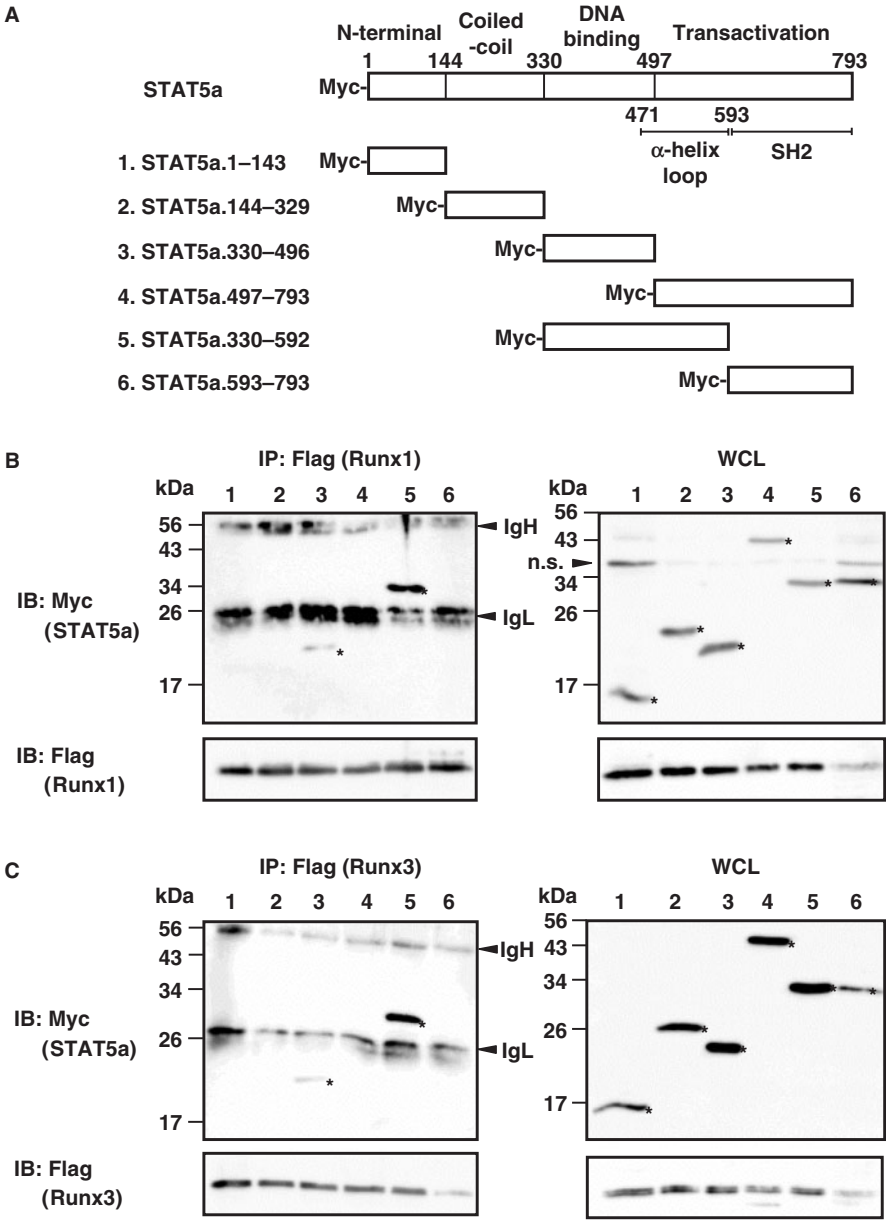

Fig. 3. **Mapping of the interaction domain of STAT5.** (A) Schematic presentation of STAT5a deletion mutants. The domain structure is shown. Numbers denote the position of amino acids. (B) HEK293T cells were transfected with the expression vectors encoding either Flag-Runx1 or Myc-STAT5a deletion mutants. The cell lysates were first checked for expression of each protein by immunoblotting. The lysates containing the equal amount of STAT5a deletion mutants and Runx1 were mixed *in vitro*, and incubated at 4°C for 1 h. The lysates were immunoprecipitated with polyclonal anti-Flag antibody, and immunoblotted with anti-Myc antibody, followed by re-probing with monoclonal anti-Flag antibody (left panels). The whole-cell lysate (WCL) (2.5% input) was blotted with anti-Myc antibody, followed by re-probing with monoclonal anti-Flag (right panels). Lane numbers correspond to those of STAT5 deletion mutants in (A). Protein size marker is shown at left. The asterisks (*) indicate STAT5a deletion mutants. 'n.s.' indicates non-specific proteins. (C) HEK293T cells were transfected with the expression vectors encoding either Flag-Runx3 or Myc-STAT5a deletion mutants. *In vitro* binding, immunoprecipitation and immunoblotting were carried out as in (B).

STAT5a and Runx1 were mainly detected in the cytosolic and nuclear fractions, respectively (Fig. 5A). In contrast, when STAT5a and Runx1 were expressed together, Runx1 was detected both in nuclear and cytosolic fractions, suggesting that Runx1 changed its localization from the nucleus to the cytosol by STAT5a. While Runx2 and Runx3 were detected in the cytosolic and nuclear fractions when expressed alone, they were detected mainly in the cytosolic fraction when expressed with STAT5a (Fig. 5B and C), suggesting that Runx2 and Runx3 also changed their localization from the nucleus to the cytosol by STAT5a. These observations were confirmed by quantitating the immunoblots (Fig. 5D). These results supported the results by immunofluorescence microscopy (Fig. 4) that Runx proteins are retained in the cytoplasm by the interaction with STAT5.

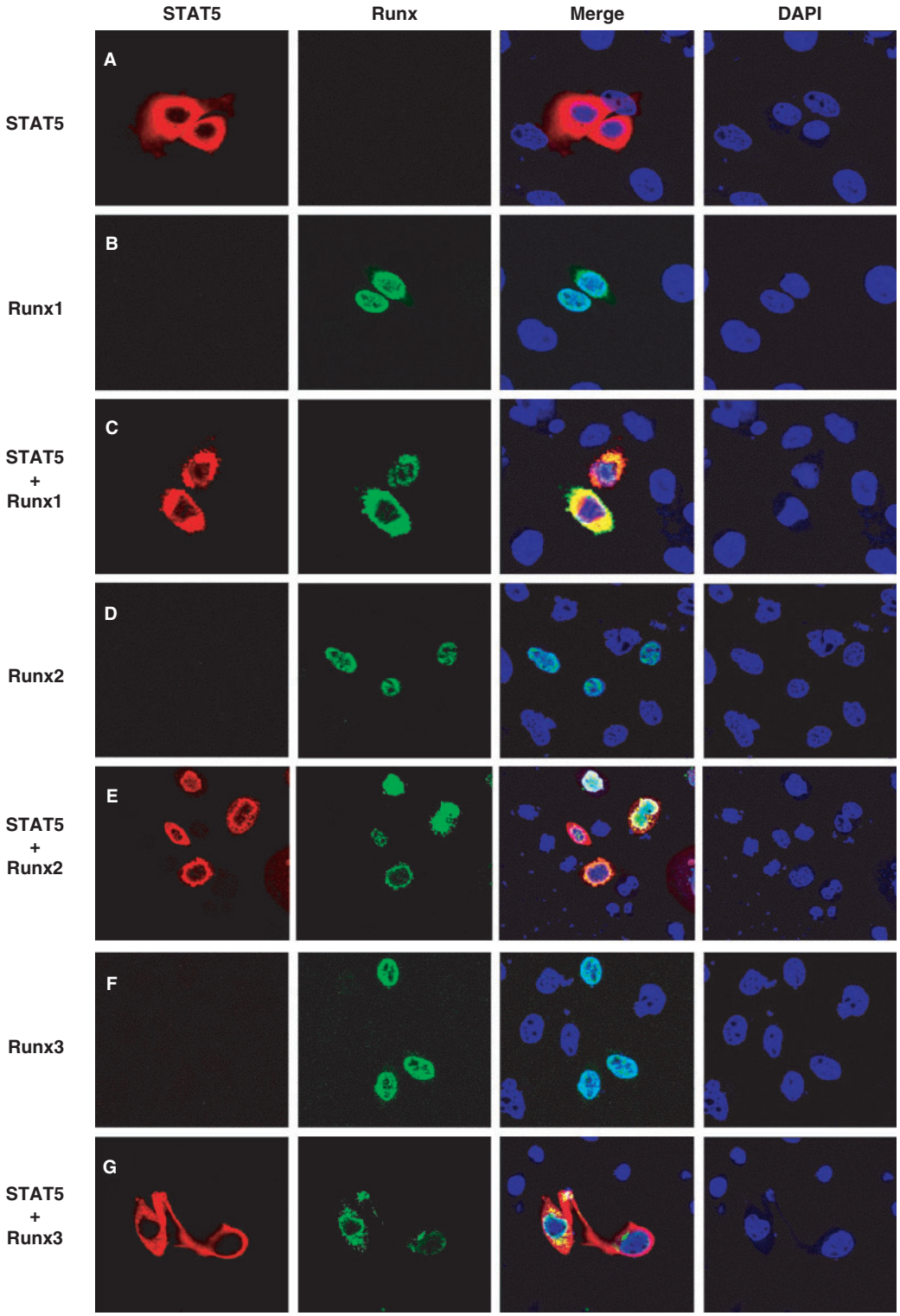

Fig. 4. **Co-localization of Runx proteins with STAT5.** The expression vectors for Myc-STAT5a (row A), Flag-Runx1, -Runx2 or -Runx3 (rows B, D and F, respectively), or the combination of both (rows C, E and G, respectively) were transiently transfected into CHO cells grown on coverslips. Twenty-four hours after transfection, the cells were fixed, permeabilized and immunostained with anti-STAT5a and monoclonal anti-Flag (Runx) antibodies. STAT5 (red) and Runx (green) were visualized with Alexa568-anti-rabbit IgG and Alexa488-anti-mouse IgG antibodies, respectively. Merged images are shown. The nucleus was detected by 4, 6-dia-midino-2-phenylindole (DAPI) staining (blue).

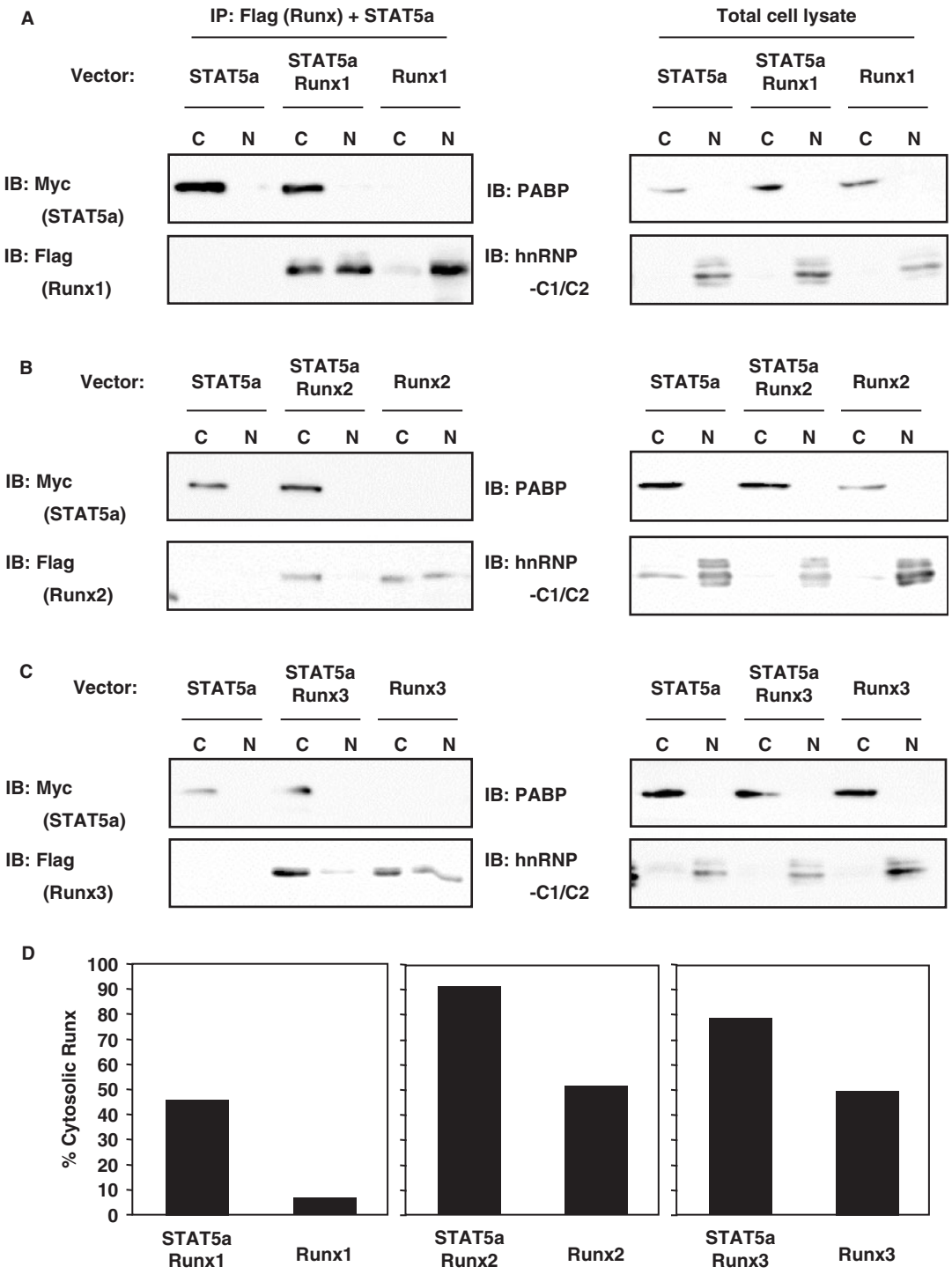

Fig. 5. **Runx proteins are retained in the cytosol by STAT5.** CHO cells were transiently transfected with the expression vectors for Myc-STAT5a and Flag-Runx1 (A), -Runx2 (B) or -Runx3 (C). Twenty-four hours after transfection, cytosolic (C) and nuclear (N) fractions were separated. Each fraction was immunoprecipitated with the mixture of anti-STAT5a and polyclonal anti-Flag (Runx) antibodies, followed by immunoblotting with anti-STAT5a and monoclonal anti-Flag antibodies (left panels). The separation was monitored by immunoblotting with a cytosolic marker, PABP, and a nuclear marker, hnRNP C1/C2 (right panels). (D) The percentage of the Runx proteins in the cytosolic fraction relative to the sum of the cytosolic and nuclear fractions was calculated by quantitating immunoblot analysis in (A) to (C).

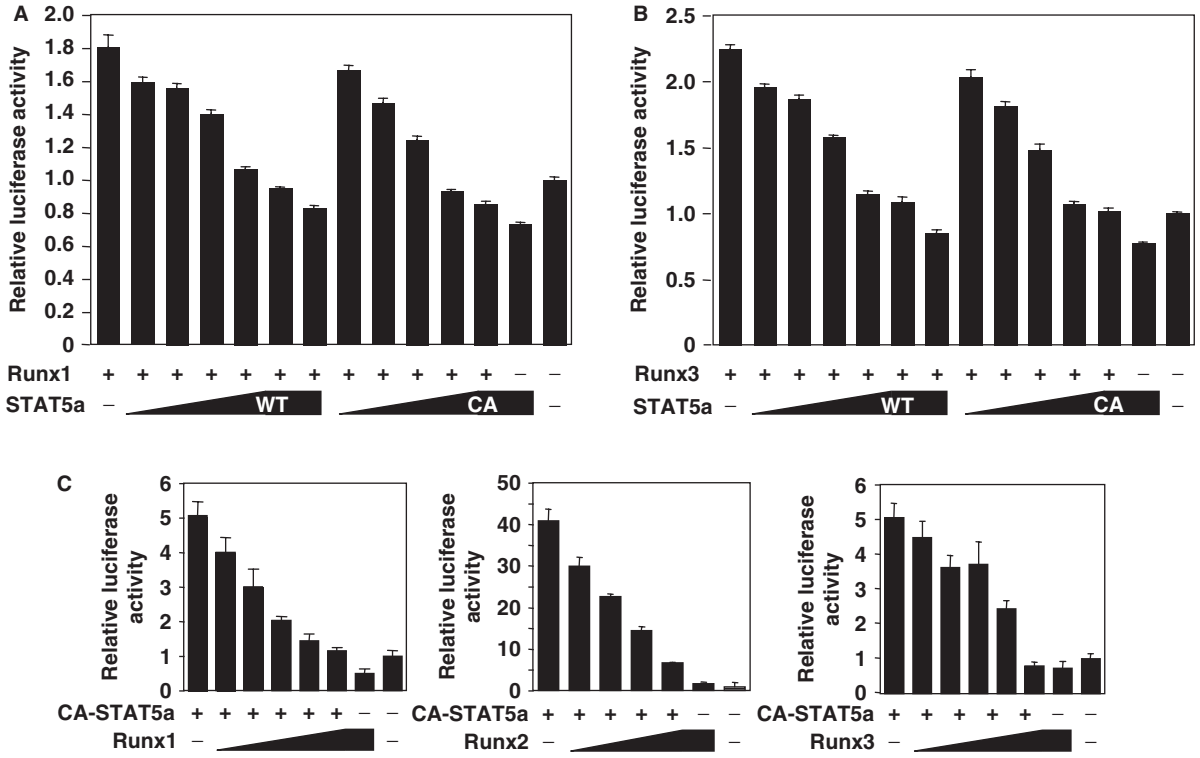

Fig. 6. **STAT5 and Runx proteins mutually suppress the transcriptional activity.** (A) HEK293T cells were transfected with Runx1-responsive Tww-tk-Luc reporter vector, pGL4.74(hRluc/TK) internal control vector, Runx1 expression vector (pcDNAFlag-Runx1: 50 ng) and an increasing amount of STAT5a expression vector (pcDNAMyc-WT-STAT5a or -CA-STAT5a: 50, 100, 150, 200 and 250 ng). The cells were recovered after 36 h, and luciferase activities were measured. Firefly luciferase activity was normalized by *Renilla* luciferase activity. The relative luciferase activity is calculated as fold induction compared with mock control. Values are the mean ± S.E. of triplicate data points from a representative experiment. Data are representative of two independent experiments. (B) HEK293T cells were transfected with the Tww-tk-Luc reporter vector, pGL4.74(hRluc/TK) internal control vector, Runx3 expression vector (pcDNAFlag-Runx3: 50 ng) and an increasing amount of STAT5a expression vector (pcDNAMyc-WT-STAT5a or -CA-STAT5a: 50, 100, 150, 200 and 250 ng). Reporter assay was carried out as in (A). (C) HEK293T cells were transfected with STAT5-responsive pGL4-Jγ1 reporter vector, EF1-Renilla internal control vector, CA-STAT5a expression vector (pcDNAMyc-CA-STAT5a: 50 ng) and an increasing amount of Runx expression vectors (pcDNAFlag-Runx1: 2, 4, 5, 10 and 20 ng; pcDNAFlag-Runx2: 10, 20, 40 and 80 ng; pcDNAFlag-Runx3: 2.5, 5, 10, 20 and 40 ng). Reporter assay was carried out as in (A).

STAT5 and Runx Proteins Mutually Suppress Transcriptional Activity—Since Runx proteins are retained in the cytoplasm by the interaction with STAT5, we next tested whether STAT5 suppresses the transcriptional activity of Runx. HEK293T cells were transiently transfected with the Runx1-responsive reporter plasmid, Runx1 and/or STAT5a expression vectors. The cells were recovered, and the promoter activity was analysed by reporter assay. As previously reported (*35*), transcriptional activation of the Tww-tk-Luc reporter was observed by Runx1 expression vector (Fig. 6A). Interestingly, this activity was inhibited by WT- and CA-STAT5a in a dose-dependent manner. We obtained similar results with STAT5b (Supplementary Fig. 3A) and an inactive STAT5a mutant with tyrosine 694 to phenylalanine substitution (data not shown). We also observed similar results with Runx3 (Fig. 6B). These results suggested that STAT5 suppresses the transcriptional activity of Runx by direct interaction and retention in the cytoplasm irrespective of sub-cellular distribution of STAT5.

Next, we checked whether Runx family proteins reciprocally suppress the transcriptional activity of STAT5. We employed a fast and efficient reporter assay for STAT5-dependent promoters (*37*). HEK293T cells were transiently transfected with STAT5-responsive Jγ1 promoter reporter plasmid (pGL4-Jγ1), and CA-STAT5a and/or Runx expression vectors. CA-STAT5 is spontaneously phosphorylated after transient expression, and transactivates STAT5-dependent promoters. The cells were recovered, and the promoter activity was analysed by reporter assay. Transcriptional activation of the Jγ1 promoter was observed with the CA-STAT5a expression vector (Fig. 6C). This activity was inhibited by Runx1, Runx2 and Runx3 in dose-dependent manners. We obtained similar results with STAT5b (Supplementary Fig. 3B). These results suggested that Runx proteins suppress the transcriptional activity of STAT5.

To determine how Runx proteins inhibit the transcriptional activity of STAT5, we next carried out EMSA. HEK293T cells were transiently transfected with

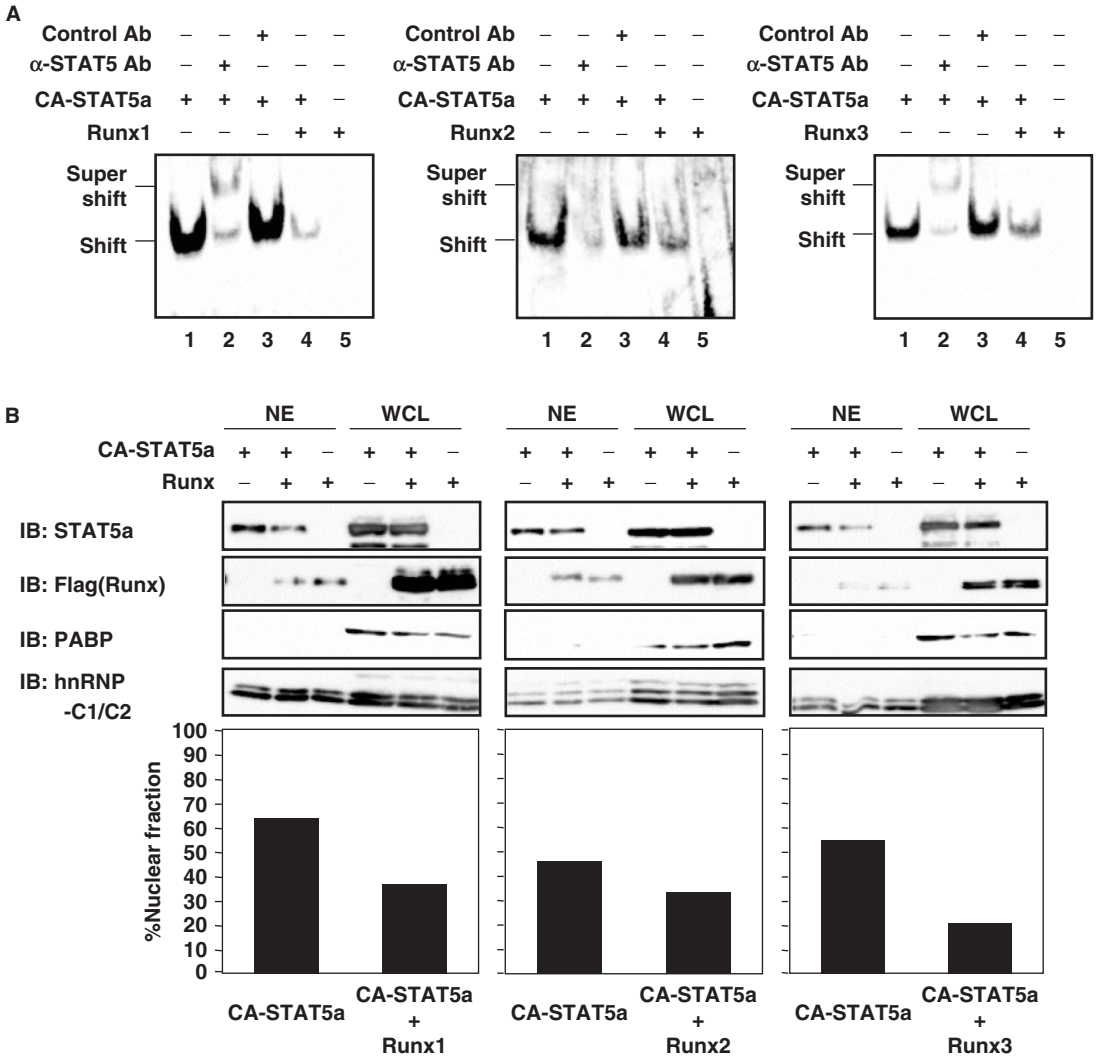

Fig. 7. **Runx proteins inhibit the DNA binding of STAT5 by retaining STAT5 in the cytoplasm.** (A) HEK293T cells were transfected with Myc-CA-STAT5a and Flag-Runx1, -Runx2 or -Runx3 expression vectors. Nuclear extract was analysed for DNA-binding activity by EMSA with the oligonucleotide probe for STAT consensus motif in mouse β-casein promoter. Shift and super-shift bands were indicated. (B) The same nuclear extract (NE) and the whole-cell lysate (WCL) were immunoblotted with anti-STAT5a antibody, followed by re-probing with monoclonal anti-Flag antibody. The separation was monitored by immunoblotting with a cytosolic marker, PABP, and a nuclear marker, hnRNP C1/C2 (upper panels). The amounts of nuclear STAT5 were quantitated and normalized with those of total STAT5 (lower panels).

CA-STAT5a and Runx expression vectors, and their nuclear extract was evaluated by EMSA with the oligonucleotide probe for a STAT consensus motif. DNA-binding activity of STAT5 was clearly detected and specifically super-shifted with anti-STAT5 antibody (Fig. 7A, lanes 1–3). This activity was reduced by co-expression of Runx proteins (lane 4). These results suggested that Runx proteins inhibit the DNA binding of STAT5. As the expressed Runx proteins form complexes with STAT5 in the cytoplasm (Fig. 4), we checked the total amount of STAT5 proteins in the nuclear extracts by immunoblotting. The amounts of nuclear STAT5a were decreased by expression of Runx1, Runx2 or Runx3 (Fig. 7B). These results suggested that Runx proteins inhibit the DNA binding of STAT5 by retaining STAT5 in the cytoplasm.

Activation Domain of Runx1 Is Necessary for Effective Suppression of STAT5—To determine which region of Runx is necessary for STAT5 suppression, we performed a reporter assay with a series of Runx1 deletion mutants (Fig. 8A). HEK293T cells were transiently transfected with the STAT5-responsive Jγ1 promoter reporter plasmid (pGL4-Jγ1) and the expression vectors for CA-STAT5a and Runx1 deletion mutants. As expected, the Runx1.178-452 deletion mutant that lacks the Runt domain failed to suppress STAT5-induced transactivation (Fig. 8B). However, the Runx1.1-177 deletion mutant that contains the Runt domain also failed to suppress the STAT5 activity. In addition, the Runx1.1-290 deletion mutant that includes a nuclear localization signal (NLS) (*38*) only weakly suppressed the activity. In contrast, the Runx1.1-369 deletion mutant that further includes

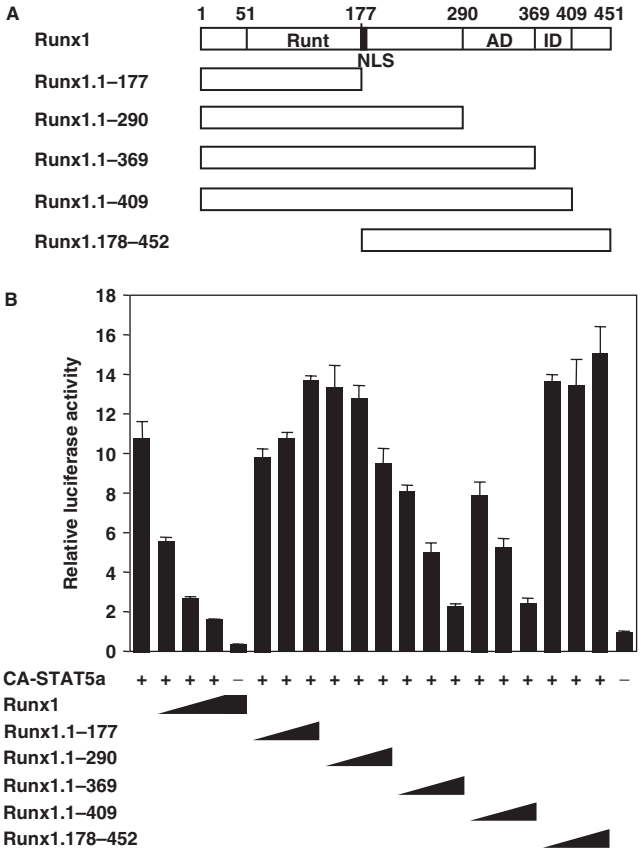

Fig. 8. **Activation domain of Runx1 is necessary for effective suppression of STAT5.** (A) Schematic presentation of Runx1 deletion mutants. Runt, Runt domain; AD, transcription activation domain; ID, transcription inhibition domain; NLS, nuclear localization signal. NLS is shown as a closed box. (B) HEK293T cells were transfected with STAT5-responsive pGL4-Jγ1 reporter vector, EF1-Renilla internal control vector, CA-STAT5a expression vector (pcDNAMyc-CA-STAT5a: 50 ng) and an increasing amount of the expression vectors for Runx1 deletion mutants (pcDNAFlag-Runx1 deletion mutants: 2.5, 5 and 10 ng). Reporter assay was carried out as in Fig. 6A.

the activation domain specifically suppressed the STAT5-induced transcription. These results suggested that the activation domain of Runx1 is necessary for effective suppression of STAT5 in addition to the Runt domain.

Runx Proteins Suppress the Transcription of an Endogenous STAT5 Target Gene—We next tested whether Runx proteins suppress the transcription of endogenous STAT5 target genes. CIS1 is one of the STAT5 target genes induced by cytokine stimulation (*39*), and an IL-3-dependent pro-B cell line, Ba/F3, induces CIS1 mRNA by IL-3 stimulation. Ba/F3 cells were introduced with Runx1, Runx2 and Runx3 cDNA by retrovirus vector. The cells were deprived of IL-3 for 8 h or stimulated with IL-3, and the levels of CIS1 mRNA were measured by real-time RT–PCR. The mock transfectants induced CIS1 mRNA by IL-3 stimulation (Fig. 9A). This induction of CIS1 mRNA was partially blocked by expression of Runx proteins. These results suggested that Runx proteins suppress the transcription of a STAT5 target gene. To confirm at which step of STAT5 activation Runx proteins impose their inhibitory effect, we first analysed the binding of STAT5 to endogenous CIS1 promoter in Ba/F3 cells by ChIP assay. STAT5 was recruited to the CIS1 promoter after IL-3 stimulation (Fig. 9B). This recruitment of STAT5 was partially blocked by expression of Runx proteins. We next analysed the phosphorylation of STAT5 by immunoblotting with anti-phosphorylated STAT5 antibody. The levels of phosphorylated STAT5 did not change by expression of Runx proteins (Fig. 9C). These results suggested that Runx proteins probably inhibit the DNA binding of STAT5 by retaining STAT5 in the cytoplasm.

DISCUSSION

In this study, we first showed that STAT5 physically interacts with Runx1, Runx2 and Runx3. The Runt domain of Runx proteins and the DNA-binding domain and α-helix loop structure of STAT5 were responsible for the interaction. We further demonstrated that STAT5 retains Runx proteins in the cytoplasm and that the interaction between STAT5 and Runx proteins mutually inhibits their transcriptional activity. Finally, we found that Runx proteins suppress the transcription of an endogenous STAT5 target gene. These results collectively suggested that STAT5 and Runx proteins physically and functionally interact to mutually inhibit their transcriptional activity.

Runx family transcription factors show synergistic and antagonistic effects by interaction with many transcriptional factors. Runx proteins have been shown to interact with PU.1, CEBP/α, Pax5, AP-1 and STAT through the Runt domain (*14*). As for the interaction with STAT, it was reported that Runx2 interacts with STAT1 through the Runt domain in a co-immunoprecipitation experiment after over-expression in HEK293T cells (*25*). In this study, we demonstrated that Runx1, Runx2 and Runx3 physically interact with STAT5 through the Runt domain, adding another example of the interaction between the Runt domain and STAT. However, the interaction with the Runt domain is not a common feature among STAT proteins, because STAT3 associates with a C-terminal 86 amino acids region of Runx2 in yeast two-hybrid experiment (*26*).

The transcriptional activity of Runx proteins can be suppressed through the interaction of the Runt domain with STAT5 by three possible mechanisms. First, Stat5 may block the dimerization of Runx proteins by competing with CBFβ, because the Runt domain is responsible for the dimerization. Second, STAT5 may mask the DNA-binding surface of Runx proteins, since the Runt domain is necessary for binding to DNA. Lastly, Stat5 sequesters Runx proteins from the nucleus, as demonstrated in this study. The first and second possibilities are still to be tested in future.

It has been reported that STAT interacts with other transcriptional factors to synergistically enhance the transcription of target genes. For example, STAT5 activated by prolactin translocates the glucocorticoid receptor into the nucleus, while ligand-bound glucocorticoid receptor transports STAT5 into the nucleus (*40*).

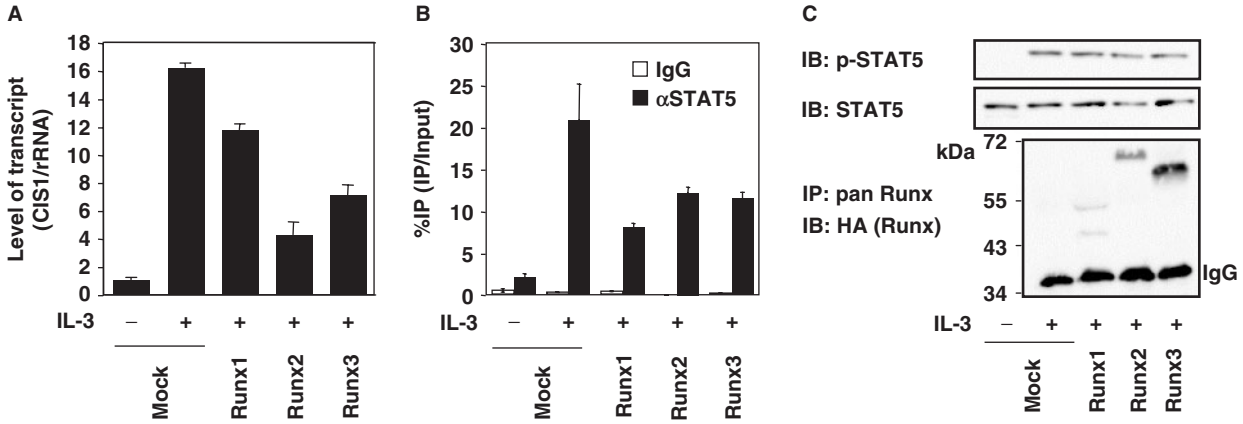

Fig. 9. **Runx proteins suppress the transcription of an endogenous STAT5 target gene.** (A) An IL-3-dependent pro-B cell line, Ba/F3, was introduced with Runx1, Runx2 and Runx3 cDNAs by retrovirus vector. The transfectants were cultured with IL-3, or starved of IL-3 for 8 h. CIS1 transcripts were measured by real-time RT–PCR. The levels of transcripts were normalized with 18 S rRNA. Values are the mean ± S.E. of triplicate data points. Data shown are representative of two independent experiments. (B) The same transfectants were cultured with IL-3, or starved of IL-3 for 8 h. Soluble chromatin preparation was immunoprecipitated with anti-STAT5 antibody or control normal rabbit IgG. Purified ChIP and input DNA were analysed by real-time PCR with the primers for a STAT5 consensus motif in the CIS1 promoter. (C) The same transfectants were cultured with IL-3, or starved of IL-3 for 8 h. The cells lysate was immunoblotted with anti-phosphorylated STAT5 antibody, followed by re-probing with anti-STAT5 antibody (upper panels). The cells lysate was also immunoprecipitated with anti-pan Runx antibody, and immunoblotted with monoclonal anti-HA antibody. Protein size marker is shown at left.

Their interaction enhances the transcription of β-casein promoter. In addition, thrombopoietin-activated STAT5 forms a complex with Oct-1, and induces the transcription of cyclin D1 promoter (*13*). In contrast, our study suggested that the interaction with Runx proteins inhibits the transcriptional activity of STAT5. This is the first report on the suppression of STAT function by interaction with other transcription factors.

The transcriptional activity of STAT5 can be suppressed through the interaction of the DNA-binding domain with Runx proteins by two possible mechanisms. One is that Runx proteins may mask the DNA-binding surface of Stat5. The other is that Runx may block the homo-dimerization of STAT5 by intercalating between two STAT5 molecules, because the DNA-binding domain of STAT is close to the interacting surface for the dimerization. These possibilities are still to be elucidated in detail.

In this study, we characterized the interaction between STAT5 and Runx, and demonstrated that STAT5 and Runx proteins physically and functionally interact to mutually inhibit their transcriptional activity. Thus, this study implies a potential role of the STAT5-Runx interaction during T-cell development in the thymus and peripheral lymphoid organs. For example, Runx1 binds to the CD4 silencer and suppresses CD4 expression in $CD4^{-}CD8^{-}$ double-negative thymocytes (*17*). When the double-negative thymocytes receive the pre-TCR signal and proceed to $CD4^{+}CD8^{+}$ double-positive stage, IL-7R/STAT5 signal is also reported to operate (*41*). Therefore, it might be possible that activated STAT5 inhibits the Runx1 binding to the CD4 silencer, thereby inducing CD4 expression.

Supplementary data are available at *JB* online.

We thank Drs T. Sudo, T. Kitamura, D. L. Wiest, and T. Saito for materials; Mses. S. Hayashi, M. Kishida, and S. Kamioka for excellent technical assistance; Drs K. Maki and S. Tani-ichi for critically reading the manuscript; and Drs M. Sugai, K. Kohu, D. Yabe, and N. Kataoka, and the members of Ikuta's lab for discussion. This work was supported by Grants-In-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by the grants provided by Novartis Foundation (Japan) for the Promotion of Science. S.O. was supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology to Graduate School of Biostudies and Institute for Virus Research, Kyoto University.

REFERENCES

1. Rekhtman, N., Radparvar, F., Evans, T., and Skoultchi, A.I. (1999) Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev.* **13**, 1398–1411
2. Zhang, P., Behre, G., Pan, J., Iwama, A., Wara-Aswapati, N., Radomska, H.S., Auron, P.E., Tenen, D.G., and Sun, Z. (1999) Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc. Natl. Acad. Sci. USA* **96**, 8705–8710
3. Nerlov, C., Querfurth, E., Kulessa, H., and Graf, T. (2000) GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood* **95**, 2543–2551
4. Caldenhoven, E., Liden, J., Wissink, S., Van de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J.A., and Van der Saag, P.T. (1995) Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol. Endocrinol.* **9**, 401–412
5. Scheinman, R.I., Gualberto, A., Jewell, C.M., Cidlowski, J.A., and Baldwin, A.S. Jr. (1995) Characterization of mechanisms

involved in transrepression of NF-κB by activated glucocorticoid receptors. *Mol. Cell. Biol.* **15**, 943–953
6. De Bosscher, K., Vanden Berghe, W., and Haegeman, G. (2006) Cross-talk between nuclear receptors and nuclear factor κB. *Oncogene* **25**, 6868–6886
7. Leonard, W.J. and O'Shea, J.J. (1998) Jaks and STATs: biological implications. *Annu. Rev. Immunol.* **16**, 293–322
8. Yao, Z., Cui, Y., Watford, W.T., Bream, J.H., Yamaoka, K., Hissong, B.D., Li, D., Durum, S.K., Jiang, Q., Bhandoola, A., Hennighausen, L., and O'Shea, J.J. (2006) Stat5a/b are essential for normal lymphoid development and differentiation. *Proc. Natl. Acad. Sci. USA* **103**, 1000–1005
9. Ye, S.K., Agata, Y., Lee, H.C., Kurooka, H., Kitamura, T., Shimizu, A., Honjo, T., and Ikuta, K. (2001) The IL-7 receptor controls the accessibility of the TCRγ locus by Stat5 and histone acetylation. *Immunity* **15**, 813–823
10. Xu, R., Spencer, V.A., and Bissell, M.J. (2007) Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors. *J. Biol. Chem.* **282**, 14992–14999
11. Stocklin, E., Wissler, M., Gouilleux, F., and Groner, B. (1996) Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* **383**, 726–728
12. Cella, N., Groner, B., and Hynes, N.E. (1998) Characterization of Stat5a and Stat5b homodimers and heterodimers and their association with the glucocortiocoid receptor in mammary cells. *Mol. Cell. Biol.* **18**, 1783–1792
13. Magne, S., Caron, S., Charon, M., Rouyez, M.C., and Dusanter-Fourt, I. (2003) STAT5 and Oct-1 form a stable complex that modulates cyclin D1 expression. *Mol. Cell. Biol.* **23**, 8934–8945
14. Ito, Y. (2004) Oncogenic potential of the RUNX gene family: 'overview'. *Oncogene* **23**, 4198–4208
15. Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., and Downing, J.R. (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**, 321–330
16. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755–764
17. Taniuchi, I., Osato, M., Egawa, T., Sunshine, M.J., Bae, S.C., Komori, T., Ito, Y., and Littman, D.R. (2002) Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **111**, 621–633
18. Woolf, E., Xiao, C., Fainaru, O., Lotem, J., Rosen, D., Negreanu, V., Bernstein, Y., Goldenberg, D., Brenner, O., Berke, G., Levanon, D., and Groner, Y. (2003) Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. *Proc. Natl. Acad. Sci. USA* **100**, 7731–7736
19. Sato, T., Ohno, S., Hayashi, T., Sato, C., Kohu, K., Satake, M., and Habu, S. (2005) Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity* **22**, 317–328
20. Meyers, S., Downing, J.R., and Hiebert, S.W. (1993) Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein–protein interactions. *Mol. Cell. Biol.* **13**, 6336–6345
21. Wang, S., Wang, Q., Crute, B.E., Melnikova, I.N., Keller, S.R., and Speck, N.A. (1993) Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. *Mol. Cell. Biol.* **13**, 3324–3339
22. Ogawa, E., Inuzuka, M., Maruyama, M., Satake, M., Naito-Fujimoto, M., Ito, Y., and Shigesada, K. (1993) Molecular cloning and characterization of PEBP2β, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2α. *Virology* **194**, 314–331
23. Kitabayashi, I., Yokoyama, A., Shimizu, K., and Ohki, M. (1998) Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. *EMBO J.* **17**, 2994–3004
24. Lutterbach, B., Westendorf, J.J., Linggi, B., Isaac, S., Seto, E., and Hiebert, S.W. (2000) A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. *J. Biol. Chem.* **275**, 651–656
25. Kim, S., Koga, T., Isobe, M., Kern, B.E., Yokochi, T., Chin, Y.E., Karsenty, G., Taniguchi, T., and Takayanagi, H. (2003) Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. *Genes Dev.* **17**, 1979–1991
26. Ziros, P.G., Georgakopoulos, T., Habeos, I., Basdra, E.K., and Papavassiliou, A.G. (2004) Growth hormone attenuates the transcriptional activity of Runx2 by facilitating its physical association with Stat3β. *J. Bone Miner. Res.* **19**, 1892–1904
27. Morita, S., Kojima, T., and Kitamura, T. (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* **7**, 1063–1066
28. Carleton, M., Ruetsch, N.R., Berger, M.A., Rhodes, M., Kaptik, S., and Wiest, D.L. (1999) Signals transduced by CD3ε, but not by surface pre-TCR complexes, are able to induce maturation of an early thymic lymphoma in vitro. *J. Immunol.* **163**, 2576–2585
29. Karasuyama, H., Rolink, A., and Melchers, F. (1988) Recombinant interleukin 2 or 5, but not 3 or 4, induces maturation of resting mouse B lymphocytes and propagates proliferation of activated B cell blasts. *J. Exp. Med.* **167**, 1377–1390
30. Onishi, M., Nosaka, T., Misawa, K., Mui, A.L., Gorman, D., McMahon, M., Miyajima, A., and Kitamura, T. (1998) Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. *Mol. Cell. Biol.* **18**, 3871–3879
31. Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T., and Kumagai, H. (2003) Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp. Hematol.* **31**, 1007–1014
32. Hayashi, K., Abe, N., Watanabe, T., Obinata, M., Ito, M., Sato, T., Habu, S., and Satake, M. (2001) Overexpression of AML1 transcription factor drives thymocytes into the CD8 single-positive lineage. *J. Immunol.* **167**, 4957–4965
33. Mili, S., Shu, H.J., Zhao, Y., and Pinol-Roma, S. (2001) Distinct RNP complexes of shuttling hnRNP proteins with pre-mRNA and mRNA: candidate intermediates in formation and export of mRNA. *Mol. Cell. Biol.* **21**, 7307–7319
34. Masui, N., Tani-ichi, S., Maki, K., and Ikuta, K. (2007) Transcriptional activation of mouse TCR Jγ4 germline promoter by STAT5. *Mol. Immunol.* **45**, 849–855
35. Tanaka, T., Tanaka, K., Ogawa, S., Kurokawa, M., Mitani, K., Nishida, J., Shibata, Y., Yazaki, Y., and Hirai, H. (1995) An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. *EMBO J.* **14**, 341–350
36. Lu, J., Maruyama, M., Satake, M., Bae, S.C., Ogawa, E., Kagoshima, H., Shigesada, K., and Ito, Y. (1995) Subcellular localization of the α and β subunits of the acute myeloid leukemia-linked transcription factor PEBP2/CBF. *Mol. Cell. Biol.* **15**, 1651–1661
37. Toda, T., Wada, H., Ogawa, S., Tani-ichi, S., and Ikuta, K. (2008) An efficient reporter assay for Stat5-dependent promoters. *Anal. Biochem.* **372**, 250–252
38. Kanno, T., Kanno, Y., Chen, L.F., Ogawa, E., Kim, W.Y., and Ito, Y. (1998) Intrinsic transcriptional activation-inhibition

domains of the polyomavirus enhancer binding protein 2/core binding factor α subunit revealed in the presence of the β subunit. *Mol. Cell. Biol.* **18**, 2444–2454

39. Matsumoto, A., Masuhara, M., Mitsui, K., Yokouchi, M., Ohtsubo, M., Misawa, H., Miyajima, A., and Yoshimura, A. (1997) CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood* **89**, 3148–3154
40. Wyszomierski, S.L., Yeh, J., and Rosen, J.M. (1999) Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. *Mol. Endocrinol.* **13**, 330–343
41. Trigueros, C., Hozumi, K., Silva-Santos, B., Bruno, L., Hayday, A.C., Owen, M.J., and Pennington, D.J. (2003) Pre-TCR signaling regulates IL-7 receptor α expression promoting thymocyte survival at the transition from the double-negative to double-positive stage. *Eur. J. Immunol.* **33**, 1968–1977