Communication

Smad Proteins Physically Interact with Calmodulin*

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The Smad family of intracellular proteins mediates signals generated by activin and other transforming growth factor β-related proteins via specific heteromeric complexes of transmembrane receptor serine kinases (1, 2). xSmad2 has been implicated as an activin signal mediator that may participate in transcriptional regulation (3, 4). We have employed an interaction cloning strategy to identify xSmad2-binding proteins and found that calmodulin directly associated with Smads. xSmad2, generated either by in vitro translation or by overexpression in COS cells, specifically bound to calmodulin-agarose; the association was calcium-dependent and required xSmad2 N-terminal residues. In the same assay, xSmad1 and hSmads 2, 3, and 4 also bound to calmodulin-agarose. Furthermore, a calmodulin antagonist, W13, increased expression of the activin-inducible transcriptional reporter, 3TP-Lux, whereas overexpression of calmodulin suppressed this reporter. These observations demonstrate that Smad proteins interact with calmodulin in a calcium-dependent way through conserved N-terminal amino acids and suggest a role for calmodulin in regulating Smad function.

Actins and structurally related family members including the transforming growth factors (TGF)β and bone morphogenetic proteins initiate signaling through heteromeric complexes of transmembrane receptors with intrinsic protein serine/threonine kinase activity (5). Extensive searches to identify receptor substrates by two-hybrid analysis have provided little insight into the mechanism of signal transmission. However, genetic analysis of both Drosophila melanogaster and Caenorhabditis elegans has led to the identification of a family of intracellular molecules that are required for signal propagation by TGFβ family members. Smad (a combination of the gene names from C. elegans, Smal, and Drosophila, Mad) homologs have now been identified in vertebrates including frogs, mice, and humans (1, 2). Functional analysis in Xenopus laevis indicates that overexpression of Smad2 mimics the effects of activin, whereas overexpression of Smad1 recapitulates bone morphogenetic protein effects (3); this result suggests that different ligands selectively employ specific Smads. The primary structure of the Smads, which contain highly conserved N-terminal and C-terminal domains separated by a divergent linker region, does not provide any clue as to the biochemical function of this family of molecules (1, 2).

Biochemical analysis of Smad2 indicates that this molecule is rapidly phosphorylated in response to both activin and TGFβ on extreme C-terminal serine residues (6). Bacterially expressed affinity-purified GST-Smad2 fusion protein can be phosphorylated by TGFβ receptor complexes immunoprecipitated from cultured cells, suggesting that Smad2 may be a direct receptor target (6). Phosphorylation of Smad2 in vivo correlates with its nuclear accumulation (6); the C-terminal domain of Smad2 expressed alone is constitutively localized in the nucleus (7). Furthermore, analysis of both Smad1 and Smad4 has shown that the conserved C-terminal domain is capable of inducing transcription when fused to a heterologous DNA-binding domain (8); based on the sequence conservation of this domain, it is expected that the other Smads may also demonstrate this function. Activin and TGFβ also lead to the specific association of Smad2 and Smad4 as assessed by immunoprecipitation from cultured cells following ligand binding (9). Smad2 has been identified as a component of an activin-specific transcriptional complex (4, 10), and there is now evidence that Mad directly binds DNA via its conserved N-terminal domain (11). These observations have led to the proposal that activin stimulates Smad2 phosphorylation via ActR complexes, which leads to nuclear accumulation and complex formation with Smad4 and participation in transcriptional activation of activin-responsive genes.

To identify proteins that physically and functionally interact with Smad2, we screened a bacteriophage expression library using radiolabeled Smad2 as probe. We have identified calmodulin as a Smad2-interacting protein; we demonstrate that the association is calcium-dependent and requires amino acids between Ile77 and Ala208 in the N-terminal half of Smad2. We also provide evidence that calmodulin can influence gene expression from an activin and TGFβ-responsive promoter.

EXPERIMENTAL PROCEDURES

Materials—Purified activin A was generously provided by Dr. Yuzuru Eto and by the National Institutes of Health Hormone Distribution Program, NIDDK, NHPP, NICHHD, and the USDA (lot 153663-36(1)). 4,5-Dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-thiazolidocarboxylic acid (d-Luciferin), N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13), N-(4-aminobutyl)-2-naphthalenesulfonamide (W12), thombin, and calmodulin-agarose were obtained from Sigma.

DNA Constructs—For expression in E. coli, xSmad2 cDNAs were subcloned into the vector pGEM-2T, which was modified to include a consensus protein kinase A phosphorylation site. For expression in mammalian cells, xSmad cDNAs provided by Jon Graff were subcloned into the vector pcDNA1 (Invitrogen) or into pcDNA1 modified to contain three iterations of the HA epitope tag at the C terminus of the protein (12); CaM cDNA (CaM A, locus MUSCAM, accession number M27844) was also subcloned into pcDNA1; a genomic CaM clone was provided by Tony Means. ActR DNAs and p3TP-Lux were as described (12). xSmad2 mutants and truncations were generated by polymerase chain reaction; all subclones were fully sequenced. Expression vectors for hSmads 2, 3, and 4 tagged with the FLAG epitope were generously provided by Dr. Rik Derynck.

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The abbreviations used are: TGF, transforming growth factor(s); CaM, calmodulin; GST, glutathione S-transferase; HA, hemaglutinin; RSK, receptor serine kinase; bHLH, basic helix-loop-helix; ALK, activin-like kinase.
xSmad2-CaM Interaction

Screening of a XElox Library—A λ XElox cDNA library from a 16-day mouse embryo was screened essentially as described (13). Phage were plated to yield 40,000 plaques/150-mm plate and incubated at 37 °C for 12 h before being overlaid with nitrocellulose circles impregnated with 1 ml isopropyl-β-D-thiogalactoside. Following an additional 12 h at 37 °C, filters were washed and probed as described (13). The xSmad2 probe was expressed in and purified from Escherichia coli as a GST fusion protein by standard methods (14) and cleaved from GST by incubation with thrombin before labeling as described (13). The labeled probe was then separated from unincorporated 32P on Sephadex G-50 Fine, Quick Spin Columns (Boehringer Mannheim) before incubation with the filters. Positive clones were plaque purified, and phage were converted to plasmids prior to double-stranded sequencing by standard methods (Sequenase version 2, U. S. Biochemical Corp./Amer sham Corp.).

In Vitro Binding Assay—Smads, generated by coupled in vitro transcription/translation using rabbit reticulocyte lysate (Promega), were mixed with 50 μl of either calcium or EGTA buffer-washed calmodulinagarose in 500 μl of either calcium or EGTA buffer. The mixture was incubated at 4 °C for 1 h with gentle rocking before being washed five times in the corresponding buffer. Bound protein was eluted by boiling in SDS sample buffer, resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions, and visualized by fluorography. Buffer consisted of 50 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM MgCl2, 0.1 mM dithiothreitol, 0.5% Triton X-100, and either 0.2 mM CaCl2 or 100 μM EGTA, with a mixture of protease inhibitors.

Luciferase Assay—The L17 cell line was cultured and transfected as described (12). Cells were transiently transfected with 0.5 μg of the p3TP-Lux reporter plasmid and combinations of 0.5 μg of calmodulin and 0.5 μg of ALK DNAs as indicated. Cells were co-transfected with a plasmid expressing β-galactosidase. Following transfection, cells recovered for 30 h and were then stimulated with 2 nM activin A, or 100 μM TGFβ in 0.25 ml of minimum essential medium containing 0.2% fetal bovine serum (v/v) with or without increasing amounts of W12 or W13 as indicated to induce transcription of 3TP-Lux. Luciferase activity was measured in 40 μl of a 200-μl lysate and normalized to β-galactosidase activity measured in 20 μl of lysate as described (12).

RESULTS

Cloning of xSmad2-binding Proteins—To identify proteins that functionally interact with xSmad2, we screened a bacteriophage expression library of 16-day mouse embryo cDNA using radiolabeled xSmad2 as probe. Screening of 1.5 × 108 total plaques resulted in the isolation of seven cDNAs encoding xSmad2-binding proteins. Partial cDNA sequencing indicated that all seven were CaM; five different cDNAs representing three CaM genes were recovered (Fig. 1). The longest cDNAs included the entire CaM coding region plus 57 5′ nucleotides that encode 19 additional amino acids. Two cDNAs encoded CaM lacking only the initial methionine, whereas one cDNA encoded only the 122 C-terminal amino acids of CaM (of 148 total), indicating that this region is sufficient for interaction with xSmad2.

The longest CaM cDNAs were also tested for interaction with two additional probes in this assay. One probe was a xSmad2 point mutant, G421D, which has been observed to result in a null phenotype in both C. elegans and D. melanogaster. The second probe consisted of only the C-terminal domain of xSmad2 between amino acids 261 and 467. Although xSmad2 (G421D) associated with phage-encoded immobilized CaM just as full-length wild-type xSmad2, the C-terminal domain of xSmad2 never associated with CaM in this assay.

In Vitro Association of Smads with Calmodulin—To determine whether xSmad2 associates with CaM in vitro, we tested xSmad2 for binding to CaM-agarose affinity resin. xSmad2, tagged with three iterations of the HA epitope at its C terminus, was transcribed and translated in vitro with [35S]methionine and then mixed with CaM-agarose in the presence of calcium or EGTA. xSmad2 was retained on CaM-agarose in the presence of calcium but not in its absence. xSmad2 was also retained on CaM-agarose in the presence of calcium when a peptide corresponding to the HA epitope was included in the binding reaction but not when a known CaM-binding peptide was present (Fig. 2). Similar results were obtained using xSmad2 generated by overexpression in COS cells (data not shown). The slight difference in mobility of in vitro transcribed/translated Smads following incubation in the binding mixture compared with input (Figs. 2 and 3) was not apparent when Smads were generated by overexpression in COS cells; furthermore, this difference was not dependent on Ca2+ or CaM, nor was it the result of phosphorylation.

We used the same assay to determine whether other members of the Smad family of proteins associate with CaM. xSmads 1 (Fig. 2), 8, and 9 tagged with the triple-HA epitope at the C termini and hSmads 2, 3, and 4 C-terminally tagged with the FLAG epitope all behaved similarly to xSmad2 with respect to calcium-dependent binding to CaM-agarose. These observations indicate that Smads can physically associate with CaM in...
a calcium-dependent manner.

Mapping the CaM-binding Domain of xSmad2—To determine which region of xSmad2 is responsible for binding to CaM, we generated deletion mutants encoding either the conserved N-terminal domain with the linker region (amino acids 1–263) or the conserved C-terminal domain (amino acids 261–467) of the molecule and tested these proteins for association with CaM as described above. In the presence of calcium, both wild-type xSmad2 and xSmad2-N were retained on CaM-agarose, whereas xSmad2-C was not (Fig. 3). This observation is consistent with the earlier observation that the purified radio-labeled C-terminal domain of xSmad2 did not bind phage-encoded CaM on nitrocellulose filters.

To further localize the CaM-binding domain of xSmad2, we used additional deletion mutants that lack increasing amounts of the N terminus: xSmad2 mutants lacking the initial 26 (xSmad2 ΔN25), 76 (xSmad2 ΔN75), or 208 amino acids (xSmad2 ΔN208) were tested for binding to CaM (Fig. 3). Wild-type xSmad2, xSmad2 ΔN25, and xSmad2 ΔN75 all were retained on CaM-agarose, whereas xSmad2 ΔN208 was not. Therefore, xSmad2 interacts with CaM through amino acids between Ile77 and Ala208 in the N-terminal half of xSmad2.

CaM Function in Activin Signaling—Because CaM physically associates with Smads and may therefore regulate Smad function, we tested whether CaM could modulate expression of an activin-responsive reporter gene. L17 mink lung epithelial cells stably transfected with the type I activin receptor, ALK4, were treated with a calmodulin antagonist, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13) or its 10-fold less active chemical analog, N-(4-aminobutyl)-2-naphthalenesulfonamide (W12) as a negative control. Activin responsiveness was evaluated by measuring expression of an activin-inducible reporter gene, 3TP-Lux. In the absence of calmodulin inhibitor, a 4-fold activin-dependent increase in luciferase expression from 3TP-Lux was observed. Similarly, at all doses of W12, activin treatment resulted in a 4–5-fold increase in luciferase expression with no changes in overall levels of luciferase expression relative to cells not treated with inhibitor. In contrast, treatment with W13 resulted in a dose-dependent proportional increase in overall luciferase expression both in the presence and the absence of activin (Fig. 4A).

Because inhibition of CaM function led to increased luciferase expression from 3TP-Lux, we tested the effect of overexpression of CaM in the same assay. L17 cells were transiently transfected with cDNAs encoding the indicated type I receptor serine kinases and CaM. Following treatment with either activin or TGFβ, luciferase expression was measured as described. Activin treatment of cells expressing only ALK4 resulted in a 10-fold induction of luciferase; although cells expressing both ALK4 and CaM responded to activin in a similar manner, overall expression of luciferase was reduced 2-fold (Fig. 4B). Treatment of cells expressing ALK5 with TGFβ yielded similar results; with no exogenous CaM, luciferase expression was induced 10-fold, whereas CaM overexpression reduced overall luciferase expression 2-fold (Fig. 4B). These data demonstrate that CaM can negatively regulate gene expression from an activin- and TGFβ-responsive promoter.

DISCUSSION

Smad proteins are responsible for mediating signals initiated by specific receptor serine kinases (RSKs) in response to activin and other TGFβ-related ligands. The central role of this family of molecules in RSK signaling is highlighted by the observation that cells compromised for either Smad2 or Smad4 activity are resistant to RSK activation. Because 1) Smads accumulate in the nucleus following ligand treatment, 2) Smad2 has been identified as a component of activin-dependent DNA-binding complexes, 3) Mad binds DNA directly, and 4) Smads1 and 4 can support transcriptional activation when fused to a heterologous DNA-binding domain, Smads are thought to act as transcriptional regulators. We have shown that Smads are capable of interacting with CaM; the interaction is dependent on calcium and requires at least residues between Ile77 and Ala208 in the N-terminal portion of Smad2. This region contains a putative (residues Leu123 to Trp132) 1-5-10 basic amphiphilic α-helix, which is a known CaM target motif (15), and may be responsible for mediating this interac-
CaM is the primary intracellular receptor for calcium ions and mediates the effects of signals that cause intracellular calcium flux (16). Calcium-saturated CaM binds to and regulates a wide variety of well-characterized enzymes, in addition to structural proteins of the cytoskeleton and transcription factors (16). Ca^{2+}/CaM can stimulate transcription factors such as c-Fos, c-Jun, cAMP response element-binding protein, and serum response factor indirectly via activation of CaM-dependent kinases (17) and can inhibit specific members of the basic helix-loop-helix (bHLH) family of transcription factors by direct protein-protein interaction (18–20). The mechanism of transcriptional inhibition of the CaM-sensitive class of bHLH proteins involves direct binding to the bHLH domains, which interferes with dimerization and prevents DNA binding (20–22). A recent report demonstrates that Mad is capable of direct sequence-specific DNA binding via an N-terminal domain requiring residues between 120 and 159 (11). This region corresponds to that of Smad2 which is required for CaM binding, and suggests that CaM may also inhibit Smad2 by interfering with DNA binding. Alternatively, CaM may interfere with assembly of the transcriptional complex formed in response to activin, which includes Smad2 and a winged helix DNA-binding protein, FAST-1 (4, 10). Although CaM is found throughout the cell, it is also possible that CaM regulates the subcellular distribution of Smads.

The potential role of calcium as a mediator of activin signaling has been investigated, but no consensus has been reached (5). We measured intracellular calcium in L17 cells stably transfected with ALK4 and did not observe any change when they were assayed under conditions identical to those that yielded induction of luciferase expression from 3TP-Lux (data not shown). The precise mechanism by which CaM negatively regulates gene expression from activin-responsive promoters remains to be elucidated; however, the identification of a calcium-dependent physical association between Smads and CaM suggests complex regulation of Smad function. Biochemical analysis of the role of CaM in regulating Smad2 activity will provide additional insight into the process of activin signal transduction.

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FIG. 4. Effect of calmodulin on activin/TGFβ-dependent 3TP-Lux expression. A, L17 cells stably expressing ALK4 were transfected with 3TP-Lux and treated with 2 nM activin A in the presence of either W12 or W13, and luciferase activity and β-galactosidase activity were measured. Black bars, – activin; hatched bars, + activin. B, L17 cells were transfected with 3TP-Lux together with various combinations of ALK4 or ALK5 and CaM. Cells were treated with 2 nM activin A or 100 μM TGFβ, and luciferase activity and β-galactosidase activity were measured. The data were normalized with the level of expression in the absence of exogenous receptors and the absence of ligand set to one. Left panel, black bars, – activin; hatched bars, + activin. Right panel, black bars, – TGFβ; hatched bars, + TGFβ. Values were determined in triplicate and represent the means ± S.D.