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Ligation of the T Cell Receptor Complex Results in Phosphorylation of Smad2 in T Lymphocytes

Mizuko Mamura,* Atsuhito Nakao,* Daisuke Goto,*,† Mitsuyasu Kato,† Yasushi Saito,* and Itsuo Iwamoto*,[1]

**Department of Internal Medicine II, Chiba University School of Medicine, Chiba, Japan; and †Department of Biochemistry, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan*

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TGF-β modulates immune responses by regulating T cell function. The Smad family of proteins has been recently shown to transduce signals for the TGF-β superfamily and Smad2 mediates TGF-β signaling. Here, we showed that TGF-β phosphorylated Smad2 and induced interaction between Smad2 and Smad4 in primary T cells and the Jurkat T cell line. Interestingly, ligation of the T cell receptor (TCR)/CD3 complex with anti-CD3 mAb also phosphorylated Smad2, but failed to induce interaction between Smad2 and Smad4 in the Jurkat T cell line. Phosphorylation of Smad2 via the TCR/CD3 complex was not abrogated by treatment with neutralizing antibody against TGF-β. Furthermore, PD98059, a MEK inhibitor, suppressed Smad2 phosphorylation by stimulation with anti-CD3 mAb in Jurkat T cell line. These findings indicated that not only TGF-β but also stimulation via the TCR/CD3 complex phosphorylated Smad2 through mitogen-activated protein (MAP) kinase cascades, suggesting that Smad2 may function in both TGF-β- and TCR/CD3 complex-mediated signaling pathways in T cells.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine, which has been shown to modulate immune responses (1–3). TGF-β regulates growth, differentiation, and function of macrophages, T cells, B cells, and NK cells (1–3). Importantly, TGF-β null mice developed extensive inflammation in various organs and died shortly after birth, suggesting that TGF-β plays a critical role in suppression of immune responses *in vivo* (4). Although TGF-β may act on multiple targets affecting immune system, downregulation of T cell function by inhibiting T cell proliferation is thought to be an important mean by which TGF-β mediates immunosuppression *in vivo* (1–3, 5).

Recent identification of the Smad family of proteins has advanced our understanding how TGF-β transduces signals from receptor serine-threonine kinases of TGF-β superfamily to nucleus (6–8). Activated TGF-β receptors phosphorylate pathway-restricted Smads, Smad2 and Smad3, and then they oligomerize with the common mediator, Smad4, and translocate to the nucleus, where they regulate transcriptional responses. Smad1, Smad5, and Smad8 are BMP pathway-restricted Smads and Smad6 and Smad7 are inhibitory Smads.

Although extensive studies have been done in the past few years on the role of Smads in TGF-β signal transduction, it is not yet determined whether Smads are involved in TGF-β signaling in T cells. Here, we showed that Smad2 was activated not only by stimulation with TGF-β, but by stimulation via the T cell receptor (TCR)/CD3 complex in T cells. Our findings suggest that Smad2 may function both in TGF-β- and TCR/CD3-mediated signal transduction in T cells.

MATERIALS AND METHODS

Purification of T cells. Human T cells and mouse CD4 or CD8 positive T cells were purified respectively from heparinized venous blood of healthy individuals and from spleen harvested from BALB/c mouse (Charles River) by Ficoll-Hypaque gradient centrifugation of lymphocytes and a magnetic cell sorting using MACS anti-CD3, anti-CD4, and anti-CD8 microbeads (Miltenyi Biotec GmbH) following manufacturer's recommendation. The purity of human CD3 T cells, CD4 and CD8 mouse T cells was confirmed by FACScalibar (Becton and Dickinson) and was consistently >99%.

Stimulation of T cells. Purified human and mouse T cells or Jurkat human T cell line (9) (1×10^6 cells/ml) were cultured in RPMI 1640 with 10% heat inactivated FCS (GIBCO) in a humidified 5% CO2 at 37°C. Upon experiments, cells were stimulated with recombinant human TGF-β1 (1–10 ng/ml) (R&D), phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) (Sigma) plus ionomycin (1 μM) (Sigma), or anti-CD3 monoclonal antibody (mAb) (OKT3; 100 μg/ml) (Ortho Biotech) cross-linked with sheep anti-mouse IgG (10 μg/ml) (Biosys, S. A.) in the presence or absence of a MEK kinase inhibitor, PD98059

[1] To whom correspondence should be addressed at Department of Internal Medicine II, Chiba University School of Medicine, 1-8-1 Inohana, Chiba City, Chiba 260-0856, Japan. Fax: 81-43-226-2095. E-mail: iwamoto@intmed02.m.chiba-u.ac.jp.

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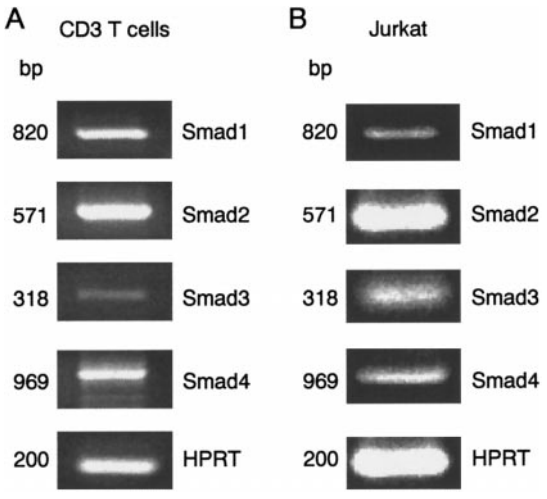

FIG. 1. Smad1-4 mRNA expression in T cells. Total RNA was extracted from human peripheral T cells (A) and Jurkat T cell line (B). cDNA of Smad1-4 mRNA and HPRT as an internal control were amplified by PCR. The PCR products were visualized by electrophoresis on 1.5% agarose gel. The data are representative of three experiments.

(New England Biolab) (10) and neutralizing antibody against TGF-β (50 μg/ml) (R&D).

RT-PCR analysis. RT-PCR analysis was performed by conventional procedure. Briefly, total RNA (0.1–10 μg) was prepared from T cells using Isogen solution (Nippon Gene) and cDNA was synthesized using 1st Strand cDNA Synthesis Kit (Boehringer Mannheim). PCR amplification (94°C for 1.5 min, 59°C for 1 min, and 72°C for 1 min; 30 cycle) was performed in a DNA thermal cycler (Perkin-Elmer). The PCR-amplified samples were run on 1.5% agarose gel, and visualized using ethidium bromide. Primers used in this study were as follows; Smad1 (5′-GTTTCCTCACTCTCCCAATAG-3′ and 3′-AAGAATGAGTTTACCCAAGTG-5′), Smad2 (5′-AGTATGGACACAGGCTCTCC-3′ and 3′-GTCTTATGGCTTCCGTCTG-5′), Smad3 (5′-TCCCCAGCACATAATAACTT-3′ and 3′-TAAAAACAGGTCAGAGGGT-5′), Smad4 (5′-ATCTGAGTCTAATGCTACCAGC-3′ and 3′-TTGTGGAACGACCTAACTT-5′) and HPRT (5′-TTCTTTGCTGACCTGCTG-3′ and 3′-TTTCTACCAGTTCCAGCG-5′).

Western blotting. Immuoblotting was performed with the antibodies against mouse Smad2 or Smad4 (Transduction Laboratories) and phosphorylated Smad2 as previously described (11). The antibody against phosphorylated Smad2 recognizes Smad2 phosphorylated by TGF-β type I receptor (12). To examine interaction between Smad2 and Smad4, immunoprecipitaton followed by immunoblotting was performed as previously described (11).

RESULTS

Smad1-4 mRNA Expression in T Cells

In order to determine whether Smads are involved in signal transduction of TGF-β in T cells, we first examined expression of mRNA of Smad1-4 in T cells. RT-PCR analysis showed that human peripheral blood T cells and Jurkat T cell line clearly expressed Smad1-4 mRNA (Fig. 1A and 1B).

TGF-β Phosphorylates Smad2 in T Cells

Because of the limited availability of antibody against Smads, we focused on Smad2 for further protein analysis. Phosphorylation of Smad2 at serines in the carboxyl-terminal motif (SSMS) by TGF-β type I receptor is a crucial event for initiation of TGF-β signal transduction (11, 13). Western blot analysis showed that human peripheral T cells and Jurkat T cell line abundantly expressed Smad2, which was phosphorylated by TGF-β (Fig. 2A and 2B). We then asked whether expression and TGF-β-mediated phosphorylation of Smad2 preferentially occurred in CD4 or CD8 T cells. As shown in Fig. 2C, both the CD4 and CD8 T cell subsets purified from mouse spleen expressed Smad2, which was phosphorylated by TGF-β in both subsets equally. These findings indicated that Smad2 was involved in TGF-β signal transduction in T cells.

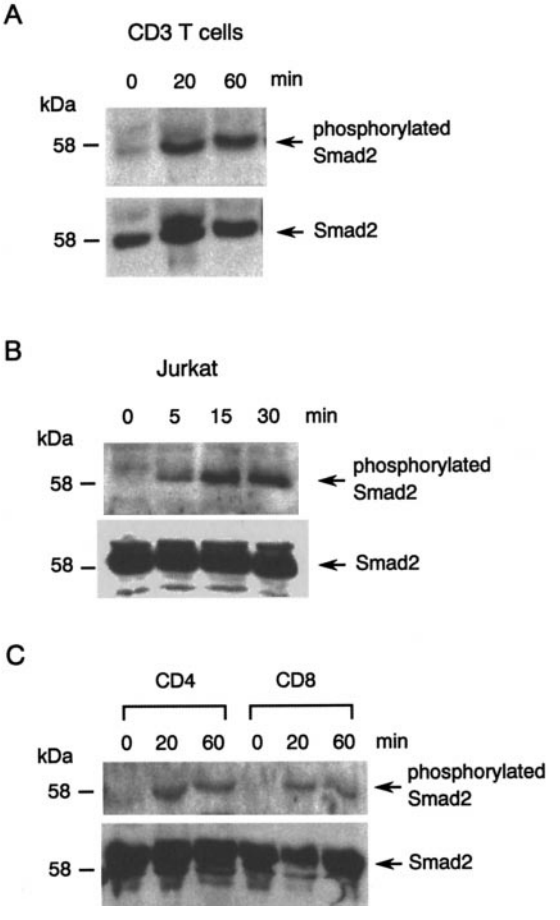

FIG. 2. TGF-β phosphorylates Smad2 in T cells. Human peripheral T cells (A), Jurkat T cell line (B), and CD4 or CD8 positive T cells from mouse spleen (C) (1×10^6/ml) were stimulated with TGF-β1 (10 ng/ml) at 37°C for indicated time. Cell lysates were immunoblotted with anti-phosphorylated Smad2 antibody (upper panel) or anti-Smad2 antibody (lower panel). The position of phosphorylated and nonphosphorylated Smad at approximately 60 kDa is shown by the arrows. The data are representative of three experiments.

Stimulation of T Cells by the Ligation of the TCR/CD3 Complex with Anti-CD3 mAb Phosphorylates Smad2 in T Cells

Recently, cross talk between receptor serine-threonine kinases and receptor tyrosine kinases at Smad level has been reported (14, 15). Caestecker *et al.* (15) showed that hepatocyte growth factor (HGF) and epidermal growth factor (EGF) activated Smad2 as well as TGF-β. Since T cells mediate signals via the TCR/CD3 complex associated with several tyrosine kinases including ZAP70 (16), we hypothesized that stimulation of the TCR/CD3 complex might activate Smad2 in T cells. Thus, we examined the effect of ligation of the TCR/CD3 complex with anti-CD3 mAb or PMA plus ionomycin on phosphorylation of Smad2 in T cells. Stimulation with PMA plus ionomycin is a potent stimulant for activation of T lymphocytes as well as ligation of the TCR/CD3 complex with anti-CD3 mAb (16). As shown in Fig. 3A, we found that stimulation of the TCR/CD3 complex with anti-CD3 mAb or stimulation with PMA plus ionomycin phosphorylated Smad2 in Jurkat T cell line. Phosphorylation of Smad2 by stimulation via the TCR/CD3 complex was detected at 5 min after the stimulation and reached a peak at 15 to 30 min (Fig. 3A). In addition, the phosphorylation of Smad2 by stimulation with anti-CD3 mAb in human peripheral T cells was not affected by treatment with neutralizing antibody against TGF-β (Fig. 3B).

However, in contrast to TGF-β, interaction between Smad2 and Smad4 was not induced by stimulation with anti-CD3 mAb in Jurkat T cell line (Fig. 3C). These findings indicated that stimulation via the TCR/CD3 complex phosphorylated Smad2 in T cells and the phosphorylation was not secondary through endogenous TGF-β. However, the phosphorylation of Smad2 by stimulation with the TCR/CD3 complex was not sufficient to induce interaction between Smad2 and Smad4.

A MEK Inhibitor, PD98059, Inhibits Phosphorylation of Smad2 by Stimulation via the TCR/CD3 Complex

Caestecker *et al.* (15) showed that a MEK inhibitor, PD98059 (10), inhibited phosphorylation of Smad2 by HGF in mink lung epithelial cells (Mv1Lu cells). Ligation of the TCR complex activates MEK/MAP kinase pathways in T cells (17). Thus, we examined the effect of PD98059 on phosphorylation of Smad2 by stimulation with anti-CD3 mAb. We found that PD98059 suppressed Smad2 phosphorylation in Jurkat T cell line by stimulation with anti-CD3 mAb, but not with TGF-β (Fig. 4). These findings indicated that TCR/CD3 complex-mediated phosphorylation of Smad2 involved MAP kinase pathways in T cells as was the case with phosphorylation of Smad2 in Mv1Lu cells by HGF (15).

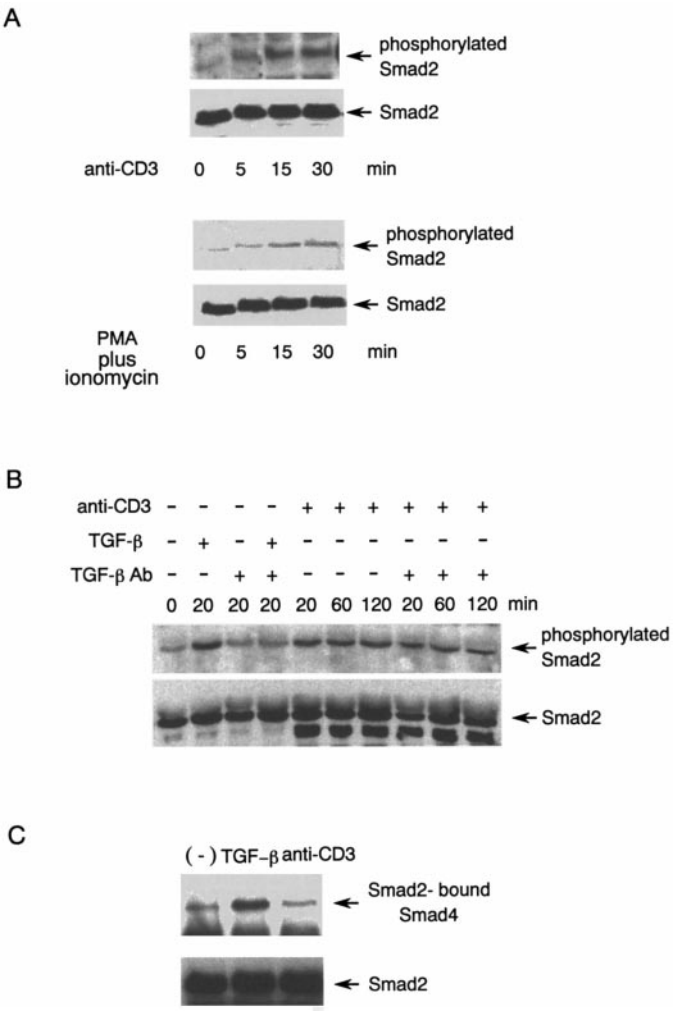

FIG. 3. Stimulation via the TCR/CD3 complex phosphorylates Smad2 in T cells. (A) Jurkat T cells (1×10^6/ml) were stimulated with anti-CD3 mAb or PMA plus ionomycin and cell lysates were immunoblotted with anti-phosphorylated Smad2 antibody (upper panel) or anti-Smad2 antibody (lower panel). (B) Human peripheral T cells (1×10^6/ml) were stimulated with anti-CD3 mAb in the presence or absence of neutralizing antibody against TGF-β (50 μg/ml) at 37°C for 20 to 60 min and cell lysates were immunoblotted as described in (A). (C) Jurkat T cells (1×10^6/ml) were stimulated with anti-CD3 mAb or TGF-β at 37°C for 60 min. Cell lysates were then immunoprecipitated with anti-Smad2 antibody followed by immunoblotting with anti-Smad4 antibody. Equal expression of Smad2 was confirmed by immunoblotting with anti-Smad2 antibody (lower panel). The data are representative of three experiments.

DISCUSSION

In this study, we showed that Smad2 was activated not only by stimulation with TGF-β, but with antibody directed towards the TCR/CD3 complex in T cells. Our findings suggest that Smad2 may function both in TGF-β- and TCR/CD3-mediated signal transduction.

We observed that Smad2 phosphorylation occurred at 5 min after stimulation with anti-CD3 mAb (Fig. 3A). Previously, Kehrl *et al.* (5) showed that biologi-

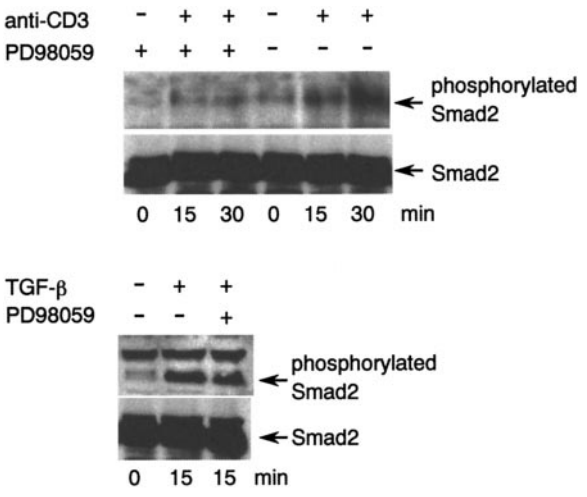

FIG. 4. Effect of a MEK inhibitor PD98059 on Smad2 phosphorylation in T cells. Jurkat T cells (1×10^6/ml) were stimulated with anti-CD3 mAb or TGF-β for indicated time in the presence or absence of PD98059 (20 μM) and cell lysates were immunoblotted as described in Fig. 3A. The data are representative of three experiments.

cally active TGF-β was significantly produced 24 hours after PHA stimulation in T cells. Thus, from the kinetics of Smad2 phosphorylation as well as the experiment using neutralizing antibody against TGF-β (Fig. 3B), it was unlikely that TGF-β produced by activated T cells phosphorylated Smad2.

Although stimulation with anti-CD3 mAb phosphorylated Smad2 in T cells, it did not induce interaction between Smad2 and Smad4 (Fig. 3C). Phosphorylation of Smad2 at serines in the carboxyl-terminal motif (SSMS) by TGF-β type I receptor is a crucial event for TGF-β signal transduction including heterodimerization of Smad2 with Smad4 (13). In addition, Kretzschmar *et al.* (18) recently suggested that phosphorylation of Smad2 by oncogenic Ras via Erk MAP kinases occurred at specific serines within the linker region of Smad2. Therefore, our findings also suggested that stimulation via the TCR/CD3 complex might phosphorylate Smad2 at different site from the SSMS motif.

What is functional role of Smad2 in TCR/CD3-mediated signaling in T cells? Dominant negative Smad2 would be helpful to address the functional role of Smad2 in the TCR/CD3 complex signaling. However, dominant negative Smad2 obtained so far affects Smad3 function (19) because Smad3 is structurally and functionally very similar to Smad2. In addition, Smad2 null mice showed embryonic lethality, which makes it hard to analyze the role of Smad2 in immune system (20, 21). Currently, we are trying to address the issue by several approaches such as usage of anti-sense Smad2 oligonucleotide.

In summary, we showed that Smad2 was activated not only by stimulation with TGF-β, but by stimulation via the TCR/CD3 complex in T cells. Our findings suggest that Smad2 may function in both TGF-β- and TCR/CD3-mediated signal transduction, which may cast a novel insight for TCR/CD3-mediated signaling.

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