SOCS-1 can suppress CD3ζ- and Syk-mediated NF-AT activation in a non-lymphoid cell line

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Abstract To elucidate T cell antigen receptor (TCR) signaling leading to activation nuclear factor of activated T cells (NF-AT), we reconstituted TCR signaling to activate NF-AT in a non-lymphoid cell line, 293T. We demonstrated that co-expression of CD8/ζ and Syk were necessary for NF-AT activation in 293T. This NF-AT response was completely inhibited by the addition of cyclosporin A or FK506, but markedly enhanced by the additional expression of Tec protein tyrosine kinase. We also show that the cytokine signaling suppressor, suppressor of cytokine signaling 1, potently inhibited this response by interacting with Syk and immunoreceptor tyrosine-based activation motifs in CD8/ζ. These results imply that this novel system may provide a useful tool to delineate or identify the regulatory molecules for CD3ζ/Syk-mediated NF-AT activation.

Key words: Signal transduction; T cell antigen receptor; Syk; Nuclear factor of activated T cell; Suppressor of cytokine signaling 1; Immunoreceptor tyrosine-based activation motif

1. Introduction

Multiple protein tyrosine kinases (PTKs) are required to convey signals from T cell antigen receptors (TCRs). Previous biochemical studies have shown that TCR signaling events involve the activation of two families of PTKs: Src PTKs (Lck and Fyn), and Syk/ZAP70 PTKs. The Src PTKs phosphorylate the two conserved tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM), which is present in each of the TCR signaling units. The phosphorylated ITAMs mediate the interaction of Syk/ZAP70 PTKs with the receptor, amplifying the TCR-triggered signal by phosphorylating additional intracellular substrates including phospholipase C-γ (PLC-γ), Cbl, Vav, LAT, and SLP76 [1,2]. Tyrosine phosphorylation of PLC-γ induces its enzymatic activity, leading to the activation of the inositol phospholipid pathway, followed by activation of protein kinase C and increase in intracellular calcium [2]. Calcineurin, a Ca^{2+}-activated phosphatase, is then activated and induces nuclear factor of activated T cell (NF-AT) activation, which finally activates transcription of the interleukin-2 (IL-2) gene [3].

Recent studies showed that Syk, but not ZAP70, alone stimulated tyrosine phosphorylation of both a chimeric ζ molecule (Tζζ) in transiently transfected COS-1 cells and a ζ-derived ITAM peptide in vitro [4]. The potential of Syk to promote ITAM phosphorylation may allow TCR-mediated signals to proceed in the absence of optimal activation of Src family kinases. This may be important for thymocytes bearing low levels of antigen receptors, for naive T cells, and for T cells lacking the appropriate co-receptor molecules (such as γδ T cells) in which Syk expression has been documented [5,6].

More recently, members of the SOCS (suppressor of cytokine signaling) family have been proposed as negative regulators of cytokine signaling [7–9]. SOCS-1 inhibits IL-6-induced phosphorylation of gp130, JAK2, and STAT3, and the subsequent differentiation of M1 cells into macrophages. In mice, SOCS-1 is constitutively expressed in the thymus, and the thymus in SOCS-1-deficient mice was markedly reduced in size due to the unregulated activation of thymocytes [10,11], suggesting that SOCS-1 is involved in the activation and development of thymocytes. SOCS-1 has also been shown to bind Tec and suppress Tec's kinase activity [12].

Here, we provide a novel system to explore the TCR signaling pathway using a non-lymphoid cell line, 293T, which is commonly used for efficient expression of various vectors. We expressed CD8/ζ chimeric molecules and various PTKs in 293T, and tyrosine phosphorylation of various substrates as well as NF-AT activity was determined. In this system, Syk, but not ZAP70, stimulated the NF-AT-mediated transcriptional response in the presence of CD8/ζ chimeric molecules. We show that this NF-AT activation was mediated by PLC-γl and completely inhibited by inhibitors of the calcineurin pathway, cyclosporin A (CsA) and FK506. We also show that the additional expression of Tec PTK markedly enhanced the NF-AT activation induced by CD8/ζ and Syk. Finally we show that one member of the SOCS family, SOCS-1, inhibited this NF-AT activation by directly binding to both Syk and ITAMs of CD3ζ.

2. Materials and methods

2.1. Cell lines and reagents

A human embryonic kidney cell line, 293T, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. pTfneo-CD8/ζ, pLGP3-mNFAT1-C, pNFAT3-Luc, porcine Syk in pEFBOS, human ZAP70 in pME18S, human Lck in pCLS, Tec in pSRα were kindly provided by Dr. A. Weiss (University of

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Abbreviations: PTK, protein tyrosine kinase; NF-AT, nuclear factor of activated T cell; PLC-γ, phospholipase C-γ; ITAM, immunoreceptor tyrosine-based activation motif; SOCS, suppressor of cytokine signaling; CsA, cyclosporin A

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California, San Francisco, CA, USA), Dr. A. Rao (Harvard Medical School, Boston, MA, USA), Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan), Dr. T. Saito (Chiba University, Chiba, Japan), Dr. M. Okada (Osaka University, Osaka, Japan) and Dr. H. Mano (Jichi Medical School, Tochigi, Japan), respectively. FK506 and CsA were kind gifts from Fujisawa (Osaka, Japan) and Sandoz (Tokyo, Japan), respectively.

2.2. Antibodies

Monoclonal anti-human CD8 (3B5) was purchased from Caltag Laboratories, Burlingame, CA, USA. Anti-ZAP70 was purchased from Transduction Laboratories, Lexington, KY, USA. Anti-human CD3ζ, anti-PLC-γ1, anti-Vav, and anti-Cbl were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine monoclonal antibody (mAb) 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-Myc mAb 9E10 was purchased from Boehringer Mannheim, Tokyo, Japan. Anti-porcine Syk was generously supplied by Dr. Y. Minami (Kobe University, Kobe, Japan). Anti-Lck antibody was kindly provided by Dr. M. Okada (Osaka University, Osaka, Japan).

2.3. Transfections and luciferase assay

293T cells were plated in 6-cm dishes or in 10-cm dishes and transfected by the standard calcium precipitation protocol. The cells were harvested 48 h after transfection and lysed in 200 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.

2.4. Immunoprecipitation and immunoblotting

The transfected 293T cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin).

The immunoprecipitates from cell lysates were resolved on 5–20% SDS–PAGE and transferred to Immobilon (Millipore, Bedford, MA, USA). The Immobilon filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced

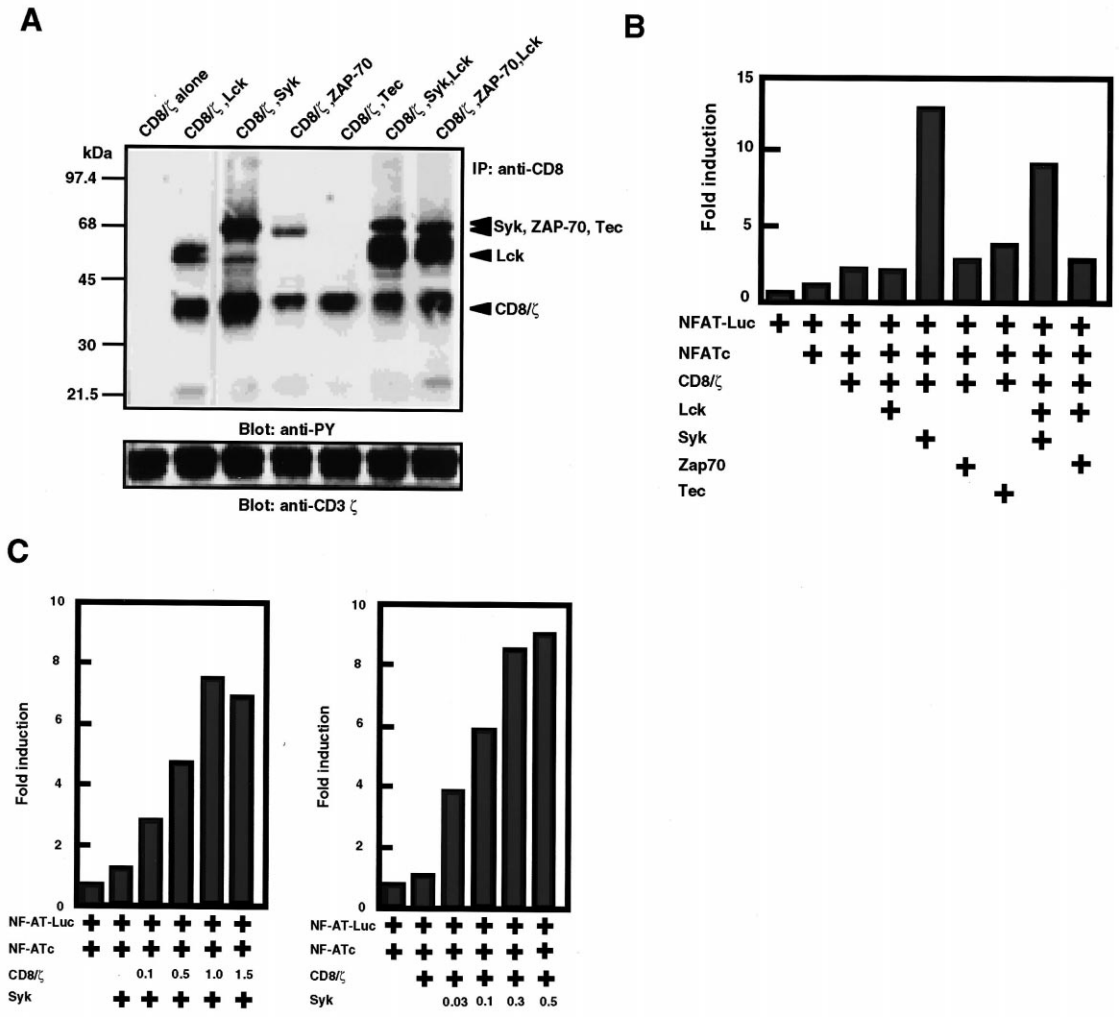

Fig. 1. Tyrosine phosphorylation of CD8/ζ and NF-AT activation in 293T cells. A: 293T cells in 10-cm dishes were transfected with each PTK expression construct (5 μg) and CD8/ζ (10 μg) by the calcium precipitation method. After 48 h, the cells were lysed and CD8/ζ was immunoprecipitated with anti-CD8 mAb. Phosphotyrosine-containing proteins were detected by immunoblotting with the anti-phosphotyrosine antibody 4G10 (upper panel). The blot was stripped and reprobed with anti-CD3ζ (lower panel). Expression of the appropriate proteins was confirmed by immunoblotting of total cell lysates with antibody against each PTK (data not shown). Molecular weight markers on the left are in kilodaltons. The results shown here are representative of at least three independent experiments. B: 293T cells in 6-cm dishes were transfected with each PTK expression construct (0.5 μg), CD8/ζ (1.5 μg), NF-ATc (1 μg), NF-AT-Luc reporter (1 μg) and 0.5 μg of pSRα-LacZ (β-gal). 48 h after transfection, cells were harvested and luciferase activity was determined. C: 293T cells in 6-cm dishes were transfected with increasing amounts of CD8/ζ or Syk (μg DNA) together with NF-ATc, NF-AT-Luc and β-gal. 48 h after transfection, cells were harvested and luciferase activity was determined. The results shown here are representative of at least three independent experiments.

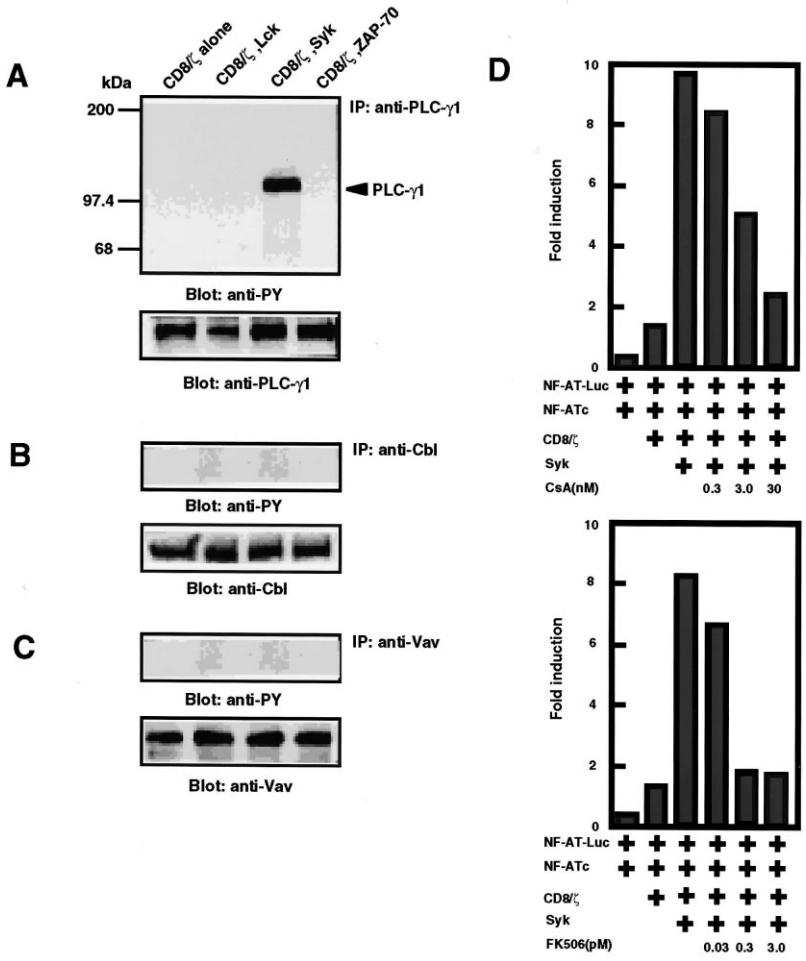

Fig. 2. Activation of PLC-γ1 and calcineurin pathway by Syk and CD8/ζ. A–C: 293T cells in 10-cm dishes were transfected with Syk (5 μg) and CD8/ζ (10 μg). After 48 h, they were lysed and immunoprecipitated with anti-PLC-γ1 (A), anti-Cbl (B), and anti-Vav (C). The immunoprecipitate with each antibody was probed with an anti-phosphotyrosine antibody as indicated (anti-PY, upper panel). The blot was stripped and reprobed with the respective antibody (lower panel). Molecular weight markers on the left are in kilodaltons. (D) NF-AT activation mediated by CD8/ζ and Syk in 293T cells can be blocked by CsA or FK506. 293T cells transfected with CD8/ζ and Syk were cultured in the presence or absence of the indicated concentrations of CsA or FK506. 48 h after transfection, cells were harvested and luciferase activity was determined. The results shown here are representative of at least three independent experiments.

chemiluminescence detection system (Amersham Pharmacia Biotech, Tokyo, Japan).

2.5. In vitro binding assay

Peptide and phosphopeptides were kindly provided by Dr. J.N. Ihle (St. Jude Children's Research Hospital, Memphis, TN, USA) and coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). Approximately 15 μmol of the peptide was coupled to 1 g dried Sepharose resin. Myc-tagged SOCS-1 and CIS in pcDNA3 were transiently expressed in 293T cells grown in 10-cm dishes. Cells were lysed in the above lysis buffer containing 10% glycerol. Cell extracts were incubated with 20 μl (50% v/v) of peptide-conjugated Sepharose, then washed three times with lysis buffer, and analyzed by immunoblotting with anti-Myc (9E10).

3. Results and discussion

3.1. Reconstitution of TCR signaling pathway in 293 T cells

It was shown that CD8/ζ chimeric protein consisting of the extracellular and transmembrane domains of CD8 and the cytoplasmic domain of the CD3ζ chain is capable of transducing signals in T cell lines in the absence of the CD3γ, δ, and ε chains [13]. To reconstitute the TCR signaling pathway in 239T cells, we first transfected a construct of CD8/ζ chimeric protein together with a variety of constructs of PTKs including Lck, Syk, ZAP70, and Tec in 293T cells, and tyrosine phosphorylation of CD8/ζ was examined after precipitation with anti-CD8 mAb. As shown in Fig. 1A, CD8/ζ was tyrosine-phosphorylated by each individual PTK examined. In addition, tyrosine-phosphorylated PTKs except Tec were co-precipitated with CD8/ζ. Although Lck is a prerequisite for activation of ZAP70 in T cells [2], co-expression of Lck with Syk or ZAP70 did not enhance phosphorylation of CD8/ζ.

To investigate whether the phosphorylation of CD8/ζ induces NF-AT activation in 293T cells, we then transfected both NF-ATc and NF-AT luciferase constructs [14] together with CD8/ζ and each individual PTK construct into 293T cells, and the NF-AT luciferase activity was assayed. As shown in Fig. 1B, the expression of Syk and CD8/ζ induced a marked acti-

vation of NF-AT, whereas expression of other PTKs and CD8/ζ showed no or very little effect on the activation. Simultaneous expression of CD8/ζ and Syk was a prerequisite for the NF-AT activation in 293T cells (Fig. 1C). This finding is in accordance with those of previous studies showing that Syk enhances TCR-mediated NF-AT activation and IL-2 production [15–17]. It is possible that the combination of Lck with ZAP70 could not transduce the signals due to the lack of T cell-specific adapter molecules, such as SLP-76 [2].

3.2. Involvement of the endogenous PLC-γl and calcineurin pathway in the reconstituted TCR signaling cascade in 293T

When CD8/ζ and Syk were co-expressed in 293T cells, various tyrosine-phosphorylated substrates other than CD8/ζ and Syk were observed (Fig. 1A), which implies that Syk might act by directly tyrosine-phosphorylating downstream substrates such as PLC-γl, Cbl or Vav [2]. To verify this possibility, CD8/ζ and Syk constructs were co-transfected into 293T cells, and tyrosine phosphorylation of the substrates was examined after immunoprecipitations. As shown in Fig. 2A, a strong phosphorylation of the endogenous PLC-γl was induced by CD8/ζ and Syk in 293T cells. Although a previous paper showed that Syk also phosphorylated the cytosolic Cbl in transiently transfected COS-1 cells [4], we could not detect tyrosine phosphorylation of either Cbl or Vav in the transfected 293T cells (Fig. 2B,C). These results suggest that endogenous PLC-γl plays a pivotal role in the NF-AT activation induced by CD8/ζ and Syk in 293T cells.

We then examined the effect of the immunosuppressive drugs CsA and FK506, which are known as potent inhibitors of TCR-mediated IL-2 gene expression, on the induction of NF-AT activation by CD8/ζ and Syk in 293T cells. As shown in Fig. 2D, both drugs inhibited the CD8/ζ- and Syk-induced NF-AT activation in a dose-dependent fashion, suggesting that this transcriptional response of NF-AT in 293T cells is mediated by the calcineurin pathway [3].

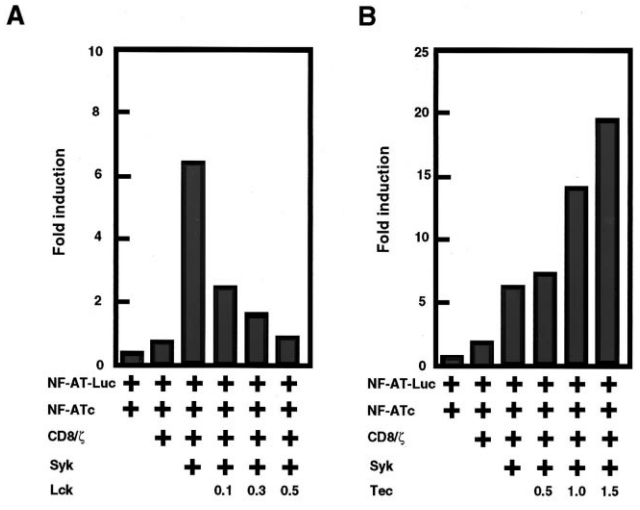

Fig. 3. Effects of other PTKs on CD8/ζ- and Syk-mediated NF-AT activation. 293T cells in 6-cm dishes were transfected with increasing amounts of Lck (A) or Tec (B) and CD8/ζ, Syk, NF-ATc, NF-AT-Luc and β-gal. 48 h after transfection, cells were harvested and luciferase activity was determined. The results shown here are representative of at least three independent experiments.

3.3. Effects of additional PTKs on CD8/ζ- and Syk-mediated NF-AT activation

In order to examine the effect of other PTKs on CD8/ζ- and Syk-mediated NF-AT activation, Lck or Tec was co-expressed together with CD8/ζ and Syk in 293T cells, and NF-AT activity was assessed. As shown in Fig. 3A, an additional expression of Lck resulted in the decrease in NF-AT activation in a dose-dependent manner. This inhibitory effect may be due to the activation of endogenous protein tyrosine phosphatases, like SHP-2 [18]. In fact, overexpression of SHP-2 partially inhibited NF-AT activation induced by CD8/ζ and Syk (data not shown). In contrast, an additional expression of Tec markedly augmented CD8/ζ- and Syk-mediated NF-AT activation (Fig. 3B). These results support previous data demonstrating that Btk/Tec and Syk family kinases exert distinct effects on calcium signaling [19,20]. These results also suggest that this novel system is useful to identify regulatory molecules which are involved in NF-AT activation triggered by CD3ζ and Syk.

3.4. Inhibition of CD8/ζ- and Syk-mediated NF-AT activation by SOCS-1

Previous studies suggested that SOCS family proteins, negative regulatory molecules for cytokine signals, may play some roles in T cell development [8,9]. To investigate the effect of the SOCS family proteins on Syk-dependent NF-AT activation in 293T cells, we co-expressed SOCS-1, CIS, or SOCS-3 together with CD8/ζ and Syk, and the luciferase activity in 293T cells was determined. Expression of SOCS-1 inhibited the NF-AT activation in a dose-dependent fashion, while CIS expression had little effect on the activation (Fig. 4A). SOCS-3 had a moderate inhibitory effect on the activation. We further examined their effect on tyrosine phosphorylation of CD8/ζ. Both SOCS-1 and SOCS-3 reduced tyrosine phosphorylation of CD8/ζ, while CIS showed little effect on the phosphorylation (Fig. 4B). Furthermore, when SOCS-1 or CIS was expressed together with CD8/ζ and Syk, SOCS-1, but not CIS, was co-precipitated with Syk as well as CD8/ζ (Fig. 4C). In the absence of Syk, SOCS-1 was not co-precipitated with CD8/ζ (data not shown), suggesting that SOCS-1 directly bound to tyrosine-phosphorylated CD8/ζ via their ITAMs.

To determine whether SOCS-1 directly interacts with the ITAMs of the ζ chain, SOCS-1 or CIS expressed in 293T cells was incubated with ITAM peptide-conjugated Sepharose beads, and the ability to bind to these peptides was examined. As shown in Fig. 4D, SOCS-1, but not CIS, showed binding ability to the phosphorylated ITAMs. It bound to either ITAM peptides singly phosphorylated at each tyrosine residue (P1 or P2) or ones doubly phosphorylated (P1, P2), but not to the unphosphorylated peptides. These results show that SOCS-1 acts as a negative regulator for the CD8/ζ- and Syk-mediated NF-AT activation by directly binding to the ITAM(s) of the ζ chain.

3.5. Concluding remarks

The present report describes a novel system to explore ITAM-mediated NF-AT activation. This novel system may be useful for exploring the regulatory molecule(s) downstream of CD3ζ or Syk. Using this system, we demonstrated that SOCS-1 inhibited this CD8/ζ- and Syk-induced NF-AT activation by directly binding to Syk and ITAM present at the ζ chain. Although the role of the SOCS family in vivo has not

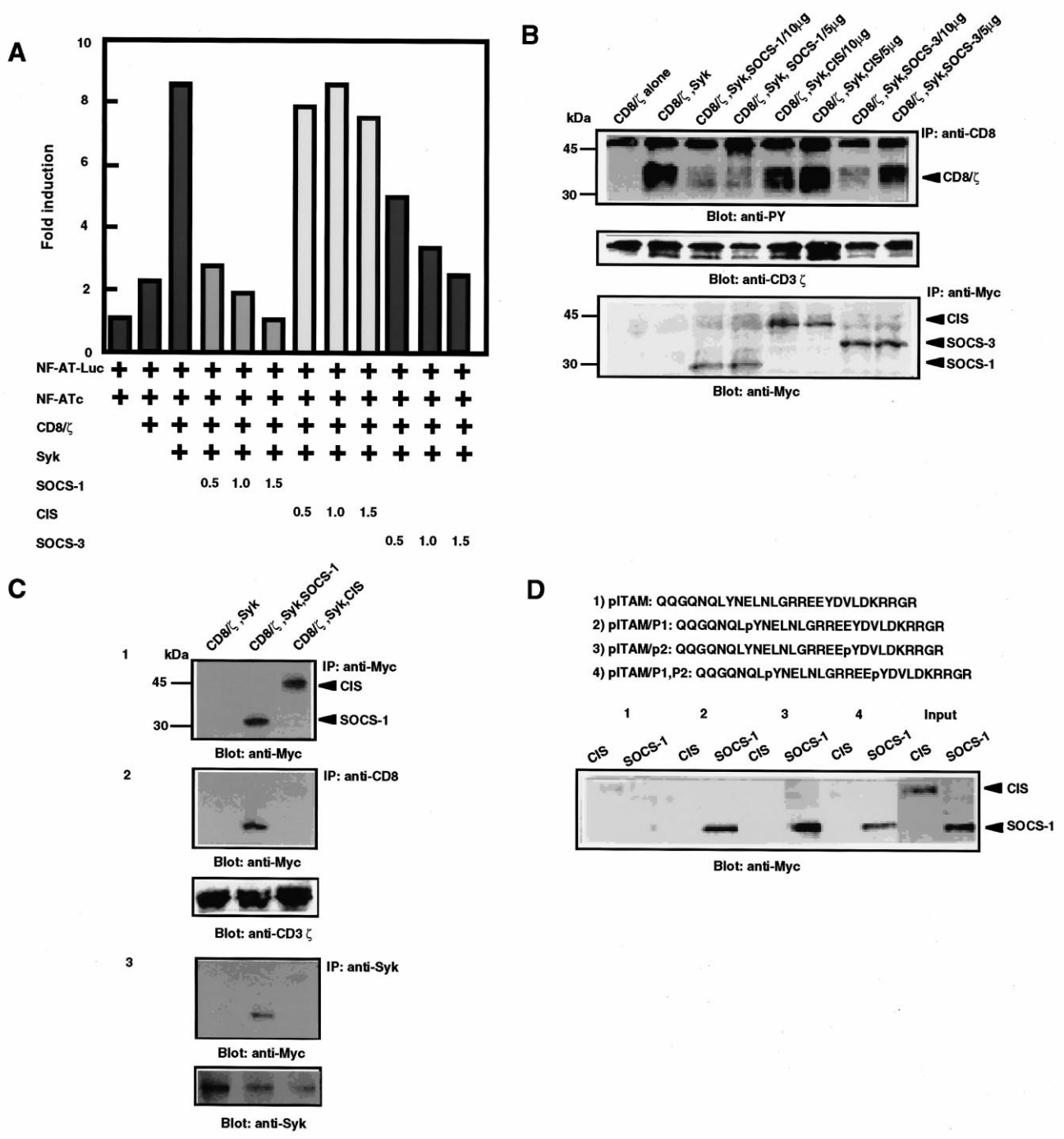

Fig. 4. SOCS-1 inhibits Syk-mediated NF-AT activation and binds to the phosphorylated ITAMs. A: 293T cells in 6-cm dishes were transfected with increasing amounts of Myc-tagged SOCS-1, CIS or SOCS-3 together with CD8/ζ, Syk, NF-ATc, NF-AT-Luc, and β-gal. 48 h after transfection, cells were harvested and luciferase activity was determined. The results shown here are representative of at least three independent experiments. B: 293T cells in 10-cm dishes were transfected with increasing amounts of Myc-tagged SOCS-1, CIS or SOCS-3 together with CD8/ζ (10 μg) and Syk (5 μg) by the calcium precipitation method. 48 h after transfection, cells were lysed and CD8/ζ was immunoprecipitated with anti-CD8 mAb. Phosphotyrosine-containing proteins were detected by immunoblotting with the anti-phosphotyrosine antibody 4G10 (upper panel). The blot was stripped and reprobed with anti-CD3ζ (middle panel). Expression of Myc-tagged proteins was detected by immunoblotting with anti-Myc (lower panel). Molecular weight markers on the left are in kilodaltons. C: 293T cells were transfected with Myc-tagged SOCS-1 (10 μg) or CIS (10 μg) together with CD8/ζ (10 μg) and Syk (5 μg) by the calcium precipitation method. 48 h after transfection, cells were lysed and immunoprecipitated with anti-Myc, anti-CD8, and anti-Syk. Myc-tagged proteins were detected by immunoblotting with anti-Myc (upper panel). The blot was stripped and reprobed with each antibody (lower panel). Molecular weight markers on the left are in kilodaltons. D: The sequences of phosphorylated and unphosphorylated ITAMs used are shown above. The beads conjugated with the indicated peptides were incubated with 293T cell lysate expressing Myc-tagged SOCS-1 and Myc-tagged CIS. After washing, beads were subjected to SDS–PAGE and blotted with anti-Myc. The input Myc-tagged proteins are shown on the right.

yet been clearly determined, our results indicate that SOCS-1 may be involved in the TCR-mediated NF-AT signaling pathway.

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