STAM2, a new member of the STAM family, binding to the Janus kinases

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Abstract We here cloned a cDNA encoding STAM2, a new member of the STAM family, which contains an SH3 domain and ITAM. STAM2 like STAM1 is associated with Jak2 and Jak3, and involved in the signaling for DNA synthesis and c-*myc* induction mediated by IL-2 and GM-CSF. Co-expression of the SH3 deletion mutants of STAM1 and STAM2 induces an additive effect on suppressing DNA synthesis upon stimulation with IL-2 and GM-CSF, suggesting that STAM1 and STAM2 exhibit compensatory effects on the signaling pathways downstream of Jak2 and Jak3 upon stimulation with GM-SCF and IL-2, respectively.

Key words: IL-2; GM-CSF; Signal transduction; Tyrosine kinase

1. Introduction

The γc chain is a common receptor subunit for cytokines such as interleukin 2 (IL-2), IL-4, IL-7, IL-9 and IL-15, and its mutations cause X-linked severe combined immunodeficiency, characterized as a profound defect of T and NK cells and impairment of B cell differentiation in humans and mice [1]. The βc chain is a common receptor subunit for cytokines such as IL-3, IL-5 and granulocyte/macrophage colony stimulating factor (GM-CSF) [2]. The cytoplasmic portions of the γc and βc chains are associated with Janus kinase 3 (Jak3) and Jak2, respectively [3,4]. Stat5 is reportedly involved in the signal transduction immediately downstream of Jak3 and Jak2, and translocated to the nucleus upon stimulation with the respective cytokines [5]. Stat5 activated by Jak2 has been shown to contribute to the signaling for DNA synthesis mediated by IL-3 [6]. However, there have been some controversial reports regarding the involvement of Stat5 in cell growth signaling mediated by Jak3 upon stimulation with IL-2; T cells derived from Stat5A/B double knockout mice do not retain ability for IL-2-induced cell growth [7], whereas T cells expressing the IL-2 receptor β chain mutant which lacks the Stat5 binding site show no activation of Stat5 upon IL-2 stimulation, are responsive to a higher dose of IL-2 to proliferate [8]. The cytokines sharing the γc and βc chains induce expression of proto-oncogenes such as c-*myc*, c-*fos* and c-*jun*, and the induction of c-*fos*/c-*jun* requires activation of Ras [9]. Although the phosphatidylinositol 3 kinase-Akt pathway is reportedly involved in IL-2-mediated c-*myc* induction [10], little is known about the c-*myc* inducing pathway immediately downstream of Jak3 in IL-2 signaling.

We recently identified a phosphotyrosine protein named STAM (signal transducing adapter molecule), which has a unique structure containing an Src homology 3 (SH3) domain and a tyrosine cluster region including an immunoreceptor tyrosine-based activation motif (ITAM) [11], and found that STAM is associated with Jak3 and Jak2 [12]. Transfection with the SH3 deletion mutant of STAM induced a reduction of DNA synthesis mediated by IL-2 and GM-CSF, and transfection of the wild-type STAM but not the STAM mutants deleted of the SH3 and ITAM, enhanced c-*myc* induction upon stimulation with IL-2 and GM-CSF, demonstrating the involvement of STAM in signaling for DNA synthesis and c-*myc* induction mediated by IL-2 and GM-CSF [12]. A variety of cytokines and growth factors such as IL-2, IL-4, IL-7, IL-3, GM-CSF, platelet-derived growth factor (PDGF) and epidermal cell growth factor (EGF) induced tyrosine phosphorylation of STAM, and STAM was expressed ubiquitously among a variety of tissues tested, suggesting a potential implication of STAM in signaling pathways from a variety of cytokine receptors [11].

We here cloned a cDNA encoding a new member of the STAM family, STAM2, and demonstrated that STAM2 as well as STAM1, the original STAM, is involved in the signaling for DNA synthesis and c-*myc* induction upon stimulation with IL-2 and GM-CSF.

2. Materials and methods

2.1. Cells

Cell lines used were human MOLT-4, ILT-Mat, TF-1, Daudi, HeLa and 293T, and a simian fibroblastoid COS7. MOLTβ is a MOLT-4 subline stably transfected with the human IL-2 receptor β

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Abbreviations: Jak, Janus kinase; SH3, Src homology 3; ITAM, immunoreceptor tyrosine-based activation motif; IL-, interleukin; GM-CSF, granulocyte/macrophage colony stimulating factor; EGF, epidermal cell growth factor; PDGF, platelet-derived growth factor; Ab, antibody; mAb, monoclonal antibody

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chain [13]. HepG2/PDGFR is a HepG2 subline stably transfected with cDNA of PDGFβ receptors [14]. BAF-B03, a mouse IL-3-dependent pro-B cell line, was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 10% WEHI-3 conditioned medium containing IL-3 and 2-mercaptoethanol.

2.2. Plasmid constructs

The wild-type STAM2 and its mutants were inserted into pCXN2 expression vectors at the *Xho*I site [15]. STAM2 mutants include 2DSH3 deleted of the SH3 domain (amino acid position Ala^{209}–Val^{256}), 2DIT deleted of the ITAM region (amino acid position Glu^{349}–Leu^{377}) and 2DY2 deleted of the C-terminal half (amino acid position Lys^{343}–Leu^{525}). Expression plasmids for the wild-type and mutants of STAM1 such as DSH3, DIT and DY2 were described previously [12]. The wild-type and mutants of STAM1 and STAM2 were subcloned into pcDNA3.1/V5-His (Invitrogen) for V5-tagging at their C-termini. Expression plasmids for Jak1 and Tyk2 were pRK5mJak1 and pRK5hTyk2, respectively [16].

2.3. Luciferase assay

Luciferase assays for c-*myc* promoter activities were performed as described previously [12]. In brief, BAF-B03 cells were incubated in the absence of IL-3 for 12 h, and then transiently transfected with expression plasmids for human IL-2 receptor β and γc, or GM-CSF receptor α and βc, together with STAMs, β-galactosidase reporter and c-*myc* promoter-driven luciferase reporter plasmids. The cells were then stimulated with 10 nM human recombinant IL-2 (provided from Ajinomoto Co., Japan) or with 20 ng/ml human recombinant GM-CSF for 6 h, and assayed for luciferase and β-galactosidase activities.

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were carried out as described previously [17]. Briefly, cells were lysed with cell extraction buffer (1% NP-40, 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 20 μg/ml aprotinin). The cell lysates were immunoprecipitated with indicated antibodies (Abs), and the immunoprecipitates were then separated by SDS–PAGE and transferred to PVDF filters (Millipore, Japan). After blocking with phosphate-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20, the filters were probed with indicated Abs and visualized by using the ECL detection system (Amersham, UK).

3. Results

3.1. Cloning of human STAM2

By screening the expression sequence tag database, we found a sequence highly homologous to the human STAM gene from a human fetal brain cDNA library (GenBank accession number M78581). Based on this information, we prepared a cDNA probe, and isolated a human cDNA clone with 3.9 kbp from a cDNA library of human phytohemagglutinin-stimulated peripheral blood leukocytes. The 3.9 kbp cDNA clone contains an open reading frame encoding a protein with 525 amino acids, which corresponds to the full length of STAM, containing an SH3 domain and tyrosine cluster region including an ITAM. Hence, we renamed STAM as STAM1, and termed the newly cloned STAM homologous molecule as STAM2. The GenBank accession number of the human STAM2 is AF042273. Amino acid sequence homologies between STAM1 and STAM2 were 50.1% in the whole, 71.9% in the N-terminal half, 89.4% in the SH3 domain and 41.7% in the C-terminal portion including the ITAM. Chicken EAST and mouse Hbp were recently cloned, and found to be homologous to STAM1 [18,19]. Our homology search of the protein databases showed that overall similarities of EAST and Hbp are 47.0% and 56% with STAM1, respectively, and 69.0% and 86% with STAM2, respectively, suggesting that

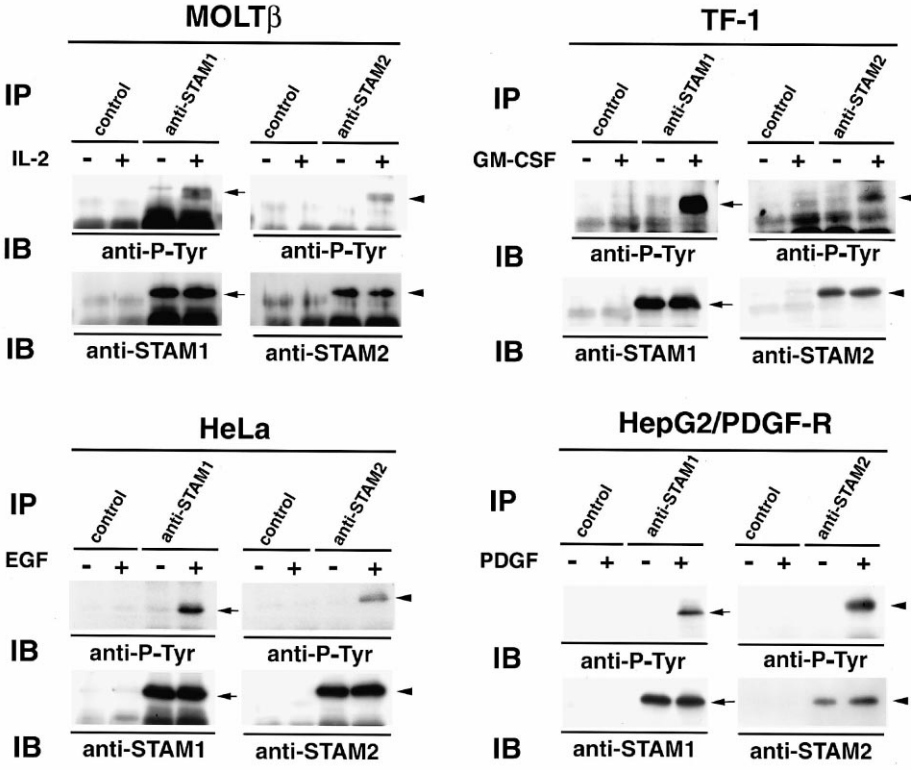

Fig. 1. Cytokine-induced tyrosine phosphorylation of STAMs. MOLTβ, TF-1, HeLa and HepG2/PDGFR cells were stimulated for 10 min with IL-2, GM-CSF, EGF and PDGF, respectively. Their lysates were then immunoprecipitated with anti-STAM2 Ab or preimmune rabbit serum as control, and immunoblotted with anti-phosphotyrosine monoclonal Ab (mAb) or anti-STAM2 Ab.

STAM2 may be the human homolog of chicken EAST and mouse Hbp.

Northern blot and immunoblot analyses revealed that STAM2 is ubiquitously expressed among a variety of human tissues and cell lines with three mRNA sizes of 1.3 kb, 3.9 kb and 6.8 kb, and 68 kDa molecular mass, respectively (data not shown).

3.2. Tyrosine phosphorylation of STAM2

Since STAM1 has been shown to be tyrosine-phosphorylated upon stimulation with a wide variety of cytokines [11], we examined the tyrosine phosphorylation of STAM2 in responses to several cytokines. MOLTβ, TF-1, HeLa and HepG2/PDGFR cells were stimulated with IL-2, GM-CSF, EGF and PDGF, respectively, for 10 min, their lysates were then immunoprecipitated with anti-STAM2 Ab followed by immunoblotting with anti-phosphotyrosine and anti-STAM2 Abs. The tyrosine phosphorylation of STAM2 was detected after treatment with all these cytokines (Fig. 1). Tyrosine phosphorylation of STAM2 was seen within 5 min of IL-2 stimulation, became maximal at 10 min and then gradually decreased (data not shown).

3.3. Association of STAM2 with the Jak family tyrosine kinases

Since STAM1 is associated with Jak3 and Jak2 via its ITAM region [12], we examined whether STAM2 is also associated with the Jaks by carrying out co-immunoprecipitation studies. IL-2-dependent ILT-Mat cells, GM-CSF-dependent TF-1 cells and IFN-α-responsive Daudi cells were

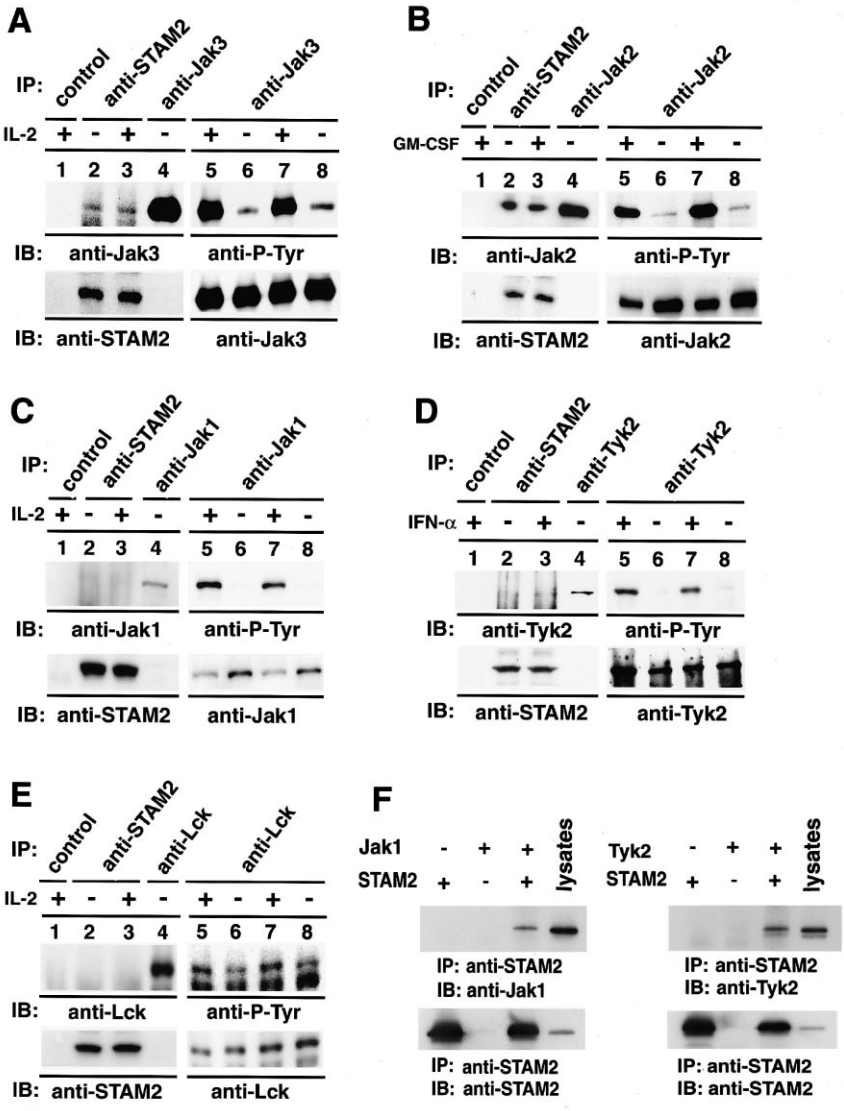

Fig. 2. Co-immunoprecipitation of STAM2 with the Jak family members and Lck. ILT-Mat (A, C, E), TF-1 cells (B) and Daudi cells (D) were incubated for 10 min in the presence (+) or absence (−) of 10 nM IL-2, 20 ng/ml GM-CSF and 0.1 ng/ml IFN-α, respectively. Their lysates were immunoprecipitated with anti-STAM2 Ab (lanes 2 and 3), preimmune rabbit serum as a control (lane 1), anti-Jak3 (A, lanes 4–8), anti-Jak2 (B, lanes 4–8), anti-Jak1 (C, lanes 4–8), anti-Tyk2 (D, lanes 4–8) and anti-Lck Ab (E, lanes 4–8). The immunoprecipitates were separated by SDS–PAGE and blotted with anti-Jak3, anti-Jak2, anti-Jak1, anti-Tyk2, anti-Lck Ab and anti-phosphotyrosine mAb (lanes 5–8) (upper blots), or with anti-STAM2, anti-Jak3, anti-Jak2, anti-Jak1, anti-Tyk2 and anti-Lck Ab (lower blots). (F) 293T cells were transfected with (+) or without (−) 2 μg each of expression plasmids for STAM2, Jak1 and Tyk2 by the calcium-phosphate method, and their lysates were immunoprecipitated with anti-STAM2 Ab followed by immunoblotting with anti-Jak1, anti-Tyk2 (upper blots) and anti-STAM2 Ab (lower blots).

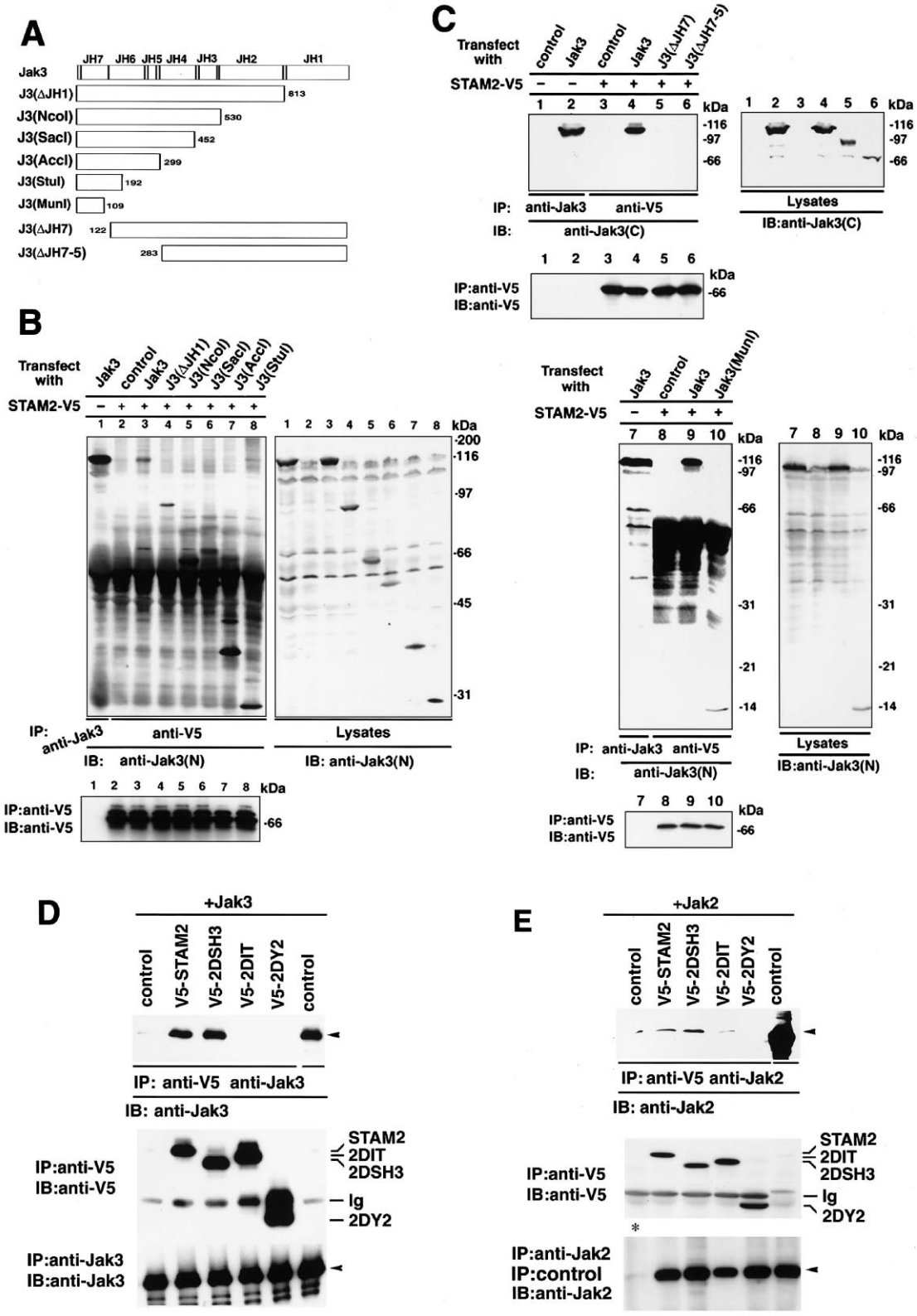

Fig. 3. Determination of the binding sites of Jak3 for STAM2, and of STAM2 for Jak3 and Jak2. (A) A schematic representation of the Jak3 mutants. The numbers indicate positions of the C-terminal or N-terminal amino acid residues of the Jak3 mutants. (B, C) Determination of the binding site of Jak3 for STAM2. COS7 cells were transfected with 10 μg each of the wild-type and mutant cDNAs of Jak3 together with V5-tagged wild-type STAM2. Their lysates were immunoprecipitated with anti-V5 mAb and immunoblotted with anti-Jak3 N-terminus (N; N-15: Santa Cruz) Ab (B, C), anti-Jak3 C-terminus Ab (C; C-21: Santa Cruz) (C) and anti-V5 mAb. (D, E) Determination of the binding site of STAM2 for Jak3 and Jak2. COS7 cells were transiently transfected with 20 μg each of V5-tagged wild-type STAM2 (STAM2-V5), SH3-deleted STAM2 mutant (V5-2DSH3), ITAM-deleted STAM2 mutant (V5-2DIT) and C-terminus half-deleted mutant STAM2 (V5-2DY2) plasmids or empty vector (pcDNA3.1-HisA-V5; Invitrogen) as control, together with 10 μg of Jak3 (D) or Jak2 plasmid (E). The cells were incubated for 48 h, and their lysates were immunoprecipitated with anti-V5 mAb and then immunoblotted with anti-Jak3 or anti-Jak2 and anti-V5 Abs.

stimulated with IL-2, GM-CSF and IFN-α, respectively, and their lysates were precipitated with anti-STAM2 Ab followed by immunoblotting with anti-Jak3, anti-Jak2, anti-Jak1, Tyk2 or anti-Lck Abs. STAM2 was significantly co-immunoprecipitated with Jak3 and Jak2 irrespective of ligand stimulation (Fig. 2A,B), but not with Jak1, Tyk2 or Lck (Fig. 2C–E). Tyrosine phosphorylation of the Jaks and Lck was significantly induced or increased after ligand stimulation (Fig. 2), indicating that the cells were responded to the ligands. Since the expression levels of Jak1 and Tyk2 were much lower than those of Jak3 and Jak2, we performed co-immunoprecipitation studies using 293T cells exogenously introduced with expression plasmids for STAM2, Jak1 and Tyk2. Both Jak1 and Tyk2 were apparently co-immunoprecipitated with STAM2 in their lysates (Fig. 2), indicating that STAM2 is able to bind to Jak1 and Tyk2 irrespective of ligand stimulation in cells expressing sufficient amounts of STAM2, Jak1 and Tyk2.

The binding site of Jak3 to STAM1 and STAM2 was determined by using Jak3 mutants [20] (Fig. 3A). The wild-type Jak3, J3(StuI) mutant and J3(MunI) mutant were co-immunoprecipitated with STAM2, whereas J3(ΔJH7) mutant was not (Fig. 3B,C). Similar results were obtained in co-immunoprecipitation between the Jak3 mutants and STAM1 (data not shown), suggesting that STAM1 and STAM2 could interact with the same binding site located at the JH7 region of Jak3. We also revealed that STAM2 like STAM1 binds to Jak2 and Jak3 through its ITAM region (Fig. 3D).

3.4. Involvement of STAM2 in c-myc induction mediated by IL-2 and GM-CSF

Next, we examined the effect of STAM2 on the cytokine-mediated signaling for the induction of proto-oncogenes such as c-*myc* and c-*fos* by using luciferase reporter gene assays with BAF-B03 cells. BAF-B03 cells transfected with the wild-type STAM2 showed a 6-fold increase of c-*myc* luciferase activity upon IL-2 stimulation compared to a barely 2.7-fold increase with the control vector, whereas the transfectant cells with 2DSH3, 2DIT and 2DY2 mutants of STAM2 showed similar increases to the control vector (Fig. 4A). The enhancement of IL-2-mediated c-*myc* induction was dose-dependent on the wild-type STAM2 plasmid (Fig. 4B). Similar results were obtained with the GM-CSF stimulation system (data not shown). These results indicate that wild-type STAM2 potentiates c-*myc* induction mediated by IL-2 and GM-CSF, suggesting the involvement of STAM2 in signal transduction for c-*myc* induction mediated by these cytokines. Both the SH3 domain and ITAM of STAM2 seem essential for such signal transduction.

The wild-type STAM2 conferred marginal effects on c-*fos* promoter-driven luciferase activities mediated by IL-2 and GM-CSF (data not shown).

3.5. The SH3 deletion mutant of STAM2 confers a dominant-negative effect on DNA synthesis mediated by IL-2 and GM-CSF

To investigate the functional significance of STAM2 in cell growth signaling mediated by cytokines, BAF-B03 cells were transiently transfected with the wild-type and mutants of STAM2 together with the human IL-2 receptor β and γc chains, and assayed for DNA synthesis after 24 h stimulation with IL-2. The BAF-B03 cells transfected with the control vector showed a significant increase of [^{3}H]thymidine incorporation, comparable with the transfectants containing the wild-type, 2DIT and 2DY2, whereas transfection with 2DSH3 induced 47.0% suppressions of [^{3}H]thymidine uptake upon stimulation with IL-2 (Fig. 5A). The suppression of IL-2-induced [^{3}H]thymidine uptake was dose-dependent on 2DSH3 plasmids (Fig. 5B). Similar results were obtained with the GM-CSF stimulation system; the 2DSH3 induced a significant suppression of GM-CSF-induced [^{3}H]thymidine uptake (data not shown). The degree of suppression of IL-2-induced [^{3}H]thymidine uptake by 30 μg of the SH3 deletion mutant of STAM1 (DSH3) alone was the same as that induced by 15 μg each of DSH3 and 2DSH3 together (Fig. 5C), indicating that there is an additive effect but not synergistic effect between the SH3-deleted mutants of STAM1 and STAM2 on IL-2-induced [^{3}H]thymidine uptake. These results suggest that both STAM1 and STAM2 contribute to the cell growth signaling mediated by IL-2 and GM-CSF through their similar mechanisms.

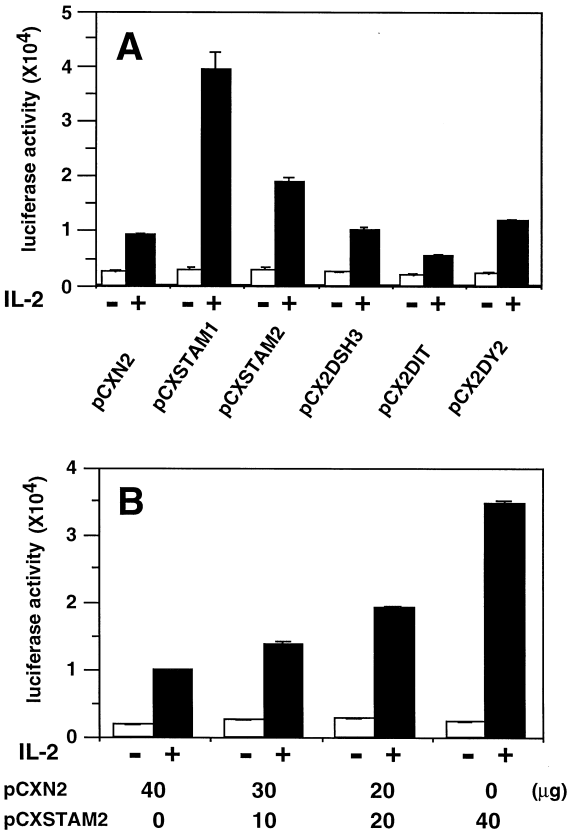

Fig. 4. Effects of STAM1 and STAM2 on induction of c-*myc* in response to IL-2. BAF-B03 cells deprived of IL-3 for 12 h were transfected with expression plasmids for the wild-type STAM1 (pCXSTAM1), the wild-type STAM2 (pCXSTAM2), 2DSH3 mutant (pCX2DSH3), 2DIT mutant (pCX2DIT), 2DY2 mutant (pCX2DY2) and empty vector (pCXN2) (A), or with combinations of pCXSTAM2 and pCXN2 (B), together with human IL-2 receptor β and γc plasmids, in addition to luciferase reporter plasmid containing c-*myc* promoter (pHXLuc) and plasmid for β-galactosidase (pENL) by electroporation. Subsequently, the cells were stimulated for 6 h with (+) or without (−) human recombinant IL-2 (A and B). The luciferase activities were displayed after correction of protein concentration and β-galactosidase activity of cell lysates. The values shown are means ± S.E.M. of triplicate determinants. Results represent one of three comparable experiments.

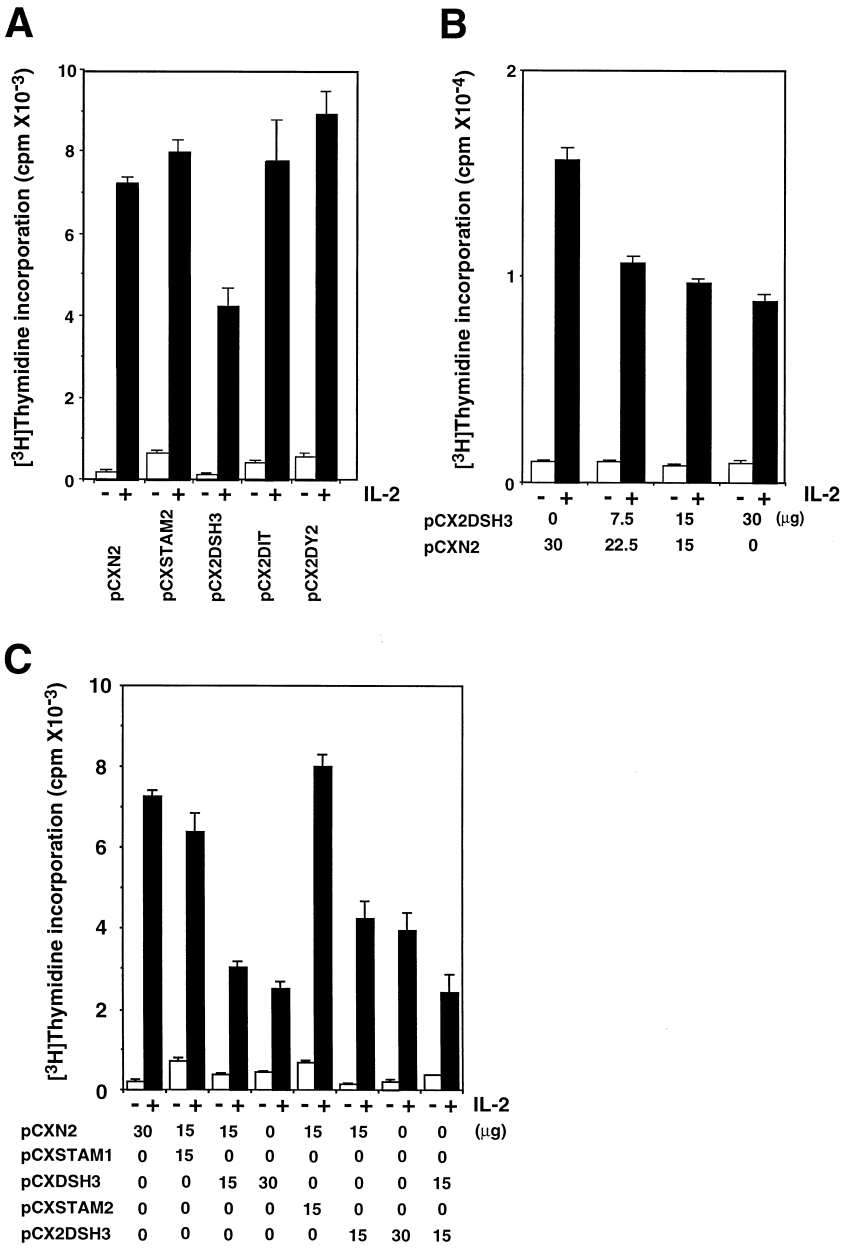

Fig. 5. The SH3 deletion mutant of STAM2 induces suppression of [^{3}H]thymidine incorporation mediated by IL-2. BAF-B03 cells maintained in the medium containing IL-3 were transfected with 15 μg each of pCXSTAM2, pCX2DSH3, pCX2DIT, pCX2DY2 and pCXN2, together with human IL-2 receptor β and γc plasmids. They were cultured for 24 h with (+) or without (−) IL-2, and assayed for [^{3}H]thymidine incorporation for the last 4 h cultivation. (B) pCX2DSH3 dose-dependent suppression of [^{3}H]thymidine incorporation mediated by IL-2. [^{3}H]thymidine incorporation assays were carried out in a similar manner to (A) with the indicated doses of pCX2DSH3. (C) An additive effect between the SH3 deletion mutants of STAM1 and STAM2 on suppression of [^{3}H]thymidine incorporation mediated by IL-2. BAF-B03 cells were transfected with the indicated plasmids together with 2 μg of human IL-2 receptor β and γc plasmids by electroporation, and were assayed for [^{3}H]thymidine incorporation in a similar manner to (A). The values shown are means ± S.E.M. of triplicate cultures. Results represent one of three comparable experiments.

4. Discussion

The present study documents the presence of transcripts encoding a new STAM1 homologous molecule, named STAM2. STAM2 was revealed to have similar characteristics to STAM1 in its ubiquitous expression among a variety of human tissues and cell lines. In addition, STAM2 contains an SH3 domain and tyrosine cluster region including an ITAM in its structure, is associated with Jak2 and Jak3, and tyrosine-phosphorylated upon stimulation with cytokines and growth factors, such as IL-2, GM-CSF, EGF and PDGF in a similar manner to STAM1 [11]. These observations suggest that a functional similarity between STAM1 and STAM2 exists.

The IL-2 receptor γc and GM-CSF receptor βc chains are associated with Jak3 and Jak2, respectively [3,4]. These kinases are indispensable for signal transduction for cell growth and in the induction of proto-oncogenes such as c-*myc* and c-*fos* [3,21]. The ITAMs of STAM1 and STAM2 were shown to both bind to the JH7 region of Jak3, suggesting that STAM1

and STAM2 bind to the same site on Jak3. This fact was further reflected in the observation that STAM1 and STAM2 are competitive in their binding to Jak3 (data not shown). Although the JH7 region of Jak3 is known to include the binding site for the γc chain, the γc binding site of Jak3 may be distinct from the binding site for the STAMs because the J3(MunI) mutant of Jak3 binds to the STAMs but not the γc chain [20]. Co-immunoprecipitation studies did not detect an association of native STAM2 with native Jak1 and Tyk2, however overexpression of Jak1 and Tyk2 along with STAM2 in 293T cells induced a significant association of STAM2 with both Jak1 and Tyk2, suggesting that STAM2 is able to bind to all the Jak family members. Since the amino acid sequence alignments of the JH7 regions of the Jak family proteins indicate that Jak3 is more homologous to Jak2 than Jak1 or Tyk2 [22,23], we suspect that the binding affinities of Jak3 and Jak2 to the STAMs may be stronger than those of Jak1 and Tyk2.

We here provided evidence for involvement of STAM2 in signal transduction mediated by IL-2 and GM-CSF. Overexpression of the wild-type STAM2 induced an enhancement of c-*myc* induction mediated by IL-2 and GM-CSF, whereas STAM2 mutants deleted of the SH3 domain and ITAM lost this ability. Similar effects on the cytokine-mediated c-*myc* induction were seen with STAM1 [12]. On the other hand, not only the SH3-deleted STAM1 mutant, but also the SH3-deleted STAM2 mutant induced significant reductions of [^{3}H]thymidine incorporation upon stimulation with IL-2 and GM-CSF. Furthermore, simultaneous transfection of the SH3-deleted mutants of STAM1 and STAM2 induced an additive effect on the reduction of [^{3}H]thymidine incorporation, suggesting that STAM1 and STAM2 contribute to the cytokine-mediated signal transduction at a similar position downstream of Jak2 and Jak3. Taken together, we suspect that STAM1 and STAM2 have compensatory functions with each other in the signal transduction for cytokine-mediated DNA synthesis and c-*myc* induction.

We previously cloned a cDNA encoding a novel molecule named AMSH, which is associated with the SH3 domain of STAM1 and possibly involved in signaling for c-*myc* induction and DNA synthesis mediated by IL-2 and GM-CSF [24]. We have confirmed that STAM2 is co-immunoprecipitated with AMSH after DSP chemical cross-linking (data not shown), suggesting the complexes of STAM1 and STAM2 with AMSH play important roles in the signal transduction mediated by these cytokines.

The amino acid sequence comparison revealed that STAM2 is a human homologue of chicken EAST and mouse Hbp. EAST was shown to bind to the EGF receptor upon EGF stimulation and to co-localize with clathrin through association with Eps15 [18]. Hbp was also found to be involved in the intracellular degradation of PDGF and its receptor [19]. These observations together with the present study suggest that the STAM family proteins are involved in regulation of not only intracellular signal transduction mediated by cytokines and growth factors but also receptor-mediated endocytosis.

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