Title: **Physical and functional interactions between STAP-2/BKS and STAT5**

Running title: Interactions between STAP-2 and STAT5

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SUMMARY

Signal-transducing adaptor protein family of proteins (STAPs), which currently contains two members, are proposed to be adaptor molecules because of their Pleckstrin homology (PH) and Src-homology 2 (SH2)-like domains. STAP-1 has been shown to interact with STAT5 and the tyrosine kinase Tec. With regard to STAP-2/BKS functions, immunoprecipitation experiments and intracellular stainings revealed STAP-2/BKS binds STAT5 in several types of cells. Mutational studies revealed that the PH and SH2-like domains of STAP-2/BKS interacted with the C-terminal region of STAT5a. STAP-2/BKS and STAT5 were found to constitutively co-localize in the cytoplasm of resting cells, but STAP-2/BKS was found to dissociate upon STAT5 phosphorylation, suggesting a role in regulating signaling of STAT5. The physiological role of these interactions is not fully understood, but in studies of overexpression of STAP-2/BKS, cytokine-induced tyrosine-phosphorylation and transcriptional activation of STAT5 was diminished. In addition, thymocytes from STAP-2/BKS-deficient mice showed the enhanced IL-2-dependent cell growth. Taken together, STAP-2/BKS is an additional modulator of STAT5-mediated signaling.

INTRODUCTION

Cytokine signaling is predominatly activated through the Janus kinase (Jak) / signal transducers and activators of transcription (STAT) pathway (1, 2, 3). The cytokine signal is initiated when Jak is transphosphorylated in response to a receptor juxaposition upon ligand binding. The activated Jak subsequently phosphorylates a number of substrates including members of the STAT protein family. Upon tyrosine phosphorylation by Jak kinases, STATs form a homodimer via SH2 domain phosphotyrosine interactions and translocates into the nucleus to activate target genes (4, 5). Seven STAT proteins have been identified, STAT1, 2, 3, 4, STAT5a, STAT5b, and STAT6. STAT1, STAT3, STAT5a, and STAT5b are activated by numerous cytokines, whereas Stat2, Stat4, and Stat6 have a narrow activation profile. Mice lacking STAT1, STAT4 or STAT6 genes are viable but lose some functions that are mediated by interferons (IFNs), interleukin 12(IL-12), or IL-4/IL-13, respectively (6, 7, 8, 9, 10), suggesting their narrow activation profile is crucial in some specific functions. Wide range of STAT3 activation suggests its multifunctionality and is supported by knockout mice fetal lethality (11) and a finding consistent with STAT3's activation by many development regulatory cytokines such as leukemia inhibitory factor (LIF), cardiotrophin-1, and IL-6 (1, 4, 5). STAT5a and STAT5b are also activated by various cytokines, which are important in growth and development, such as growth hormone (GH), prolactin (PRL), erythropoietin (EPO), IL-2, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and thrombopoietin. The phenotypes of STAT5a and STAT5b single-gene knockout mice and double-knockout mice revealed important roles of STAT5s (12, 13, 14, 15, 16, 17). STAT5a is critical in GM-CSF signaling (12), PRL signaling (14), and IL-2 signaling (15), while STAT5b is necessary to maintain the sexual dimorphism of body growth rates and liver gene expression (16), IL-2-mediated T-cell proliferation, and NK cell development (13, 17).

The regulation of Jaks and STATs is important for the control of cytokine signaling. Because of the critical role of cytokines in mediating inflammation and immunity, it could be proposed that the

upregulated activation of Jaks could contribute to hematopoietic disorders, autoimmunity, and inflammatory diseases. In these regards, recent studies have identified several molecules that regulate cytokine signaling. Suppressors of cytokine signaling (SOCS), cytokine-inducible SH2-containing protein (CIS) and protein inhibitor of activated STAT (PIAS) are a novel Src homology 2 (SH2)-containing protein family, which is induced by various cytokines suppress Jak-STAT signaling through a direct interaction with Jak kinases or cytokine receptors (18, 19). CIS is involved in regulating cytokine signal transduction mediated by STAT5 (18, 20, 21, 22) through binding to the tyrosine-phosphorylated IL-3 and EPO receptors causing a negative regulation of their signals (20). CIS transgenic mice exhibited a phenotype similar to STAT5 deficient mice, supporting its importance in cytokine signaling by directly influencing the STAT5 signal (22). PIAS family proteins also inhibit DNA binding activity of activated STAT and subsequent gene expression (23). Regulation of cytokine signaling has also been shown to be controlled by several protein tyrosine phosphatases (PTPs) (24, 25, 26). The mechanisms that terminate or down-modulate the Jak-STAT pathway are not fully understood. However, it has also been suggested that specific regulation of cytokine signaling may involve additional SH2-containing molecules.

Recently, we have cloned a novel adaptor molecule, STAP-2 (Signal Transducing Adaptor Protein-2) as a c-fms interacting protein (27) and STAP-1 as a c-kit interacting protein (28). STAP-2 and STAP-1 contain an N-terminal Pleckstrin homology (PH) and a region distantly related to the Src homology 2 (SH2) domain (overall 33% amino acid identity) (28). However, STAP-2 has a C-terminal proline-rich region that is not present in STAP-1. The N-terminal PH domain of STAP-2 and STAP-1 shared 36% identity and 58% similarity. The central region of STAP-2 is distantly related to the SH2 domain. This region of STAP-2 shared 40% sequence identity with that of STAP-1 and 29% sequence identity with the SH2 domain of human PLC□2 (27, 28). Human STAP-2 is identical to a recently cloned adaptor molecule, BKS, a substrate of BRK (breast tumor kinase) tyrosine kinase (27). STAP-1 has been shown to have hematopoietic specific expression and associate with STAT5 (28). STAP-1 was also identified as a Tec-interacting protein, which is tyrosine phosphorylated in response to B-cell

receptor (BCR) stimulation and termed as BRDG1 (BCR Downstream Signaling 1)(29). Unlike STAT1, STAP-2/BKS is expressed in a variety of tissues and its C-terminal region contains the proline-rich, tyrosine phosphorylation motifs and a YXXQ motif. We previously showed STAP-2/BKS expression was strongly induced in hepatocytes in response to lipopolysaccharide (LPS) or inflammatory cytokines like interleukin-6 (IL-6), while its expression of myeloid cells is constitutive. Furthermore, we demonstrated that STAP-2/BKS modulated IL-2/STAT3-mediated transcriptional activation through its YXXQ motif (27).

In the present study, we address the involvement of STAP-2/BKS with the STAT5-mediated signaling. We demonstrate STAP-2/BKS physically and functionally interacted with STAT5. Furthermore, thymocytes from STAP-2/BKS-deficient mice showed an enhanced IL-2-dependent growth. These results indicate that STAP-2/BKS is a negative modulator in the STAT5-mediated signaling.

EXPERIMENTAL PROCEDURES

Reagents, antibodies and mice

Erythropoietin (EPO) was a kind gift from Kirin Co. (Tokyo, Japan). Expression vectors for FLAG-tagged Jak2, JaK2KE (30), STAT5a, STAT5b and EPOR (31) as well as plasmids for STAT5-LUC, Casein-LUC (32), were kindly provided by Dr. J. N. Ihle (St. Jude CRH, Memphis, TN), Dr. D. Wang (The Blood Res. Inst., Milwaukee, WI) and Dr. H. Wakao (Helix Res. Inst., Chiba, Japan), respectively. Myc-tagged STAP-2/BKS constructs were described previously (27). To obtain mammalian expression vectors for glutathione S-transferase (GST)-fusion STAP-2 mutants, STAP-2 mutant cDNAs were generated by PCR methods and sequenced (primer sequences are available upon request) and inseted into pEBgs vector (33). Myc-tagged STAT5a or STAT5b mutants were also generated by PCR methods and sequenced (primer sequences are available upon request). Anti-Myc, anti-GST and anti-STAT5a/b antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 antibody and rabbit polyclonal anti-FLAG antibody were purchased from Sigma (St Louis, MO). Anti-phosphotyrosine monoclonal antibody (PY20) was purchased from Cosmobio (Tokyo, Japan). Anti-human STAP-2/BKS antibody was described previously (34). The generation of STAP-2/BKS-deficient mice was described previously (27). Mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University.

Cell culture, transfection, luciferase assays and cell proliferation assays

An IL-3-dependent murine pro-B cell line, Ba/F3 was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and 10% conditioned medium from WEHI-3B cells as a source of IL-3. A stable transformant expressing STAP-2/BKS or a series of STAP-2/BKS mutants was established as described previously (35) and maintained in the above medium in the presence of G418 (1mg/ml). Human T cell leulemia cell line, Jurkat was maintained in RPMI1640 medium supplemented with 10% (FCS). A stable transformant expressing STAP-2/BKS was

established as described previously (35) and maintained in the above medium in the presence of G418 (1mg/ml). Human T cell lymphoma, HUT78 was maintained in RPMI1640 medium supplemented with 10% (FCS). Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol (36). The cells were harvested 48 hrs after transfection and lysed in 100 μl of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions. Luciferase activities were normalized to the β-galactosidase activities. Three or more independent experiments were carried out for each assay. Cell proliferation was determined by [^{3}H] thymidine incorporation assays. Ba/F3 ($2x10^3$/well) cells were cultured in 96-well plate with the increasing amounts of WEHI-3B supernatant. The cells were cultured for 48 hrs and then were pulsed for 12 hrs with [^{3}H] thymidine (1μCi/well) (Amersham Biosciences, Piscataway, NJ) and then harvested. [^{3}H] thymidine incorporation was determined using a Direct Beta Counter Matrix-96 (Packard, Meriden, CT).

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed as described previously (36). The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μM sodium orthovanadate, 1 μM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to Immobilon filter (Millipore; Bedford, MA). The filters were then immunoblotted with the respective antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Indirect Immunofluorescence

Monkey COS7 or human HeLa cells were maintained in DMEM containing 10% FCS transfected with FLAG-STAT5a and Myc-STAP-2/BKS together with Jak2WT or Jak2KE, EPOR by the

calcium phosphate precipitation protocol (37). Forty-eight hrs after transfection, cells were fixed with a solution containing 4% paraformaldehyde and reacted with anti-FLAG antibody or anti-Myc antibody. The cells were then reacted with FITC-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG (CHEMICON, Temecula, CA) and observed under a confocal laser fluorescent microscope. Images were obtained by using a Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom.

IL-2-induced proliferative responses in thymocytes

Thymocytes from wild-type or STAP-2/BKS-deficient mice were prepared as described previously (38). Thymocytes ($5x10^5$/well) were cultured in 96-well plate with the increasing amounts of IL-2 (PeproTech, London, England) in the presence of 5 g/ml anti-CD3 (2C11) and 1 g/ml anti-CD28 (37.51) (BD Biosciences Pharmingen, San Diego, CA). Thymocytes were also cultured with the increasing amounts of phytohemagglutinin (PHA) (Wako, Osaka, Japan). The cells were cultured for 48 hrs and then pulse-labeled with 1 Ci of [^{3}H] thymidine for an additional 8 hrs.

RESULTS

Association of STAP-2/BKS with STAT5 in vivo

Studies examining the members of the family of STAP proteins have suggested that association with the STAT family of latent transcriptional factors may have regulational significance (27, 28). In these regards, STAP-2/BKS association with STAT3 up-regulated the ability of STAT3 to induce transcription (27). The observation that STAP-1 interacts with STAT5 (28) prompted this study to evaluate the functional significance of the interaction. Myc-tagged STAP-2/BKS was co-transfected with either FLAG-tagged STAT5a or FLAG-tagged STAT5b into 293T cells. The cell lysates were immunoprecipitated with anti-Myc antibody and blotted with anti-FLAG antibody. The immunoprecipitates with STAP-2/BKS contained STAT5a/b proteins (Fig. 1A). Similarly, 293T cells were transfected with FLAG-tagged STAT5a or FLAG-tagged STAT5b alone. The cell lysates were immunoprecipitated with anti-Myc antibody and blotted with anti-FLAG antibody. Non-specific bands for STAT5a or STAT5b were not detected (Fig. 1A). We further examined their interaction in a reverse situation. The transfectants were lysed and immunoprecipitated with anti-FLAG antibody and blotted with anti-Myc antibody. The immunoprecipitate with STAT5a/b also contained STAP-2/BKS proteins (Fig. 1B). Similar interaction was observed in human T cell leulemia cell line, Jurkat expressing Myc-tagged STAP-2/BKS. Jurkat/STAP-2/BKS cells were lysed and immunoprecipitaed with anti-STAT5 antibody and blotted with anti-Myc antibody. The immunoprecipitates with anti-STAT5 antibody contained STAP-2/BKS proteins (Fig. 1C). The Western blot analysis of STAP-2 protein showed two bands in Fig. 1A, B. This is derived from the C-terminal region of STAP-2, because both GST-fused PH and SH2-like domain did not show two bands. Only GST-fused the C-terminal region (GST-STAP-2 C) showed two bands as shown Fig. 3H. The STAP-2 protein with a deletion of the C-terminal region also showed a single band (Fig. 3G). At the present time, we do not know the precise mechanisms. This may be due to the posttranscriptional modification such as protein phosphorylation, although any tyrosine phosphorylation was not observed when we overexpressed STAP-2 alone in 293T cells (data not shown).

To examine the physiological interaction between STAP-2/BKS and STAT5, human T cell lymphoma, HUT78, which endogenously expressed both proteins, were employed. HUT78 cells were unstimulated or stimulated with IL-2 for 15 min and the cells were lysed and immunoprecipitated with

control antibody or anti-STAP-2/BKS antibody. The same lysates were also immunoprecipitated with anti-STAT5 antibody. In HUT78 cells, endogenous STAT5 was well tyrosine phosphorylated by IL-2 stimulation (Fig. 1D, lower panel). Furthermore, the immunoprecipitate with anti-STAT5 antibody contained STAP-2/BKS (Fig. 1D; upper panel). It is worthy to note, the amounts of STAP-2/BKS bound to STAT5 decreased as a result of STAT 5 activation/phosphorylation in response to IL-2. The dissociation of STAP-2/BKS from activated STAT5 was also observed in 293T cells overexpressing Jak2. Increasing amounts of tyrosine phosphorylated STAT5 resulted in a decrease of the STAP-2/BKS bound to STAT5 (data not shown). Therefore, STAP-2/BKS can directly associate with STAT5. However, their interaction is reduced when STAT5 is tyrosine-phosphorylated.

Co-localization of STAP-2/BKS with STAT5

We next examined co-localization of STAP-2/BKS with STAT5a in COS7 cells. To activate STAT5a in COS cells, we transfected expression vectors for wild-type Jak2 (Jak2WT) or Jak2KE, an inactive form of Jak2. COS7 cells were transfected with FLAG-tagged STAT5a and Myc-tagged STAP-2/BKS together with Jak2KE or Jak2WT. Forty-eight hrs after transfection, the transfectants were fixed and reacted with rabbit anti-FLAG polyclonal antibody or mouse anti-Myc monoclonal antibody, and visualized with rhodamine-conjugated anti-rabbit antibody or fluorescein isothiocyanate-conjugated anti-mouse antibody. As shown in Fig. 2A, STAT5a remained localized to the cytoplasm in the presence of Jak2 KE, whereas STAT5a translocated into nucleus in the presence of Jak WT. STAP-2/BKS was observed in both the cytoplasm and nucleus in the Jak2WT- and Jak2KE-transfected cells. We also examined co-localization of STAP-2/BKS with STAT5 after EPO stimulation in COS7 cells. COS7 cells were transfected with FLAG-tagged STAT5a and Myc-tagged STAP-2/BKS together with an expression vector for EPO receptor. Forty-eight hrs after transfection, the cells were stimulated with EPO for 30 min and fixed, and then stained with rabbit anti-FLAG polyclonal antibody or mouse anti-Myc monoclonal antibody, and visualized with rhodamine-conjugated anti-rabbit antibody or fluorescein isothiocyanate-conjugated anti-mouse antibody. As shown in Fig. 2B, only small amounts of STAP-2/BKS translocated into nucleus, while most of STAT5 existed in nucleus after EPO-stimulation. Therefore, unphosphorylated STAT5 co-localized with STAP-2/BKS in cytoplasm, but phosphorylated STAT5 translocated into nucleus without STAP-2/BKS-association.

Molecular mechanisms of STAP-2/BKS interactions with STAT5

We also determined the interacting domain of STAP-2/BKS on STAT5 using a series of STAT5a or STAT5b deletion mutants. As depicted for STAT5 in Fig. 3A, C, STAT5 components include the N-terminal domain, the coiled-coil domain, the DNA binding domain, the linker domain, the SH-2 domain, and the C-terminal transactivation domain. 293T cells were transfected with FLAG-tagged STAP-2/BKS and/or a series of Myc-tagged STAT5a or STAT5b deletion mutants. The tranfectants were lysed and immunoprecipitated with anti-FLAG antibody and blotted with anti-Myc antibody. As shown in Fig. 3A, B, C and D, several parts of C-terminal region of STAT5a (145-330, 331-496, 467-793, respectively) or STAT5b (145-330, 331-496, 467-786, respectively) interacted with STAP-2/BKS, suggesting the multiple binding sites of STAP-2/BKS exist on STAT5a or STAT5b. We next determined the interacting domain on STAP-2/BKS. 293T cells were transfected with FLAG-tagged STAT5a or STAT5b and/or a series of Myc-tagged STAP-2/BKS deletion mutants. The transfectants were lysed and immunoprecipitated with anti-FLAG antibody and blotted with anti-Myc antibody. As shown in Fig. 3E and 3F, a deletion mutant of the PH domain of STAP-2/BKS showed a reduced interaction with STAT5a or STAT5b, while full-lengh and deletion mutants of the SH2 or C-terminal domain of STAP-2/BKS retained a strong binding to STAT5a. To further confirm this, a series of deletion mutants of STAP-2/BKS fused with GST (GST-STAP-2 PH, GST-STAP-2 SH2 and GST-STAP-2 C) were constructed (Fig. 3G). The respective mutants together with FLAG-tagged STAT5a or STAT5b were transiently expressed in 293T cells. The binding potential of these proteins with FLAG-tagged STAT5a or STAT5b was examined by immunoprecipitation with anti-FLAG antibody followed by western blotting with anti-GST. As shown in Fig. 3H, both GST-STAP-2 PH and GST-STAP-2 SH2 interacted with STAT5a or STAT5b. Therefore, both PH and SH2 domains of STAP-2/BKS can bind to STAT5, but the interaction of the PH domain was stronger than that of the SH2 domain.

STAP-2/BKS suppresses EPO-induced tyrosine-phosphorylation and transcriptional activation of STAT5

To elucidate whether the interactions between STAP-2/BKS and STAT5 have functional significance, the interaction was examined in EPO-stimulated or unstimulated 293T cells. 293T cells

expressing exogenous EPO receptor were transfected with or without STAP-2/BKS and then activated with EPO. The stimulated cells were harvested and immunoprecipitated with anti-STAT5 antibody followed by western blotting. EPO-induced tyrosine-phosphorylation of STAT5 decreased in the cells transfected with STAP-2/BKS (Fig. 4A), suggesting that STAP-2/BKS interaction with STAT5 influences STAT5 activation. To address whether this reduction in tyrosine phosphorylation correlated to transcriptional regulation, a transient reporter assay was performed using STAT5 reporter constructs, STAT5-LUC or Casein-LUC. 293T cells were transfected with STAT5-LUC or Casein-LUC and/or increasing amounts of STAP-2/BKS. Thirty-six hrs after transfection, the cells were stimulated with EPO. As shown in Fig, 4B, STAP-2/BKS suppressed the STAT5 transcriptional activation by EPO in parallel with its expression. Therefore, STAP-2/BKS regulates STAT5 transcriptional activity.

STAP-2/BKS suppresses IL-3-dependent growth and tyrosine-phosphorylation of STAT5 in Ba/F3 cells

To further assess the functional relevance between STAP-2/BKS and STAT5, we established the stable transformants expressing wild-type STAP-2/BKS or its deletion mutants in an IL-3-dependent murine pro-B cell line, Ba/F3 (Fig. 5A) in which STAT5 has been shown to exert pleiotropic functions regulating cell growth, differentiation and apoptosis (39, 40). We examined the effects of wild-type STAP-2/BKS or its mutants on IL-3-dependent cell growth. As shown in Fig. 5B, all transformants expressing wild-type STAP-2/BKS and its mutants showed significant reduction in cell growth induced by IL-3, whereas the transformant with a deletion of either the PH or SH2 domain showed only small amount of reduction in cell growth. This result indicates that both the PH and SH2-like domain are important for the growth suppression mediated by STAP-2/BKS. We also assessed the tyrosine-phosphorylation of STAT5 induced by IL-3 in Ba/F3 cells expressing control vector or a high amount (H) or a low amount (L) of wild-type STAP-2/BKS. Reduced tyrosine-phosphorylation of STAT5 by IL-3 was observed in Ba/F3 cells expressing a high amount of STAP-2/BKS (Fig. 5C), but not in Ba/F3 cells expressing control vector or a low amount of STAP-2/BKS. Therefore, the STAP-2/BKS expression affected not only IL-3-dependent cell growth but also tyrosine phosphoylation of STAT5 induced by IL-3 in Ba/F3 cells.

Enhanced IL-2-induced proliferation in STAP-2/BKS-deficient thymocytes

Predicting that STAP-2 deficient mice should have a greater response to growth factor because of the reduced negative regulation imposed by STAP-2/BKS, we examined the effect of IL-2/STAT5-mediated T-cell growth in the STAP-2/BKS deficient thymocytes. STAP-2/BKS deficient thymocytes showed the enhanced response to IL-2 (Fig. 6A). However, PHA stimulation, which is STAT5-independent, induced the similar growth response in the wild-type and STAP-2/BKS deficient thymocytes (Fig. 6B). Together, these results demonstrate a role for STAP-2/BKS in negatively regulating STAT5-signaling in vivo.

DISCUSSION

STAP-2/BKS. STAP-2/BKS is expressed in a variety of tissues, and it has the C-terminal proline-rich and tyrosine phosphorylation motifs in an addition to the motifs defining the STAP family protein. In our previous study, we also demonstrated STAP-2/BKS interacted with STAT3 through its YXXQ motif and enhanced STAT3 transcriptional activity (27). Association of STAP-2/BKS with STAT3 was dependent on tyrosine phosphorylation of YXXQ motif in STAP-2/BKS. However, STAP-2/BKS interacted with STAT5 through its PH and SH2-like domains, while the binding potential of the PH domain of STAP-2/BKS was stronger than that of SH2-like domain. Furthermore, STAP-2/BKS associated with unphosphorylated STAT5 in cytoplasm of resting cells and dissociated with STAT5 by stimuli. The difference between STAP-2/BKS-STAT5 and STAP-2/BKS-STAT3 interactions may mediate the different functions of STAP-2/BKS on each STAT protein. STAP-2/BKS is localized throughout cytoplasm and nucleus. Indeed, PSORT database search revealed that nuclear localization signal (NLS) exists in STAP-2/BKS (amino acid 344-347 in murine STAP-2/BKS). Our previous study demonstrated that STAP-2/BKS was rapidly translocated to plasma membrane through its PH domain by EGF-stimulation, suggesting that the PH domain of STAP-2/BKS may play a role on its functions (27). However, we do not know whether nuclear localization of STAP-2/BKS is required for its functions, and further studies will be necessary for the significance of the NLS of STAP-2/BKS. On the other hand, the strength of the binding capacity may explain why the deletion mutants of the PH domain but not those of the SH2-like domain lost the suppression activity of EPO/STAT5-mediated luciferase induction in 293T cells (data not shown). This possibility is likely to be supported by the facts that EPOR was overexpressed in experiments for EPO/STAT5-mediated luciferase activity and endogenous IL-3R was used in experiments for IL-3/STAT5-mediated cell proliferation assay and that the suppression of STAT5 signaling wasSTAP-2/BKS-dose-dependent. Alternatively, the participations of the PH and SH2-like domains in the association with STAT5 may depend on uncertain cellular circumstances. Present studies showed that the PH domain of STAP-2/BKS was necessary for the functional interactions with STAT5. The data indicate that endogenous STAP-2/BKS

associates with unphosphorylated form of STAT5 and negatively modulates tyrosine-phosphorylation and transcriptional activation of STAT5.

Like other STAT proteins, STAT5 contains the N-terminal domain, the coiled-coil domain, the DNA binding domain, the linker domain, the SH2 domain, and the C-terminal transactivation domain. Crystal structural study of STAT1 revealed that a STAT1 fragment including the coiled-coil domain, the DNA binding domain, the linker domain and the SH2 domain, except for the N-terminal and the C-terminal transactivation domain showed a core structure and formed a DNA complex (41). As shown in Fig. 3, STAP-2/BKS interacted with STAT5a or STAT5b through the coiled-coil domain, the DNA binding domain, and the C-terminal region containing the linker and SH23 domain, although the interaction between STAT5a/b and the coiled-coil domain was faint (Fig. 3A, B). Furthermore, both PH and SH2-like domain of STAP-2/BKS bound to STAT5a/b (Fig. 3H). These data suggest that STAP-2/BKS contains at least two binding sites for the core fragment of STAT5, and binds to and masks STAT5 protein. The conformational change of the core structure of STAT5 by tyrosine-phophorylation may reduce the binding affinity for STAP-2/BKS. Crystal structural analysis of STAT5-STAP-2/BKS complex will be required to clarify the details of their interactions.

STAT5a-deficient mice showed a marked decreased proliferative response to low concentration of IL-2 (15). There was a much greater decreased proliferation in response to IL-2 in STAT5b-deficient mice than was seen in STAT5a-deficient mice (13). The STAT5a/b double knockout mice showed a profoundly deficiency in peripheral T-cells (17), indicating that STAT5 proteins plays vital role in IL-2 signaling. Recent study using STAT5a/b knockout mice also demonstrated STAT5 is required for embryonic thymocyte production, TCR gene transcription, and Peyer's patch development (42). In STAP-2/BKS deficient mice, cellularity in the thymus and T-cell development assessed by expression of CD4 and CD8 appeared normal in the STAP-2/BKS-deficient mice (data not shown). Expression levels of CD3 on thymocytes were also indistinguishable between the wild-type and STAP-2/BKS-deficient mice (data not shown). However, STAP-2/BKS deficient thymocytes showed the enhanced

response to IL-2. This result suggests that STAP-2/BKS may play a role in the regulation of STAT5 in thymocytes.

Although the molecular mechanism of STAT5 modulation by STAP-2/BKS has not been clarified yet, one of the mechanisms is that STAP-2/BKS simply masks the STAT5 molecule to form a latent STAT5-STAP-2/BKS complex in the absence of ligand stimulation. This model may be supported by our observation that IL-2 stimulation in HUT78 cells can decrease the amounts of STAP-2/BKS bound to STAT5 (Fig. 1C). The other possibility is that STAP-2/BKS recruits other regulatory molecules, such as protein tyrosine phosphatases (PTPs) or ubiquitin ligases. They dephosphorylate tyrosine-phosphorylated STAT5 or accelerate the degradation of STAT5 by the ubiquitin-proteasome pathway. Recently APS, adaptor molecule containing PH and SH2 domains, was shown to be tyrosine-phosphorylated by Jak2 at its C-terminal tyrosine residue and interacted with c-Cbl (43). Overexpression of APS in an EPO-dependent hematopoietic cell line resulted in a reduced activation of STAT5 but not cell proliferation in response to EPO. Furthermore, co-expression of APS and c-Cbl, but not expression of either alone inhibited EPO-dependent STAT5 activation in 293 cells, suggesting that one of the major functions of APS is in recruitment of c-Cbl into the receptor/Jak complex. STAP-2/BKS may also recruit PTPs or ubiquitin-proteasome signaling molecules like c-Cbl proteins to block STAT5 activation. However, overexpression of STAP-2/BKS resulted in no alteration of endogenous STAT5 contents, suggesting that STAP-2/BKS may not recruit ubiquitin-proteasome signaling molecules but PTPs for STAT5. SHP-2 but not SHP-1 has been demonstrated to directly dephosphorylate STAT5 (25). Indeed, SHP-1 and SHP-2 interacted with STAP-2/BKS in 293T cells (Y. Sekine and T. Matsuda, unpublished data). Further detailed study is necessary to elucidate the molecular mechanisms of STAT5 modulation by STAP-2/BKS.

The overall structure of STAP-2/BSK resembles that of adaptor/docking proteins such as the insulin receptor substrates (IRS)(44), Grb2-associated binder (Gab)(45), and Downstream of Kinase (Dok)(46), since these adaptor molecules contain the PH domain at the N-terminal region, a phosphotyrosine-binding domain, such as the PTB domain, or a Met-binding domain (MBD), in the

middle, and tyrosine phosphorylation sites in the C-terminal region. The most of these molecules expresses ubiquitously. However, the knockout works showed that some of them are indispensable for the functions in a specific tissue or organ. An ubiquitously expressed adaptor molecule, Gab2 is recently shown to be essential in the FcεRI-mediated signaling pathway in mast cells (47). Our previous study demonstrated that STAP-2/BKS affected on the FcεRI signaling pathway in rat basophilic leukemia cells (34). In the STAP-2/BKS-deficient thymocytes, we could detect a little bit enhanced proliferation compared to the wild-type thymocytes, when we stimulated them with anti-CD3 and anti-CD28 antibodies (Fig. 6B). These results suggest that STAP-2/BKS may have some effects on TCR signaling. However, at the present time, we have not obtained any results on the FcεRI signaling in the STAP-2/BKS-deficient mast cells. The STAP-2/BKS deficient mice will also provide a powerful tool for these investigations.

In conclusion, our studies describe a novel function for STAP-2/BKS in regulating STAT5-mediated signaling. STAP-2/BKS interacted with STAT5 in vivo and suppressed EPO/STAT5-mediated transcriptional activation or IL-3-depedent growth in pro-B cells. Furthermore, thymocytes from STAP-2/BKS-deficient mice showed the enhanced proliferation in response to IL-2. We propose that STAP-2/BKS may play a role in modulating the STAT5-mediated signaling in immune cells.

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FIGURE LEGEND

Fig. 1. **Association of STAP-2/BKS with STAT5 in vivo.**

(A) 293T cells ($1x10^7$) were transfected with FLAG-tagged STAT5a (10□g) or STAT5b (10□g) with or without Myc-tagged STAP-2/BKS (5□g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-Myc antibody and immnoblotted with anti-FLAG (upper panel) or anti-Myc antibody (middle panel). Total cell lysates (1%) were blotted with anti-FLAG antibody (bottom panel) to monitor the expression of STAT5a or STAT5b. Protein molecular size markers are shown at left (in kilodaltons; kDa).

(B) 293T cells ($1x10^7$) were transfected with FLAG-tagged STAT5a (10□g) or STAT5b (10□g) and/or Myc-tagged STAP-2/BKS (5□g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc (bottom panel) to monitor the expression of STAP-2/BKS. Protein molecular size markers are shown at left (in kilodaltons; kDa).

(C) Stable transformants of Myc-tagged STAP-2/BKS in Jurkat cells ($1x10^7$) were lysed, and immunoprecipitated with anti-STAT5 antibody and immnoblotted with anti-Myc antibody (upper panel) or anti-STAT5 antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (bottom panel) to monitor the expression of STAP-2/BKS. IgH, heavy chain of immunoglobulin. Protein molecular size markers are shown at left (in kilodaltons; kDa).

(D) Human T cell lymphoma, HUT78 cells ($2x10^7$) were stimulated with or without IL-2 (20U/ml) for 15 min. The stimulated cells were lysed, and immunoprecipitated with control IgG or anti-STAP-2/BKS antibody and immnoblotted with anti-STAT5a/b antibody or anti-STAP-2/BKS antibody (upper panels). The immunoprecipitate with anti-STAT5a/b antibody was also blotted with anti-phosphotyrosine (PY) antibody or anti-STAT5 antibody (bottom panels). Protein molecular size markers are shown at left (in kilodaltons; kDa).

Fig.2. **Co-localization of STAP-2/BKS with STAT5.**

(A) COS7 cells were transfected with FLAG-tagged STAT5a and Myc-tagged STAP-2/BKS together with Jak2 kinase-dead (KE) or Jak2 wild-type (WT). Forty-eight hrs after transfection, cells were fixed and reacted with rabbit anti-FLAG polyclonal antibody and mouse anti-Myc monoclonal antibody, and visualized with rhodamine-conjugated anti rabbit antibody or fluorescein isothiocyanate-conjugted anti-mouse antibody. These figures were merged. The same slide was also stained with DAPI for the nuclei staining.

(B) COS7 cells were transfected with FLAG-tagged STAT5a and Myc-tagged STAP-2/BKS together with an expression vector of EPOR. Forty-eight hrs after transfection, cells were stimulated with EPO (1U/ml) for 30 min, and then fixed and reacted with rabbit anti-FLAG polyclonal antibody or mouse anti-Myc monoclonal antibody, and visualized with rhodamine-conjugated anti-rabbit antibody or fluorescein isothiocyanate-conjugted anti-mouse antibody. These figures were merged. The same slide was also stained with DAPI for the nuclei staining.

Fig. 3. **Molecular mechanisms of STAP-2/BKS interactions with STAT5**

(A) Domain structure of STAT5a and mutant fragments are schematically shown.

(B) 293T cells ($1x10^7$) were transfected with FLAG-tagged STAP-2/BKS (5□g) and/or a series of Myc-tagged STAT5a mutants (10□g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-Myc antibody (middle panel) or anti-FLAG antibody (lower panel). Total cell lysates (1%) were blotted with anti-Myc antibody to monitor the expression of wild-type STAT5a or its mutants. IgH, heavy chain of immunoglobulin. Protein molecular size markers are shown at left (in kilodaltons; kDa).

(C) Domain structure of STAT5b and mutant fragments are schematically shown.

(D) 293T cells ($1x10^7$) were transfected with FLAG-tagged STAP-2/BKS (5□g) and/or a series of Myc-tagged STAT5b mutants (10□g). Forty-eight hrs after transfection, the cells were lysed, and

immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-Myc antibody (middle panel) or anti-FLAG antibody (lower panel). Total cell lysates (1%) were blotted with anti-Myc antibody to monitor the expression of wild-type STAT5b or its mutants. Protein molecular size markers are shown at left (in kilodaltons; kDa).

(E) Domain structure of STAP-2/BKS and mutant fragments are schematically shown.

(F) 293T cells ($1x10^7$) were transfected with FLAG-tagged STAT5a or STAT5b (10□g) and/or a series of Myc-tagged STAP-2 mutants (5□g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-Myc antibody (upper panels) or anti-FLAG antibody (middle panels). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panels) to monitor the expression of wild-type STAP-2/BKS or its mutants.

(G) Domain structure of STAP-2/BKS and GST-fused mutant fragments are schematically shown.

(H) 293T cells ($1x10^7$) were transfected with FLAG-tagged STAT5a or STAT5b (5□g) and/or GST or a series of GST-fused STAP-2/BKS mutants (10□g). Forty-eight hrs after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-GST antibody (upper panels) or anti-FLAG antibody (middle panels). Total cell lysates (1%) were blotted with anti-GST antibody (lower panels) to monitor the expression of GST or its fusion proteins. Protein molecular size markers are shown at left (in kilodaltons; kDa).

Fig. 4. **STAP-2/BKS suppresses EPO-induced tyrosine-phosphorylation and transcriptional activation of STAT5.**

(A) 293T cells ($1x10^7$) were transfected with or without Myc-tagged STAP-2/BKS (5□g) together with EPOR (2□g) and treated with EPO (1U/ml) for the indicated time. The cells were lysed and immunoprecipitated with anti-STAT5 antibody, and blotted with anti-phosphotyrosine (PY) antibody (upper panel) or anti-STAT5 antibody (middle panel). Total cell lysates (1%) were blotted

with anti-Myc antibody (lower panel) to monitor the expression of STAP-2/BKS. Protein molecular size markers are shown at left (in kilodaltons; kDa).

(B) 293T cells in a 12-well plate were transfected with STAT5-LUC (0.4□g) or Casein-LUC (0.4□g) and/or the increasing amounts of STAP-2/BKS together with EPOR (50ng) as indicated. Thirty-six hrs after transfection, the cells were stimulated with EPO (1U/ml) for additional 12 hrs. The stimulated cells were harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. Total extracts (5%) from cells transfected with the above constructs were blotted with anti-Myc antibody (lower panel) to monitor the expression of wild-type STAP-2/BKS.

Fig. 5. **STAP-2/BKS suppresses IL-3-dependent growth and tyrosine-phosphorylation of STAT5 in Ba/F3 cells.**

(A)Stable BaF/3 transformants with a series of STAP-2/BKS mutants were established. Total extracts (1%) of each transformant were examined by Western blot using anti-Myc antibody to monitor the expression of wild-type STAP-2/BKS or its mutants. Protein molecular size markers are shown at left (in kilodaltons; kDa).

(B) BaF/3 transformants (2×10^3) were cultured in a 96-well plate with the increasing amounts of WEHI3B supernatant (as a source of IL-3) at the indicated concentration for 48 hrs. The cell growth was determined by [^{3}H] thymidine incorporation assays.

(C) STAP-2/BKS transfected Ba/F3 cells (2×10^6) were treated with IL-3 (10% of WEHI3B sup.) for the indicated time. The cells were lysed, and immunoprecipitated with anti-STAT5 antibody and immnoblotted with anti-PY antibody (upper panel) or anti-STAT5 antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel) to monitor the expression of STAP-2/BKS. IgH, heavy chain of immunoglobulin. Protein molecular size markers are shown at left (in kilodaltons; kDa).

Fig. 6. **Enhanced IL-2-dependent growth in STAP-2/BKS-deficient thymocytes**.

Wild-type (WT) or STAP-2-deficient (KO) thymocytes ($5x10^5$ per well) were cultured in a 96-well plate for 48 hrs in the presence of PHA (A) or anti-CD3 (5 g/ml) / anti-CD28 (1 g/ml) together with the increasing amounts of IL-2 (B). The cells were then pulse-labeled with 1 Ci of [^{3}H] thymidine for an additional 8 hrs. Results shown are representative of three experiments performed in triplicate.

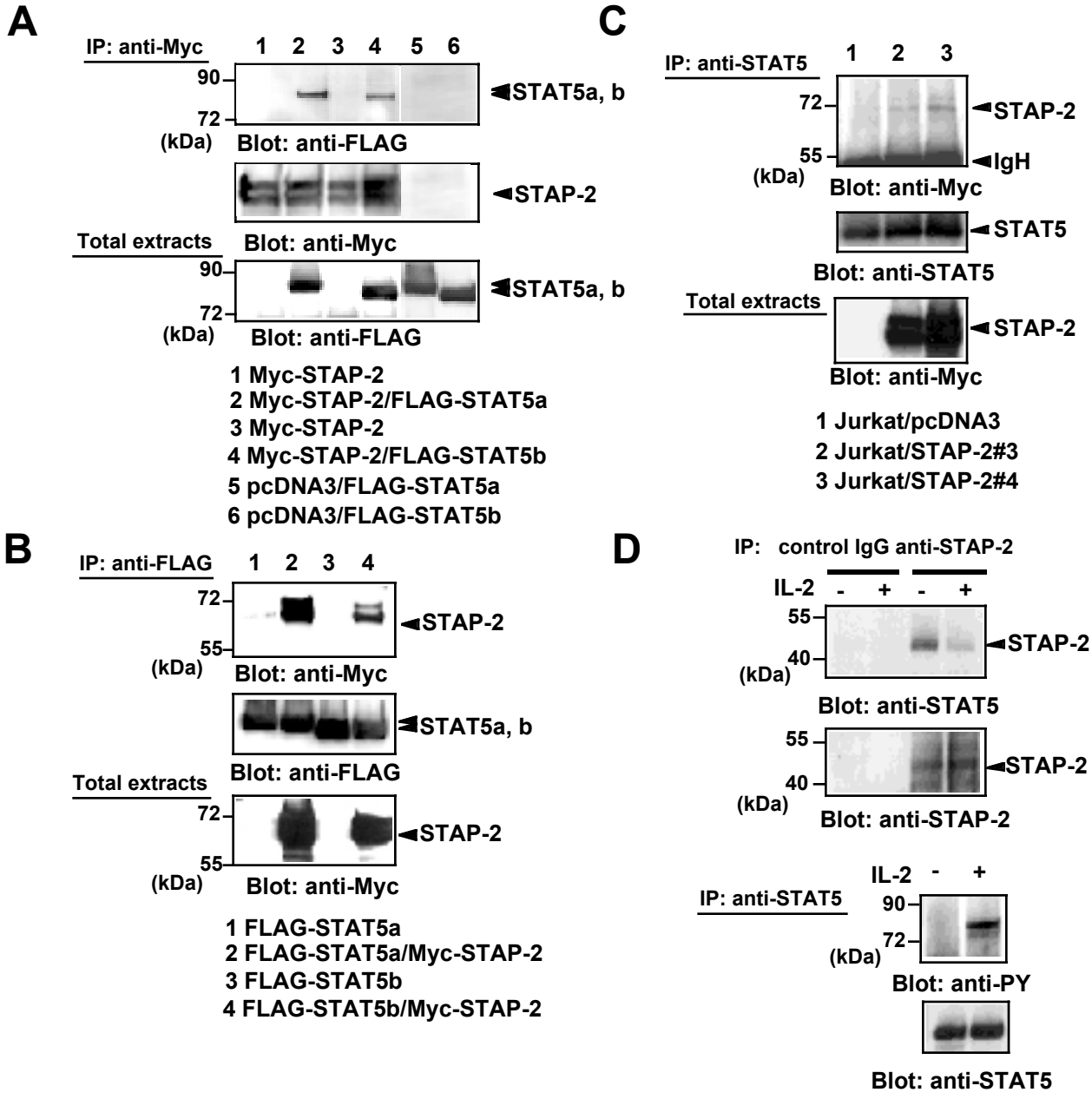
A
IP: anti-Myc
1 2 3 4 5 6
90
72
(kDa)
STAT5a, b
Blot: anti-FLAG
STAP-2
Total extracts
Blot: anti-Myc
90
72
(kDa)
STAT5a, b
Blot: anti-FLAG
1 Myc-STAP-2
2 Myc-STAP-2/FLAG-STAT5a
3 Myc-STAP-2
4 Myc-STAP-2/FLAG-STAT5b
5 pcDNA3/FLAG-STAT5a
6 pcDNA3/FLAG-STAT5b
B
IP: anti-FLAG
1 2 3 4
72
55
(kDa)
STAP-2
Blot: anti-Myc
STAT5a, b
Blot: anti-FLAG
Total extracts
72
55
(kDa)
STAP-2
Blot: anti-Myc
1 FLAG-STAT5a
2 FLAG-STAT5a/Myc-STAP-2
3 FLAG-STAT5b
4 FLAG-STAT5b/Myc-STAP-2
C
IP: anti-STAT5
1 2 3
72
55
(kDa)
STAP-2
IgH
Blot: anti-Myc
STAT5
Blot: anti-STAT5
Total extracts
STAP-2
Blot: anti-Myc
1 Jurkat/pcDNA3
2 Jurkat/STAP-2#3
3 Jurkat/STAP-2#4
D
IP: control IgG anti-STAP-2
IL-2 - + - +
55
40
(kDa)
STAP-2
Blot: anti-STAT5
55
40
(kDa)
STAP-2
Blot: anti-STAP-2
IL-2 - +
IP: anti-STAT5
90
72
(kDa)
Blot: anti-PY
Blot: anti-STAT5

Fig. 1

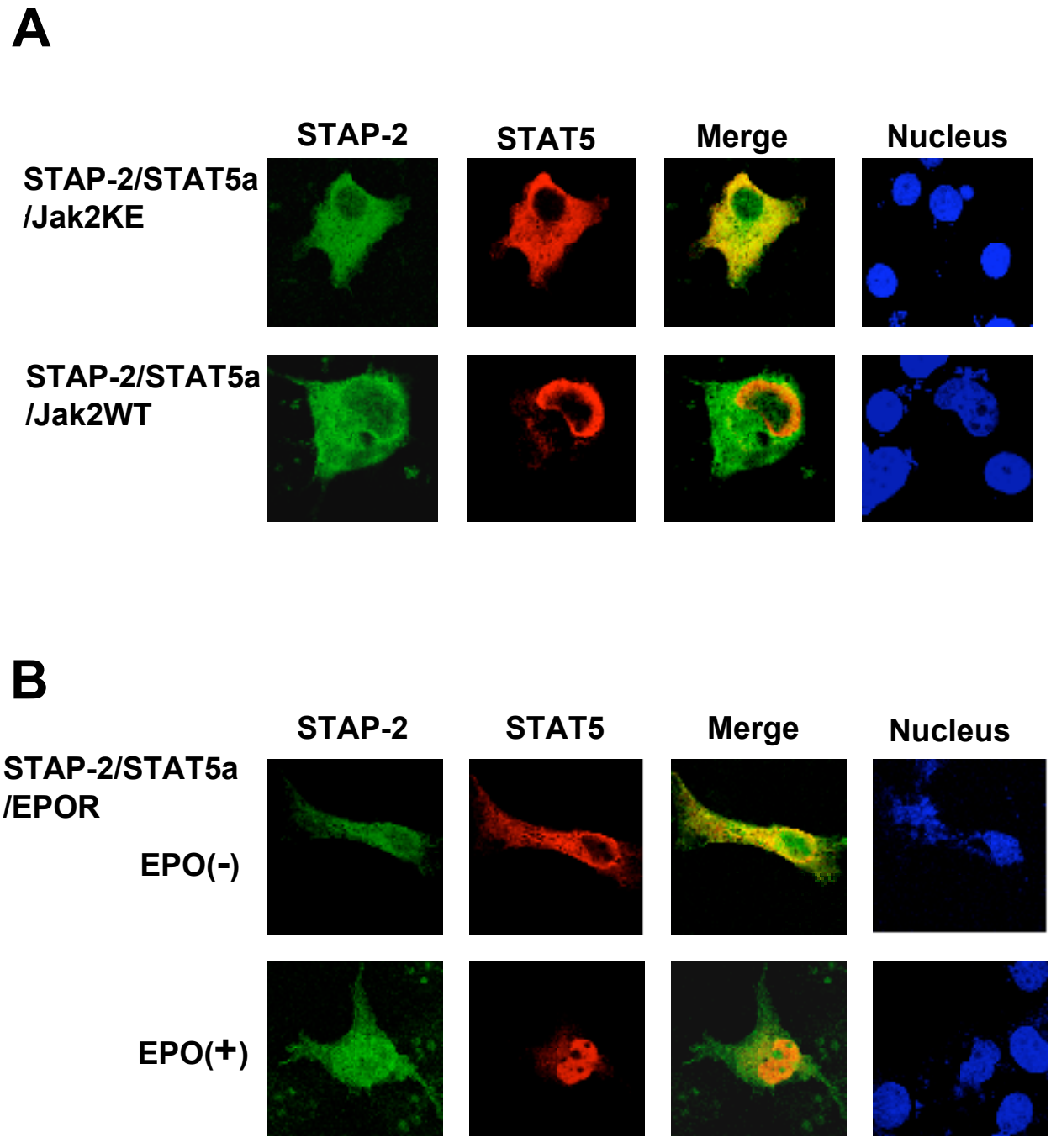
A
STAP-2
STAT5
Merge
Nucleus
STAP-2/STAT5a /Jak2KE
STAP-2/STAT5a /Jak2WT
B
STAP-2
STAT5
Merge
Nucleus
STAP-2/STAT5a /EPOR
EPO(-)
EPO(+)

Fig. 2

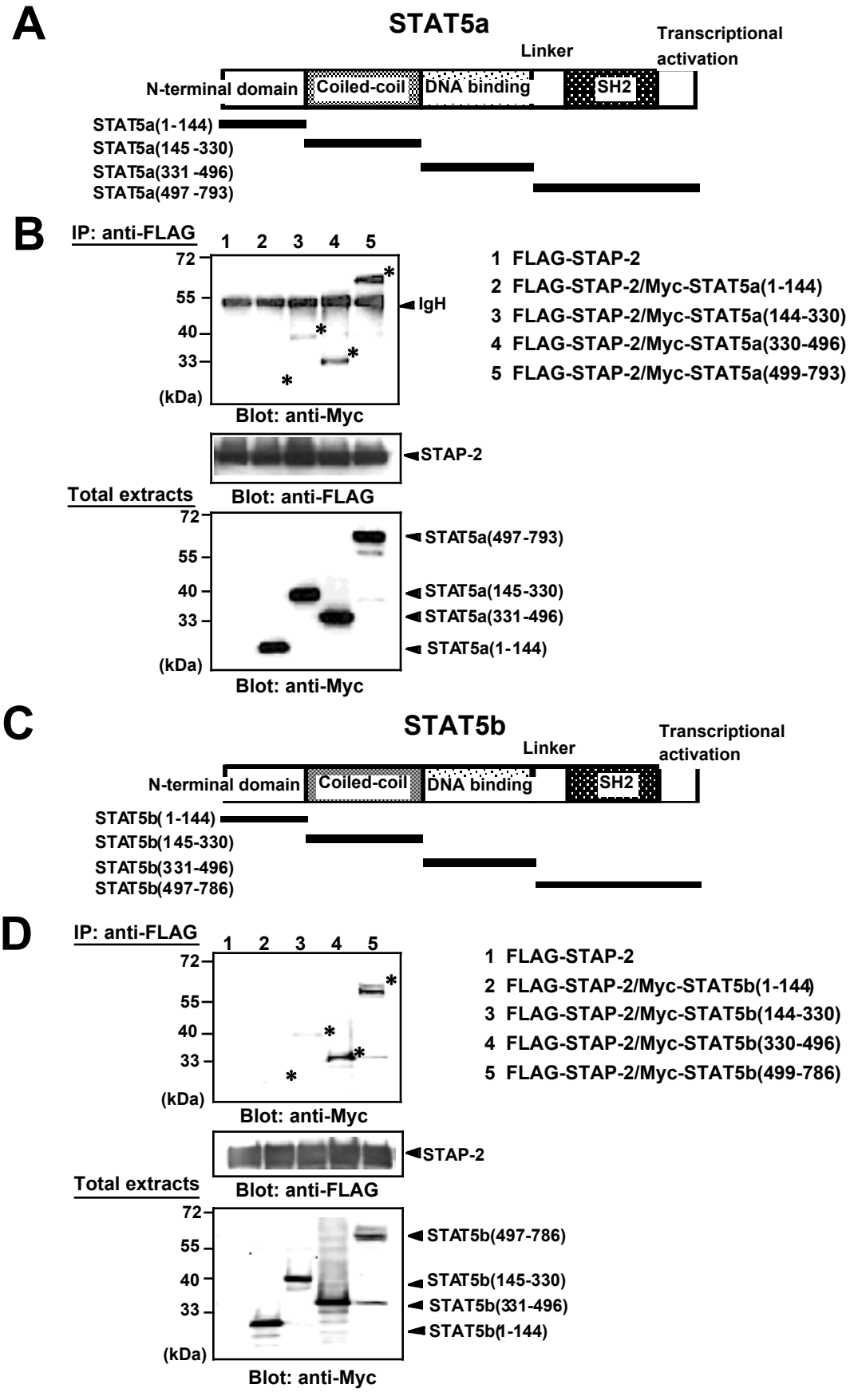
A
STAT5a
Linker
Transcriptional activation
N-terminal domain
Coiled-coil
DNA binding
SH2
STAT5a(1-144)
STAT5a(145-330)
STAT5a(331-496)
STAT5a(497-793)
B
IP: anti-FLAG
1 2 3 4 5
72
55
40
33
(kDa)
IgH
Blot: anti-Myc
STAP-2
Blot: anti-FLAG
Total extracts
STAT5a(497-793)
STAT5a(145-330)
STAT5a(331-496)
STAT5a(1-144)
Blot: anti-Myc
1 FLAG-STAP-2
2 FLAG-STAP-2/Myc-STAT5a(1-144)
3 FLAG-STAP-2/Myc-STAT5a(144-330)
4 FLAG-STAP-2/Myc-STAT5a(330-496)
5 FLAG-STAP-2/Myc-STAT5a(499-793)
C
STAT5b
Linker
Transcriptional activation
N-terminal domain
Coiled-coil
DNA binding
SH2
STAT5b(1-144)
STAT5b(145-330)
STAT5b(331-496)
STAT5b(497-786)
D
IP: anti-FLAG
1 2 3 4 5
72
55
40
33
(kDa)
Blot: anti-Myc
STAP-2
Blot: anti-FLAG
Total extracts
STAT5b(497-786)
STAT5b(145-330)
STAT5b(331-496)
STAT5b(1-144)
Blot: anti-Myc
1 FLAG-STAP-2
2 FLAG-STAP-2/Myc-STAT5b(1-144)
3 FLAG-STAP-2/Myc-STAT5b(144-330)
4 FLAG-STAP-2/Myc-STAT5b(330-496)
5 FLAG-STAP-2/Myc-STAT5b(499-786)

Fig. 3a

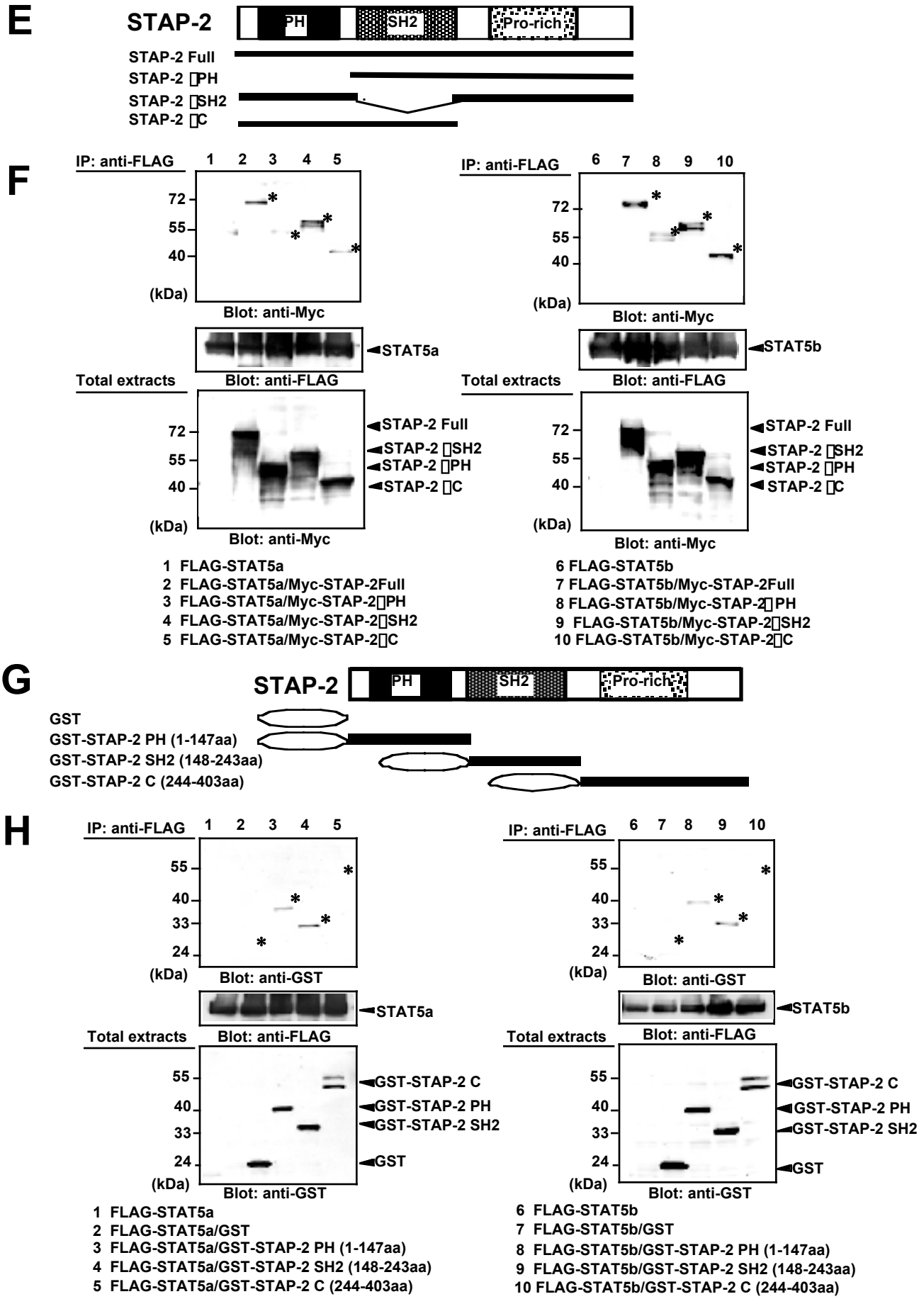
E
STAP-2
PH
SH2
Pro-rich
STAP-2 Full
STAP-2 ΔPH
STAP-2 ΔSH2
STAP-2 ΔC
F
IP: anti-FLAG
1 2 3 4 5
72
55
40
(kDa)
Blot: anti-Myc
STAT5a
Blot: anti-FLAG
Total extracts
STAP-2 Full
STAP-2 ΔSH2
STAP-2 ΔPH
STAP-2 ΔC
Blot: anti-Myc
IP: anti-FLAG
6 7 8 9 10
72
55
40
(kDa)
Blot: anti-Myc
STAT5b
Blot: anti-FLAG
Total extracts
STAP-2 Full
STAP-2 ΔSH2
STAP-2 ΔPH
STAP-2 ΔC
Blot: anti-Myc
1 FLAG-STAT5a
2 FLAG-STAT5a/Myc-STAP-2Full
3 FLAG-STAT5a/Myc-STAP-2ΔPH
4 FLAG-STAT5a/Myc-STAP-2ΔSH2
5 FLAG-STAT5a/Myc-STAP-2ΔC
6 FLAG-STAT5b
7 FLAG-STAT5b/Myc-STAP-2Full
8 FLAG-STAT5b/Myc-STAP-2ΔPH
9 FLAG-STAT5b/Myc-STAP-2ΔSH2
10 FLAG-STAT5b/Myc-STAP-2ΔC
G
STAP-2
PH
SH2
Pro-rich
GST
GST-STAP-2 PH (1-147aa)
GST-STAP-2 SH2 (148-243aa)
GST-STAP-2 C (244-403aa)
H
IP: anti-FLAG
1 2 3 4 5
55
40
33
24
(kDa)
Blot: anti-GST
STAT5a
Blot: anti-FLAG
Total extracts
GST-STAP-2 C
GST-STAP-2 PH
GST-STAP-2 SH2
GST
Blot: anti-GST
IP: anti-FLAG
6 7 8 9 10
55
40
33
24
(kDa)
Blot: anti-GST
STAT5b
Blot: anti-FLAG
Total extracts
GST-STAP-2 C
GST-STAP-2 PH
GST-STAP-2 SH2
GST
Blot: anti-GST
1 FLAG-STAT5a
2 FLAG-STAT5a/GST
3 FLAG-STAT5a/GST-STAP-2 PH (1-147aa)
4 FLAG-STAT5a/GST-STAP-2 SH2 (148-243aa)
5 FLAG-STAT5a/GST-STAP-2 C (244-403aa)
6 FLAG-STAT5b
7 FLAG-STAT5b/GST
8 FLAG-STAT5b/GST-STAP-2 PH (1-147aa)
9 FLAG-STAT5b/GST-STAP-2 SH2 (148-243aa)
10 FLAG-STAT5b/GST-STAP-2 C (244-403aa)

Fig. 3b

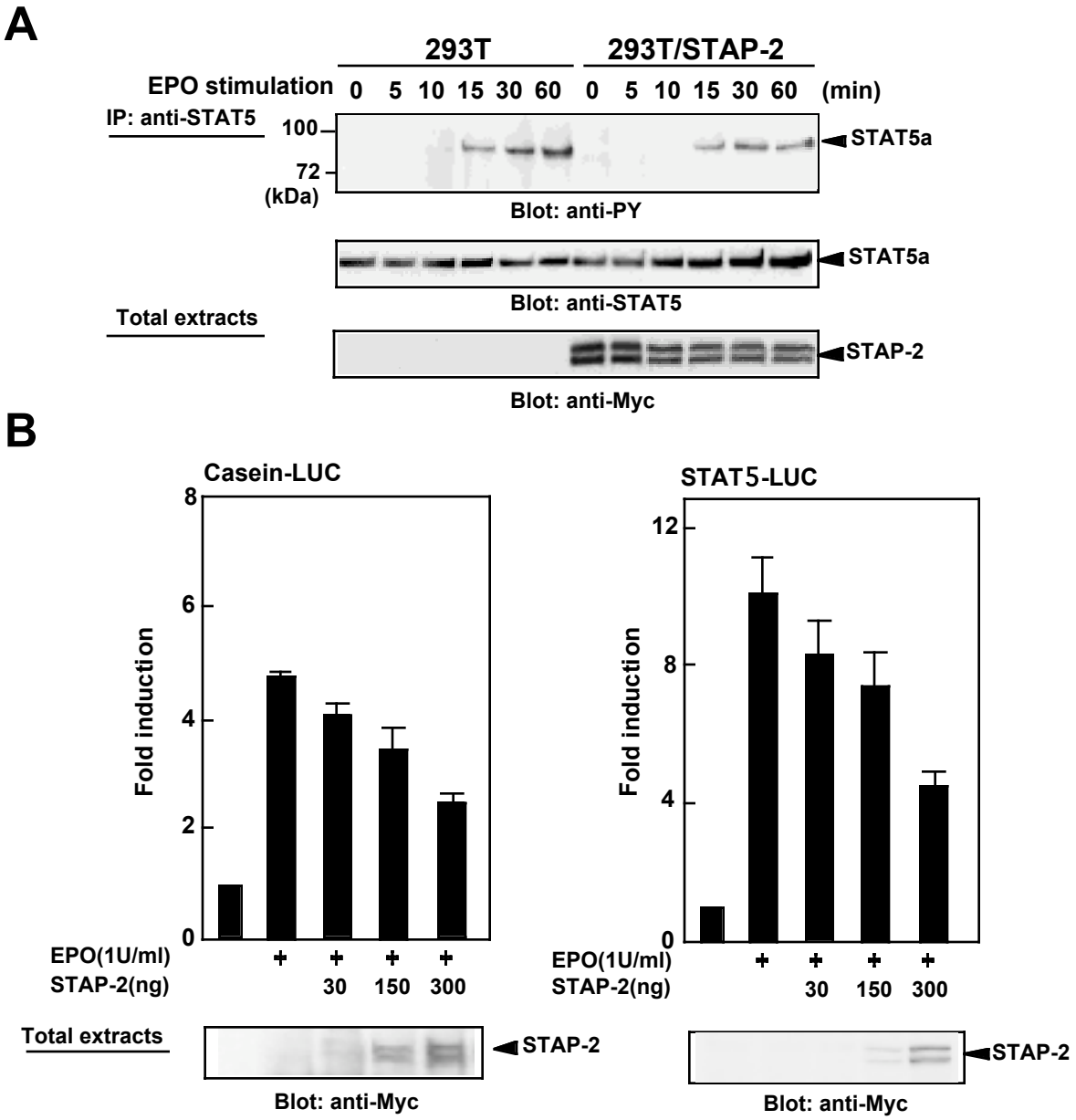
A
293T
293T/STAP-2
EPO stimulation
0 5 10 15 30 60 0 5 10 15 30 60 (min)
IP: anti-STAT5
100
72
(kDa)
STAT5a
Blot: anti-PY
STAT5a
Blot: anti-STAT5
Total extracts
STAP-2
Blot: anti-Myc
B
Casein-LUC
Fold induction
0
2
4
6
8
EPO(1U/ml)
STAP-2(ng)
+ + + +
30 150 300
Total extracts
STAP-2
Blot: anti-Myc
STAT5-LUC
Fold induction
0
4
8
12
EPO(1U/ml)
STAP-2(ng)
+ + + +
30 150 300
STAP-2
Blot: anti-Myc

Fig. 4

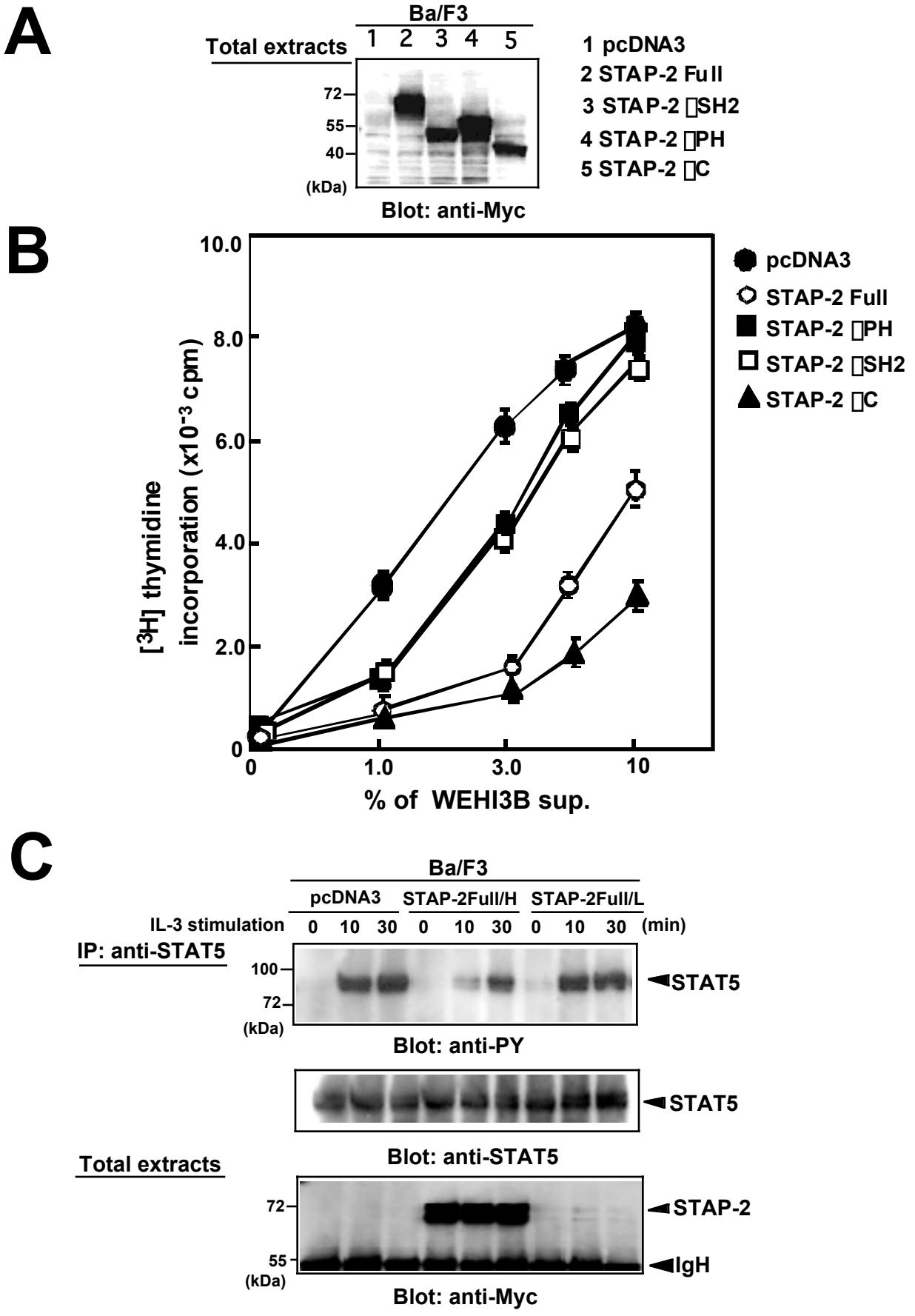
A
Ba/F3
Total extracts
1 2 3 4 5
72
55
40
(kDa)
Blot: anti-Myc
1 pcDNA3
2 STAP-2 Full
3 STAP-2 ΔSH2
4 STAP-2 ΔPH
5 STAP-2 ΔC
B
10.0
8.0
6.0
4.0
2.0
0
[3H] thymidine incorporation (x10-3 cpm)
0
1.0
3.0
10
% of WEHI3B sup.
pcDNA3
STAP-2 Full
STAP-2 ΔPH
STAP-2 ΔSH2
STAP-2 ΔC
C
Ba/F3
pcDNA3
STAP-2Full/H
STAP-2Full/L
IL-3 stimulation
0 10 30 0 10 30 0 10 30 (min)
IP: anti-STAT5
100
72
(kDa)
STAT5
Blot: anti-PY
STAT5
Blot: anti-STAT5
Total extracts
72
55
(kDa)
STAP-2
IgH
Blot: anti-Myc

Fig. 5

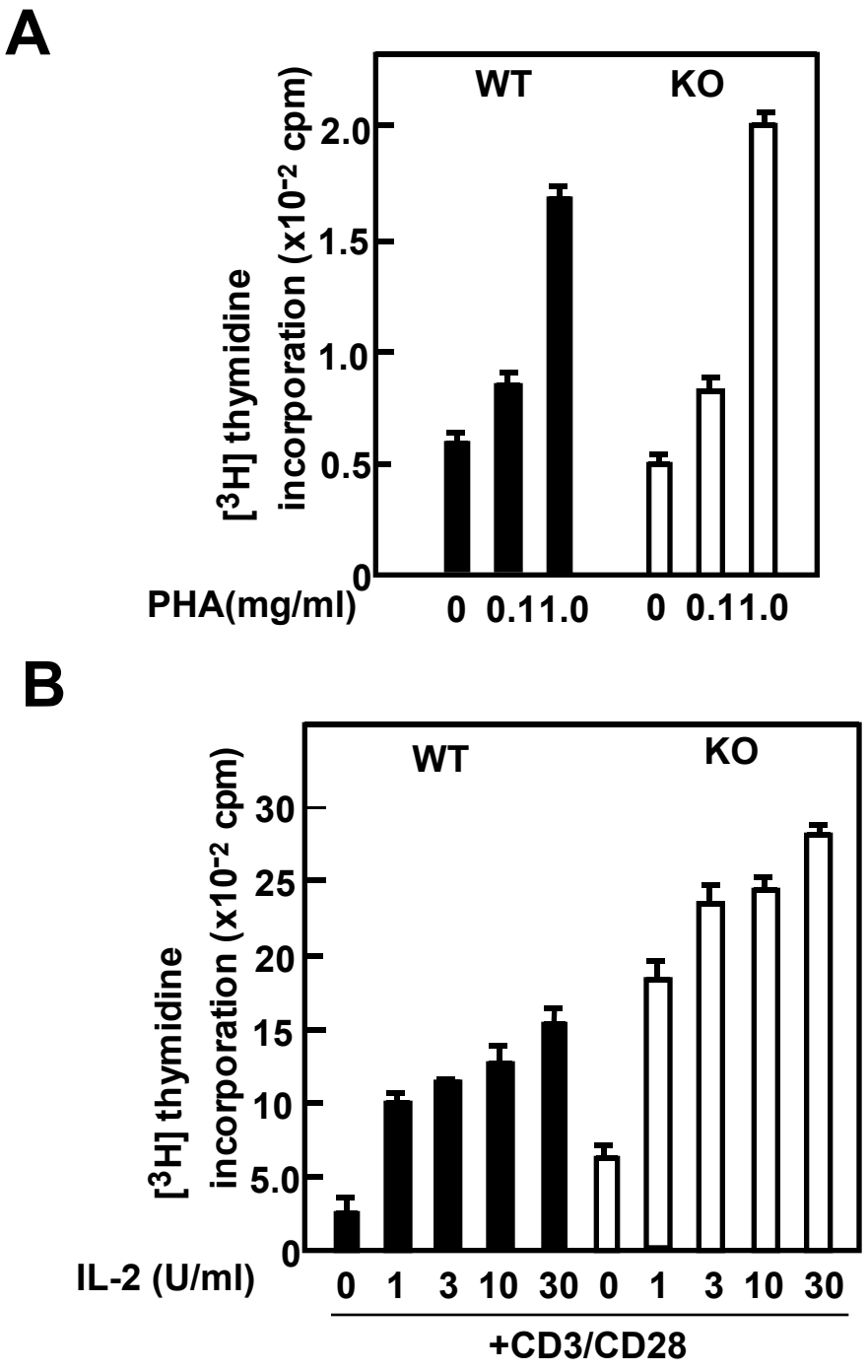
A
WT
KO
[3H] thymidine incorporation (x10-2 cpm)
2.0
1.5
1.0
0.5
0
PHA(mg/ml)
0 0.1 1.0 0 0.1 1.0
B
WT
KO
[3H] thymidine incorporation (x10-2 cpm)
30
25
20
15
10
5.0
0
IL-2 (U/ml)
0 1 3 10 30 0 1 3 10 30
+CD3/CD28

Fig. 6