Calmodulin Differentially Modulates Smad1 and Smad2 Signaling*

The members of the Smad protein family are intracellular mediators of transforming growth factor β (TGF-β) signaling. Smad1 transduces bone morphogenetic protein signals, inducing formation of ventral mesoderm in Xenopus embryos, whereas Smad2 transduces activin/ TGF-β signals, generating dorsal mesoderm. Calmodulin directly binds to many Smads and was shown to downregulate Smad activity in a cell culture system (Zimmerman, C. M., Kariapper, M. S. T., and Mathews, L. S. (1997) J. Biol. Chem. 273, 677–680). Here, we extend those data and demonstrate that calmodulin alters Smad signaling in living embryos, increasing Smad1 activity while inhibiting Smad2 function. To characterize this regulation, we undertook a structure-function analysis and found that calmodulin binds to two distinct and conserved regions in both Smad1 and Smad2. Receptor tyrosine kinase signaling also modifies Smad activity (Kretzschmar, M., Doody, J., and Massagué, J. (1997) Nature 389, 618–622; Kretzschmar, M., Doody, J., Timokhina, I., and Massagué, J. (1999) Genes Dev. 13, 804–816; de Caestecker, M. P., Parks, W. T., Frank, C. J., Castagnino, P., Bottaro, D. P., Roberts, A. B., and Lechleider, R. J. (1998) Genes Dev. 12, 1587–1592). We show that calmodulin binding to Smads inhibits subsequent Erk2-dependent phosphorylation of Smads and vice versa. These observations suggest the presence of a cross-talk between three major signaling cascades as follows: Ca²⁺/calmodulin, receptor tyrosine kinase, and TGF-β pathways.

The transforming growth factor β (TGF-β) superfamily plays essential roles in many diverse biological processes including development, physiologic homeostasis, and oncogenesis (5–8). Signaling events, initiated by individual TGF-β superfamily ligands, result in characteristic cell type-specific responses, such as cell proliferation, apoptosis, and differentiation (9, 10). The receptors for the TGF-β superfamily are transmembrane serine kinases that phosphorylate members of the Smad family (11–13). In turn, the Smads transduce TGF-β signals and thereby regulate many important biological decisions including the development of a wide range of organisms from nematodes to mammals (10, 14–20). In addition, the critical role of Smads in controlling cell proliferation is highlighted by the fact that Smads are mutated in many human cancers including colon cancer, pancreatic cancer, and breast cancer (21–24).

The identification of the Smads as key intracellular mediators of TGF-β signals has begun to shed light on how these extracellular signals are transduced from the cell membrane to the nucleus, where they elicit intracellular responses (14, 25, 26). Structurally, Smads consist of two highly conserved globular amino- and carboxyl-terminal domains (Mad homology (MH) domain 1 and 2, respectively) that are connected via a divergent linker region. The vertebrate Smads can be divided into three classes. Pathway-restricted Smads (Smad1, Smad2, Smad3, Smad5, and Smad8) are phosphorylated by the serine kinase receptors upon ligand stimulation (27). Then, they associate with Smad4 or Smad4β, the only known vertebrate members of the second class of Smads, termed common Smads (28–31). Together, this complex translocates to the nucleus, binds DNA and other transcription factors (32–37), and alters gene expression (15, 26, 35, 38). The third class of Smads, Smad6 and Smad7, lack the key MH1 domain and inhibit, rather than activate, TGF-β signaling (39–43). Biochemical approaches and studies in Xenopus embryos are concordant and have revealed that pathway-restricted Smads function in distinct and specific pathways (11, 44). Smad1 and Smad5 transduce BMP signals, whereas Smad2 and Smad3 function in the activin or TGF-β pathways (10, 16, 32, 45, 46).

The discovery that Smads are signal transduction molecules involved in physiological responses to extracellular signals has increased the focus of research on Smad regulation with two signaling systems possibly involved, receptor tyrosine kinase (RTK) and calcium/calmodulin (Ca²⁺/CaM) (1–4). For example, cell culture-based experiments have shown that stimulation of RTK pathways, via Erk2, leads to increased phosphorylation of Smad1 and Smad2 (2–4). The linker region of Smad1 contains several canonical Erk sites (PXSP) that become phosphorylated after epidermal growth factor stimulation of R-1B/L17 cells and by Erk2 in vitro (2). As a consequence of this phosphorylation, nuclear accumulation of Smad1 is inhibited, thus blocking Smad1 activity. Smad2 and Smad3 are also substrates for Erk2, and one report showed that epidermal growth factor stimulation of Mv1Lu cells partially excluded these Smads from the nucleus and hence decreased their function (3). These data contrast with an earlier report in which Erk2-dependent phosphorylation of Smad2 increased both nuclear localization and activity of Smad2 (4).

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The abbreviations used are: TGF-β, transforming growth factor β; BMP, bone morphogenetic protein; CaM, calmodulin; RTK, receptor tyrosine kinase; MH, Mad homology; S1, Smad1; S2, Smad2; CBR, calmodulin binding region; BBM1, brush border myosin 1; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; PBS, phosphate-buffered saline; DTT, dithiothreitol; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

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In addition to the RTK cascade, another major signaling pathway, calcium/calmodulin, has also been suggested to alter Smad function. It was recently reported that calmodulin binds to the amino-terminal half of several Smads including Smad1 and Smad2 (1). This binding might have functional consequences as inhibiting calmodulin increased activin-dependent induction of marker gene expression in L17 mink lung epithelial cells, whereas overexpression of calmodulin decreased activin- and TGF-β-dependent induction of a transcripational reporter (1). From these data, it was concluded that calmodulin negatively regulates Smad2 and hence activin/TGF-β signaling. These observations prompted us to examine the functional relationship between calmodulin binding and Erk2 phosphorylation of Smads. In the present study, we show the following: 1) calmodulin increases the activity of Smad1 and decreases the activity of Smad2 in Xenopus embryos and explants; 2) Smad1 and Smad2 have two calmodulin-binding sites in their MH1 domains; and 3) calmodulin binding and Erk2 phosphorylation are reciprocally regulated in vitro. Taken together, these observations provide further evidence for cross-talk between Ca²⁺/CaM, TGF-β, and RTK signaling.

**EXPERIMENTAL PROCEDURES**

**Embryological Methods**—Embryos were obtained, microinjected, and cultured, and animal caps were dissected, cultured, and harvested as described (47). Embryos were either uninjected (control) or injected with mRNA as described in the figure legends.

**Formation of Synthetic mRNA for Microinjection**—The Smad1 and Smad2 full-length constructs used for making synthetic mRNA have been described previously (16). The calmodulin construct used for making synthetic mRNA, a generous gift of Lawrence Mathews, was linearized with BamHI. Capped mRNA was transcribed in vitro as described (48).

**RT-PCR Analysis**—RNA extraction and RT-PCR analyses have been described previously (47, 49, 50). The intensity of the radioactive bands amplified by RT-PCR reflects the abundance of the mRNA. The conditions of PCR detection of RNA transcripts and the primer sequences have been described elsewhere (49–52).

**Deletion Constructs**—Deletion constructs of Smad1 and Smad2 were prepared by subcloning the corresponding PCR fragments into pcDNA3+ at the BamHI and EcoRI restriction sites. PCR reactions consisted of primer, plasmid template, 5 μl of 10× Vent DNA polymerase buffer, 2.5 μl of dNTP mix (5 μM each); 40.5 μl of H₂O, and 0.5 μl of Vent DNA polymerase (Promega). PCR conditions were as follows: 2 min at 94 °C, 19 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by 5 min at 72 °C. The reaction products were digested with the appropriate restriction enzymes, gel-purified, and ligated into the preexisting vector. The constructs were verified by sequencing, and the primers used for the constructs are shown in Table I.

**Introduction of Internal Point Mutations by PCR**—To create point mutations within a cDNA, amino- and carboxyl-terminal PCR fragments were amplified using synthetic oligonucleotides that carried the desired mismatches. The primers were complementary to opposite strands of the plasmid, so that the resulting cDNA fragments overlapped in the region of these primers. The purified fragments were combined, denatured at 95 °C, and then allowed to anneal for 5 min on ice. Thereafter, Vent polymerase (Promega) extended the overlapping regions toward the amino and the carboxyl termini of the cDNA (5 cycles under the same conditions as the PCR described above). The contiguous, mutated cDNA was amplified in a subsequent PCR with primers 5’ and 3’ of the mutated area. PCR conditions were as described above. The fragment was then subcloned as described above, and the primers used for the constructs are shown in Table II.

**Cloning of GST-Smad Fusion Proteins**—The coding regions of Smad1, Smad2, and hSmad1(4SP/AP) were PCR-amplified and cloned into the polylinker of the vector pGEX-2T (Promega). The sequences and restriction sites of the forward primers were chosen to render in-frame fusion with the GST gene of the vector, and the primers used are shown in Table III.

**Purification of GST-Smad Fusion Proteins**—GST fusion proteins were expressed in E. coli DH5α cells (Stratagene). The bacteria were grown in LB medium supplemented with 100 μg/ml ampicillin. Expression of recombinant proteins was induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37 °C at an A₆₀₀ of 0.5. The bacteria were pelleted and washed 3× with 10 volumes of ice-cold PBS before they were lysed with 100 μg/ml lysozyme for 1 h on ice. After sonication, cellular debris was pelleted. To purify the fusion proteins, the supernatant, which contains the fusion proteins, was collected and mixed with 300 μl of glutathione-Sepharose 4B beads. After a 20-min incubation at room temperature, the beads were pelleted and washed 5× with 10 ml of ice-cold PBS containing 150 mM NaCl. The beads were resuspended in 500 μl of ice-cold PBS and stored for further use. To elute the fusion proteins off the beads, they were pelleted and mixed with 5 bed volumes of glutathione elution buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 20 mM MgCl₂, 1 mM DTT, 5% Triton X-100, 20 mM CaCl₂, or 20 mM EGTA). In *Vitro* Phosphorylations—Phosphorylation reactions were performed in a 50-μl volume that contained 1× kinase buffer, 10 μM ATP, 0.5 mg/ml substrate, and 200 ng/ml activated Erk2. 10× kinase buffer consists of 200 mM Tris-HCl, pH 8.0, 100 mM MgCl₂, 1 mM DTT, and 1 mM benzamidine. In some experiments, 2.5 μM of [γ⁻³²P]ATP (10–30 cpm/nmol, Amersham Pharmacia Biotech) was added per reaction.
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| Construct     | Primer sequence |
|---------------|-----------------|
| S1(A)-C46      | 318 5'-GAAGATCTCTCATCGAGGAACTGGAAGAACCCACACCAAGGAAATATAC-3' |
| S1(A)-C165     | 119 5'-CGGAGATCTTCTTAAGACGAGGAGGATGC-3' |
| S1(B)-C46      | 118 5'-GGGGATCCATGAAATGTGACAG-3' |
| S1(B)-C165     | 741 5'-CGGAGATCTCTCATCGAGGAACTGGAAGAACCCACACCAAGGAAATATAC-3' |
| S1(AB)-C46     | 740 5'-ATTACCTAGGTGCTGCGGCTGAGATCAGAGCCTGAGACTGAAACC-3' |
| S1(AB)-C165    | 119 5'-GGGGATCCATGAAATGTGACAG-3' |
| S2(A)-C58      | 318 5'-GAAGATCTCTCATCGAGGAACTGGAAGAACCCACACCAAGGAAATATAC-3' |
| S2(A)-C46      | 740 5'-ATTACCTAGGTGCTGCGGCTGAGATCAGAGCCTGAGACTGAAACC-3' |
| S2(A)-C165     | 119 5'-GGGGATCCATGAAATGTGACAG-3' |
| S2(B)-C46      | 122 5'-CGGAGATCTCTCATCGAGGAACTGGAAGAACCCACACCAAGGAAATATAC-3' |
| S2(B)-C165     | 122 5'-GGGGATCCATGAAATGTGACAG-3' |
| S2(AB)-C46     | 720 5'-CACAATTTCTGCTTTCTCAGTGTGCGACGTGACGATGGCTGCCATGCCACAGGTGCCAGTA-3' |
| S2(AB)-C165    | 19 5'-CACAATTTCTGCTTTCTCAGTGTGCGACGTGACGATGGCTGCCATGCCACAGGTGCCAGTA-3' |
| S2(AB)-C58     | 603 5'-GAAGATCTCTCATCGAGGAACTGGAAGAACCCACACCAAGGAAATATAC-3' |
| S2(AB)-C46     | 19 5'-CACAATTTCTGCTTTCTCAGTGTGCGACGTGACGATGGCTGCCATGCCACAGGTGCCAGTA-3' |
| S2(AB)-C165    | 603 5'-GAAGATCTCTCATCGAGGAACTGGAAGAACCCACACCAAGGAAATATAC-3' |

 reactions were incubated at 30 °C and then used for other assays or stopped by the addition of 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8; 200 mM DTT; 4% SDS; 0.2% bromophenol blue; 20% glycerol). The reactions were then analyzed by SDS-PAGE.

**Calmodulin/GST-Smad Fusion Protein Binding Assays**—GST-Smad fusion proteins, bound to glutathione-Sepharose 4B beads, were phosphorylated using 10 mM ATP and 200 ng/ml activated Erk2 for 5 min at 4 °C. The reaction mixture was incubated with 0.2 nM 125I-CaM (PerkinElmer Life Sciences) for 1 h at 4 °C in the presence of either 200 mM CaCl2 or 100 mM EGTA. The beads were pelleted, and the supernatant was replaced with 50 μl of 2× SDS sample buffer, boiled, and analyzed by SDS-PAGE.

Soluble GST-Smad fusion proteins were phosphorylated according to the procedure described above (see under In Vitro Phosphorylations) using [γ-32P]ATP. Following the addition of 4% (v/v) calmodulin-Sepharose 4B beads, the reaction mixture was incubated for 20 min at 4 °C with either calcium or EGTA. Afterward the beads were washed and processed as described above.

To determine if calmodulin binding to Smads influences their phosphorylation by Erk2, GST-Smad proteins, bound to glutathione-Sepharose 4B beads, were mixed with 500 nM calmodulin (Calbiochem) in the presence of kinase buffer, 10 mM ATP, and either 200 μM CaCl2 or 100 μM EGTA. The reaction mixture was incubated at 4 °C for 1 h, after which [γ-32P]ATP (10–30 cpm/fmol, Amersham Pharmacia Biotech) and 200 ng/ml activated Erk2 were added. Following a 5 min incubation at 4 °C, the beads were pelleted, washed and processed as described above.

**TABLE III**

| cDNA     | Primer constructs | Primer sequence |
|----------|-------------------|-----------------|
| Smad1(1–467) | 294 5'-ATAGGATCCTGAATGTCAGGGC-3' |
| Smad2(1–467) | 329 5'-GATGAATCTCTCTAGCAGGAGAGATGC-3' |
| hS1(4SP/AP)(1–464) | 304 5'-GATGAATCTCTCTAGCAGGAGAGATGC-3' |
|          | 694 5'-TCCGAGATCTCATGAGGAGGAG-3' |
|          | 695 5'-TCCGAGATCTCATGAGGAGGAG-3' |

RESULTS

**Calmodulin Blocks Smad2-dependent Morphogenesis in Xenopus Embryos**—To study the in vivo effects of calmodulin on Smad2 activity, we chose a model system of calmodulin signaling, the Xenopus embryo. We synthesized mRNAs encoding full-length Smad2 and calmodulin, injected the mRNAs alone or together into one-cell stage Xenopus embryos, allowed the embryos to develop, and then examined the embryos for any phenotypic changes. Consistent with previous findings, Smad2-injected embryos underwent drastic morphological changes due to the induction of dorsal mesoderm (Fig. 1A) (16). Strikingly, when Smad2 mRNA was co-injected with calmodulin mRNA, this effect was prevented, and the embryos were phenotypically indistinguishable from uninjected sibling controls (Fig. 1A).

**TABLE II**

| Primer constructs |
|-------------------|
| Smad2(1–467)      |
| Smad1(1–467)      |
| hS1(4SP/AP)(1–464)|

**Embryos injected with calmodulin mRNA alone developed normally (data not shown). These data suggest that calmodulin not only down-regulates Smad2 activity in cell culture systems but that it can rescue a Smad2-dependent phenotype in a complex developing organism.

To characterize further this phenomenon, we used the Xenopus animal cap assay. In this assay, mRNA is injected into the prospective ectoderm of one-cell stage embryos. At the blastula stage, animal poles are dissected and cultured until the appropriate stage for morphological or molecular analysis (16, 42).

Normally, cultured animal pole explants (animal caps) form ciliated epidermis (skin), but they can be converted to other cell fates such as ventral mesoderm, dorsal mesoderm, or neural tissue, depending on the activity of the effector molecule(s) that is (are) expressed in the animal caps (42, 53–58).

To study the effect of calmodulin on Smad2 activity in animal cap explants, we injected synthetic Smad2 mRNA with or without calmodulin mRNA into the animal pole of fertilized eggs, removed the animal pole tissue, and cultured the animal caps. Control and calmodulin-injected animal caps are round, but that it can rescue a Smad2-dependent phenotype in a complex developing organism.

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**Calmodulin Blocks Smad2-dependent Induction of Dorsal Mesoderm**—The results presented in Fig. 1, A and B, suggest that calmodulin inhibits Smad2 activity in vivo. However, the morphologic changes might not reflect a change in gene expression (59, 60). To analyze molecularly the effect that calmodulin
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Fig. 1. Calmodulin inhibits Smad2 and activates Smad1. A, calmodulin blocks Smad2-dependent whole embryo phenotypes. Synthetic mRNA encoding Smad2 (S2; 0.025 ng) was injected with or without 4 ng of mRNA encoding CaM into the animal poles of fertilized eggs. The embryos were allowed to develop until stage 35 and then photographed. While Smad2-injected embryos underwent morphological changes, embryos co-injected with both Smad2 and calmodulin (S2 + CaM) did not and were indistinguishable from un.injected control (ctrl) embryos. B, calmodulin blocks Smad2-dependent morphogenesis in animal caps. Animal poles were injected with mRNA-encoding Smad2 (S2; 0.025 ng) with or without 4 ng of calmodulin, and animal caps were dissected at stage 8 (blastula) and cultured until stage 19 and photographed. Smad2-injected animal caps elongated. In contrast, Smad2/CaM co-injected animal caps did not elongate (S2 + CaM) and appeared identical to un injected control (ctrl) animal caps. C, calmodulin inhibits the dorsal mesoderm inducing activity of Smad2. Animal poles were injected with increasing doses of synthetic mRNA encoding Smad2 with (+) or without (−) 4 ng of mRNA-encoding calmodulin. Animal caps were dissected at stage 8 and cultured. At tadpole stage 38, RNA from 5 animal caps was pooled and analyzed by RT-PCR for the presence of the indicated transcripts. At all doses (0.0125, 0.025, and 0.05 ng), Smad2 (S2) induced the expression of the dorsal mesodermal marker muscle actin (M.Actin) but neither the ventral mesodermal marker globin nor the neural marker neural cell adhesion molecule (NCAM). Of note, calmodulin abolished the Smad2-dependent induction of muscle actin (lane CaM +). EF-1α is ubiquitously expressed and serves as a negative control. Lane C corresponds to control animal caps and demonstrates that mesodermal markers are not normally expressed in the animal cap. D, calmodulin increases Smad1 activity. Animal caps expressing different amounts (1, 2, and 4 ng) of Smad1 with (+) or without (−) 4 ng of calmodulin were cultured until tadpole stage 38, and the RNA was analyzed as described (C). Smad1 (S1) induced the expression of the ventral mesodermal marker globin. When calmodulin was co-expressed, the activity of Smad1 increased (lane CaM +). Calmodulin never induced the expression of globin when it was expressed alone. The lanes and markers are as described in the legend to C.

has on Smad2 function, we injected increasing amounts of synthetic Smad2 mRNA alone or with calmodulin mRNA and then analyzed animal caps for the differential expression of tissue-specific markers. When expressed alone, calmodulin did not induce the expression of any markers tested (Fig. 1C). In contrast, Smad2 induced, in a dose-dependent fashion, the expression of the dorsal mesodermal marker, muscle actin. Of note, co-injection with calmodulin abrogated the Smad2-dependent induction of muscle actin. These results demonstrate that calmodulin blocks Smad2 signaling in vivo.

Calmodulin Increases Smad1 Activity—It was previously demonstrated that calmodulin binds not only to Smad2 but also to other Smads including Smad1 (1). However, the functional consequences of this interaction were not addressed. As we found that calmodulin altered Smad2 function in vivo, we sought to determine whether calmodulin might also affect the activity of other Smads. We chose Smad1 because, in contrast to Smad2, it transduces BMP signals and induces ventral mesoderm formation in Xenopus embryos (10, 11, 16). To determine if calmodulin could alter Smad1 activity, we injected increasing amounts of mRNA encoding Smad1 alone or with calmodulin mRNA and assayed the animal caps for the expression of tissue-specific markers. In animal caps, Smad1 induced the ventral mesodermal marker globin, but neither the dorsal mesodermal marker, muscle actin, nor the neural marker neural cell adhesion molecule (Fig. 1D, NCAM). Surprisingly, calmodulin enhanced the dose-dependent induction of globin by Smad1 (Fig. 1D). Thus, calmodulin has opposite effects on the activities of Smad1 and Smad2, increasing Smad1 activity and decreasing Smad2 function. These results imply a distinct and specific regulation of the BMP and activin/TGF-β signaling pathways by Ca²⁺/CaM.

Smad1 and Smad2 Contain Two Calmodulin Binding Domains—To characterize further the mechanism of calmodulin-dependent changes in Smad signaling, we attempted to delineate the calmodulin binding domain(s). Prototypic calmodulin binding regions consist of amphipathic helices of approximately 20 amino acids that contain clusters of basic amino acids neighboring by hydrophobic residues. Previously, it was shown that calmodulin could bind to the amino-terminal region of Smad2 between amino acids 77 and 204 (1). In order to expand already existing data, we chose to elucidate the calmodulin-binding site(s) in Smad1. To that end, we generated constructs in which the amino- or carboxyl-terminal regions of Smad1 were serially deleted. Next, the deletion constructs were transcribed and translated in vitro in the presence of [³⁵S]methionine/cysteine. These radiolabeled Smad peptides were then tested for the ability to bind to calmodulin-Sepharose 4B beads (Fig. 2A). As virtually all physiologically relevant calmodulin processes are calcium-dependent, the binding reactions were carried out in the presence of calcium or EGTA as internal specificity controls.
When we tested the carboxyl-terminal deletion constructs (ΔCterm) for the ability to bind calmodulin, we found that a peptide consisting of amino acids 1–49 (Smad1(N1–49)) bound to calmodulin-Sepharose beads (Fig. 2, A and B). So, the very amino-terminal region, consisting of amino acids 1–49, is a calmodulin binding region (CBR). Of note, this CBR is conserved among Smads, and we call it CBR-A. Calmodulin typically binds to regions of proteins that contain α-helices, and CBR-A contains two amino acid stretches that have been predicted to form helices, based on the Smad3 crystal structure (61). We also tested a series of amino-terminal deletion constructs (ΔNterm) for calmodulin binding. A fragment lacking the amino-terminal 86 residues (Smad1(C86–465)) still bound calmodulin, while deleting nine more amino acids (Smad1(C95–465)) eliminated calmodulin binding (Fig. 2, A). This indicated that amino acids 86–95 of the MH1 domain of Smad1 might contain at least part of a second calmodulin-binding site. This region is also conserved among Smads and corresponds to helix 4 in the Smad3 crystal structure (61). Taken together, these experiments revealed the presence of two distinct CaM binding domains in Smad1 (Fig. 2C).

The overall high degree of sequence conservation of the Smad1 CBR-A and CBR-B with their homologous Smad2 sequences (Fig. 2D) prompted us to test whether these regions in Smad2 also bound calmodulin. To that end, we generated similar deletion constructs for Smad2, and we tested them for calmodulin binding. We found that Smad2 also contained the same two binding domains (not shown). Next, we attempted to determine whether these regions were necessary for calmodulin binding. We therefore prepared constructs of Smad1 and Smad2 in which we either deleted the putative binding sites or introduced point mutations into them and tested them for their ability to bind calmodulin (Fig. 3). Constructs in which the CBR-As were deleted bound to calmodulin, as did constructs in which the basic amino acids within the CBR- Bs and the surrounding region were mutated to alanines (Fig. 3). Deleting the CBR-As (S1-(A) and S2-(A)) drastically reduced calmodulin binding, whereas mutating the basic residues of the CBR-Bs has only a weak effect (S1-(B) and S2-(B); Fig. 3, A and C). This might indicate that CBR-As have a higher affinity for calmodulin than the CBR-Bs. Deletion of the CBR-As in combination with point mutations in two arginines in the CBR-Bs markedly decreased but did not eliminate calmodulin binding (not shown). We then also mutated two neighboring histidine residues that are conserved between Smad1 and Smad2. This leads to a complete loss of calmodulin binding, confirming the locations of the binding regions and extending them to the histidines (Fig. 3). Taken together, our data suggest that two distinct binding motifs are present in Smad1 and Smad2 and that the binding domains are conserved.

**Reciprocal Relationship of CaM Binding and Erk2-dependent Phosphorylation**—It was recently shown that RTK pathways regulate Smad activity. This occurs via Erk2 phosphorylation of Smad1 and Smad2 on serine residues within their linker region and leads to a down-regulation of Smad1 activity and, as initially reported, to an increase in Smad2 signaling (2–4). Together with our data, this suggested that regulation of TGF-β signaling through Ca2+/CaM and through RTK pathways had opposite effects on Smad function and prompted us to determine if this reciprocal relationship was mechanistically based. Of note, a more recent report suggests that RTK signaling can also decrease Smad2 activity (3).
To explore the possibility that the RTK, Ca\(^{2+}\)/CaM, and Smad signaling pathways might interact biochemically, we determined whether prior Erk2 phosphorylation of the Smads would alter subsequent calmodulin binding. To that end, we expressed GST fusion proteins of Smad1 and Smad2 in bacteria and purified the proteins. The GST-Smad fusion proteins were incubated with or without active Erk2, and then CaM-Sepharose beads were added in the presence of calcium or EGTA. The CaM-Sepharose beads were then precipitated, and the pellet was subjected to SDS-PAGE. Co-precipitation of the GST-Smad fusion proteins was determined by a Western blot with an antibody directed against the GST tag. As a control, we performed the same experiment with a construct encoding a mutant Smad1 protein (GST-S1(4SP/AP)) that cannot be phosphorylated by Erk2 because all four serines that are phosphorylated by Erk2 (PXSP sites) have been mutated to alanines (PXAP) (2). SDS-PAGE and autoradiography of the samples following the pull down demonstrated that Smad1 and Smad2 are substrates for Erk2 and that the Smad1 4SP/AP construct is not (Fig. 4A, a). As shown in Fig. 4A, phosphorylation of Smad1 and Smad2 with Erk2 reduced their potential to associate with calmodulin (left and center, respectively). This effect is a consequence of Erk2-dependent phosphorylation of the Smads and not just on the presence of Erk2 in the reaction mix, as inhibition of the calmodulin-Smad association was not observed with the non-phosphorylatable mutant Smad (GST-S1(4SP/AP)) (Fig. 4A, right).

In a second approach, we phosphorylated the purified fusion proteins with active Erk2 or, as a control, did a mock phosphorylation (Fig. 4B). Then, we performed binding studies with \(^{125}\text{I}-\text{CaM}\) in the presence of calcium, pulled down the GST-Smad fusion proteins with glutathione beads, and then determined whether \(^{125}\text{I}-\text{CaM}\) co-precipitated with the Smads. As shown in Fig. 4B, \(^{125}\text{I}-\text{CaM}\) bound, in a Ca\(^{2+}\)-dependent fashion, to both Smad1 (left) and Smad2 (center). Notably, Erk2 phosphorylation of either Smad1 or Smad2 abrogated calmodulin binding. In this instance, prior incubation with Erk2 had no effect on \(^{125}\text{I}-\text{CaM}\) binding to GST-S1(4SP/AP) (right), again demonstrating the specificity of the effect. These experiments are consistent with the idea that calmodulin preferentially binds to non-phosphorylated forms of Smad1 or Smad2.

Next, we tested the possibility that calmodulin binding might interfere with subsequent Erk2 phosphorylation of the Smads. In these experiments, calmodulin was incubated with GST-Smad fusion proteins in the presence of either calcium or EGTA, and then Erk2 and \([\gamma-^{32}\text{P}]\text{ATP}\) were added to the reaction mix, which was then subjected to SDS-PAGE and autoradiography. Erk2 phosphorylated both Smad1 and Smad2 but not Smad1(4SP/AP) (Fig. 4C). Notably, prior calmodulin binding lowers the level of Erk2-dependent phosphorylation of both Smad1 (left) and Smad2 (center). The specificity of this inhibition was demonstrated by the lack-of-effect of calmodulin when EGTA, rather than calcium, was included in the binding reaction. As a further control, we assessed the level of Erk2-phosphorylation of the transcription factor ATF2, to which calmodulin does not bind. In this case, the amount of Erk2-dependent phosphorylation was unaffected by the presence of calmodulin (data not shown). Therefore, calmodulin does not inhibit Erk2 directly but rather inhibits the ability of Erk2 to phosphorylate Smad1 and Smad2. Taken together, these data suggest that calmodulin binding to Smads and Erk2 phosphorylation of Smads are reciprocally regulated.

**DISCUSSION**

Smad proteins are transcriptional activators, placed at a crucial position for developmental processes of vertebrates and invertebrates. Hence, it is conceivable that they may be subject to fine-tuned regulation by proteins within the TGF-\(\beta\) pathway (e.g. Smad4, Smad6, and Smad7) as well as proteins in other pathways. A new member of the group of Smad-regulating proteins is calmodulin (1). Calmodulin is involved in a wide range of diverse cellular processes such as cell cycle control, cell motility, smooth muscle contraction, and intracellular signaling (62–65). It activates various kinases (CaM kinase I and II and myosin light chain kinase), phosphatases (calcineurin), ion channels, and other cytosolic enzymes (66). In the present study, we demonstrate that calmodulin specifically decreases Smad2-dependent effects and increases Smad1 actions in *Xenopus* embryos and explants. Both Smad1 and Smad2 contain two distinct calmodulin-binding sites. RTK signaling also mod-
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**Fig. 4. Calmodulin binding to Smads and their phosphorylation by Erk2 are reciprocally regulated.** A, Erk2 phosphorylation of Smads decreases calmodulin binding. Purified GST fusions of Smad1, Smad2, and a control, non-phosphorylatable version of Smad1, S1(4SP/AP), were incubated with or without active Erk2 in the presence of [γ-32P]ATP and then tested for their ability to bind calmodulin. Prior phosphorylation by Erk2 reduced the ability of Smads to subsequently bind calmodulin. SDS-PAGE and autoradiography of the samples following the pull down demonstrated that Smad1 and Smad2, but not the mutant S1(4SP/AP), were phosphorylated by Erk2 (a). GST-Smad proteins that bound calmodulin-Sepharose in the pull down were detected by Western blot analysis with antibodies directed against GST (b). The experiments were performed in the absence of either Ca2+ or EGTA to test for the specificity of calmodulin binding. – and + indicate the absence or presence of the indicated compound in the assay. B, Erk2 phosphorylation of Smads inhibits calmodulin binding. GST-Smad1, GST-Smad2, or GST-S1(4SP/AP) were incubated with or without active Erk2 and then mixed with 125I-labeled calmodulin in the presence of either calcium or EGTA. The GST fusion proteins and any associated protein were precipitated with glutathione-Sepharose beads, washed to remove nonspecific interactions, and analyzed on a 15% SDS-protein gel, and 125I-CaM was detected by autoradiography (a). A Western blot of the lysates probed with antibodies directed against GST (b) demonstrated that roughly equal amounts of the GST fusion Smads were incubated in each reaction. Of note, calmodulin preferentially bound to non-phosphorylated forms of Smad1 or Smad2. C, calmodulin binding to Smads reduces the level of Erk2 phosphorylation. GST-Smad fusion proteins were tested for binding to calmodulin in buffer containing either calcium or EGTA, and the proteins were then incubated with [γ-32P]ATP in the presence or absence of Erk2. The level of Smad phosphorylation was evaluated by autoradiography (a) and a Western blot probed with antibodies directed against GST demonstrated the presence of relatively equal amounts of GST-Smad fusion proteins (b). The analysis of the assays was performed as described in A and B. Labels are as in A.

ifies Smad function. We found that calmodulin binding inhibits Erk2 phosphorylation and that Erk2 phosphorylation inhibits calmodulin binding *in vitro*. These data suggest cross-talk between Ca2+/CaM, TGF-β, and RTK signaling.

Calmodulin enhanced the ventral mesoderm forming activity of Smad1 and blocked the activity of Smad2 *in vivo*. This is most likely due to the direct Ca2+-dependent association of these proteins observed in the *in vitro* experiments. These data suggest that Ca2+/CaM may be involved in the formation of mesoderm, not by acting as an inducer itself but rather as a modulator of known pathways. In contrast to our findings, a recent report suggested that calmodulin could reverse a Smad1-dependent whole embryo phenotype (67). One notable difference is that we microinjected mRNA encoding calmodulin, whereas in the report calmodulin protein was injected. Our results have been reproduced in many independent experiments, and we have not observed an inhibition of Smad1 action. The data presented here are consistent with a model in which activating calcium/calmodulin signaling should increase dorsal mesoderm formation. The experiments were performed with antibodies directed against GST demonstrated the presence of relatively equal amounts of GST-Smad fusion proteins (b). The analysis of the assays was performed as described in A and B. Labels are as in A.

with our data that increasing levels of Ca2+/CaM decreased dorsal mesoderm formation. However, recent data suggest that lithium may also act by inhibiting glycogen synthase kinase β (71). However, the initial studies with a lithium-dependent increase in dorsal mesoderm formation demonstrated that the effects were rescued by injection of inositol 1,4,5-trisphosphate, which suggests that the effect was due to an alteration in Ca2+/CaM signaling. Further support for a role of Ca2+/CaM in dorsal-ventral patterning is provided by experiments performed with a serotonin receptor. Ectopic expression of the serotonin 1C receptor, which activates both the phosphatidylinositol cycle and calcium/calmodulin signaling, ventralized whole embryos (72). In addition, the serotonin 1C receptor blocked the activin-dependent induction of the dorsal mesoderm in *Xenopus* embryos (72). This result is in concert with our findings that calmodulin inhibits Smad2-dependent generation of dorsal mesoderm and activates Smad1-dependent ventral mesoderm formation.

Further support for the idea that calcium/calmodulin signaling is important in embryonic patterning is garnered from studies on the early development of *Drosophila* embryos. In *Drosophila*, the dorsal ventral axis is reversed with respect to vertebrates, and the fly orthologue of Smad1, mothers against decapentaplegic, is critical to formation of dorsal fates (73). Hence, by analogy with our results with Smad1 in *Xenopus*, mothers against decapentaplegic activity and thus fly dorsal fates should be increased by calcium/calmodulin signaling. In concert with that notion, Creton et al. (74) reported the exist-
ence of a calcium gradient in Drosophila embryos with high calcium levels on the dorsal side. Of note, an elevated level of calcium was required for expression of dorsal-specific genes, and this increased calcium level-specified dorsal development. The authors (74) proposed that a high calcium level enhances decapentaplegic action. The reversal of the dorsal-ventral axis in Drosophila compared with chordates along with the evolutionary conservation of the decapentaplegic/BMP pathways implies a similar physiological mechanism for the specification of embryonic patterning in Xenopus (73, 75). Our data are consistent with that idea and suggest a role for calcium/calmodulin in dorsal-ventral patterning in vertebrates.

Through structure-function studies, we found that calmodulin binds to two distinct, and conserved, regions in both Smad1 and Smad2. Despite the large sequence diversity among calmodulin binding domains, some overall consensus features of these regions have been revealed (76). These features include the following: 1) a net positive charge; 2) clusters of basic residues, neighbored by hydrophobic residues; and 3) the tendency to form amphipathic α-helices. Another feature that is used to identify CaM-binding sites is the presence of hydrophobic residues at positions 1–8-14 or positions 1–5-10 of a given helical sequence. All of these attributes are found in both of the binding regions contained in Smad1 and Smad2. Both CBR-A and CBR-B have net positive charges (CBR-A: +4 in Smad1, +5 in Smad2; CBR-B: +2 in Smad1 and Smad2) and encompass clusters of basic amino acids that are flanked by hydrophobic residues. In addition, the Smad1 and Smad2 CBR-A and CBR-Bs contain stretches of amino acids that have been predicted to form α-helices based on the Smad3 crystal structure (61). CBR-A contains two helices, helix 1 and helix 2, either of which could bind calmodulin. However, whereas helix 1 fulfills all the calmodulin binding domain requirements, helix 2 shows some sequence variation between Smad1 and Smad2, and this increased calcium level-specified dorsal development. Of note, an elevated level of calcium was required for expression of dorsal-specific genes, and this, in turn, determines the calcium/calmodulin concentrations. Such regulation has been a mechanism for Smads to interpret intracellular changes in calcium, as binding of calmodulin blocks Erk2-dependent phosphorylation and, conversely, phosphorylation inhibits binding of calmodulin. Although the CBPs do not contain the Erk2 target sites in the primary structure, CaM binding and Erk2-dependent phosphorylation could possibly influence each other at a distance by changing the tertiary structure of the Smad protein or by physically blocking the accessibility of the phosphorylation sites.

The physiological relevance of our finding that calmodulin binding and Erk2 phosphorylation are reciprocally regulated remains unknown. The mechanistic basis of how calmodulin regulates the in vivo activity of Smad1 and Smad2 will require further study. The studies presented here may help explain the cross-talk taken between these three critical signaling cascades.

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