Elucidation of Smad Requirement in Transforming Growth Factor-β Type I Receptor-induced Responses*

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Transforming growth factor-β (TGF-β) elicits cellular effects by activating specific Smad proteins that control the transcription of target genes. Whereas there is growing evidence that there are TGF-β type I receptor-initiated intracellular pathways that are distinct from the pivotal Smad pathway, their physiological importance in TGF-β signaling is not well understood. Therefore, we generated TGF-β type I receptors (also termed ALK5s) with mutations in the L45 loop of the kinase domain, termed ALK5(D266A) and ALK5(3A). These mutants showed retained kinase activity but were unable to activate Smads. Characterization of their signaling properties revealed that the two L45 loop mutants did not mediate Smad-dependent transcriptional responses, TGF-β-induced growth inhibition, and fibronectin and plasminogen activator-1 production in R4-2 mink lung epithelial cells lacking functional ALK5 protein. Mutation in the L45 loop region did not affect the binding of inhibitory Smads but did abrogate the weak binding of X-linked inhibitor of apoptosis protein and Disabled-2 to ALK5. This suggests that the L45 loop in the kinase domain is important for docking of other binding proteins. Interestingly, JNK MAP kinase activity was found to be activated by the ALK5(3A) mutant in various cell types. In addition, TGF-β-induced inhibition of cyclin D1 expression and stimulation of PMEPA1 (androgen-regulated prostatic mRNA) expression were found to occur, albeit weakly, in an Smad-independent manner in normal murine mammary gland cells. However, the TGF-β-induced epithelial to mesenchymal transdifferentiation was found to require an intact L45 loop and is likely to be dependent on the Smad pathways.

Transforming growth factor-β (TGF-β) Belongs to a family of cytokines that regulate cell proliferation and differentiation of

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§ The abbreviations used are: TGF-β, transforming growth factor-β; ALK, activin receptor-like kinase; AP, activating protein; Dab-2, Disabled-2; DMEM, Dulbecco's modified Eagle's medium; FAST, forkhead activin signal transducer; FCS, fetal calf serum; GFP, green fluorescent protein; GMP-140, granulocyte/macrophage colony-stimulating factor; GST, glutathione S-transferase; IRES, intra-ribosomal entry site; JNK, c-Jun N-terminal kinase; MAP, mitogen protein kinase; Mv1Lu, mink lung epithelial cells; NMuMG, normal murine mammary gland cells; PAI-1, plasminogen activator inhibitor-1; PMEPA1, androgen-regulated prostate specific mRNA; PBS, phosphate-buffered saline; pS2, phospho-Smad2 antibody; Smad, Smad-related protein; TAK1, many different cell types (1). TGF-β family members, which include TGF-βs, activins, and bone morphogenetic proteins, were found to possess critical roles during embryogenesis and in maintaining tissue homeostasis during adult life. Deregu-

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TGF-β-activating kinase 1; TjR, TGF-β receptor; XIAP, X-chromosome-linked inhibitor of apoptosis; HA, hemagglutinin; CMV, cytomegalovirus; MOPS, 4-morpholineproanesulfonic acid.
Both rapid (within 10–30 min) as well as delayed (after several hours) activation of MAP kinase have been reported, of which the delayed activation has been shown to depend on Smad pathways (19). The mitogen-activated protein kinase TGF-β-activated kinase 1 (TAK1) has been shown to be phosphorylated upon TGF-β stimulation and can lead to the activation of JNK and p38 MAP kinase pathways (17, 23, 24). Interestingly, TAK-1 also activated TGF-β-induced expression of fibronectin and inhibition of insulin-like growth factor-binding protein-5 or NOV, a secreted glycoprotein, were shown to cooperate with each other in transcriptional responses by forming direct protein interactions (32–37). In addition, TGF-β-induced activation of p38 MAP kinase was shown to induce ATF-2 phosphorylation, which acted synergistically with Smads in transcriptional activation (38). JNK was found to be rapidly activated by TGF-β in mink lung epithelial cells (Mv1Lu) and to facilitate TGF-β-induced Smad activation (19). In contrast, activation of extracellular-regulated kinase MAP kinase has also been shown to induce the phosphorylation in the linker region of R-Smads and, thereby, to inhibit the ligand-induced nuclear accumulation of R-Smads (39–41).

To investigate the pathways that are activated in the absence of Smad activation we have generated a TGF-β type I receptor (ALK5) with mutations in the L45 loop of the kinase domain that are defective in Smad activation and characterized the signaling properties of these mutated type I receptors.

### EXPERIMENTAL PROCEDURES

#### Expression Plasmids—
All of L45 mutants in human ALK5 were made by the QuikChange site-directed mutagenesis kit (Stratagene) using pCDNA3-ALK5/HA and pCDNA3-ALK5/HA as templates. pMEP-ALK5/HA, pMEP-ALK5/2D66A/HA, and pMEP-ALK5/3A/HA were constructed by ligation of the insert from pCDNA3-ALK5/HA or its mutants with pMEP4 (Invitrogen). The chimeric constructs between GM-CSF αR and ALK5 (pNA3) and between GM-CSF βR and TβRII (pHA-βRII) were provided by Dr. E. B. Leof (42). To create chimeric constructs between human GM-CSF αR and human ALK5/HA, the intracellular domain of ALK5 from pNA3 was substituted into the corresponding domain of ALK5 from pCDNA3-ALK5/HA or its mutants. Subsequently, the inserts of chimeric constructs were ligated into the LZRS-pBMN-ms(-IRES)-eGFP retroviral vector constructed from LZRS-pBMN-LacZ, which was obtained from Dr. G. Nolan. pBabe-LZRS-pBMN-ms(-IRES)-eGFP retroviral vector constructed from pMEP-ALK5/HA, pMEP-ALK5(D266A)/HA, and pMEP-ALK5(3A)/HA was subcloned into the pAdEasy-1, the obtained plasmids were transfected into 293T cells with lysis buffer three times, the proteins in immunoprecipitates and protein G-Sepharose beads were added to the reaction mixture and incubated with FLAG M5 antibody (Sigma) for 2 h at 4 °C. Subsequently, protein G-Sepharose beads were added to the reaction mixture and incubated with FLAG M5 antibody (Sigma) for 2 h at 4 °C. The lysates were precleared with protein G-Sepharose beads. The lysates were precipitated with anti-ALK5 and anti-βRII antibodies. Western blotting was performed using 4–20% SDS-PAGE. The expression of αALK5 or its mutants was linked to that of GFP, both GFP- and βRII-positive cells were collected by fluorescence-activated cell sorter to get double-positive cells.

#### Transcriptional Reporter Assays—
One day before transfection, pMv1Lu or R4-2 cells were seeded at 4.0 × 10⁵ cells/well in 12-well plates. The cells were transfected using FuGENE 6. Where indicated, TGF-β3 was added into the dishes 24 h after transfection. Subsequently, the cells were cultured in DMEM containing 0.3% FCS for 18 h. For luciferase (pCH10) assays, luciferase activity was measured to normalize for transfection efficiency. Each transfection was carried out in triplicate and repeated at least twice.

#### Immunoprecipitation and Western Blotting—To detect Smad2 phosphorylation by constitutively active (ca) ALK5 or its mutant derivatives in COS7 cells, 9 μg of pDE3-FLAG-Smad2 and 3 μg of caALK5 or its mutant derivatives were transfected in COS7 cells at 1.5 × 10⁵ cells/10-cm dish using FuGENE 6. Forty hours after transfection, the cells were lysed in 1 ml of lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2.5 μg/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF, and 20 mM β-glycerolphosphate). The cell lysates were pre clarified with protein G-Sepharose. The lysates were precipitated with FLAG M5 antibody (Sigma) for 2 h at 4 °C. Subsequently, protein G-Sepharose beads were added to the reaction mixture and incubated for 30 min at 4 °C. After washing the immunoprecipitates with lysis buffer three times, the proteins in immunoprecipitates and aliquots of total cell lysates were separated by SDS-PAGE and transferred to a Hybond-C Extra membrane (Amersham Biosciences). The membrane was subsequently probed with phosphorylated Smad2-specific antibodies (pS2) (8) or FLAG M5 antibody. Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Amersham Biosciences) and chemiluminescent substrate. The expression of ALK5 receptors was determined by immunofluorescence of the (CAGA)₁₂-luc reporter. The detection of the interactions between caALK5 and 1-Smads, between caALK5 and XIAP, or between ALK5 and Dab-2 was performed by immunoprecipitation followed by Western blotting according to the above method, except that cells were lysed in TNE buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2.5 μg/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF, and 20 mM β-glycerolphosphate). The expression of proteins in R4-2 or NuMuG stable transfectants was detected by Western blotting of total cell lysates in TNE buffer. pS2 antibody for phosphorylated Smad2, anti-βRII antibody (Transduction Laboratories) for total Smad2, anti-HA12CA5 antibody (Roche Molecular Biochemicals) for HA, and anti-FLAG M5 antibody for βRII chimera were used as primary antibodies.

#### Receptor Autophosphorylation Assay—
One day before transfection, COS7 cells were seeded at 2 × 10⁵ cells/3.5-cm dish. The cells were transfected with pCDNA3-caALK5/HA or its mutant derivatives by the above procedure. Because the expression of αALK5 or its mutants was monitored by fluorescence-activated cell sorter to get double-positive cells.
ties by FuGENE 6. Forty hours later, the cells were lysed with lysis buffer, precleared with protein G-Sepharose beads, and incubated with anti-HA12CA5 antibody for 2 h at 4 °C. Protein G-Sepharose beads were then added to the reaction mixture, and incubation was continued another 30 min at 4 °C. The immunoprecipitates were washed with lysis buffer three times and with the kinase reaction buffer (10 mM Tris (pH 7.4), 10 mM MgCl₂, and 2 mM MnCl₂) twice and then incubated with kinase buffer (10 mM Tris (pH 7.4), 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 25 °C. Immunoprecipitates were separated by SDS-PAGE. The expression of receptor proteins was determined by 35S-metabolic labeling and immunoprecipitation (44).

**Growth Inhibition Assay**—R4-2 transformants were seeded at 1 × 10⁶ cells/well in 24-well plates. Before the addition of TGF-β3, the cells were simultaneously treated (or not treated) with 100 μM ZnCl₂ to inhibit JNK activity. Two hours later, the cells were washed once with PBS, fixed with PBS, incubated with 148 kBq/ml 35S-labeling mixture containing 14C-17; Santa Cruz) was used for immunoprecipitations. The anti-HA (pH 7.4), 10 mM MgCl₂, and 2 mM MnCl₂) twice and then incubated with kinase buffer (10 mM Tris (pH 7.4), 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 25 °C. Immunoprecipitates were separated by SDS-PAGE. The expression of receptor proteins was determined by 35S-metabolic labeling and immunoprecipitation (44).

**Protein G-Sepharose beads** and incubated with FLAG M2 (Sigma) antibody for 30 min at 4 °C. The cells were then washed twice with PBS, incubated 1 μCi of [3H]thymidine (Amersham Biosciences) for 30 min at 25 °C. Immunoprecipitates were separated by SDS-PAGE. The expression of receptor proteins was determined by 35S-metabolic labeling and immunoprecipitation (44).

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FIG. 1. Identification of ALK5 L45 mutants that are defective in Smad activation, but with retained kinase activity. A, schematic representation of ALK5 and location of L45 loop in the kinase domain. TM, transmembrane domain. GS, glycine-serine rich domain. BMPR, bone morphogenetic protein receptor. B, comparison of amino acid sequences in L45 loop region among different human ALKs. The amino acid residues that were selected for mutation into alanine residues are indicated with numbers, referring to their position in the ALK5 sequence. The two β-strands (β4 and β5) that flank the L45 loop are shown as arrows. C, in vitro autophosphorylation activity of caALK5 and wild type ALK5 and various L45 mutant derivatives. ALK5 constructs were transfected into COS7 cells, immunoprecipitated (IP) with anti-HA antibody, and subjected to in vitro autophosphorylation. D, in vitro autophosphorylation activity of caALK5 and wild type ALK5 and various L45 mutant derivatives. ALK5 constructs were transfected into COS7 cells, immunoprecipitated (IP) with anti-HA antibody, and subjected to in vitro autophosphorylation.
Smad2 in transfected COS7 cells. Among the three mutants with equivalent kinase activity compared with caALK5, caALK5(D266A) and caALK5(3A) did not phosphorylate Smad2 (Fig. 1D). In addition, we could not see any enhancement of Smad1 phosphorylation by caALK5 or caALK5 mutants (data not shown).

To examine whether ALK5(D266A) and ALK5(3A) can activate Smad-dependent reporter activity, we first transfected caALK5 or its mutants in Mv1Lu cells. As expected, caALK5 dramatically induced (>70-fold) Smad-dependent luciferase activity. However, caALK5(D266A) and caALK5(3A) exhibited only 4.1- and 2.3-fold activation, respectively (Fig. 1E). ALK5 is known to be present in cells as a dimer in the absence of ligand (61). It is possible that the exogenous caALK5 L45 mutants can make complexes with endogenous ALK5, which can explain the low residual activity of caALK5 L45 mutants. Thus, introduction of ALK5 L45 mutant in the cells, which possess endogenous functional ALK5, may not lead to a complete Smad-independent signaling. Therefore, we transfected ALK5 L45 mutants in R4-2 cells that lack functional ALK5. As seen in Fig. 1F, neither ALK5(D266A) nor ALK5(3A) activated (CAGA)12-Luc (i.e. a readout for the activation of the Smad3/4 pathway) or 2×ARE-luc reporter (i.e. a read-out for the Smad2/Smad4 pathway). These results were fully consistent with the inability of ALK5(D266A) and ALK5(3A) to induce Smad2 phosphorylation. We therefore used ALK5(D266A) and ALK5(3A) in the following experiments to examine Smad-(in)dependent signaling.

ALK5 L45 Mutants Do Not Rescue TGF-β-induced Growth Inhibition, Fibronectin, and PAI-1 Production in Mink Cells That Are Deficient in ALK5—Mv1Lu cells are potently inhibited in their growth and produce high levels of fibronectin and PAI-1 upon TGF-β stimulation. To elucidate the abilities of ALK5(D266A) and ALK5(3A) to mediate these responses, we stably transfected these receptors in R4-2 cells that are deficient in functional ALK5. All receptor constructs were placed to an autophosphorylation reaction. Expression controls for ALK5 are shown below by immunoprecipitation of 35S-labeled cell lysates from parallel transfected COS7 cells with anti-HA antibody. D, effect of caALK5 and L45 mutant derivatives on Smad2 phosphorylation. caALK5 or L45 mutant derivatives were transiently co-transfected with FLAG-Smad2 into COS7 cells. The level of C-terminal Smad2 phosphorylation was determined by Western blotting (WB) with anti-phospho-Smad2 (pS2) antibody. Expression controls for ALK5 and Smad2 are shown below. E, caALK5 L45 mutants activate Smad-dependent luciferase reporter weakly in Mv1Lu cells. caALK5 and its derivatives were transiently transfected with (CAGA)12-Luc into Mv1Lu cells. Luciferase values were normalized for transfection efficiency. Part of the original figure is expanded in the inset to compare the values of caALK5 L45 mutants with those of mock transfection. All values represent the mean ± S.D. Significantly different from the mock: *p < 0.05; **p < 0.005; F, effect of ALK5 and L45 mutant derivatives on TGF-β-induced transcriptional responses. ALK5 and L45 mutant derivatives were transiently transfected with (CAGA)12-Luc (left panel) or ARE-Luc (right panel) into R4-2 cells that lack functional ALK5 and treated with TGF-β, and transcriptional response was determined by measuring luciferase activity. Luciferase values were normalized for transfection efficiency. All values represent the mean ± S.D.
Fig. 3. ALK5(3A) mutant interacts with I-Smads but not with XIAP nor Dab-2. A, caALK5 or caALK5(3A) mutant was cotransfected with 6×Myc-Smad6 or 6×Myc-Smad7. To show interaction between ALK5 and I-Smads, the cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody followed by Western blotting (WB) with anti-Myc antibody. Expression controls for ALK5 or I-Smads are shown below by Western blotting on total cell lysates. B, caALK5 without or with 3A mutation of the L45 loop was cotransfected with expression constructs for GST-XIAP. To show interaction between components, cell lysates were first subjected to immunoprecipitation with anti-HA antibody followed by Western blotting with anti-GST antibody. Expression controls are shown below by Western blot analysis of total cell lysates. C, ALK5 or ALK5(3A) was cotransfected with FLAG-Dab-2 in COS7 cells. The cell lysates were immunoprecipitated with anti-HA antibody followed by Western blotting with FLAG M5 antibody. The expression of ALK5 and Dab-2 is shown below by Western blotting of total lysates.

under the transcriptional control of the metallothionein promoter, which can be induced by ZnCl2. Expression analysis of type I receptors in R4-2 stable transfectants revealed ZnCl2-inducible expression of wild type ALK5, ALK5(D266A), and ALK5(3A) (Fig. 2A). Consistent with the experiment in COS7 cells, Smad2 phosphorylation by TGF-β was observed only in wild type ALK5-expressing R4-2 cells after the addition of ZnCl2 (data not shown). We then investigated whether ALK5(D266A) and ALK5(3A) mutants could be substituted for wild type ALK5 with respect to TGF-β-induced growth inhibition (Fig. 2B), fibronectin, and PAI-1 protein production (Fig. 2C). However, ALK5(D266A) and ALK5(3A) mutants were not able to mediate these responses. Thus, they appear to depend on an intact L45 loop and are likely to be Smad-dependent responses. Consistent with the results obtained from R4-2 cells that express ALK5 L45 mutants, TGF-β did not induce fibronectin mRNA levels in MDA-MB-468 cells that are deficient in Smad4. This TGF-β-induced response with delayed kinetics (induction after 12 h, but not 2 h) could be rescued after Western blotting with anti-GST antibody. Expression controls are shown below by Western blot analysis of total cell lysates. C, ALK5 or ALK5(3A) was cotransfected with FLAG-Dab-2 in COS7 cells. The cell lysates were immunoprecipitated with anti-HA antibody followed by Western blotting with FLAG M5 antibody. The expression of ALK5 and Dab-2 is shown below by Western blotting of total lysates.

ALK5 L45 Mutant Interacts with I-Smads but Not XIAP or Dab-2—I-Smads bind to activated type I receptors and can compete with R-Smads for receptor binding (62–64). However, the domain(s) in the type I receptor responsible for I-Smad binding have not been characterized. To test the interaction of Smad6 or Smad7 with ALK5(3A) mutant, I-Smads and receptors were transfected into COS7 cells and subjected to immunoprecipitation followed by Western blotting. The ALK5(3A) mutant was found to interact with Smad6 or Smad7 as efficiently as wild type ALK5 (Fig. 3A). This suggests that the L45 loop is not important for interaction with I-Smads. However, when we analyzed the interaction of two other components known to bind type I receptors, i.e. XIAP (47) (Fig. 3B) and Dab-2 (10) (Fig. 3C), we found that none of them interacted with the ALK5(3A) mutant using a similar strategy as above. The L45 loop region may, thus, not only be important for Smad binding but also for interaction with other signaling components.

ALK5 L45 Mutant Activates JNK MAP Kinase—TGF-β has been shown to activate JNK MAP kinase in certain cell types (18, 19, 21, 27–30). We investigated the ability of caALK5 or caALK5(3A) to induce phosphorylation of c-Jun in 293T cells. 293T cells were chosen because they have very little endogenous ALK5 and can be efficiently transfected. Expression constructs for HA-tagged caALK5 or caALK5(3A) was co-transfected with an expression construct for JNK in 293T cells, and cell lysates were subjected to immunoprecipitation with antiFLAG M5 antibody followed by in vitro kinase reaction using GST-c-Jun 1–135 as a substrate. Like caALK5, caALK5(3A) was found to potently activate JNK (Fig. 4A). In contrast, the phosphorylation of Smad2 in 293T cells was not enhanced by caALK5(3A) but was dramatically induced by caALK5 (data not shown). Consistent with these results, TGF-β induced a significant JNK activation in R4-2 cells that were infected with wild type ALK5 or ALK5(3A) mutant adenoviruses. Peak levels of JNK activation were reached 10 min after TGF-β challenge (Fig. 4B). TGF-β-induced Smad2 phosphorylation in R4-2 cells expressing wild type ALK5 but not in cells expressing ALK5(3A) mutant (Fig. 4B). Thus, TGF-β-induced JNK activation occurs in a Smad-independent manner. Further support for ALK5(3A)-induced activation of JNK was obtained by analyzing the activation of Gal4-c-Jun in R4-2 cells. caALK5(3A) significantly induced the Gal4-c-Jun transcriptional reporter, albeit weaker than caALK5 (Fig. 4C, upper panel). Consistent with the Gal4-c-Jun assay, the activation of pAP-1-Luc, which can be activated by JNK, was also weakly induced by caALK5 (Fig. 4C, middle panel). Transfection of ALK5(3A) in R4-2 cells mediated a 2-fold activation of pAP-1-Luc reporter in response to TGF-β (Fig. 4D). In contrast, the Gal4-CHOP assay, which exhibits a readout of the activated p38 pathway (65), was not significantly affected by caALK5(3A) but was influenced by caALK5 (Fig. 4C, lower panel).

Characterization of Chimeric GM-CSF/TGF-β Receptors—GM-CSF is known to be a species-specific ligand. Chimeric receptors between GM-CSF and TGF-β receptors have shown that TGF-β signaling can be reconstituted in a system independent of TGF-β ligand (42, 66). We generated a chimeric receptor between the extracellular domain of GM-CSF αR and the intracellular domain of ALK5(3A) or ALK5(D266A), which
allowed us to study TGF-β signaling in a cell upon stimulation with GM-CSF when co-transfected with GM-CSF βRII/ALK5 chimera. Both chimeric receptor chains were subcloned in retroviral expression vectors. After infection of NMuMG cells, chimeric receptor-expressing clones were sorted by fluorescence-activated cell sorter analysis. We analyzed TGF-β- or GM-CSF-induced Smad2 phosphorylation in wild type NMuMG cells and in cells expressing GM-CSF βRII/ALK5 chimera alone (termed βII) or together with GM-CSF αRI/ALK5 (termed αRI/βII), GM-CSF αRI/ALK5(D266A) (termed αRI(D266A)/βII), or GM-CSF αRI/ALK5(3A) (termed αRI(3A)/βII) (Fig. 5A). As expected, TGF-β induced Smad2 phosphorylation in all cells, whereas GM-CSF only induced Smad2 phosphorylation in cells expressing αRI/βII but not in other cell clones (Fig. 5B). Thus, consistent with previous results, the intracellular domain of neither ALK5(D266A) nor ALK5(3A) induced Smad2 phosphorylation when the chimeric receptors were activated. In addition, GM-CSF stimulated a significant increase in AP1-Luc activity in αRI(3A)/βII cells. In-

Fig. 4. The ALK5(3A) mutant activates JNK MAP kinase. A, to test the effect of ALK5 and the ALK5(3A) mutant on JNK activation, HA-tagged expression constructs encoding these receptors were cotransfected with FLAG-tagged JNK into 293T cells. Cell lysates were immunoprecipitated with anti-FLAG M2 antibody and subjected to an in vitro phosphorylation assay using GST-c-Jun 1-135 as a substrate (upper panel). Expression controls for ALK5 (middle panel) and JNK (lower panel) are shown by Western blots (WB) on total cell lysates with anti-HA and anti-FLAG M2 antibodies, respectively. Kinase activity was calculated relative to the value in the absence of ALK5. B, R4-2 mink cells were infected with ALK5 adenoviruses. Infected cells were stimulated with 10 ng/ml TGF-β3 for the indicated times. Cell lysates were immunoprecipitated with anti-JNK1 antibody and subjected to an in vitro phosphorylation assay using GST-c-Jun 1-135 as a substrate (upper panel). Expression for ALK5 (middle panel) and phosphorylated Smad2 (lower panel) are shown by Western blots on total cell lysates with anti-HA and anti-pS2 antibodies, respectively. Relative kinase activity was calculated with each value of non-stimulated cells. C, R4-2 mink cells were transfected with Gal4-cJun, pFR-Luc reporter, and JNK together with constructs for caALK5 or caALK5(3A) (top panel), AP-1-Luc reporter and JNK together with expression constructs for caALK5 or caALK5(3A) (middle panel), and Gal4-CHOP, pFA-chop, and HA-p38 together with expression constructs for caALK5 or caALK5(3A) (bottom panel). Luciferase values normalized for transfection efficiency are shown. All values represent the mean ± S.D. Significantly different from the mock: *, p < 0.05; **, p < 0.005. D, R4-2 mink cells were transfected with AP-1-Luc reporter and JNK together with expression constructs for ALK5(3A). Twenty-four hours after transfection, the cells were stimulated with 10 ng/ml TGF-β3 for 18 h. Luciferase values normalized for transfection efficiency are shown. All values represent the mean ± S.D. Significantly different from the mock in the absence of TGF-β3: *, p < 0.05.
Interestingly, the luciferase activity was higher after 6 h compared with 18 h of GM-CSF stimulation (Fig. 5).

**GM-CSF/ALK5 L45 Mutants Can Modulate Gene Expression but Fail to Induce Stress Fibers in NMuMG Cells**—Transcriptional analysis on \( \beta II \)- and \( \alpha III \)-expressing NMuMG cells upon stimulation with GM-CSF indicated that the genomic response is much stronger in the presence than the absence of Smad signaling. Among the limited group of genes that were found to be up-regulated and down-regulated by GM-CSF (and TGF-\( \beta \)) in \( \beta II \)- and \( \alpha III \)-expressing cells are PMEPA1 and cyclin D1, respectively (Fig. 6, A and B). Wild type ALK5 was found to mediate a stronger signal than ALK5(3A) mutant; this suggests that both Smad-dependent and Smad-independent signaling are needed to efficiently regulate these genes by TGF-\( \beta \). PMEPA1 was initially identified as an androgen-regulated prostatic mRNA (67) without functional annotation. Cyclin D1 was previously shown to be a target of TGF-\( \beta \) (68) and has been implicated in TGF-\( \beta \)-induced growth arrest.

NMuMG cells transdifferentiate from an epithelial phenotype to a spindle-shaped morphology in response to TGF-\( \beta \) as can be demonstrated by a reorganization of the actin cytoskeleton (69, 70). We therefore analyzed \( \beta II \), \( \alpha III \)- and \( \alpha III \)-expressing Swiss3T3 transformants for TGF-\( \beta \) and GM-CSF-induced stress fiber formation. As expected, we found that TGF-\( \beta \) induced stress fiber formation in all cell lines (Fig. 6C), whereas GM-CSF induced stress-fiber formation in \( \beta II \)- but not in \( \alpha III \)- or \( \alpha III \)-expressing Swiss3T3 transformants (data not shown). Thus, an intact L45 loop is critical for ALK5-mediated induction of stress fibers.

**DISCUSSION**

Smads are pivotal intracellular mediators of TGF-\( \beta \)-induced responses by relaying the signal from the activated TGF-\( \beta \).
receptor to the nucleus, where they affect the transcription of target genes. TGF-β-induced responses that are independent of Smads have been reported (11). However, their physiological importance is not well understood. Therefore, we investigated the pathways that are activated in mutant TGF-β type I receptors (ALK5s) defective in Smad activation but with retained kinase activity. Most of the TGF-β-induced responses in a variety of cell lines that we examined, including growth inhibition, fibronectin and PAI-1 protein production, and stress fiber formation, were dependent on having an ALK5 with in-

Fig. 6. GM-CSF/ALK5 L45 mutants can mediate effects on gene expression of specific genes but fail to induce stress fibers in NMuMG cells. The expression of PMEPA1 and cyclin D1 were enhanced and decreased in the chimeric GM-CSF/ALK5 L45 mutant stimulated with GM-CSF, respectively. A, Northern blot using total RNA from each transformant stimulated with 10 ng/ml TGF-β3 (T) or 50 ng/ml GM-CSF (G) for 1 and 6 h was performed using PMEPA1 as a probe (upper panel). Relative expression levels (normalized using 28 S) compared with non-stimulated cells are indicated. Equal loading of RNA samples is shown by ethidium bromide stain of gel before Northern blotting (lower panel). B, cyclin D1 mRNA in NMuMG transformants stimulated with 10 ng/ml TGF-β3 (T) or 50 ng/ml GM-CSF (G) for the indicated times was detected by Northern blot analysis (upper panel). Relative expression levels (normalized using 28 S) compared with non-stimulated cells are indicated. Equal loading of RNA samples is shown by ethidium bromide stain of gel before Northern blotting (lower panel). C, cells stably expressing βII in the absence or presence of α wt and α(3A) or nontransfected cells treated without or with TGF-β3 or GM-CSF were stained with phalloidin for polymeric actin.
tact L45 loop and are likely to be Smad-dependent. ALK5-mediated activation of JNK was found to be independent of Smads. In addition, TβR-I was found to weakly regulate the expression of specific genes in a Smad-independent manner. Taken together these results indicate that different independent signaling pathways are initiated from the activated type I receptor. Although our results show that Smad-independent signaling through TGF-β receptor is not sufficient to mediate many of the regulatory effects of TGF-β on cell proliferation and differentiation, further studies are under way to investigate the effects of the mutants in more short term responses, like mobilization of the actin cytoskeleton. In addition, our data certainly do not rule out an important role for TGF-β-induced Smad-independent signaling in either promoting, inhibiting, or redirecting the Smad pathway or an important role for Smad-independent signaling in TGF-β-induced responses that are induced via Smad-mediated changes in expression of genes, e.g., growth factors, their receptors, and AP1 family members.

Consistent with previous data that implicate Smads in TGF-β-induced growth arrest (71, 72), we found that ALK5 L45 mutants defective in Smad activation were unable to mediate growth arrest despite being able to down-regulate cyclin D1 (Fig. 6B). Cyclin D1 has been implicated in TGF-β-induced growth arrest. However, either TGF-β-induced down-regulation of cyclin D1 in Smad-independent manner is not efficient enough or Smad-dependent regulation of other cell cycle regulators, e.g., up-regulation of CDK inhibitors p15 and p21 and down-regulation of c-Myc, are needed for anti-proliferative action of TGF-β. In EpH4 polarized mammary epithelial cells ALK5-initiated signaling to growth arrest has been shown to occur in part independently of the Smad pathway via phosphorylation 2A-mediated inactivation of p70S6K (73). Whether our ALK5 L45 mutants can mediate (partial) growth arrest in EpH4 cells remains to be investigated. The dependence for intact L45 loop for the observed ALK5-induced PAI-1 and fibronectin protein production in R4-2 cells (Fig. 2C) is consistent with the inability of ALK5 L45 mutants to activate the PAI-1 promoter based reporter (CAGA)_12-Luc assay (Fig. 1F) and the lack of TGF-β-induced fibroectin mRNA levels in Smad4 deficient MDA-MB-468 cells (Fig. 2D). The observed TGF-β-induced fibroectin has been previously shown to be dependent on JNK activation (29). However, the ability of the ALK5 L45 mutant to activate JNK without inducing fibroectin levels indicates that JNK activation alone by TGF-β is not sufficient for the induction of fibroectin production. TGF-β has been reported to induce fibroectin protein levels independent of Smad4 (29). However, we found that TGF-β-induced fibroectin mRNA levels require Smad4 (Fig. 2D). The reason for this discrepancy remains to be elucidated.

The ALK5(3A) mutant was found to interact with I-Smads (Fig. 3). I-Smads have been shown to compete with R-Smads for this discrepancy remains to be elucidated. The reason for this discrepancy remains to be elucidated.

in Fig. 3). I-Smads have been shown to compete with R-Smads for interaction with activated type I receptors (62–64). Taken together, these results suggest that R-Smads and I-Smads interact also with a region in the receptor, such as glycine-serine-rich domain (74). The weak binding of XIAP and Dab-2 was found to be independent on the intact L45 loop in ALK5. Thus, the L45 loop region may be important for interaction not only R-Smads but also with other signaling components. Therefore, to complement our studies on Smad-independent signaling using ALK5 L45 mutant-expressing cells, we are currently examining the responses on TGF-β in cells that lack certain R-Smads or Smad4.

Activation of JNK was shown to be enhanced by the caALK5(3A) mutant (Fig. 4). Thus, this response is independent of the Smad pathway. The observation that ALK5(3A) is weaker than wild type ALK5 in activating c-Jun-based tran-
scriptional reporters suggests that Smad signaling contributes to the activation of this reporter, as previously shown (32–37). XIAP has been implicated as a link between the type I receptor and the JNK pathway (26). However, ALK5(3A), which is not capable of binding to XIAP, can induce moderate JNK-mediated reporter activities (Fig. 4B). This suggests that XIAP is dispensable for JNK activation mediated by TGF-β in R4-2 cells. Although the TGF-β receptor-interacting protein Xav has been shown to mediate JNK activity (21), we were unable to show enhancement of JNK activity in the presence of caALK5 or caALK5(3A) in 293T cells (data not shown). Receptor-mediated activation of Smads may occur at early endosomes as SARA (Smad anchor for receptor activation), the molecule presenting R-Smads to the type I receptor, is exclusively located in this organelle (75–77). It will be of interest to examine whether TGF-β-induced JNK activation is initiated at the plasma membrane or at early endosomes.

The TGF-β-induced change of epithelial cells into fibroblastoid-shaped cells and formation of actin stress fibers were found to require an intact L45 loop. This result is consistent with previous data that show that Smads alone can weakly induce stress fiber formation and cooperate with activated ALK5 for full epithelial-to-mesenchymal transition (70). While our manuscript was in preparation, Yu and co-workers reported on the characterization of a similar ALK5 L45 mutant capable of inducing apoptosis but not epithelial-to-mesenchymal transition via a p38 pathway in NMuMG cells (48). We observed a weak but not consistent p38 phosphorylation in response to activation of mutant chimeric GM-CSF/TGF-β receptor. The differences between our observations and those of Yu et al. (48) may be because of the use of constitutively active ALK5 L45 mutant by Yu et al. (48) compared with ligand-mediated activation of chimeric GM-CSF/TGF-β receptor complex by us. An advantage of the use of constitutively active receptor is that the signal is built-up in the cell, allowing easier detection. However, we found that caALK5 L45 mutants, when expressed in Mv1Lu cells expressing endogenous ALK5, very weakly activated a Smad-dependent reporter (Fig. 1E). In addition, caALK5 L45 mutant receptors will induce a sustained response, which does not occur when ALK5 L45 mutant receptors are activated by ligands.

In conclusion, using Smad-activation-defective L45 loop mutants of ALK5, we have shown that ALK5-mediated JNK activation, and certain gene responses can occur independent of the Smad pathway. The ALK5 L45 mutants will be important tools to examine the requirement of Smads, and other signaling components that bind to the L45 loop for the various effects by TGF-β.

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