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Award Number: DAMD17-01-1-0592

TITLE: Mechanism of Ras Activation by TGFβ

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REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Mechanism of Ras Activation by TGFβ

5. FUNDING NUMBERS
DAMD17-01-1-0592

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
We have shown that TGFβ regulates Ras and Mapk signaling pathways. Dynein is a molecular motor protein that mediates intracellular transport of cargo along polarized microtubules (MTs) toward the minus ends. Activation of a motor may occur by several mechanisms, which can be regulated by growth factors. Thus, the receptors and signaling pathways for these polypeptides are potential mediators of motor protein activation and organelle trafficking, events which ultimately determine the collective spatial organization of the signaling pathways within the cell. Here we describe a novel TGFβ receptor-interacting protein, termed km23, which is also a dynein light chain. km23 interacts with TGFβ receptors and is phosphorylated after ligand-receptor engagement. Forced expression of km23 induces specific TGFβ responses. TGFβ induces the recruitment of km23 to the intermediate chain of dynein, which is blocked by a kinase-deficient form of TGFβ RII. This is the first demonstration of a link between dynein and a natural, growth inhibitory cytokine. Further, our results indicate km23 may function as a motor receptor for the recruitment and transport of TGFβ signaling components along MTs. Alterations in km23 would alter such transport and disrupt TGFβ growth inhibitory signals, thereby increasing the malignant behavior of the cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
TGFβ, growth factors, signal transduction, motor proteins, dynein, Mapk, Ras	136
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

We previously demonstrated that TGFβ rapidly activates Ras (3-6 min), as well as both the Erk and Sapk/JNK Mapk's (within 5-10 min). These effects occurred in untransformed epithelial cells and in human breast cancer cells (BCCs) that had retained TGFβ responsiveness in terms of TGFβ's growth inhibitory effects. The effects are not observed in cells resistant to TGFβ-mediated growth inhibition, and both TGFβ receptors (RI and RII) are required for these effects. We have more recently provided definitive evidence for the biological significance of these pathways in mediating several TGFβ responses, including cell cycle regulation, autoinduction, urokinase receptor induction, and positive regulation of the Smad pathway (for reviews, see Mulder, 2000; Yue and Mulder, 2001). Since TGFβ receptors are serine/threonine kinases, the mechanisms for activation of Ras by TGFβ would differ from those observed for tyrosine kinase receptors. Signaling components with SH2 domains would not be expected to directly bind. The objective of the current proposal was to identify TGFβ signaling components that may regulate the activation of Ras/Mapk/JNK pathways by TGFβ. These studies are highly relevant to BC, since TGFβ proteins are the major endogenous growth inhibitors secreted by human BCCs. More advanced BCCs lose responsiveness to TGFβ's growth inhibitory effects. This is largely mediated by alterations in TGFβ signaling pathways, such as those under study in this proposal. Elucidation of the diverse signals that are activated by TGFβ will facilitate our understanding of the mechanisms by which this important growth inhibitor mediates its effects, as well as how these signaling pathways are subverted to produce malignant breast cancer.

BODY: In order to identify TGFβ signaling components that mediate TGFβ responses through the Ras/JNK pathway, we have developed a novel method. This method utilizes the phosphorylated, activated cytoplasmic domains of the TGFβ receptors as probes to screen an expression library. The library was prepared from a highly TGFβ-responsive epithelial cell line (Tang et al., 2002). The cytoplasmic regions of both receptors were phosphorylated *in vitro* using a kinase assay prior to screening. One of the novel TGFβ signaling intermediates we identified, termed km23, is a 96-amino acid protein encoded by a 291-bp open reading frame. It is a ubiquitously expressed, cytoplasmic protein.

We have found that km23 is also a light chain of the molecular motor protein dynein. TGFβ stimulates not only the phosphorylation of km23, but also the recruitment of km23 to the dynein intermediate chain (DIC). Kinase-active TGFβ receptors are required for km23 phosphorylation and interaction with DIC. Recruitment of dynein light chains (DLCs) to the dynein complex is important not only for specifying the cargo that will bind (Vaughan and Vallee, 1995), but also for the regulation of intracellular transport itself (Karcher et al., 2002). Thus, km23 appears to function as a motor receptor, linking the dynein motor to specific cargo. We also demonstrate that km23 can mediate specific TGFβ responses, including JNK activation, c-Jun phosphorylation, and growth inhibition.

Collectively, our results are consistent with a role for km23 in both TGFβ signaling and dynein-mediated transport along microtubules (MT's). It is likely that the binding of km23 to the DIC after TGFβ receptor activation is important for specifying the nature of the cargo that will be

transported along the MTs. Any disruption in km23 would be expected to prevent or alter movement of specific cargo along MT's. In this way, alterations in km23 might result in a mis-localization of these proteins, with a disruption of TGFβ growth inhibitory signals. Along these lines, protein traffic direction is required for the maintenance of cell polarity, which, if lost, can result in tumor formation (Peifer, 2000; Bilder et al., 2000). Accordingly, sequence alterations at specific regions of km23 in human tumors might play a role in tumor development or progression.

Accordingly, we are examining BCC lines to determine whether any km23 alterations can be found. We have identified km23 alterations in 45% of human tumor tissues that were examined as part of another proposal. We are in the process of expressing these alterations to determine whether these can increase the malignant phenotype or potential of human cancers.

KEY RESEARCH ACCOMPLISHMENTS:

- We have extensive functional data to demonstrate that km23 is an important component of TGFβ signaling pathways such as Ras/JNK.
- We also have functional data to indicate that km23 is an important component of the cell's motor machinery, responsible for moving TGFβ signaling components to their appropriate destinations in the cell.
- km23 alterations may occur at specific "hot spots" in km23 that are critical for the cellular functions of km23.
- We are preparing cellular models that mimic the alterations in km23 found in tumor cells to validate km23 as a critical anti-cancer therapeutic target for human cancer.

REPORTABLE OUTCOMES:

1. Yue, J. and Mulder, K.M. Transforming growth factor-beta signal transduction in epithelial cells. Pharmacology & Therapeutics 91:1-34, 2001.
2. Yue, J. and Mulder, K.M. Requirement of TGFbeta receptor-dependent activation of c-Jun N-terminal kinases (JNKs)/Stress-activated protein kinases (Sapks) for TGFbeta up-regulation of the urokinase-type plasminogen activator receptor. Submitted, 2002.
3. Tang, Q., Staub, C.M., Gao, G., Jin, Q., Wang, Z., Ding, W., Aurigemma, R.E., and Mulder, K.M. km23: A novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein. Molecular Biology of the Cell, In Press, 2002.
4. Tang, Q., Liu, X., and Mulder, K.M. Enhancement of TGFbeta-mediated transcriptional activation of the Smad binding element by Smad1. In preparation, 2002.
5. Tang, Q., Staub, C.M., Ding, W., Jin, Q., Wang, Z., and Mulder, K.M. km23: A novel TGFbeta signaling component that is truncated in human cancers. In preparation, 2002.

6. Mulder, K.M. km23: A TGFβ signaling intermediate that interacts with the molecular motor dynein. FASEB Summer Research Conference: The TGFβ Superfamily: Signaling and Development, July 7-12, 2001.

7. Tang, Q., Staub, C.M., and Mulder, K.M. km23: A TGFβ signaling intermediate that interacts with dynein. Molecular Motors Meeting, Woods Hole, MA, Sept. 2001.

8. Tang, Q., Staub, C.M., Ding, W., Jin, Q., Wang, Z., and Mulder, K.M. km23: A novel TGFβ signaling component that is truncated in human cancers. Penn State Univ., University Park, PA, April 2002.

9. Tang, Q., Staub, C.M., Gao, G., Jin, Q., Wang, Z., Ding, W., Aurigemma, R., and Mulder, K.M. km23: A novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein. Anti-Cancer Drug Discovery and Development Summit, Princeton, NJ, June 17-19, 2002.

10. Mulder, K.M. km23—A novel TGF-beta-related target that is altered in human cancers. Drug Discovery Technology, IBC Life Sciences, Boston, MA, August 3-8, 2002.

11. Funding Applied for Based on Work Supported by this Award: National Cancer Institute, National Institutes of Health, # 1 R01 CA100239-01, *Role of km23 in Human Cancer*, Principal Investigator: Kathleen M. Mulder, 04/01/03-03/31/08.

12. Funding Applied for Based on Work Supported by this Award: Department of Defense, Idea Award, *Development of km23-based diagnostics and therapeutics*, Principal Investigator: Kathleen M. Mulder, 01/01/03-12/31/05.

CONCLUSIONS:

1. The phosphorylated, activated cytoplasmic domains of the TGFβ receptors were used as probes to screen an expression library that was prepared from a highly TGFβ-responsive epithelial cell line.

2. The TGFβ receptor-interacting protein km23 was isolated and identified to be a light chain of the motor protein dynein.

3. This 11-kD cytoplasmic protein is associated with the TGFβ receptor complex intracellularly, and it is phosphorylated on serine residues after ligand-receptor engagement.

4. A kinase-deficient form of TGFβ RII prevents km23 from being phosphorylated after TGFβ treatment.

5. Cellular expression of km23 can mimic specific TGFβ responses, namely the activation of JNK, the phosphorylation of c-Jun, and the inhibition of cell growth.

6. TGFβ induces the recruitment of km23 to the intermediate chain of dynein, and the kinase activity of the TGFβ receptors is required for this interaction.

7. km23 specifies the signaling intermediates that are transported along microtubules by dynein after TGFβ receptor activation. This is the first demonstration of a link between cytoplasmic dynein and a natural, growth inhibitory cytokine.

SO WHAT SECTION:

km23 as a "Drugable" Target: Several Different Theapeutic Approaches are Applicable

- km23 appears to function as a tumor suppressor, blocking cancer cell growth under normal conditions. In contrast, km23 alterations in human breast cancer should abrogate the tumor suppressive function of km23.

- Several therapeutic approaches have been employed to repair or replace the loss of tumor suppressor protein functions (i.e., gene therapy approaches, blockade of binding proteins, etc.); these are also applicable to km23.

- Pharmacological screens are underway to identify novel agents that can restore the normal functions of km23, or replace the altered forms/functions of km23.

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Yue, J. and **Mulder, K.M.** Requirement of Ras/MAPK pathway activation by transforming growth factor β for transforming growth factor β_1 production in a Smad-dependent pathway. *J. Biol. Chem.* 275:30765-30773, 2000.

Yue, J. and **Mulder, K.M.** Transforming growth factor-beta signal transduction in epithelial cells. *Pharmacology & Therapeutics*, 91:1-34, 2001.

NOTE: Please see references contained within these papers

APPENDICES:

Yue, J. and **Mulder, K.M.** Requirement of Ras/MAPK pathway activation by transforming growth factor β for transforming growth factor β_1 production in a Smad-dependent pathway. *J. Biol. Chem.* 275:30765-30773, 2000.

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Tang, Q., Staub, C.M., Gao, G., Jin, Q., Wang, Z., Ding, W., Aurigemma, R., and Mulder, K.M. km23: A novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein. Anti-Cancer Drug Discovery and Development Summit, Princeton, NJ, June 17-19, 2002.

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Curriculum Vitae for Kathleen M. Mulder

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 40, Issue of October 6, pp. 30765–30773, 2000
Printed in U.S.A.

Requirement of Ras/MAPK Pathway Activation by Transforming Growth Factor β for Transforming Growth Factor β_1 Production in a Smad-dependent Pathway*

Received for publication, January 3, 2000, and in revised form, June 7, 2000
Published, JBC Papers in Press, June 7, 2000, DOI 10.1074/jbc.M000039200

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Our previous results have shown that transforming growth factor β (TGFβ) rapidly activates Ras, as well as both ERKs and SAPKs. In order to address the biological significance of the activation of these pathways by TGFβ, here we examined the role of the Ras/MAPK pathways and the Smads in $TGF\beta_3$ induction of $TGF\beta_1$ expression in untransformed lung and intestinal epithelial cells. Expression of either a dominant-negative mutant of Ras (RasN17) or a dominant-negative mutant of MKK4 (DN MKK4), or addition of the MEK1 inhibitor PD98059, inhibited the ability of $TGF\beta_3$ to induce AP-1 complex formation at the $TGF\beta_1$ promoter, and the subsequent induction of $TGF\beta_1$ mRNA. The primary components present in this $TGF\beta_3$-inducible AP-1 complex at the $TGF\beta_1$ promoter were JunD and Fra-2, although c-Jun and FosB were also involved. Furthermore, deletion of the AP-1 site in the $TGF\beta_1$ promoter or addition of PD98059 inhibited the ability of $TGF\beta_3$ to stimulate $TGF\beta_1$ promoter activity. Collectively, our data demonstrate that $TGF\beta_3$ induction of $TGF\beta_1$ is mediated through a signaling cascade consisting of Ras, the MAPKKs MKK4 and MEK1, the MAPKs SAPKs and ERKs, and the specific AP-1 proteins Fra-2 and JunD. Although Smad3 and Smad4 were not detectable in $TGF\beta_3$-inducible AP-1 complexes at the $TGF\beta_1$ promoter, stable expression of dominant-negative Smad3 could significantly inhibit the ability of $TGF\beta_3$ to stimulate $TGF\beta_1$ promoter activity. Transient expression of dominant-negative Smad4 also inhibited the ability of $TGF\beta_3$ to transactivate the $TGF\beta_1$ promoter. Thus, although the Ras/MAPK pathways are essential for $TGF\beta_3$ induction of $TGF\beta_1$, Smads may only contribute to this biological response in an indirect manner.

Transforming growth factor β (TGFβ)[1] is a natural growth inhibitor for epithelial-derived cells and a pleiotropic polypeptide for a variety of other cell types (1). TGFβ initiates its signaling by binding and activating TGFβ receptor types I (RI) and II (RII), which then form heterocomplexes and activate downstream components (1–3). Recently, members of the Sma and Mad homologue (Smad) family of signaling intermediates have been cloned and appear to play an important role in TGFβ signal transduction (1–3). Thus far, nine mammalian Smads (1–9) have been identified (3–5). The binding of TGFβ to TGFβ receptor complexes induces the phosphorylation of receptor-activated Smads, including Smads 1, 2, and 3 (3, 6, 7). The phosphorylated receptor-activated Smads form a heteromeric complex with the co-Smad Smad4 and translocate to the nucleus (3–5). This heteromeric complex may either directly bind the promoters of its target genes, or associate with other transcription factors to induce gene transcription (8, 9).

The mitogen-activated protein kinases (MAPKs) represent another major type of signaling intermediate for TGFβ (1, 2). We were the first to demonstrate that TGFβ could activate Ras, ERK1/2, and Sapks/JNKs within 3–5 min of TGFβ addition (10–13). Recently, other groups have confirmed the finding that TGFβ can activate ERKs and Sapks/JNKs (14–17). We were also the first to demonstrate that Ras was required for TGFβ-mediated activation of ERKs (18) and for the up-regulation of $p21^{Cip1}$ and $p27^{Kip1}$ (19). In addition, we have provided evidence of transmodulation of the Smad1 pathway by the Ras/MAPK pathways (6, 7).[2] However, the cellular events targeted by these TGFβ-mediated kinase activation events have not been widely studied.

TGFβ regulates the growth of cancer cells in both an autocrine and a paracrine fashion (1, 21). In TGFβ-sensitive tumor cells, autocrine TGFβ inhibits the growth and diminishes the tumorigenic potential of the cells (2, 21–24). In TGFβ-resistant tumor cells, which still secrete large amounts of TGFβ, the secreted TGFβ can enhance tumorigenesis by increasing cell migration, connective tissue formation, immunosuppression, and angiogenesis in a paracrine fashion (1, 21). Specifically blocking the production of TGFβ would inhibit the paracrine, tumor-enhancing effects of TGFβ in adenocarcinomas that have become refractory to TGFβ-mediated growth inhibition. Thus, it is important to explore the signaling cascades mediating TGFβ production.

Previous work has demonstrated that $TGF\beta_1$ can induce its own production (25–27). In the current report, we demonstrate for the first time that $TGF\beta_3$ induction of $TGF\beta_1$ is mediated through the Ras → → MAPKKs (MKK4 and MEK1) → MAPKs (Sapks and ERKs) signaling cascades. Moreover, we demonstrate that these pathways are required for the ability of TGFβ to regulate specific AP-1 proteins, namely Fra-2 and JunD, thereby leading to $TGF\beta_1$ production. Finally, although the

* This work was supported by National Institutes of Health Grants CA51424, CA54816, and CA68444 (to K. M. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TGFβ, transforming growth factors β; RasN17, dominant-negative Ras mutant; IECs, intestinal epithelial cells; MAPK, mitogen-activated protein kinase; Smads, Sma and Mad homologues; JNK/SAPK, c-Jun N-terminal kinases/stress-activated protein kinases; ERKs, extracellular signal-regulated kinases; EMSAs, electrophoretic mobility shift assays; RPAs, RNase protection assays; MAPKK, mitogen-activated protein kinase kinase; RI, receptor type I; DN, dominant-negative; SBE, Smad-binding elements.

[2] X. J. Liu and K. M. Mulder, submitted for publication.

Smads did not directly bind the relevant AP-1/SBE site in the $TGF\beta_1$ promoter, Smads 3 and 4 may be indirectly involved in $TGF\beta_3$ induction of $TGF\beta_1$.

MATERIALS AND METHODS

Cell Culture—The untransformed rat intestinal epithelial cell (IEC) clone IEC 4-1 (TGFβ-sensitive) was isolated as described previously (28). Cells were routinely maintained in SMIGS medium, consisting of McCoy's 5A (Life Technology, Inc.) supplemented with amino acids, pyruvate, and antibiotics (streptomycin, penicillin), and containing insulin (4 μg/ml), glucose (4.5 mg/ml), and 5% fetal bovine serum. RasN17-transfected IEC 4-1 clone E3, isolated and characterized as described previously (18), was routinely maintained in SMIGS plus G418 (131 μg/ml). DN MKK4-transfected IEC 4-1 clones N10 and N12, isolated and characterized as described previously,[3] were routinely maintained in SMIGS plus G418 (500 μg/ml). CCL64-L20 and CCL64-Smad3C mink lung epithelial cells, gifts from Dr. H. Lodish (Cambridge, MA), were routinely maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

Multiprobe RNase Protection Assay—The rCK-3 (45631P, PharMingen) and hCK-3 (45033P, PharMingen) Multi-Probe template sets contain multiple probes, including $TGF\beta_1$, L32, and glyceraldehyde-3-phosphate dehydrogenase. The probes were synthesized as described in the user manual (Multi-probe RNase Protection Assay System, PharMingen). Total RNA (15 μg) from proliferating cultures of IEC 4-1, IEC-P3, RasN17 E3, DN MKK4 clones N10 or N12, CCL64-L20, or CCL64-Smad3C cells were incubated with the labeled probe set overnight at 56 °C. The samples were then digested by RNase and resolved on 5% denaturing gels as described in the user manual (Multi-probe RNase protection assay system, PharMingen). Gels were dried and exposed to x-ray film at −70 °C.

Luciferase Reporter Assays—CCL64 cells were transfected with 0.5 μg of phTG5-Lux or 3TP-Lux, and 0.125 μg of renilla luciferase control reporter (pRL-SV40), using SuperFect (301305, QIAGEN) as described in the user manual. $TGF\beta_3$ (10 ng/ml) was added 21 h after transfection, and luciferase activity was measured at 24 h after TGFβ treatment. The dual luciferase assay (E1910, Promega) was performed according to the manufacturer's instructions. Transfection efficiency was determined by co-transfecting renilla luciferase.

Site-directed Mutagenesis—The plasmid phTG5-Lux, containing a 450-base pair fragment of the human $TGF\beta_1$ gene promoter, was provided by S. J. Kim (Bethesda, MD). The AP-1 consensus site (−362 to −355, 5′-TGTCTCA-3′) was changed into 5′-TGcagCA-3′ by Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer used was: 5′-CCTCTGGTCGGCTCCCCTGTGCAGCATCCCCC-GGATTAAGCCTTC-3′. The mutant clones were verified by DNA sequencing.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts were prepared as described previously (30). Briefly, cells were plated and treated with TGFβ as described above. Cells were washed with ice-cold phosphate-buffered saline twice, and lysed in 1 ml of ice-cold hypotonic lysis buffer (20 mM Hepes, pH 7.4, 20% glycerol, 0.01 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM glycerophosphate, 1 mM NaV_3O_4, 1 × protease inhibitor mixture). After a 20-min incubation on ice, samples were centrifuged at 10,000 × *g* at 4 °C. The pellets were resuspended in 200 μl of nuclear extraction buffer (hypotonic buffer + 500 mM NaCl). The nuclear lysates were cleared by centrifugation, and protein concentrations were determined by the bovine serum albumin assay as described previously (19).

Oligonucleotides were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Nuclear extracts (6 μg) were incubated with binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.25 mg/ml poly(dI-dC)-poly(dI-dC)) for 10 min at room temperature, followed by addition of 1 μl (500,000 cpm) of ^{32}P-labeled oligonucleotides to each reaction. The reactions were incubated at room temperature for 20 min. For supershift assays, 1 μl of antibodies anti-pan-Jun (sc-44X, Santa Cruz, CA), anti-pan-Fos (sc-253x), anti-c-Jun (sc45x), anti-c-Fos (sc-52x), anti-FosB (sc-48x), anti-Fra-1 (sc-183x), anti-Fra-2 (sc-604x), anti-JunB (sc-46X), anti-JunD (sc-74x), anti-Smad4 (sc-1909x), anti-Smad4 (sc-7154x), or anti-Smad3 (51-1500, Zymed Laboratories Inc., South San Francisco, CA) were then added to reactions. The reactions were incubated at room temperature for 45–60 min, stopped by addition of 1 μl of gel loading (× 10) buffer, and analyzed by native polyacrylamide gels (4%) at 150 volts for 2 h. The gels were dried and exposed to x-ray film at −80 °C. The $TGF\beta_1$ probe corresponding to the AP-1 site (−362 to −355) was: $^{-372}$GGCT-CCCCTGTGTCTCATCCCCCGGAT^{-345}. The mutant $TGF\beta_1$ AP-1 probe was: $^{-381}$GAAGGCTTAATCCGGGGGATgctgCACAGGGGAGCC-GACCAGAGG^{-336}. The $TGF\beta_1$ probe corresponding to the second potential SBE (+21 to +25) was: $^{+8}$TCCGCGGAGCAAGACAGCGAGGG-CCC^{+38}. The control SBE probe used was: 5′-GGAGTATGTCTAGAC-TGACAATGTAC-3′ (38).

RESULTS

Requirement of Ras, MEK1, and MKK4 for $TGF\beta_3$ Induction of $TGF\beta_1$—Untransformed IECs, which are exquisitely sensitive to the growth inhibitory effects of TGFβ (28), have been shown to display autoinduction of $TGF\beta_1$ mRNA expression (31). Furthermore, we have previously demonstrated that TGFβ resulted in a rapid activation of Ras, ERKs, and SAPK/JNK in these cells (10–13). However, the biological significance of the activation of these components by TGFβ was not entirely clear. Thus, it was of interest to determine whether $TGF\beta_3$ induction of $TGF\beta_1$ was mediated through the Ras/MAPK signaling cascades, thereby linking the activation of these cytoplasmic effects to an important biological response of TGFβ.

The role of Ras in mediating $TGF\beta_3$ induction of $TGF\beta_1$ was examined by RNase protection assays (RPAs) (Fig. 1*A*). For these studies, we utilized the IEC 4-1 clone E3, which had been stably transfected with a dominant-negative mutant of Ras (RasN17) under the control of an inducible metallothionein promoter (18). Our previous results demonstrated that a 4-fold induction of RasN17 expression, after a 36-h treatment with $ZnCl_2$ in these cells, was sufficient to completely block TGFβ downstream events mediated by the Ras pathway (18, 19).

We performed RPAs to examine the effects of RasN17 on $TGF\beta_3$ induction of $TGF\beta_1$ expression. As shown in Fig. 1*A*, *left side*, in the absence of $ZnCl_2$, $TGF\beta_1$ mRNA expression was increased to values 10-fold above initial baseline levels after 4 h of $TGF\beta_3$ treatment. In contrast, in the presence of $ZnCl_2$, $TGF\beta_1$ mRNA expression was increased by only 2.5-fold after the same time period of $TGF\beta_3$ treatment (Fig. 1*A*, *right side*). Thus, the induction of RasN17 by $ZnCl_2$ inhibited the ability of $TGF\beta_3$ to induce $TGF\beta_1$ mRNA expression by 75%. Similar results have been observed for $TGF\beta_1$ mRNA expression after 24 h of $TGF\beta_3$ treatment and by Northern blot analysis (data not shown). Taken together, our results clearly demonstrate that TGFβ activation of Ras is required for $TGF\beta_3$ induction of $TGF\beta_1$.

TGFβ activates ERKs through a Ras-dependent pathway (18). Thus, it is conceivable that $TGF\beta_3$ may regulate $TGF\beta_1$ production by activating the MEK1/ERK pathway as one of the pathways downstream of Ras. We have previously employed the MEK1 inhibitor PD98059 to block Erk1 activation by TGFβ in IEC 4-1 cells. A concentration of PD98059 of 10 μM resulted in complete blockade of the ability of TGFβ to activate Erk1, without affecting basal Erk1 activity levels (7). Here, we utilized this MEK1 inhibitor in RPAs to examine the requirement of MEK1 activation for $TGF\beta_3$ induction of $TGF\beta_1$ expression. As shown in Fig. 1*B*, in the absence of PD98059, $TGF\beta_1$ mRNA expression was increased to levels 9.8-fold above initial baseline values by 4 h after $TGF\beta_3$ treatment. In contrast, in the presence of PD98059, $TGF\beta_1$ mRNA expression was increased by only 3-fold above initial baseline levels over the same time period. Thus, MEK1 blockade by PD98059 inhibited the ability of $TGF\beta_3$ to induce $TGF\beta_1$ mRNA expression by 70%. Accordingly, our results indicate that $TGF\beta_3$ induction of $TGF\beta_1$ is mediated through the MEK1/ERK pathway as one of the events downstream of Ras.

We have previously shown that TGFβ activated the SAPK/JNK pathway (11, 12), and that Ras was required for TGFβ-mediated SAPK/JNK activation.[3] Thus, it is conceivable that

[3] J. Yue and K. M. Mulder, submitted for publication.

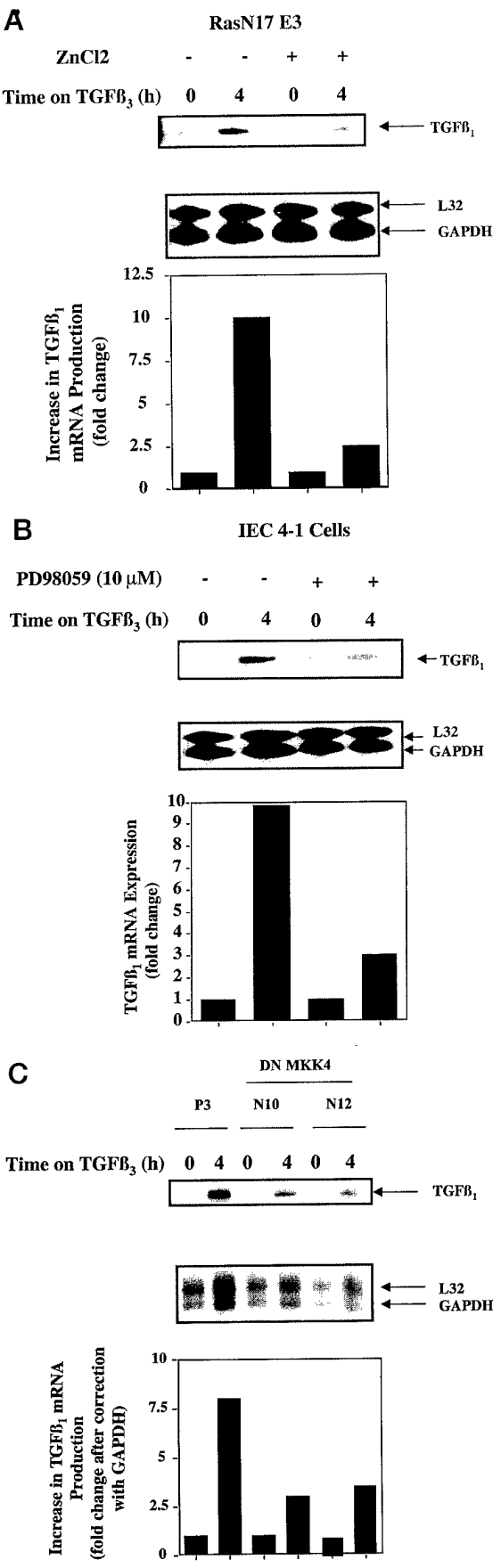


FIG. 1. **Expression of a dominant-negative mutant of Ras (RasN17) or a dominant-negative mutant of MKK4 (DN MKK4), or addition of the MEK1 inhibitor PD98059, inhibits TGFβ3 induction of TGFβ1.** *A,* proliferating cultures of IEC RasN17 E3 cells were incubated in serum-free medium either in the absence or presence of $ZnCl_2$ for 36 h to induce RasN17 expression. Cells were then treated with or without TGFβ3 (10 ng/ml) for 4 h. Total RNA was isolated. RNase protection assays were performed as described under "Materials and Methods." Representative of four experiments. *B,* proliferating cultures of IEC 4-1 cells were incubated in serum-free medium with or without TGFβ3 (10 ng/ml) for 4 h in the presence of the MEK1 inhibitor PD98058 (10 μM). Total RNA was isolated and RNase protection assays were performed. Representative of two experiments. *C,* the DN MKK4 expressing IEC clones N10 and N12, and empty vector-transfected IEC-P3 cells were plated and treated with TGFβ for 4 h. Total RNA was isolated and RNase protection assays were performed. Representative of two experiments. *Bottom panels,* densitometric scan of results shown in *top panels*. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

TGFβ could regulate TGFβ1 production by activating the MKK4/SAPK pathway as another pathway downstream of Ras. For these studies, we utilized the IEC 4-1 clones N10 and N12, which had been stably transfected with a dominant-negative mutant of MKK4 (DN MKK4). We have shown that overexpression of DN MKK4 in these clones completely blocked the ability of TGFβ to activate SAPK/JNK and phosphorylate c-Jun.[3] Thus, expression of DN MKK4 in these DN MKK4 clones was sufficient to completely inhibit TGFβ-mediated SAPK/JNK activation and its downstream events.

The effects of DN MKK4 on TGFβ3 induction of TGFβ1 were examined by RPAs as shown in Fig. 1*C*. TGFβ3 increased TGFβ1 mRNA expression by 8.5-fold in empty vector-transfected IEC P3 cells. In contrast, in the DN MKK4-expressing N10 or N12 clones, TGFβ3 increased TGFβ1 mRNA expression by only 3- or 3.6-fold above initial baseline levels, respectively (Fig. 1*C*). Thus, expression of DN MKK4 significantly inhibited the ability of TGFβ3 to induce TGFβ1 mRNA expression. Accordingly, our results indicate that TGFβ activation of SAPK/JNK is also required for TGFβ3 induction of TGFβ1.

Requirement of the AP-1 Site in the TGFβ1 Promoter and the ERK Pathway for TGFβ3 Stimulation of TGFβ1 Promoter Activity—The TGFβ1 promoter contains an AP-1 site at −362 to −355 (25–27). Although previous results have suggested that TGFβ1 autoinduction could be mediated through this AP-1 site in the TGFβ1 promoter (25–27), there is no definitive evidence that this AP-1 site is essential for this effect. Here, we examined the requirement for this AP-1 site in the transactivation of the TGFβ1 promoter by TGFβ in CCL64 mink lung epithelial cells. As shown in Fig. 2, TGFβ3 treatment increased TGFβ1 promoter luciferase activity by 7-fold in CCL64 cells. However, TGFβ3 failed to increase the activity of the TGFβ1 promoter containing a mutated AP-1 site in the same cell type (the *right two bars* in Fig. 2*A*). In addition, the basal TGFβ1 promoter activity was decreased by 50% when the AP-1 site was mutated (Fig. 2*A*). Taken together, our results demonstrate that the AP-1 site in the TGFβ1 promoter is essential for TGFβ transactivation of the TGFβ1 promoter.

Since PD98059 significantly inhibited TGFβ3 induction of TGFβ1 mRNA, it was of interest to examine whether PD98059 could inhibit the ability of TGFβ3 to stimulate TGFβ1 promoter activity. As shown in Fig. 2*B*, TGFβ3 treatment increased TGFβ1 promoter luciferase activity by 6.5-fold in CCL64 cells. In the presence of PD98059 (20 μM), TGFβ3 treatment only increased TGFβ1 promoter luciferase activity by 3.5-fold; basal TGFβ1 promoter activity was not affected by this concentration of PD98059. Although higher concentrations of PD98059 resulted in a further inhibition of TGFβ3 stimulation of TGFβ1 promoter activity, basal TGFβ1 promoter activity was also decreased at concentrations above 20 μM (data not shown). These results indicate that the ERK pathway is, indeed, required for TGFβ3 induction of TGFβ1. The data also suggest that the ERK pathway may contribute to TGFβ3 induction of TGFβ1 through

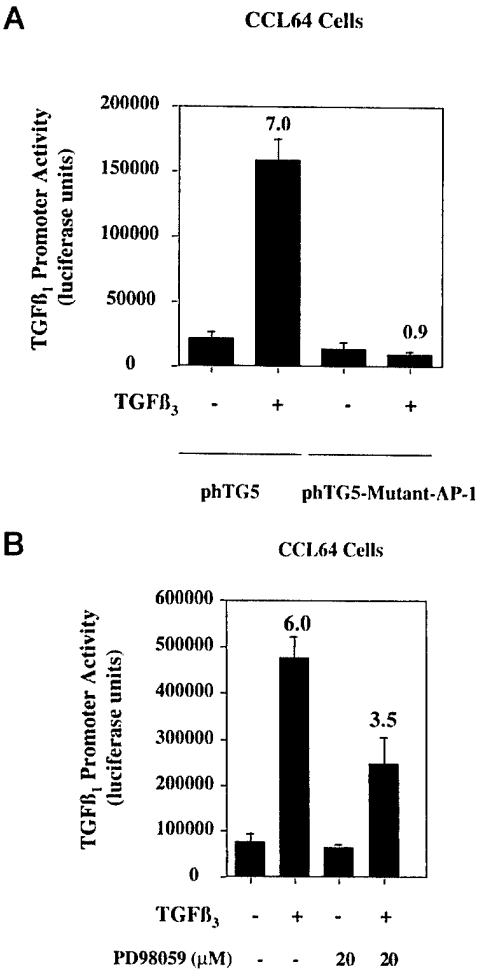


FIG. 2. **Requirement of the AP-1 site in the TGFβ1 promoter and the ERK pathway for TGFβ3 stimulation of TGFβ1 promoter activity.** *A*, proliferating cultures of CCL64 cells were transfected with 0.5 μg of phTG5-Lux or phTG5-Mutant-AP-1-Lux, and 0.125 μg of renilla luciferase control reporter (pRL-SV40) by Superfect (301305, QIAGEN). Twenty-one hours after transfection, cells were incubated in serum-free medium for 1 h. Thereafter, TGFβ3 (10 ng/ml) was added, and luciferase activity was measured 24 h after TGFβ treatment using the dual luciferase assay (E1910, Promega) according to the manufacturer's instructions. The results plotted represent the $\bar{x} \pm$ S.D. for triplicate transfections. The *number above* the *bar* represents the fold change by comparing the luciferase activity in the presence or absence of TGFβ. *B*, proliferating cultures of CCL64 cells were transfected with 0.5 μg of phTG5-Lux and 0.125 μg of renilla luciferase control reporter (pRL-SV40). TGFβ3 (10 ng/ml) was added 21 h after transfection in the presence of MEK inhibitor PD98059 (20 μM). Luciferase activity was measured as described in the legend to Fig. 2*A*. Representative of four experiments.

the AP-1 site in the TGFβ1 promoter.

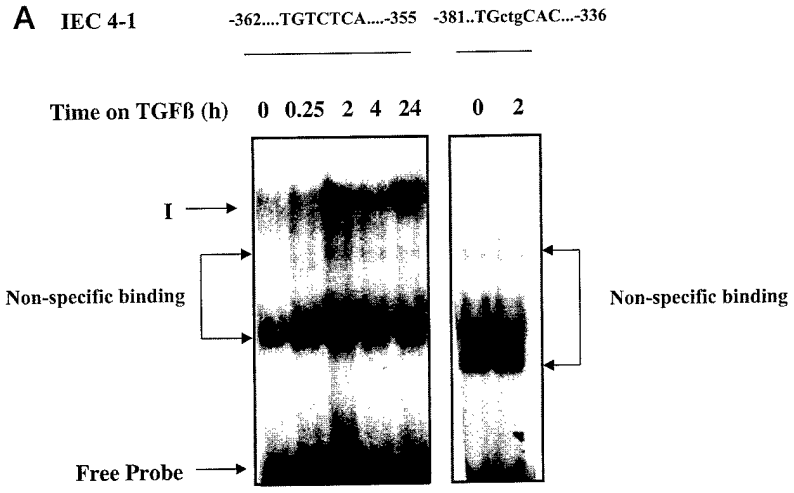
Temporal Relationship between TGFβ3-stimulated Transcription Factor Binding to the TGFβ1 Promoter and TGFβ1 mRNA Expression—If TGFβ3 induction of TGFβ1 is mediated through the AP-1 site in the TGFβ1 promoter, then TGFβ induction of complex formation at this site should kinetically precede the increase in production of TGFβ1 mRNA. In order to verify whether this was the case, we performed EMSAs using an oligonucleotide (−372 to −345) spanning the AP-1 site in the TGFβ1 promoter as a probe. The results in the *left panel* of Fig. 3*A* indicate that the levels of Complex I began increasing by 15 min after TGFβ treatment and were maximal by 2 h post-TGFβ addition. After this time the levels began to decline. However, the levels of Complex I were still much higher than initial baseline levels by 4 and 24 h after TGFβ addition. In contrast, a gel-shifted Complex I was not observed in response to treatment of the cells with TGFβ when an oligonucleotide containing a mutant AP-1 site was used as a probe (Fig. 3*A*, *right panel*). Similar results were observed in CCL64 mink lung cells (Fig. 3*B*). Therefore, the AP-1 site in the TGFβ1 promoter is essential for TGFβ to stimulate the formation of Complex I.

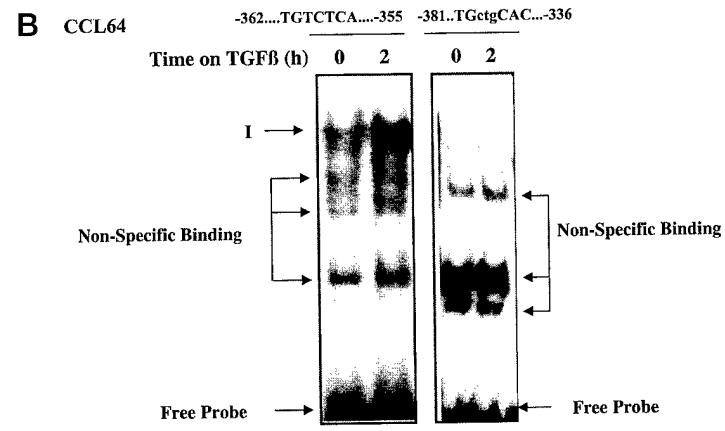
In order to determine whether the TGFβ3 induction of TGFβ1 mRNA expression was preceded by TGFβ-inducible complex formation at the AP-1 site, we also examined the kinetics for TGFβ3 effects on TGFβ1 mRNA expression by RPAs. As shown in Fig. 3*C*, TGFβ1 mRNA expression was increased to levels 2.4-fold above initial baseline values by 30 min after TGFβ3 treatment. By 2 h of TGFβ3 treatment, maximal TGFβ1 mRNA expression levels of 9-fold above initial baseline values were reached. TGFβ1 mRNA expression levels were then maintained at levels 8.5-fold above initial baseline values for at least 4 h. Thereafter, TGFβ1 mRNA expression levels declined, so that expression levels were 5-fold above initial baseline values by 24 h after TGFβ3 treatment. Thus, formation of the TGFβ3-inducible complex at the AP-1 site in the TGFβ1 promoter temporally preceded TGFβ3 induction of TGFβ1 mRNA expression in IEC 4-1 cells. Similar kinetics have been observed for the ability of TGFβ3 to stimulate TGFβ1 secretion into the medium of IECs using a TGFβ1 enzyme-linked immunosorbent assay system (data not shown). Our results provide strong evidence of a tight association between TGFβ3 stimulation of transcription factor binding at the AP-1 site in the TGFβ1 promoter and TGFβ3 induction of TGFβ1. Our results support the requirement of this AP-1 site for mediating this biological response to TGFβ.

Requirement of Ras, ERK, and SAPK/JNK for TGFβ3 Stimulation of Transcription Factor Binding at the TGFβ1 Promoter—We have demonstrated in Figs. 1–3 that Ras/MAPK signaling cascades and the AP-1 site in the TGFβ1 promoter are required for TGFβ3 induction of TGFβ1, and that TGFβ3 can induce transcription factor binding at this AP-1 site. Accordingly, it was of interest to examine whether TGFβ3 activation of the Ras/MAPK pathways was required for TGFβ stimulation of Fos and Jun binding at this site. An oligonucleotide (−372 to −345) spanning the AP-1 site in the TGFβ1 promoter was utilized in EMSAs. In RasN17E3 cells, TGFβ3 increased the formation of protein-DNA Complex I (see *arrow*) after TGFβ3 treatment in the absence of $ZnCl_2$ (Fig. 4*A*, *left side*). In contrast, in the presence of $ZnCl_2$ (Fig. 4*A*, *right side*), the induction of RasN17 blocked the formation of this TGFβ3-inducible complex. In addition, as shown in Fig. 4*A*, *left side*, addition of either a pan-Fos or pan-Jun antibody completely blocked the induction of Complex I by TGFβ3, while normal rabbit IgG had no effect on this complex. Accordingly, our results indicate that TGFβ3 activation of Ras is required for TGFβ3 induction of Fos-Jun complex formation at this AP-1 site.

We also examined whether the MEK1 inhibitor PD98058 would inhibit the ability of TGFβ3 to induce Fos and Jun binding at the TGFβ1 promoter (Fig. 4*B*). EMSAs demonstrated that in the absence of PD98059, the induction of Fos-Jun complex formation at the TGFβ1 promoter (Complex I) was prominent after 2 h of TGFβ treatment. In the presence of PD98059, however, TGFβ3 failed to induce the formation of this complex. Thus, the MEK1/ERK pathway is required for TGFβ3 induction of AP-1 protein binding at the TGFβ1 promoter as well.

EMSAs were also performed to determine whether MKK4 was required for TGFβ3 induction of AP-1 protein binding at the TGFβ1 promoter. As shown in Fig. 4*C*, in the parental IEC 4-1 cells, TGFβ3 induced the formation of the Fos-Jun complex, similar to that seen in Fig. 4, *A* and *B*. In contrast, in DN





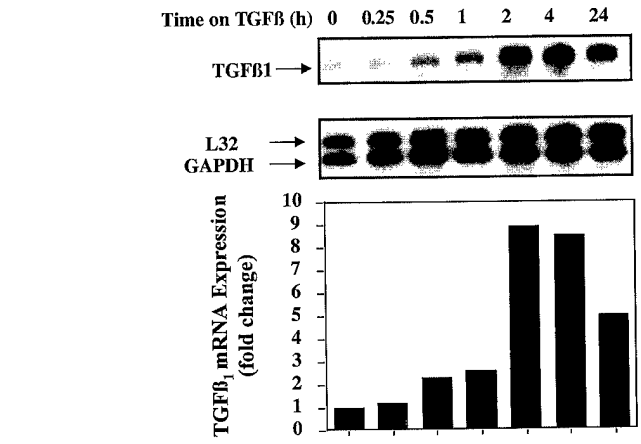


FIG. 3. **Kinetics for TGFβ$_3$ induction of transcription factor binding at the TGFβ$_1$ promoter and for TGFβ$_3$ induction of TGFβ$_1$ mRNA expression.** Proliferating cultures of IEC 4-1 cells (*A* and *C*) or CCL64 cells (*B*) were incubated in serum-free medium for 1 h. Cells were then treated with or without TGFβ$_3$ (10 ng/ml) for the indicated times. *A* and *B*, nuclear extracts were prepared as described under "Materials and Methods." EMSAs were performed using oligonucleotides containing the AP-1 site in the TGFβ$_1$ promoter (−372 to −345) or the mutant AP-1 site in the TGFβ$_1$ promoter (−381 to −336) as probes. *C*, total RNA was isolated, and RPAs were performed as described under "Materials and Methods." *Bottom panel*, densitometric scan of results shown in *top panel*. *GADPH*, glyceraldehyde-3-phosphate dehydrogenase.

MKK4-transfected IECs, TGFβ$_3$ failed to increase the formation of Complex I. Thus, overexpression of DN MKK4 blocked the ability of TGFβ$_3$ to induce the formation of this complex at the AP-1 site in the TGFβ$_1$ promoter. Collectively, our data indicate that TGFβ activation of SAPK/JNK is also required for TGFβ$_3$ stimulation of Fos-Jun complex formation at the TGFβ$_1$ promoter.

Specific AP-1 Proteins Are Present in the TGFβ$_3$-inducible Complex at the TGFβ$_1$ Promoter—Previous results demonstrated that expression of antisense c-Jun and c-Fos constructs inhibited TGFβ$_1$ autoinduction (25). However, there is no definitive evidence to indicate which specific Fos or Jun members constitute this TGFβ-inducible AP-1 complex. Since the Jun and Fos families include multiple proteins in addition to c-Jun

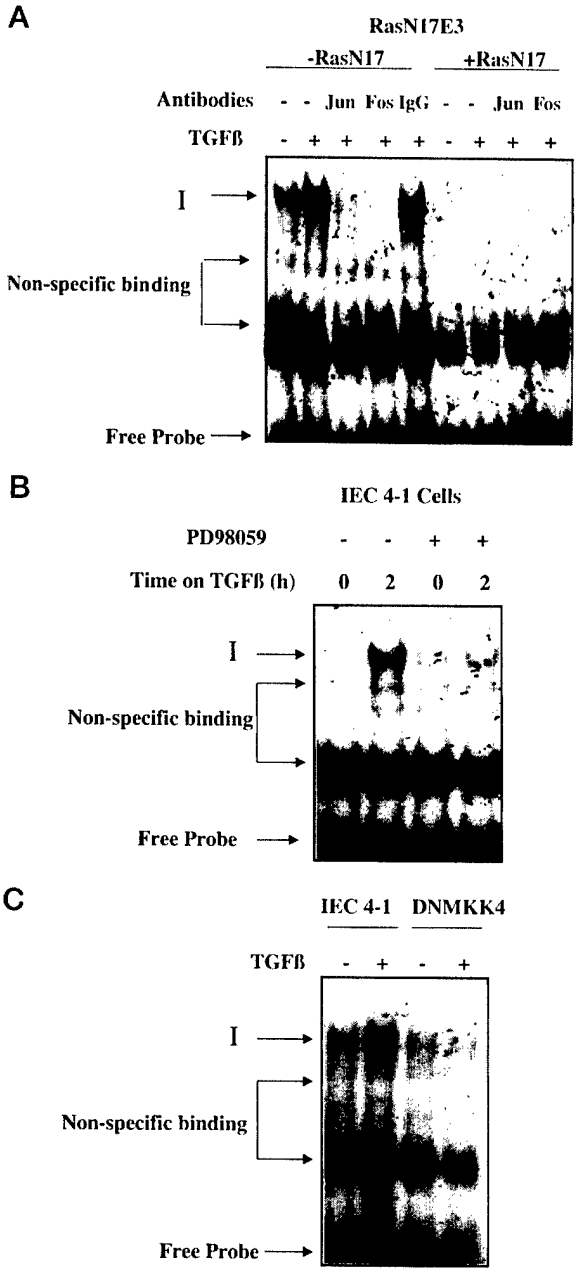


FIG. 4. **Requirement of Ras, MKK4, and MEK1 for TGFβ$_3$ induction of transcription factor binding at the TGFβ$_1$ promoter.** *A*, proliferating cultures of IEC RasN17 E3 cells were incubated in serum-free medium either in the presence or absence of $ZnCl_2$ (100 μg/ml) for 36 h to induce RasN17 expression. Cells were then treated with or without TGFβ$_3$ (10 ng/ml) for 2 h. Nuclear extracts were prepared as described under "Materials and Methods," and EMSAs were performed using a probe containing the AP-1 site in the TGFβ$_1$ promoter (−372 to −345). Supershifts were performed using antibodies prepared against pan-Jun (sc-44 X) and pan-Fos (sc-413 X). Normal rabbit IgG was used as a control. The TGFβ-induced protein-DNA Complex I is indicated by the *arrow*. Representative of three experiments. *B*, EMSAs were performed using nuclear extracts from IEC 4-1 cells that were pretreated with or without the MEK1 inhibitor PD98058 (10 μM) in the presence or absence of TGFβ$_3$. The AP-1 site in the TGFβ$_1$ promoter (−372 to −345) was used as a probe. Representative of two experiments. *C*, EMSAs were performed using nuclear extracts from IEC 4-1 cells or DN MKK4 cells that were treated with TGFβ$_3$ or left untreated. The AP-1 site in the TGFβ$_1$ promoter (−372 to −345) was used as a probe. Representative of two experiments.

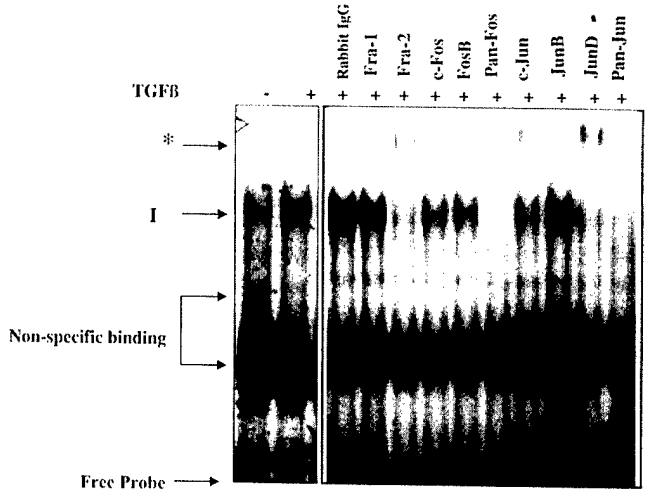


FIG. 5. **Involvement of JunD, Fra-2, FosB, and c-Jun in TGFβ$_3$ stimulation of transcription factor binding at the TGFβ$_1$ promoter.** EMSAs were performed using nuclear extracts from IEC 4-1 cells that were treated with or without TGFβ$_3$ for 2 h. The AP-1 site in the TGFβ$_1$ promoter (−372 to −345) was used as a probe. Supershifts were performed using specific antibodies against c-Jun, JunB, JunD, c-Fos, Fos-B, Fra-1, or Fra-2. The pan-Jun and pan-Fos antibodies were used as controls. The TGFβ-inducible protein-DNA Complex I and supershifts are indicated by *arrows* or *asterisks*, respectively. Representative of three experiments.

and c-Fos (32), it was of interest to determine which Fos and Jun family members mediated this complex formation. Fig. 5 depicts the results of supershift assays performed using antibodies specific for each Fos and Jun family member. The pan-Jun and pan-Fos antibodies were also used as controls. As expected, the pan-Jun and pan-Fos antibodies blocked the formation of Complex I. Furthermore, specific anti-JunD and anti-Fra-2 antibodies supershifted and blocked Complex I as shown in Fig. 5. Addition of the specific anti-c-Jun or anti-FosB antibodies also resulted in a supershifted band above Complex I, yet the extent of the shift was less prominent than that observed for the anti-JunD and anti-Fra-2 antibodies. In contrast, the specific anti-JunB and anti-Fra-1 antibodies had no significant effect on this complex. In summary, our data indicate that the primary components present in the TGFβ$_3$-inducible AP-1 complex at the TGFβ$_1$ promoter are Fra-2 and JunD, although c-Jun and FosB are also present. It is noteworthy that TGFβ can directly phosphorylate JunD, and that expression of RasN17 completely blocked the ability of TGFβ to phosphorylate JunD (data not shown).

Smad3 and Smad4 Are Not Present in the TGFβ$_3$-inducible AP-1 Complex at the TGFβ$_1$ Promoter—Recently, Smad-binding elements (SBEs) have been identified by several groups (33–39). Although the reported SBEs vary among different genes, it would appear that most SBEs contain either AGAC or its complementary sequence GTCT (33–39). We have searched the TGFβ$_1$ promoter sequence (26) and found two potential minimal SBE sites. One potential SBE site (GTCT, −363 to −359) overlaps the AP-1 site (TGTCTCA, −362 to −355) in the TGFβ$_1$ promoter. The second potential SBE is located from +21 to +25 (AGAC) in the TGFβ$_1$ promoter. Thus, it was of interest to determine whether Smads could bind these potential SBEs in the TGFβ$_1$ promoter.

A control SBE probe was also prepared, which was a consensus Smad3/Smad4-binding site identified by random oligonucleotide screening (38). As shown in Fig. 6, after 2 h of TGFβ treatment, TGFβ increased the formation of the complex at the control SBE probe (Fig. 6, *left panel*). Addition of either Smad3 or Smad4 antibodies resulted in a supershift, as indicated by the *arrow* at the top (Fig. 6, *left panel*). For the TGFβ$_1$ probe spanning both the AP-1 site and the first potential SBE (−372 to −345), TGFβ increased the formation of the AP-1 complex

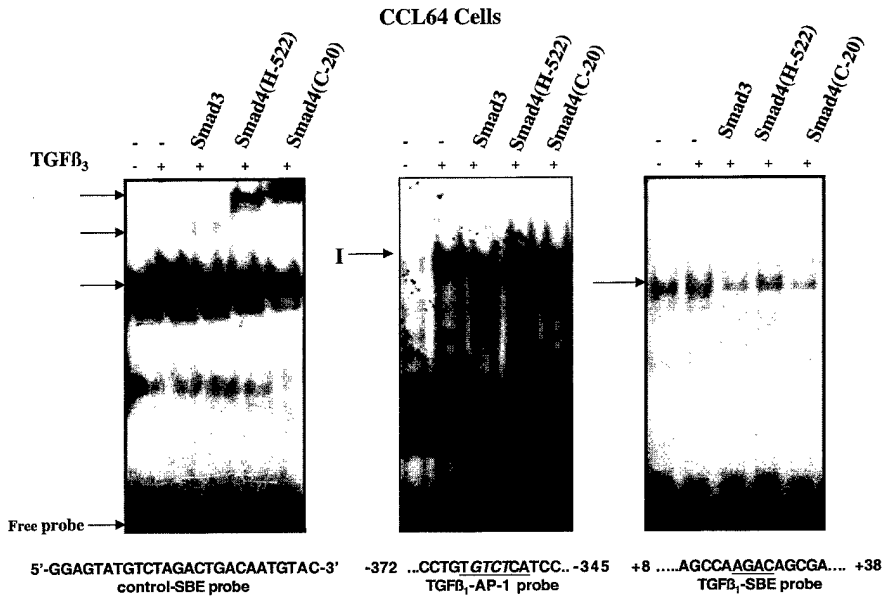


FIG. 6. **Smad3 and Smad4 are not present in the TGFβ3-inducible AP-1 complex at the TGFβ1 promoter.** EMSAs were performed using nuclear extracts from CCL64 cells that were treated with TGFβ3 or left untreated. The probes used were: the control SBE (*left panel*), oligonucleotides spanning the AP-1 site in the TGFβ1 promoter (−372 to −345) (*middle panel*), or oligonucleotides spanning the second potential SBE in the TGFβ1 promoter (+8 to +38) (*right panel*). Supershifts were performed using anti-Smad4 (H-522), anti-Smad4 (C-20), or anti-Smad3 (51-1500, Zymed Laboratories Inc.) antibodies. The TGFβ-inducible protein-DNA complexes and supershifts are indicated by *arrows*.

after 2 h of TGFβ treatment (Fig. 6, *middle panel*), similar to the results in Fig. 3*A*. However, neither Smad3 nor Smad4 antibodies affected the formation of this complex (Fig. 6, *middle panel*). Thus, Smads do not appear to be involved in this TGFβ3-inducible AP-1 complex at the TGFβ1 promoter. For the TGFβ1 probe spanning the second potential SBE (+8 to +38), none of the protein-DNA complexes were inducible by TGFβ, and none of the Smad3 or Smad4 antibodies had any significant effect on complex formation (Fig. 6, *right panel*). Thus, this potential SBE in the TGFβ1 promoter is not utilized for TGFβ3 induction of TGFβ1.

TGFβ3 Induction of TGFβ1 Is Dependent on Smads—Since Smads play an important role in TGFβ signaling (1–3) and TGFβ sometimes activates Sapks/JNKs, JunB, or c-Jun via Smad-dependent pathways (33, 36, 40), it was of interest to determine whether Smads were required in any respect for TGFβ3 induction of TGFβ1. Accordingly, the ability of TGFβ3 to stimulate TGFβ1 promoter activity was examined in Smad3C-CCL64 cells, which stably express a dominant-negative form of Smad3 (41). It has been previously reported that TGFβ failed to induce phosphorylation of Smad3 in this cell line, and that overexpression of this dominant-negative mutant of Smad3 blocked the ability of TGFβ to inhibit cell growth (41). Thus, normal Smad3 function in this cell line is lost. As expected, TGFβ3 stimulated 3TP-Lux activity by 11-fold in the parental CCL64-L20 cells, but only increased 3TP-Lux activity by 3.4-fold in the CCL64-Smad3C cells (Fig. 7*A*, *left panel*). Similarly, as shown in the *right panel* of Fig. 7*A*, TGFβ3 stimulated TGFβ1 promoter activity by 9.8-fold in the parental CCL64-L20 cells, but only increased TGFβ1 promoter activity by 2.1-fold in the CCL64-Smad3C cells. Thus, overexpression of this dominant-negative mutant of Smad3 inhibited the ability of TGFβ3 to increase TGFβ1 promoter activity by 75%. The results from RPAs also indicated that overexpression of this dominant-negative mutant of Smad3 significantly inhibited the ability of TGFβ3 to stimulate TGFβ1 mRNA expression in the CCL64-Smad3C cells (data not shown). Accordingly, these results indicate that TGFβ3 induction of TGFβ1 requires Smad3. It is possible that expression of this DN Smad3 may also interfere with Smad2 function in CCL64 cells (41). However, previous results have shown that TGFβ1 autoinduction was also blocked in Smad3-null macrophages and keratinocytes (42).

To determine whether TGFβ3 induction of TGFβ1 required Smad4, a dominant-negative mutant of Smad4 (DN Smad4) was co-transfected with phTG5-Luc into CCL64 cells. As shown in Fig. 7*B*, TGFβ3 stimulated TGFβ1 promoter activity by 5.5-fold; expression of DN Smad4 inhibited the ability of TGFβ3 to increase TGFβ1 promoter activity in a dose-dependent manner. Thus, Smad4 is required for TGFβ3 induction of TGFβ1 in CCL64 cells as well.

DISCUSSION

This report is the first to demonstrate that TGFβ activation of the Ras/MAPK pathways is essential for TGFβ3 induction of TGFβ1 expression. Blockade of TGFβ activation of Ras, MKK4/JNK, or MEK/ERK, each inhibited TGFβ3 induction of TGFβ1. We also demonstrate that Ras, MKK4/JNK, and MEK/ERK were required for TGFβ3-stimulated AP-1 complex formation at the TGFβ1 promoter. JunD and Fra-2 were the primary components present in this TGFβ3-inducible AP-1 complex, but c-Jun and FosB were also involved. Deletion of the AP-1 site in the TGFβ1 promoter abolished TGFβ3-inducible Fos-Jun complex formation and TGFβ3 transactivation of the TGFβ1 promoter. Although neither Smad3 nor Smad4 were detectable in the TGFβ3-inducible AP-1 complex in the TGFβ1 promoter, expression of a dominant-negative mutant of Smad3 inhibited the ability of TGFβ3 to increase TGFβ1 promoter activity and TGFβ1 mRNA expression. Furthermore, expression of a dominant-negative mutant of Smad4 inhibited TGFβ3 stimulation of TGFβ1 promoter activity. Thus, TGFβ3 induction of TGFβ1 is dependent upon TGFβ activation of the Ras/MAPK pathways, and Smads may also indirectly contribute to this biological response.

Our results reveal that TGFβ activation of Ras and the MAPK pathways is required for an important biological response to TGFβ, namely, autocrine production of TGFβ1. Although it has been shown previously that the H-*ras* oncogene can stimulate TGFβ1 promoter activity (43), oncogenic Ras may activate additional different downstream targets than those regulated by TGFβ specifically (2). Furthermore, overexpression of activated *ras* genes can result in TGFβ resistance and Smad inactivation (44). Thus, it is important to make the distinction between TGFβ activation of normal cellular Ras (as we are examining here) *versus* activation of Ras by overexpression, activating mutations, or other factors (reviewed in Ref. 2).

The activation of the Ras/MAPK pathways by TGFβ can mediate biological effects of TGFβ in addition to autocrine amplification of TGFβ1 production. For example, we have pre-

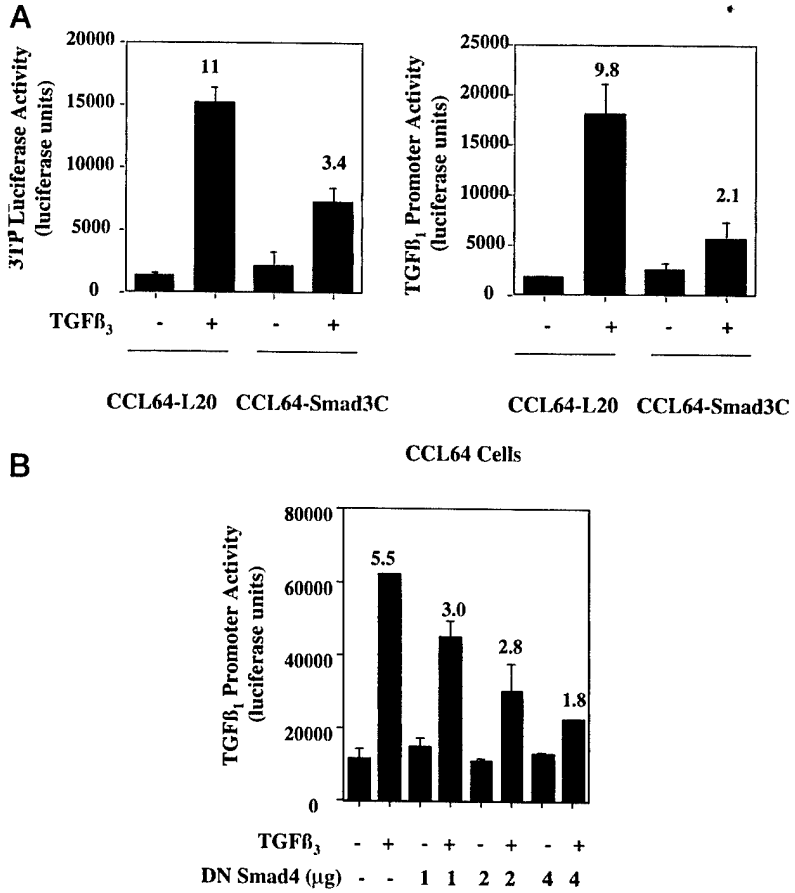


FIG. 7. **TGFβ$_3$ induction of TGFβ$_1$ is dependent upon Smad3 and Smad4 in CCL64 cells.** *A*, proliferating cultures of CCL64-L20 cells or CCL64-Smad3C cells were transfected with 0.5 μg of 3TP-Lux (*left panel*) or phTG5-Lux (*right panel*), and 0.125 μg of renilla luciferase control reporter (pRL-SV40) by Superfect. Luciferase activity was measured as described in the legend to Fig. 2*A*. The results plotted represent the $\bar{x} \pm$ S.D. for triplicate transfections. Representative of three independent experiments. *B*, proliferating cultures of CCL64 cells were transfected with 0.5 μg of phTG5-Lux, 0.125 μg of renilla luciferase control reporter (pRL-SV40), and different doses of DN Smad4 or empty vector by Superfect. Luciferase activity was measured as described in the legend to Fig. 2*A*. The results plotted represent the $\bar{x} \pm$ S.D. for triplicate transfections. Representative of three independent experiments.

viously shown that Ras is essential for TGFβ up-regulation of CKIs that contribute to TGFβ-mediated growth inhibition (18, 19). Results from two other groups have confirmed our original findings that the Ras/MAPK pathways were required for TGFβ induction of p21^{Cip1} expression (45, 46). Furthermore, we have demonstrated a requirement of the Ras/MEK1 pathway for the positive modulation of the Smad1 pathway (6, 7). TGFβ activation of SAPK/JNK has also been demonstrated to be required for the induction of fibronectin synthesis by TGFβ (16). Additional cellular functions of Ras/MAPK activation by TGFβ are likely to be elucidated as well.[4]

It is of considerable interest that JunD and Fra-2 are the primary components present in the TGFβ$_3$-stimulated AP-1 complex at the TGFβ$_1$ promoter. TGFβ has been shown previously to activate AP-1 proteins and to regulate the expression of downstream target genes (47–51). However, with respect to TGFβ-inducible genes, the involvement of JunD and Fra-2 has only been observed for the murine *laminin α3A* gene (50). Although JunD has also been reported to be involved in TGFβ induction of interleukin-6, collagenase-1, and collagenase-3 expression (47, 51), Fra-2 was not involved in the regulation of these genes. Moreover, the transactivation properties of Fra-2·JunD complexes are enhanced relative to JunD homodimers (52). Thus, the involvement of Fra-2/JunD in the regulation of TGFβ-inducible genes appears to be somewhat specific. Selective targeting of this combination of AP-1 members could block TGFβ$_1$ production specifically. Moreover, our results suggest that TGFβ may activate different AP-1 proteins to specifically regulate the expression of its target genes.

Although some AP-1 proteins have been shown to be important in mediating transformation of cells induced by oncogenes, JunD has actually been shown to inhibit the transformation of cells *in vitro* (53). Furthermore, overexpression of JunD can decrease the growth of mesenchymal cells (53). Thus, it is conceivable that the growth inhibitory effects of JunD may partially be due to the role of JunD in mediating TGFβ$_1$ production. That is, the induction of TGFβ$_1$ production by JunD may amplify the growth inhibitory effects of TGFβ in TGFβ-sensitive cells. In contrast, in some cancer cells, JunD and Fra-2 may still stimulate TGFβ$_1$ production, yet the cells would be resistant to TGFβs growth inhibitory effects. In this case, the elevated TGFβ would actually enhance the tumor-promoting effects of TGFβ via paracrine mechanisms. Along these lines, Fra-2 expression levels are high in breast cancer cells (54).

In *Drosophila*, the production of the TGFβ superfamily member Dpp requires DJNK, DFos, and DJun during dorsal closure (55–60). Here we demonstrate that Ras, MKK4/JNK, MEK/ERK, and specific AP-1 proteins are required for TGFβ$_3$ induction of TGFβ$_1$ in mammalian cells. Thus, the requirement of the Ras/MAPK pathways for production of TGFβ superfamily members appears to be evolutionarily conserved. Moreover, our results have indicated that TGFβ can directly activate JNK (reviewed in Refs. 1 and 2). Thus, it may also be possible for Dpp to directly activate DJNK and to establish an autocrine loop during dorsal closure. Along these lines, it has been shown that Dpp is required for Dfos expression during dorsal closure (58). However, the ability of Dpp to induce its own expression through DJNK/DJun/DFos has not been reported thus far.

Here, we demonstrate that TGFβ$_3$ induction of TGFβ$_1$ was also dependent upon Smads in CCL64 cells. Similarly, in *Drosophila*, it has been found that Mad is required for the maintenance of dpp expression during embryonic midgut development (61, 62). However, we were unable to detect binding of

[4] J. Yue and K. M. Mulder, unpublished results.

Smad3, or Smad4 to the AP-1 site in either IECs or CCL64 cells (Fig. 6). Moreover, pretreatment of CCL64 cells with cycloheximide (10 μg/ml) did not affect the ability of TGFβ to increase AP-1 complex formation under conditions where protein synthesis was inhibited by 97% (data not shown). Thus, it is possible that Smads may not function as transcription factors in mediating $TGF\beta_3$ induction of $TGF\beta_1$. Instead, Smads, in this context, may function as cytoplasmic modulators for other signaling components. Indeed, it has been reported that Smads can associate with other cytoplasmic proteins, including Smad anchor for receptor activation (SARA) (63), calmodulin (29), and microtubules (20).

In summary, $TGF\beta_3$ induction of $TGF\beta_1$ appears to occur via the following mechanism: TGFβ activates Ras, leading to induction of both the MKK4/SAPK an MEK1/ERK signaling cascades. These cascades, in turn, stimulate AP-1 protein complex formation at the AP-1 site in the $TGF\beta_1$ promoter. JunD and Fra-2 are the major AP-1 proteins that bind at this site to induce $TGF\beta_1$ transcription, thereby resulting in increased $TGF\beta_1$ protein expression and secretion. In addition, although Smads appear to contribute to this important cellular response of TGFβ, their effects are likely to be indirect in this context. Perhaps Smads assume novel intracellular functions not currently represented by the accepted Smad activation cascade.

Acknowledgments—We thank M. Morin (Pfizer Pharmaceuticals, Groton, CT) for generously supplying the $TGF\beta_3$, S. J. Kim (National Cancer Institute, Bethesda, MD) for the phTG5 construct, H. F. Lodish (MIT, Cambridge, MA) for the CCL64-Smad3C cells, R. Derynck (University of California, San Francisco, CA) for the DN Smad3 and DN Smad4 constructs, and R. Davis (University of Massachusetts, Worcester, MA) for the DN MKK4 construct.

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TRANSFORMING GROWTH FACTOR-β SIGNAL TRANSDUCTION IN EPITHELIAL CELLS

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Reprinted From: Pharmacology and Therapeutics
Volume 91 Number 1 2001

ELSEVIER

Pharmacology & Therapeutics 91 (2001) 1–34

Pharmacology & Therapeutics

Associate editor: E. Vesell

Transforming growth factor-β signal transduction in epithelial cells

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Abstract

Transforming growth factor (TGF)-β is a natural and potent growth inhibitor of a variety of cell types, including epithelial, endothelial, and hematopoietic cells. The ability of TGF-β to potently inhibit the growth of many solid tumors of epithelial origin, including breast and colon carcinomas, is of particular interest. However, many solid tumor cells become refractory to the growth inhibitory effects of TGF-β due to defects in TGF-β signaling pathways. In addition, TGF-β may stimulate the invasiveness of tumor cells via the paracrine effects of TGF-β. Accordingly, in order to develop more effective anticancer therapeutics, it is necessary to determine the TGF-β signal transduction pathways underlying the growth inhibitory effects and other cellular effects of TGF-β in normal epithelial cells. Thus far, two primary signaling cascades downstream of the TGF-β receptors have been elucidated, the Sma and mothers against decapentaplegic homologues and the Ras/mitogen-activated protein kinase pathways. The major objective of this review is to summarize TGF-β signaling in epithelial cells, focusing on recent advances involving the Sma and mothers against decapentaplegic homologues and Ras/mitogen-activated protein kinase pathways. This review is particularly timely in that it provides a comprehensive summary of both signal transduction mechanisms and the cell cycle effects of TGF-β.

Keywords: TGF-β; Ras; MAPK; Smad; Cell cycle; Cancer; Signal transduction

Abbreviations: ActRI, Type I activin receptor; ActRIB, Type II binding activin receptor; ActRII, Type II activin receptor; ALK, activin receptor-like kinase; AP, activator protein; ATF, activating transcription factor; BAMBI, bone morphogenetic protein and activin membrane-bound inhibitor; BMP, bone morphogenetic protein; CAK, Cdk-activating kinase; cAMP, cyclic AMP; Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; co-Smad, common-partner Sma and mothers against decapentaplegic homologue; CRE, cyclic AMP response element; CREB, Ca^{2+}/cyclic AMP response element-binding protein; dpp, decapentaplegic; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; GDF, growth and differentiation factor; GDNF, glial cell-derived neurotrophic factor; HDAC, histone deacetylase; IEC, intestinal epithelial cell; Ig, immunoglobulin; JNK, Jun N-terminal kinase; LAP, latency-associated peptide; Mad, mothers against decapentaplegic; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MH1, N-terminal domain; MH2, C-terminal domain; MIS, Müllerian-inhibiting substance; M phase, mitosis phase; NF, nuclear factor; PAI, plasminogen activator inhibitor; PKA, protein kinase A; PKC, protein kinase C; PL, phospholipase; pRb, retinoblastoma protein; RasN17, dominant-negative Ras mutant; Rb, retinoblastoma; RI, Type I receptor; RII, Type II receptor; RSmad, receptor-activated Sma and mothers against decapentaplegic homologue; SAPK, stress-activated protein kinase; SARA, Sma and mothers against decapentaplegic homologue anchor for receptor activation; SBD, Sma and mothers against decapentaplegic homologue-binding domain; SBE, Sma and mothers against decapentaplegic homologue-binding element; Smad, Sma and mothers against decapentaplegic homologue; Sp, stimulatory protein; S phase, synthesis phase; SRE, serum response element; STAT, signal transducer and activator of transcription; TAB1, TGF-β-activated kinase-binding protein-1; TAK1, TGF-β-activated kinase-1; TβRI, transforming growth factor-β Type I receptor; TβRII, transforming growth factor-β Type II receptor; TCF, ternary complex factor; TGF, transforming growth factor; TRE, 12-O-tetradecanoylphorbol-13-acetate response element; uPAR, urokinase receptor.

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0163-7258/01/$ – see front matter
PII: S0163-7258(01)00143-7

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1. Introduction

Transforming growth factor (TGF)-β is the prototype of over 40 family members, which share many structural and functional similarities. TGF-β superfamily members play an essential role in almost every aspect of cellular processes, including early embryonic development, cell growth, differentiation, cell motility, and apoptosis (reviewed in Roberts & Sporn, 1990; Hartsough & Mulder, 1997; Massagué, 1998; Piek et al., 1999; Derynck & Feng, 1997). The ability of TGF-β to potently inhibit the growth of many solid tumors of epithelial origin, including breast and colon carcinoma cells, is well established (Hartsough & Mulder, 1997; Massagué, 1998; Piek et al., 1999; Derynck & Feng, 1997; Markowitz & Roberts, 1996). However, many solid tumor cells develop resistance to the growth inhibitory effects of TGF-β due to defects in TGF-β signaling pathways (Hartsough & Mulder, 1997). Thus, it is important to elucidate TGF-β signal transduction pathways and to identify defects in the signaling components that are critical to the ability of tumor cells to subvert the growth inhibitory and other cellular effects of TGF-β.

Over the last few years, tremendous progress has been made in identifying signal transduction pathways involving TGF-β superfamily members. The complicated nature of TGF-β biology is unraveling slowly. TGF-β initiates its signals by binding and activating a tetrameric receptor complex consisting of Type I and II serine/threonine kinase receptors. The active receptor complex then transduces signals to downstream cellular components, including the

Sma and mothers against decapentaplegic (Mad) homologues (Smads) and members of the Ras/mitogen-activated protein kinase (MAPK) pathways. Moreover, TGF-β may utilize multiple intersecting pathways to regulate downstream cellular events. The sum total effect of this intricate network of pathways functionally defines the TGF-β molecule.

This review will first introduce briefly the structure, activation, and cellular functions of TGF-β superfamily members. The mechanisms of TGF-β-mediated growth inhibition in epithelial cells will then be summarized. A major part of the review will focus on recent advances involving TGF-β signaling, including the Smad and Ras/MAPKs pathways. Finally, the role of TGF-β in human cancer will be discussed.

2. Transforming growth factor-β

2.1. Transforming growth factor-β superfamily members

Over the last 17 years, over 40 TGF-β superfamily members have been identified (reviewed in Massagué, 1998). Almost all TGF-β superfamily members have an N-terminal precursor sequence and a mature C-terminal sequence containing seven to nine conserved cysteines. The homology between the mature C-terminal sequence of different TGF-β superfamily members ranges from 20% to 90%, suggesting that all of these TGF-β members may originate from the same ancestral genes (reviewed in Massagué, 1998; Kingsley, 1994; Hogan, 1996). As shown in Fig. 1, the TGF-β superfamily members can be grouped by sequence similarity. Among them, TGF-β1-3, bone morphogenetic proteins (BMPs) and their *Drosophila* homologue decapentaplegic (Dpp), activins, and Müllerian-inhibiting substance (MIS) are the most widely studied members.

TGF-β1-3 are the three mammalian TGF-β isoforms. They are 25-kDa homodimers, with each subunit consisting of ~ 112 amino acids. TGF-β1 was the first TGF-β superfamily member identified (Roberts et al., 1981, 1982, 1983). The amino acid sequence identity between the mature human TGF-β1 and TGF-β2, TGF-β1 and TGF-β3, or TGF-β2 and TGF-β3 is 71%, 76%, or 80%, respectively (Roberts & Sporn, 1990). In general, no significant differences are observed among the biological activities of these three isoforms in vitro. In vivo, however, these three isoforms are not functionally redundant, demonstrated convincingly by the non-overlapping phenotypes of three different isoform null mice (Bottinger et al., 1997). As the prototype of the TGF-β superfamily, the structures, functions, and signaling pathways of TGF-β will be discussed in detail in the following sections.

Subfamily	TGF-ß	BMP	Activin	Others
	TGF-ß1 TGF-ß2 TGF-ß3	BMP2 BMP4 Dpp BMP5 BMP6/Vgr-1 BMP7/OP1 BMP8a/OP2 BMP8b 60A, CDMP2 GDF5/CDMP1 GDF6/BMP13 Radar GDF7/BMP12 Vg-1, Univin GDF1 GDF3/Vgr-2 Dordalin BMP9 BMP10 CDMP BMP3/Osteogenin GDF10 Nodal Xnr1 Xnr2 Xnr3	Activin A Activin B Activin C Activin D Activin E	MIS GD9 Inhibin-α Lefty GDNF

Fig. 1. TGF-β superfamily members. CDMP, cartilage-derived morphogenetic protein; OP, osteogenic protein; Vgr, Vg-1 related; Xnr, *Xenopus* nodal-related protein.

BMPs originally were identified as osteogenic proteins that induce new bone formation (Kingsley, 1994; Hogan, 1996). These proteins were later found to play an important role in diverse biological processes, including cell differentiation, cell-fate determination, cell growth, neurogenesis, morphogenesis, apoptosis, and early embryonic development. Based upon sequence identity, many of the growth and differentiation factors (GDFs) were also grouped into the BMP family. Thus far, there are over 20 BMP family members; the sequence homology among them ranges from 42% to 92%.

After cleavage of the N-terminal precursor domain, the C-terminal domain of BMPs normally forms a disulfide-linked homodimer (except GDF3 and GDF9). Unlike TGF-β, the C-terminal domain of BMPs normally contains N-linked glycosylation sites and does not form a latent complex with its N-terminal precursor. Natural heterodimers, such as BMP2/BMP7 and BMP2/BMP6, have even higher binding affinities for BMP receptors. *Drosophila* Dpp is a close homologue of BMP2 and BMP4. Dpp plays an essential role in both dorsoventral axis and endoderm induction in the early *Drosophila* embryo, and also plays a role in the formation of adult appendages, including eyes and wings. BMP2/4 and Dpp can replace each other functionally (Kingsley, 1994; Hogan, 1996). Nodal, a novel BMP member, plays an important role in determining the left-right axis during embryonic development (Levin et al., 1995).

Activin members originally were identified as endocrine regulators for pituitary function. They also play an important role in mesoderm induction in *Xenopus* (Gaddy-Kurten et al., 1995). Activin is comprised of two inhibin β chains. Five activin family members have been identified thus far: activins A, B, C, D, and E. Inhibin, a heterodimeric glycoprotein, is comprised of an α chain and a β chain. Inhibin antagonizes the actions of activin and inhibits follicle-stimulating hormone production (Gaddy-Kurten et al., 1995). MIS, a distantly related TGF-β family member, regulates regression of the Müllerian duct and blocks formation of the uterus and oviducts. Like TGF-β, the mature C-terminal MIS segment is a 25-kDa disulfide-linked dimer; it can form a non-covalent link with its N-terminal precursor (Donahoe, 1992).

Lefty, a novel distantly related TGF-β superfamily member, lacks the cysteine residue required for homodimerization (Hogan, 1996). Lefty plays an important role in vertebrate embryogenesis, and has been suggested to function as a feedback inhibitor of Nodal signaling during vertebrate gastrulation (Meno et al., 1999). The most divergent TGF-β superfamily member is the glial cell-derived neurotrophic factor (GDNF), which shares little sequence similarity with other members. The receptor for GDNF is a tyrosine kinase (Lin et al., 1993).

Since TGF-β superfamily members play an important role in almost every aspect of growth and development, more TGF-β superfamily members may well be identified. However, the precise mechanisms by which TGF-β family members function remain to be elucidated. The studies summarized below have expanded our knowledge of the biological functions and signaling pathways for TGF-β.

2.2. Transforming growth factor-β structure

Most TGF-β family members are initially synthesized as larger (390–412 amino acid) precursor proteins containing three domains: an N-terminal signal domain; a variable pro-domain; and the mature, small C-terminal fragment (reviewed in Piek et al., 1999). The signal domain targets the precursor to the secretory pathways. The pro-domain may help the folding, dimerization, and regulation of the biological activity of the C-terminal small fragment. There is limited similarity between the sequence of the pro-domain of TGF-β superfamily members. The actual signaling molecule is the small C-terminal fragment that normally contains 7–9 cysteines (GDF3, GDF9, and lefty contain only 6 cysteines). The mature TGF-β molecule is a disulfide-bonded homodimer. Heterodimers have also been found, such as TGF-β1.2, TGF-β2.3, BMP2/BMP7, and BMP2/BMP6 (Roberts & Sporn, 1990; Massagué, 1998).

The crystal structure of TGF-β2 has been determined. The TGF-β2 monomer is a butterfly-like knot structure held together by four intrachain disulfide bonds formed by 8 cysteines; the last cysteine residue in each monomer forms an additional disulfide bond linking two monomers into a dimer (Sun et al., 1992; Schlunegget & Grutter, 1992). The conserved 7–9 cysteine residues in most TGF-β superfamily members suggest that they all share similar three-dimensional structures. Along these lines, no essential differences exist in the tertiary structure among the three TGF-β isoforms, as shown by the three-dimensional structures of TGF-β1 and TGF-β3 (Hinck et al., 1996; Mittl et al., 1996). Minor differences in the tertiary structure among TGF-β isoforms were detected. For example, residues 69–72 form a Type II β-turn in TGF-β1, but a Type I β-turn in TGF-β2. Also, the surface-exposed turn formed by residues 92–95 is a flexible Type II β-turn in TGF-β1, but is not well-defined in TGF-β2. These minor differences may play a role in the significant differences in receptor-binding affinities between TGF-β2 and other TGF-β isoforms (Hinck et al., 1996; Mittl et al., 1996).

2.3. Transforming growth factor-β activation from its latent form

Mechanisms controlling the activation of latent TGF-β are important for regulating the activity of TGF-β (Roberts, 1998). As mentioned in Section 2.2, TGF-βs are synthesized as 390–412 amino acid precursors. These precursors are processed proteolytically in the Golgi apparatus at the conserved dibasic RXXR site by furin, a convertase family of endoproteases. The mature C-terminal fragment of 110–114 amino acids is then released

(Blanchette et al., 1997). After processing, the mature TGF-β non-covalently binds to its N-terminal pro-domain, termed latency-associated peptide (LAP). This results in the formation of a small latent complex that prevents TGF-β from interacting with its receptors. The carbohydrate structures in the LAP are important in rendering TGF-β inactive in the latent complex (Miyazono & Heldin, 1989). The functions of the pro-domain of other TGF-β members remain unknown. In most cases, LAP covalently binds to a matrix protein, termed latent TGF-β-binding protein, to form a large latent complex. Latent TGF-β-binding proteins are important in TGF-β secretion and in attachment to the extracellular matrix (ECM) (Taipale et al., 1994).

The means by which latent TGF-β is activated are not fully understood. Proteases, such as plasmin or cathepsin, are thought to cleave the LAP to release the active TGF-β dimer (Grainger et al., 1994, 1995; Nunes et al., 1995). Binding of the LAP to the mannose-6 phosphate receptor, an insulin-like growth factor II receptor, can activate TGF-β as well (Flaumenhaft et al., 1993). Reactive oxygen species, radiation treatment, or acidic cellular microenvironments can also disrupt the interaction between the LAP and TGF-β to release the active TGF-β (Barcellos-Hoff et al., 1994; Barcellos-Hoff & Dix, 1996; Jullien et al., 1989). In vivo, it has been shown that thrombospondin is a major activator of TGF-β1. Binding of thrombospondin to the LAP can cause a conformational change in the latent complex. This unmasks the receptor-binding epitope of latent TGF-β, thereby activating the latent molecule (Crowford et al., 1998; Ribeiro et al., 1999). Binding of the LAP to immunoglobulin (Ig)G can also activate latent TGF-β, either by directly changing the conformation of the latent complex, or indirectly, by releasing the active TGF-β following internalization of the latent TGF-β-IgG complex (Stach & Rowley, 1993).

More recently, it has been shown that the binding of LAP to integrin αVβ6 can cause the β6 cytoplasmic domain to become accessible for binding to the actin cytoskeleton. This may induce a change in the conformation of the latent complex and permit access of mature TGF-β to its receptors. This mechanism of latent TGF-β activation has been suggested to play a role in regulating pulmonary inflammation and fibrosis (Munger et al., 1999). It has also been suggested that the interaction between latent TGF-β and αVβ1 integrin may initiate integrin signaling pathways (Munger et al., 1998).

Some TGF-β-associated proteins have also been shown to play an important role in regulating TGF-β bioactivity (Piek et al., 1999). Decorin, biglycan, and a 60-kDa protein in the ECM have been shown to bind TGF-β and inhibit TGF-β activity (Yamaguchi et al., 1990; Xu et al., 1998; Piek et al., 1997). Similarly, binding proteins for other TGF-β superfamily members have been identified, and can inhibit the bioactivity of these molecules. For example, noggin, chordin, DAN, follistatin, or gremlin can bind to BMP and prevent it from interacting with its receptors (Hsu et al., 1998; Blader et al., 1997; Francios & Bier, 1995; Iemura et al., 1998). Follistatin can also bind to activins to block the binding site in activins for their receptors (Mather, 1996).

2.4. Transforming growth factor-β expression

Although the three TGF-β isoforms, TGF-β1-3, share the same receptors and have similar cellular effects in vitro, TGF-β isoforms are differentially expressed during embryogenesis (reviewed in Roberts & Sporn, 1992). Their differential expression pattern during development may well explain the lack of phenotypic overlap among TGF-β-isoform null mice (Bottinger et al., 1997). Due to the maternal supply of TGF-β1, the TGF-β1-/- mice survived gastrulation, but died by the fourth week of birth due to an excessive inflammatory response. The main phenotypes included defects in yolk-sac vasculogenesis and breakdown of immune homeostasis (Shull et al., 1992; Kulkarni et al., 1993; Diebold et al., 1995). TGF-β2 null mice displayed a wide range of developmental defects in the heart, lung, craniofacial, limb, spinal column, eye, inner ear, and urogenital tract (Sanford et al., 1997). In contrast, TGF-β3 null mice displayed more specific defects in pulmonary development and palatogenesis, and died perinatally (Proetzel et al., 1995; Kaartinen et al., 1995).

The promoters for these TGF-β isoforms may determine their differential expression patterns and their differential responses to various stimuli (Roberts, 1998). The promoter of TGF-β1 lacks a TATA box, but has multiple regulatory enhancers, including activator protein (AP)-1, stimulatory protein (Sp)-1, and early growth response protein-1 sites. Accordingly, TGF-β1 isoforms can be induced by a variety of extracellular stimuli, including multiple oncogenes and viral proteins (Kim et al., 1989a, 1989b, 1990; Birchenall-Roberts et al., 1990; Yoo et al., 1996; Michelson et al., 1994). In contrast, the promoters of TGF-β2 and TGF-β3 both contain a TATA box and a cyclic AMP (cAMP) response element (CRE)/activating transcription factor (ATF) site, indicating that TGF-β2 and TGF-β3 may be more developmentally and hormonally regulated (O'Reilly et al., 1992; Lafyatis et al., 1990). High levels of expression of TGF-β3 in certain cells have been associated with transactivation of the TGF-β3 promoter via a repeated TCCC motif in its promoter; this may also be associated with the developmental and tissue-specific expression of TGF-β3 (Lafyatis et al., 1991). It has been shown that a raloxifene response element in the TGF-β3 promoter was required for TGF-β3 induction by estrogen in bone (Yang et al., 1996). The mRNA transcripts of all three isoforms have long 3′ and 5′ untranslated regions. This suggests that TGF-β isoforms can be post-transcriptionally regulated as well (Romeo et al., 1993).

2.5. Transforming growth factor-β biological function

TGF-βs regulate numerous biological activities, including cell proliferation, differentiation, adhesion, apoptosis,

ECM production, immune regulation, neuroprotection, and early embryonic development (reviewed in Roberts & Sporn, 1990; Hartsough & Mulder, 1997). As a pleiotropic cytokine, the multifunctional nature of TGF-β is context-dependent. Its effects are dependent on cell type, stage of differentiation, growth conditions, concentration of ligands, and presence of other growth factors (reviewed in Roberts & Sporn, 1990).

TGF-β is a potent growth inhibitor for a wide variety of cell types, including epithelial, endothelial, fibroblast, neuronal, lymphoid, osteoblast, and hematopoietic cells. Tremendous progress has been made during the last few years regarding the mechanisms mediating the growth inhibitory effects of TGF-β. These will be summarized in detail in the next section. However, TGF-β has also been shown to induce mitogenesis of mesenchymal cells, which may be due to the release of other growth factors in response to TGF-β (reviewed in Roberts & Sporn, 1990; Hartsough & Mulder, 1997).

TGF-β tightly regulates the production of the ECM by balancing new synthesis and turnover of ECM components (Hartsough & Mulder, 1997). TGF-β can increase the synthesis of ECM components, including collagen, fibronectin, laminin, and proteoglycans. TGF-β can also increase the expression of protease inhibitors, such as plasminogen activator inhibitor (PAI)-1 and the tissue specific inhibitor of metalloprotease. On the other hand, the expression of proteolytic enzymes, such as collagenase, transin, and thiol proteinase, are inhibited by TGF-β. Overall, TGF-β promotes the production and deposition of ECM. In response to injury, the production of TGF-β can stimulate cell proliferation and the production of additional ECM components to heal the tissue. Thus, TGF-β plays an important role in wound healing. However, overexpression of TGF-β in a pathological situation may produce pathological amounts of ECM and cause fibrosis (reviewed in Border & Noble, 1994).

TGF-βs can alter cell differentiation depending upon the cell type and growth conditions. For example, it has been shown that TGF-β can stimulate the differentiation of keratinocytes, bronchial epithelial cells, and colon carcinoma cells (reviewed in Mulder & Brattain, 1989). Other TGF-β superfamily members, including Dpp, BMPs, and activin, also play an important role in cell differentiation, especially during development (reviewed in Hogan, 1996).

TGF-βs play an important role in the immune system and are potent immunoregulatory factors (reviewed in Letterio & Roberts, 1998). Similarly, depending upon the cell type, state of differentiation, and the absence or presence of other growth factors or cytokines, TGF-β can either positively or negatively control the growth and activity of leukocytes, T-cells, and macrophages. For example, TGF-β induces a chemotactic response in human peripheral blood monocytes, which plays an essential role in the recruitment of mononuclear cells into sites of inflammation or injury (Wahl et al., 1987; Wiseman et al., 1988). In contrast, TGF-β suppresses the actions of tissue macrophages, which contributes to the resolution of an inflammatory response (Fargeas et al., 1992; Turner et al., 1991). Moreover, TGF-β has been demonstrated to play a role in autoimmune diseases. TGF-β1 null mice displayed multiple syndromes of autoimmune disease, which resulted in the death of the mice several weeks after birth (Shull et al., 1992; Kulkarni et al., 1993; Diebold et al., 1995).

TGF-βs have also been implicated in neuroprotection (reviewed in Pratt & McPherson, 1997). Both glial and neural cells produce various TGF-β isoforms (Unsicker et al., 1991; da Cunha et al., 1993). TGF-β isoforms can potently affect the proliferation, actions, and survival of neurons, glial cells, astrocytes, microglia, and oligodendrocytes (Martinou et al., 1990; Gouin et al., 1996). Thus, TGF-βs play an important role in the development and homeostasis of the CNS. In addition, TGF-βs also have been associated with several neurodegenerative diseases, including multiple sclerosis, Parkinson's disease, and Alzheimer's disease (Peress et al., 1996; Link, 1994; Mogi et al., 1995; Vawter et al., 1996; van der Wal et al., 1993).

3. Transforming growth factor-β-mediated growth inhibition in epithelial cells

3.1. Introduction

TGF-β is a potent growth inhibitor for epithelial cells. Addition of TGF-β arrests cells in the late G1 phase of the cell cycle. However, once cells pass the restriction point, TGF-β has no effect on cell cycle (reviewed in Hocevar & Howe, 1998). Due to its pleiotropic nature, the mechanisms involving TGF-β-mediated growth inhibition vary for different cell types, and also depend upon the experimental conditions. In summary, TGF-β-mediated growth inhibition has been associated with effects on G1 phase cyclins, cyclin-dependent kinases (Cdks), Cdk-activating kinase (CAK), Cdc25A, and Cdk inhibitors (CKIs).

3.2. Mammalian cell cycle

The eucaryotic cell cycle is divided into four distinct phases termed G1, synthesis (S), G2, and mitosis (M). After the M phase, the newly divided cells can enter the DNA S phase from G1. The commitment of cells to entry into the S phase from the G1 phase occurs at the restriction point late in the G1 phase, and depends upon cell growth conditions, such as the presence of mitogenic growth factors or growth inhibitors. In the absence of mitogenic factors, cells may exit from the cell cycle and remain arrested in a state of quiescence, called G0. The presence of serum mitogenic growth factors is required for cells to continuously progress through the cell cycle, and for cells in the G0 phase to re-enter the cell cycle.

Progress through the cell cycle is controlled by protein kinase complexes, consisting of two families of proteins. One is the family of Cdks, which phosphorylate their downstream targets on serines and threonines. The second is the family of cyclins, which associate with Cdks to control their activity (Morgan, 1995).

In different phases of the cell cycle, distinct Cdk-cyclin complexes are formed to promote progression through the cell cycle. From the early G1 phase to the end of the G1 phase, cyclin D-Cdk4 and 6 complexes are active. Starting from the late G1 phase until the end of the S phase, cyclin E-Cdk2 complexes are activated. Cyclin A-Cdk2 complexes start to be active from the early S phase to the mid G2 phase. Finally, cyclin A- and cyclin B-Cdc2 complexes are required from the mid G2 phase until the end of the M phase (reviewed in Morgan, 1995).

The assembly, activation, and disassembly of cyclin-Cdk complexes serve as the critical components of cell cycle progression. Cyclins are synthesized and degraded during each division of the cell cycle. In addition to binding to cyclins, full activation of Cdks requires the phosphorylation of a conserved threonine residue by CAK and dephosphorylation of one or two threonine/tyrosine residues by a dual phosphatase, Cdc25. The activity of the G1 cyclin/Cdk complexes can also be inhibited by CKIs (Fig. 2) (reviewed in Sherr & Roberts, 1999).

G1 progression is tightly controlled by the activity of Cdk-cyclin complexes, the products of the retinoblastoma (Rb) gene [retinoblastoma proteins (pRbs)], and transcription factors such as E2Fs. The Rb gene is a tumor suppressor gene. Its products include p107, p110, and p130, each of which is hypophosphorylated at the early G1 phase. The hypophosphorylated pRbs are associated with the E2F family of transcription factors to prevent the binding of E2Fs to DNA targets, thereby inhibiting the expression of a variety of genes. E2F transcription factors include E2Fs 1-5. For G1 progression, active cyclin D-Cdks 4 and 6 complexes are induced by mitogenic growth factors, and Cdk2-cyclin E complexes phosphorylate pRb sequentially. The hyperphosphorylated pRbs disassociate from E2Fs, allowing the E2Fs to bind to target DNA and activate transcription of a number of genes that are required for G1 to S phase transition (Fig. 2) (reviewed in Sherr & Roberts, 1995).

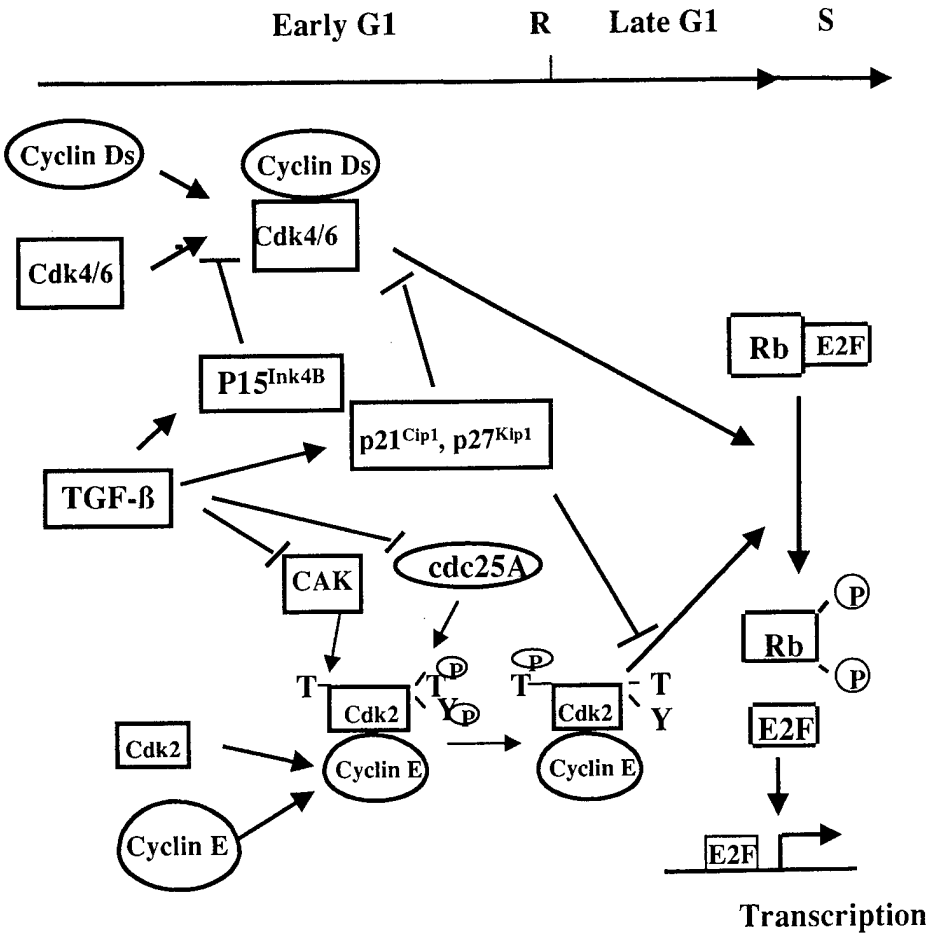

Fig. 2. Schematic representation of the relevant intracellular targets that mediate the growth inhibitory effects of TGF-β in epithelial cells. P, phosphorylation; R, restriction point; T, threonine; Y, tyrosine.

3.3. G1 cyclin-dependent kinase inhibitors

There are two families of CKIs: Ink family inhibitors and Cip/Kip family inhibitors. The Ink family includes $p16^{Ink4A}$, $p15^{Ink4B}$, $p18^{Ink4C}$, and $p19^{Ink4D}$. They can specifically bind to Cdks 4 and 6 to sequester cyclin D, thereby inhibiting the activity of Cdks 4 and 6. Among them, $p16^{Ink4A}$ is thought to be a tumor suppressor gene because it is mutated in a variety of human tumors (Sherr & Roberts, 1995). Since $p16^{Ink4A}$ is expressed in the late G1 phase, it was proposed that pRb phosphorylation by Cdks 4 and 6 may stimulate $p16^{Ink4A}$ expression, which then would function as a negative feedback regulator to block the activity of Cdks 4 and 6 (Tam et al., 1994). In contrast, extracellular signals such as TGF-β can directly up-regulate the expression of $p15^{Ink4B}$ to inhibit the activities of Cdks 4 and 6 (Hannon & Beach, 1994).

The Cip/Kip family includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. $p21^{Cip1}$ was the first CKI identified (Harper et al., 1993). Overexpression of $p21^{Cip1}$ can arrest cells in the late G1 phase by inactivating the G1 and S phase cyclin-cdk activities (Harper et al., 1993). $p21^{Cip1}$ is a universal growth inhibitor, and can form complexes with cyclin D-Cdk4/6, cyclin E-Cdk2, and cyclin A-Cdk2 in order to inhibit their activities (Sherr & Roberts, 1999). Moreover, at low concentrations, $p21^{Cip1}$ can act as an assembly factor to promote formation of active Cdk complexes (LaBaer et al., 1997). In some cell lines, $p21^{Cip1}$ expression is dependent on p53 (El-Deiry et al., 1993, 1994); in other cells, $p21^{Cip1}$ expression is p53-independent (Alpan & Pardee, 1996; Michieli et al., 1994). The expression of $p57^{Kip2}$ is tissue-restricted, and overexpression of $p57^{Kip2}$ can also arrest cells in the late G1 phase (Lee et al., 1995; Matsuoka et al., 1995).

$p27^{Kip1}$ was cloned as an inhibitor of cyclin E-Cdk2 (Polyak et al., 1994; Toyoshima & Hunter, 1994). $p27^{Kip1}$ can also bind to cyclin D-Cdk4/6 complexes to inhibit their activities. The abundance of $p27^{Kip1}$ is regulated at both translational and post-translational levels (Clurman & Porter, 1998). It has been shown that $p27^{Kip1}$ can be degraded by the ubiquitin-proteasome system (Pagano et al., 1995). Increased new synthesis of $p27^{Kip1}$ is due to an increased rate of translation (Hengst & Reed, 1996). Overexpression of $p27^{Kip1}$ in cultured cells arrests the cell cycle in the G1 phase (Polyak et al., 1994; Toyoshima & Hunter, 1994). *$p27^{Kip1}$* null mice exhibited gigantism due to increased cell number. The female mice were sterile, and the rate of tumorigenesis was increased in the pituitary gland (Fero et al., 1996, 1998; Nakayama et al., 1996; Kiyokawa et al., 1996). Additional data suggests that low or absent $p27^{Kip1}$ protein levels in tumor cells is an important clinical marker of disease progression (Porter et al., 1997; Tan et al., 1997; Catzavelos et al., 1997; Tsihilias et al., 1998; Cote et al., 1998; Esposito et al., 1997; Florenes et al., 1998; Jordan et al., 1998; Lloyd et al., 1997; Sanchez-Beato et al., 1997). However, most of these data are correlative in nature and do not identify $p27^{Kip1}$ loss as a causal event in multistep tumorigenesis. The only direct data implicating *$p27^{Kip1}$* as a tumor suppressor gene came from studies in *$p27^{Kip1}$* null mice (Nakayama et al., 1996; Fero et al., 1996; Kiyokawa et al., 1996). Not only did the *$p27^{Kip1}$* null mice develop pituitary adenomas, but they were hypersensitive to both radiation and chemical carcinogen-induced tumorigenesis when compared with *$p27^{Kip1}$* wild-type mice. Moreover, the sensitivity to tumorigenesis in these mice depended upon the copy number of the *$p27^{Kip1}$* gene (p27-/- > p27-/+ > p27+/+) (Fero et al., 1998). Accordingly, $p27^{Kip1}$ is considered to be haplo-insufficient for tumor suppression.

3.4. Transforming growth factor-β effects on G1 cyclin-dependent kinase and cyclin

TGF-β can potently inhibit the activities of both cyclin D-Cdk4/6 complexes and cyclin E/Cdk2 complexes, thereby resulting in the hypophosphorylation of pRbs and decreased transcriptional activity by E2Fs (reviewed in Hocevar & Howe, 1998). It has been shown that active transcriptional repression by the Rb-E2F complex mediates G1 arrest by TGF-β (Zhang et al., 1999). Due to differences in both cell type and experimental conditions, there were some contradictory results during the early years of research regarding whether or not TGF-β could regulate the expression levels of cyclin E, Cdk4, cyclin D, and Cdk2. For example, TGF-β treatment of synchronized cells released from the G0 state inhibited the expression of Cdk4 and Cdk2 (Ewen et al., 1993; Geng & Weinberg, 1993), whereas the expression levels of Cdk4, Cdk6, or Cdk2 were not affected by TGF-β in exponentially proliferating cells (Hannon & Beach, 1994; Reynisdóttir et al., 1995). In addition, TGF-β can decrease the expression levels of cyclin A. This effect, however, may be secondary to the TGF-β-mediated cell cycle arrest in the late G1 phase (Geng & Weinberg, 1993; Hartsough et al., 1996).

3.5. Transforming growth factor-β effects on cyclin-dependent kinase inhibitors

With the discovery of CKIs, the growth inhibitory effects of TGF-β have been associated with up-regulation of the CKIs $p15^{Ink4B}$, $p21^{Cip1}$, and $p27^{Kip1}$. TGF-β treatment of HaCaT keratinocytes and CCL64 mink lung epithelial cells leads to induction of $p15^{Ink4B}$, increased binding of $p15^{Ink4B}$ to Cdk4/6, and inhibition of the activities of Cdk4/6 (Reynisdottir et al., 1995). Up-regulation of $p15^{Ink4B}$ by TGF-β also leads to the release of $p27^{Kip1}$ from Cdk4/6 complexes, with a corresponding increase in $p27^{Kip1}$ present in Cdk2-cyclin E complexes. The different subcellular locations of $p15^{Ink4B}$ (cytoplasm) and $p27^{Kip1}$ (nuclear) have been suggested to enable $p15^{Ink4B}$ and $p27^{Kip1}$ to coordinately inhibit Cdk2 and Cdk4/6 kinase activities (Reynisdóttir & Massagué, 1997). Moreover, it has been shown that TGF-β up-regulation of $p15^{Ink4B}$ is Smad3-dependent (Rich et al., 1999). However, overexpression of Smad3 in Smad3 null mouse astrocytes failed to increase

$p15^{Ink4B}$ expression, suggesting that Smad3 may not be transcriptionally involved in TGF-β up-regulation of $p15^{Ink4B}$ (Rich et al., 1999). Another report indicated that TGF-β down-regulation of c-myc was required for TGF-β up-regulation of $p15^{Ink4B}$ (Warner et al., 1999). It is still not clear whether TGF-β up-regulation of $p15^{Ink4B}$ is directly related to c-myc down-regulation. Along these lines, it has been shown that c-myc can induce cyclins D1 and 2 to sequester $p21^{Cip1}$ and $p27^{Kip1}$, thereby leading to cell proliferation (Perez-Roger et al., 1999). Accordingly, it is possible that overexpression of c-myc can indirectly diminish TGF-β up-regulation of $p15^{Ink4B}$ by increasing expression of specific cyclin Ds to promote cell proliferation.

In the absence of up-regulation of $p15^{Ink4B}$ by TGF-β, regulation of Cdc25A, $p27^{Kip1}$, and/or $p21^{Cip1}$ by TGF-β appears to play a more dominant role in the regulation of Cdk activity (Florenes et al., 1996; Malliri et al., 1996; Yue et al., 1998; Iavarone & Massagué, 1997). Since TGF-β-mediated down-regulation of Cdc25A has been observed in only a few cell types thus far (Iavarone & Massagué, 1997, 1999; Kang et al., 1999b), regulation of $p21^{Cip1}$ and $p27^{Kip1}$ by TGF-β appears to be more universal (Hartsough & Mulder, 1997; Hocevar & Howe, 1998; Yue et al., 1998). It has been shown that TGF-β increases the expression of $p21^{Cip1}$ in a p53-independent pathway (Datto et al., 1995). Moreover, we have demonstrated that TGF-β activation of Ras is required for TGF-β up-regulation of $p21^{Cip1}$ (Yue et al., 1998). Recently, two other groups have confirmed our finding in their systems (Hu et al., 1999; Kivinen & Laiho, 1999). Additionally, it has been reported that TGF-β may activate the Ras/MAPK pathways to activate c-Jun, which can then transactivate the promoter of the $p21^{Cip1}$ gene via the Sp-1 site, thereby acting as a superactivator of the Sp-1 transcription factor (Kardassis et al., 1999; Kivinen et al., 1999).

We and other groups have demonstrated that TGF-β can regulate new synthesis, localization, and/or protein expression levels of $p27^{Kip1}$ in a variety of cell types (Reynisdóttir & Massagué, 1997; Florenes et al., 1996; Yue et al., 1998). Mal and co-workers (1996) reported that the adenovirus oncoprotein E1A can reverse the growth-inhibitory effects of TGF-β in mink lung epithelial cells by inactivating $p27^{Kip1}$, thereby suggesting that $p27^{Kip1}$ is required for TGF-β-mediated growth inhibition. Moreover, we have reported that dominant-negative Ras partially blocks the growth-inhibitory effects of TGF-β in intestinal epithelial cells (IECs), and that Ras is required for the rapid TGF-β-mediated increase in new synthesis of $p27^{Kip1}$ (Hartsough et al., 1996; Yue et al., 1998). These findings also support the contention that $p27^{Kip1}$ is required for TGF-β- mediated growth inhibition. Although the inhibitory effects of TGF-β on T-cells and mouse embryonic fibroblasts prepared from p27-/- knockout mice were maintained (Nakayama et al., 1996; Fero et al., 1996; Kiyokawa et al., 1996), a potential requirement of $p27^{Kip1}$ for TGF-β growth inhibitory effects in epithelial cells was not examined. Thus, whether $p27^{Kip1}$ is essential for TGF-β-mediated growth inhibition in epithelial cells remains to be established.

With regard to $p57^{Kip2}$, there is no evidence that TGF-β can up-regulate $p57^{Kip2}$ expression or increase its association with the Cdk complexes to inhibit Cdk activity. However, it has been shown recently in osteoblasts that TGF-β can actually inhibit $p57^{Kip2}$ expression and increase Cdk2 activity, leading to cell proliferation (Urano et al., 1999). These delayed effects of TGF-β on $p57^{Kip2}$ expression suggest that the down-regulation of $p57^{Kip2}$ may be secondary to TGF-β-stimulation of cytokine or growth factor secretion in these cells.

3.6. Transforming growth factor-β effects on other G1 regulators

The c-*myc* proto-oncogene has been implicated in TGF-β-mediated growth inhibition (reviewed in Alexandrow & Moses, 1997). Our laboratory was the first to demonstrate that c-myc is repressed in response to TGF-β; this effect was observed in the human colon carcinoma cell line MOSER (Mulder et al., 1988; Mulder & Brattain, 1988). Later, it was shown that TGF-β could rapidly inhibit c-myc expression in a wide variety of cell types (Mulder et al., 1990a, 1990b; Munger et al., 1992; Zentella & Massagué, 1992; Bang et al., 1996; Pietenpol et al., 1990a, 1990b). The down-regulation of c-myc by TGF-β may increase the association of CKIs with Cdk2 or Cdk4/6, thereby inhibiting the activities of these Cdks. Along these lines, it has been shown that TGF-β down-regulation of c-myc is required for TGF-β up-regulation of $p15^{Ink4B}$ (Hu et al., 1999). TGF-β down-regulation of c-myc may also affect other G1 components, and it has been shown that c-myc may function as a transcription factor to regulate the expression of cyclins A, E, and D1 (reviewed in Alexandrow & Moses, 1997). However, whether TGF-β down-regulation of c-myc is required for the growth inhibitory effects of TGF-β remains to be determined, although it has been suggested that TGF-β inhibition of c-myc expression may be essential for growth inhibition by TGF-β1 in some cell lines (Pietenpol et al., 1990a). In addition, it was shown that TGF-β can inhibit lung branching morphogenesis, which is correlated with the ability of TGF-β to inhibit N-myc expression in lung bud organ culture (Serra et al., 1994). Whether this TGF-β down-regulation of N-myc is required for TGF-β inhibition of lung development remains to be determined.

The ability of TGF-β to suppress the expression of the Cdk tyrosine phosphatase Cdc25A has also been associated with the growth inhibitory effects of TGF-β (Reynisdottir et al., 1995; Reynisdóttir & Massagué, 1997; Kang et al., 1999b). However, a rapid decrease in Cdc25A expression by TGF-β was only observed in one mammary epithelial cell line that lacks expression of $p15^{Ink4B}$ (Reynisdóttir & Massagué, 1997). In keratinocyte and gastric carcinoma cells, the effects of TGF-β on Cdc25A were delayed; down-regulation of Cdc25 occurred 10-15 hr after TGF-β

treatment (Reynisdottir et al., 1995; Kang et al., 1999b). Moreover, in keratinocytes, there are indications that TGF-β may induce an E2F4-p130 complex with histone deacetylase (HDAC)1 in order to bind to the repressor site in the Cdc25A promoter, thereby leading to a decrease in Cdc25A expression (Reynisdottir et al., 1995). However, since the effects of TGF-β on Cdc25A were delayed in keratinocytes, the induction of this E2F4-p130-HDAC1 repressor complex may also be secondary to the growth inhibitory effects of TGF-β.

More recently, TGF-β has been shown to inhibit Cdk2-cyclin E complexes by inhibiting CAK activity in human HepG2 hepatocellular carcinoma cells (Nagahara et al., 1999). In HepG2 cells, TGF-β treatment did not affect p21Cip1, p27Kip1, p15Ink4B, or Cdc25A expression levels, but resulted in an inactive Cdk2/cyclin E complex due to the absence of Thr160 phosphorylation on Cdk2. It has been shown that TGF-β can specifically down-regulate Cdk2 CAK activity without affecting Cdk4 CAK activity (Nagahara et al., 1999). Given the pleiotropic nature of TGF-β, it is not surprising that TGF-β may regulate different G1 components in different cell types. The mechanisms mediating TGF-β regulation of these G1 components remain to be determined.

4. Transforming growth factor-β receptor signaling

4.1. Three types of transforming growth factor-β receptors

TGF-β initiates its signaling by binding to its receptor complex. Three major types of TGF-β receptors have been identified; namely, Type I (65–70 kDa), Type II (85–110 kDa), and Type III (280–300 kDa) (reviewed in Derynck & Feng, 1997). Among them, the Type I (RI) and Type II (RII) receptors are the signaling receptors. Although other TGF-β cell surface-binding proteins have also been identified, their role, if any, in TGF-β signaling has not been identified clearly (reviewed in Derynck & Feng, 1997).

Six different RII receptors have been identified; each can bind to different ligands (Derynck & Feng, 1997). TGF-β RII (TβRII) binds only to TGF-β isoforms, with a higher affinity for TGF-β1 and TGF-β3, and a lower affinity for TGF-β2. TβRII is a 567 amino acid serine/threonine kinase and contains a 136 amino acid long N-glycosylated and cysteine-rich extracellular domain, a single transmembrane domain, a short juxtamembrane domain, a long kinase domain, and a short C-terminal tail (Lin et al., 1992).

Other Type II receptors are also serine/threonine kinases. They are structurally similar, but have some variations. The kinase domain is the most conserved domain (Derynck & Feng, 1997). ActRII is a Type II activin receptor; its ligands include activins, BMP7/OP-1, and GDF-5 (Mathews & Vale, 1993). ActRIIB is another Type II activin receptor; its ligands include activins, BMP7/OP-1, GDF-5, and BMP2 (Mathews et al., 1992; Attisano et al., 1992). The Type II BMP receptor can bind to BMP2/4 and BMP7/OP-1 (Rosenzweig et al., 1995; Nohno et al., 1995). MIS is the only ligand for its Type II receptor AMHR (Baarends et al., 1994; di Clemente et al., 1994). Punt, identified in *Drosophila*, is a Dpp Type II receptor (Letsou et al., 1995). The ligand for the Type II receptor Daf-4, identified in *Canorhabditis elegans*, has not been identified, although BMP2 and BMP4 can bind to Daf-4 in vitro (Estevez et al., 1993).

With regard to Type I receptors, 11 of them have been identified. The TGF-β Type I receptor (TβRI) TβRI/ activin receptor-like kinase (ALK)5/R4 only binds to TGF-βs (Franzen et al., 1993; Yamashita et al., 1994). There are two activin Type I receptors, ActRI and ActRIB. ActRIB, also known as ALK4 or R2, can only bind to activins (ten Dijke et al., 1993; Carcamo et al., 1994). ActRI, also known as ALK-2, Tsk7L, SKR1, or R1, can bind to various ligands, including activins, TGF-β, BMP2/4, and BMP7/OP1 (Yamashita et al., 1995; ten Dijke et al., 1994a, 1994b). Both BMPRIA/ALK3/BRK1/TRII and BMPRIB/ALK-6/BRK2 are BMP family Type I receptors. Their ligands include BMP2/4, BMP7/OP-1, and GDF-5 (Yamashita et al., 1995; Koenig et al., 1994).

ALK1, also known as TSR-1 or R3, is another Type I receptor that can bind to both TGF-β and activins, but the physiological functions of ALK-1 are unknown (Attisano et al., 1993; ten Dijke et al., 1994a). A deficiency in ALK-1 expression has been associated the hereditary haemorrhagic telangiectasis Type I in humans (Johnson et al., 1996). In *Drosophila*, two Type I receptors, Tkv and Sax, have been found to bind to Dpp (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994; Xie et al., 1994). The ligands for three other Type I receptors, ALK7, AtRI, and Daf-1 (a *C. elegans* Type I receptor) have not been identified yet (Tsuchida et al., 1996; Georgi et al., 1990; Wrana et al., 1994).

All Type I receptors, like Type II receptors, are single transmembrane serine/threonine kinases (reviewed in Derynck & Feng, 1997). Type I and Type II receptors share many structural similarities, although Type I receptors do have a shorter extracellular domain, a much shorter C-terminal tail, and a highly conserved GS-rich region immediately preceding the kinase domain. The SGSGLP sequence is considered to be a signature of Type I receptors. The phosphorylation of serines and threonines in the GS domain by TβRII is required for the activation of RI and TGF-β signaling (reviewed in Derynck & Feng, 1997).

Type III receptors include betaglycan and endoglin (reviewed in Derynck & Feng, 1997). Betaglycan is abundantly expressed in a wide variety of cell types, including mesenchymal, epithelial, neuronal, and other cell types. Endoglin is expressed primarily in vascular endothelial cells and hematopoietic cells (Yamashita et al., 1994; Cheifetz et al., 1992). Betaglycan has higher affinity for TGF-β2 than for TGF-β1 and TGF-β3 (Wang et al., 1991; Lopez-Casillas et al., 1991). Both betaglycan and endoglin are single transmembrane proteins that exist as homodimers. The endoglin homodimer is disulfide-bond linked (Yamashita

et al., 1994; Cheifetz et al., 1992), while monomers of the betaglycan homodimer are non-covalently linked (Henis et al., 1994).

The biological functions of both betaglycan and endoglin are not clear. It was thought that betaglycan may function to present the TGF-βs, especially TGF-β2, to TβRII (Lopez-Casillas et al., 1993; Moustakas et al., 1993). More recently, it has been shown that betaglycan is responsible for endocardial cell transformation (Brown, C. B. et al., 1999). In addition, some cell lines can secrete soluble betaglycan, corresponding to the extracellular domain of betaglycan, to inhibit the binding of TGF-β to the TβRI-TβRII complex (Lopez-Casillas et al., 1994). Indeed, it has been shown that this soluble betaglycan can inhibit tumorigenesis and metastasis of MDA-MB-231 human breast cancer cells by reducing the activity of TGF-β1 and TGF-β2 secreted by these cells (Bandyopadhyay et al., 1999). For endoglin, deficient endoglin function has also been suggested to be the cause of hereditary haemorrhagic telangiectasis Type I in humans (McAllister et al., 1994).

4.2. Mechanisms of transforming growth factor-β receptor activation

All Type I and Type II TGF-β receptors exist as homodimers in the absence of ligand (Derynck & Feng, 1997; Henis et al, 1994; Chen & Derynck, 1994). Two modes of ligand-receptor binding exist (Fig. 3) (reviewed in Massagué, 1998). For TGF-β receptors, TβRI binds poorly to TGF-β alone (Franzen et al., 1993; ten Dijke et al., 1993). TβRII homodimer must bind to TGF-β first, which then recruits TβRI to form a heterotetrameric complex (Wrana et al., 1994; Attisano et al., 1996). TβRII is constitutively hyperphosphorylated at several serine residues (Mathews & Vale, 1993; Wrana et al., 1994). The formation of TGF-β receptor heterotetrameric complex results in the phosphorylation of serine and threonine residues in the TβRI GS domain by TβRII (Wrana et al., 1994). The phosphorylated TβRI then becomes activated, and signals are transduced to cytoplasmic targets (Luo & Lodish, 1996; Wieser et al., 1995). Activin and MIS bind to their receptors in a similar manner (Mathews & Vale, 1993; di Clemente et al., 1994). Since TGF-β2 has only low affinity for TβRII, the binding of TGF-β2 to Type III receptors can facilitate the binding of TGF-β2 to TβRII, which, in turn, can recruit TβRI to form an active receptor complex (Lopez-Casillas et al., 1993; Moustakas et al., 1993). However, TGF-β2 can also directly bind to TGF-β receptor complexes in the absence of RIII in some cell types (Zhou et al., 1995a, 1995b; Mulder et al., 1993).

For BMP members, BMPs (including BMPs and Dpp) bind poorly to their Type I or Type II receptors at basal levels, but have high affinity for the receptor complexes (Rosenzweig et al., 1995; Nohno et al., 1995; Letsou et al., 1995, Liu et al., 1995; Nishitoh et al., 1996). Accordingly, it is thought that BMPs need to simultaneously bind to both Type I and Type II receptors in order to form an active heterotetrameric receptor complex.

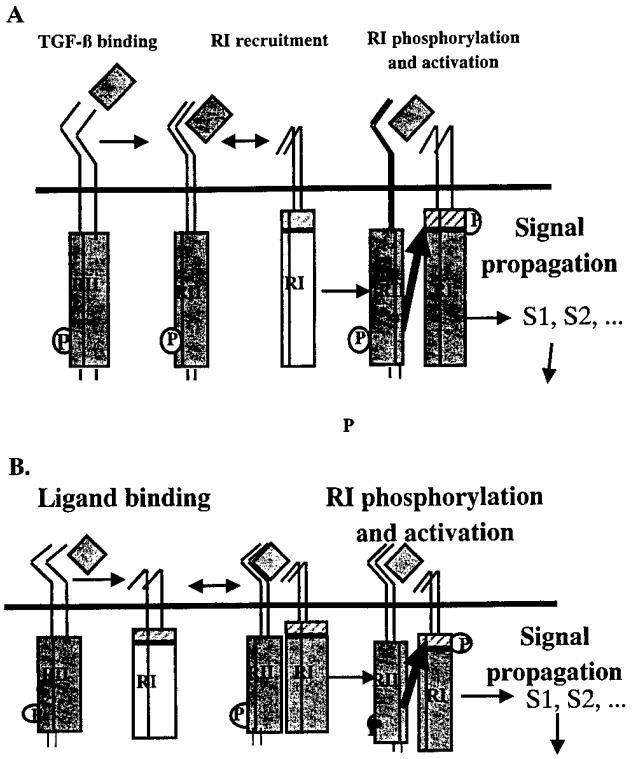

Fig. 3. Models for ligand binding to receptor complexes. A: Sequential binding, as for TGF-βs and activins. B: Cooperative binding, as for BMPs and Dpp. P, phosphorylation. Adapted from Wrana et al. (1994).

4.3. Structural requirements for transforming growth factor-β receptor activation

Specific domains in TβRI and TβRII are important for TGF-β signaling (reviewed in Massagué, 1998). The extracellular domains of both receptors are required for ligand binding (Feng et al., 1995; Okadome et al., 1994). The cytoplasmic domains of both TβRI and TβRII are required for the dimerization of the receptors, which is essential for TGF-β signaling (Luo & Lodish, 1996; Feng et al., 1995; Feng & Derynck, 1996). In addition, the kinase domain of TβRII is required for TβRII to phosphorylate TβRI, while the kinase domain of TβRI is required for TβRI to phosphorylate downstream targets (Feng et al., 1995; Wrana et al., 1992, 1994; Carcamo et al., 1995). It is also possible that TβRII can directly phosphorylate other downstream targets. The GS domain, as mentioned in Section 4.1, is essential for the activation of TβRI by TβRII (Wrana et al., 1992, 1994; Feng et al., 1995; Wieser et al., 1993). It has been suggested that the L45 loop, between kinase subdomains IV and V of TβRI, is required for substrate recognition (Feng & Derynck, 1997; Chen et al., 1998).

Some of the serine and threonine residues in the Type I and Type II receptors are important for TGF-β receptor signaling as well (reviewed in Derynck & Feng, 1997). TβRII primarily autophosphorylates threonines in vitro, but serines in vivo (Wrana et al., 1994). TβRII autophosphorylates on at least three serine residues: Ser409 and Ser416 in the protein kinase domain and Ser213 in the juxtamembrane domain (Luo & Lodish, 1997). Phosphorylation of Ser213 and Ser409 is essential for TβRII kinase signaling, while phosphorylation of Ser416 inhibits TβRII function (Luo & Lodish, 1997).

Phosphorylation of TβRII has also been observed on Ser551 and Ser553 of the C-terminus and Ser225, Ser228, and Ser229 in the juxtamembrane domain (Wrana et al., 1994; Souchelnytskyi et al., 1996). Since C-terminal tail deletion did not affect TβRII actions (Feng et al., 1995; Carcamo et al., 1995), the phosphorylation of the C-terminal tail is not thought to play a critical role in TβRII signaling. However, it is still not known whether the phosphorylation of the serine residues in the juxtamembrane domain plays a role in TβRII signaling. In vitro, TβRII can autophosphorylate on Tyr259 in the kinase domain, Tyr336 in subdomain V, and Try424 in subdomain VIII (Lawler et al., 1997). The role of TβRII tyrosine autophosphorylation in TGF-β signaling is not clear. TβRII also phosphorylates TβRI at four sites in the GS domain (Thr186, Ser187, Ser189, and Ser191) and at Ser165 in the juxtamembrane domain. The phosphorylation of Ser165 was thought to be the direct effect of TβRII, and may modulate the signaling specificity of TGF-β receptors (Souchelnytskyi et al., 1996).

4.4. Transforming growth factor-β receptor interacting proteins

Before the discovery of Smads, a few TGF-β receptor-associated cytoplasmic proteins had been identified. Both FKBP12 and the α-subunit of farnesyltransferase were isolated as TβR1 cytoplasmic-associated proteins using the yeast two-hybrid system (Wang et al., 1996a, 1996b; Chen, X. et al., 1997; Ventura et al., 1996). FKBP12 is an abundant 12-kDa cytosolic protein with cis-trans peptidyl-prolyl isomerase activity. FKBP12 binds to a conserved Leu-Pro motif in the TβRI juxtamembrane domain, and functions as an inhibitor for TGF-β signaling by preventing the phosphorylation of TβRI by TβRII at the basal level. The binding of TGF-β to the receptor complex appears to release the FKBP12 from TβRI (Wang et al., 1996b; Chen, Y. G. et al., 1997; Huse et al., 1999). As for α-farnesyltransferase, its role, if any, in TGF-β signaling is not clear. TGF-β does not appear to affect farnesyltransferase activity (Wang et al., 1996a; Ventura et al., 1996; Kawabata et al., 1995).

More recently, BMP and activin membrane-bound inhibitor (BAMBI), a transmembrane protein, has been shown to stably interact with TGF-β family receptors (Onichtchouk et al., 1999). BAMBI can inhibit TGF-β, BMP, and activin signaling by preventing the formation of functional TGF-β-family signaling receptor complexes (Onichtchouk et al., 1999). Activin receptor-interacting protein-1, a mouse PDZ protein, has also been shown to interact with the activin Type IIA receptor and Smad3 (Shoji et al., 2000). It has been suggested that activin receptor-interacting protein-1 may play a role in activin signaling at specific subcellular sites and in regulating signal transduction in neuronal cells (Shoji et al., 2000).

Other TGF-β receptor-associated proteins have been identified. TGF-β receptor interacting protein-1 associates with TβRII; serine threonine kinase receptor-associated protein interacts with TβRI and TβRII; TβRI-associated protein-1 associates with TβRI; B α, a WD-40 repeat subunit of phosphatase 2A, associates with TβRII and TβRI; RI; and apolipoprotein J associates with TβRII and TβRI (reviewed in Piek et al., 1999). The roles of these proteins in TGF-β signaling have not been clearly elucidated.

5. Sma and Mad homologues

5.1. Identification of Smads

The first member of the Smad family of proteins, Mad, was identified by suppressor/enhancer genetic screening for genes that can enhance the effect of weak dpp alleles (Raftery et al., 1995; Sekelsky et al., 1995). Later, three Mad homologues in *C. elegans*, Sma-2, -3, and -4, were isolated; these share similar mutant phenotypes with Daf-1, a Type II TGF-β receptor in *C. elegans* (Savage et al., 1996). Thereafter, several Mad homologues were identified in vertebrates; they are termed Smads. Thus far, 9 mammalian Smads (Smads 1-9) have been cloned by either screening cDNA libraries or searching EST databases (reviewed in Massagué, 1998; Heldin et al., 1997; Riggins et al., 1996). Smad4, also known as DPC4, originally was identified by screening for gene mutations in pancreatic cancers (Hahn et al., 1996). All the genetic and functional studies indicate that Smads play an important role in TGF-β signaling (reviewed in Massagué, 1998; Piek et al., 1999; Heldin et al., 1997).

Smads can be divided into three groups based upon their structure and function (Fig. 4). The first group, referred to as receptor-activated Smads (RSmads), includes Smads 1-3, 5, and 8. Mad is a *Drosophila* homologue of Smad1; Sma2 and Sma3 are the RSmads in *C. elegans*. The second group is referred to as common-partner Smads (co-Smads), including Smad4, Medea (a homologue of Smad4 in *Drosophila*), and Sma-4 (the co-Smad in *C. elegans*). In *Xenopus*, two co-Smads have been identified: Smad4α and Smad4β (Masuyama et al., 1999). The last group is referred to as the inhibitory Smads, including Smads 6 and 7, and Dad (a *Drosophila* inhibitory Smad) (reviewed in Massagué, 1998; Piek et al., 1999; Heldin et al., 1997).

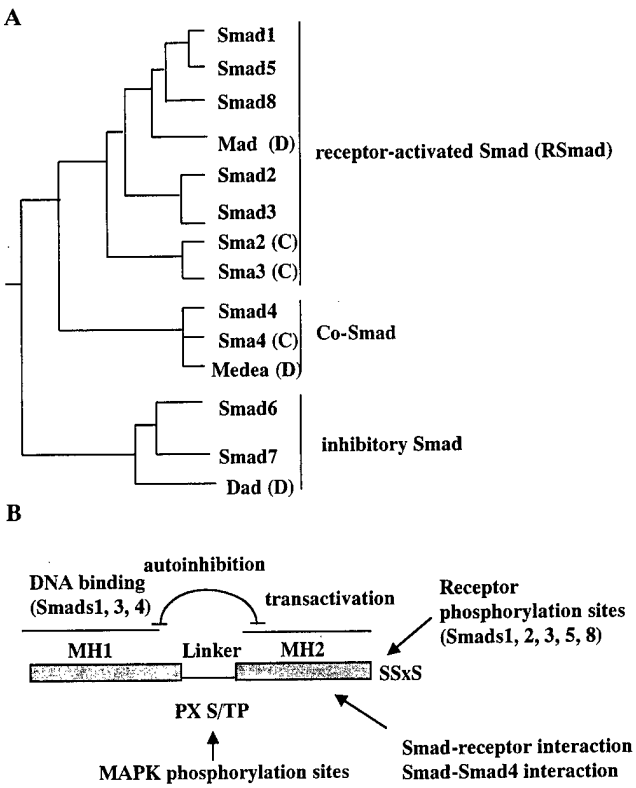

Fig. 4. The Smad superfamily. A: Phylogenetic relationships of Smad members. B: Schematic structure of Smads. C, *C. elegans*; D, *Drosophila*; P, proline; S, serine; T, threonine.

5.2. Structure of Smads

The molecular weight of Smads ranges from 42 to 60 kDa (reviewed in Massagué, 1998; Piek et al., 1999; Heldin et al., 1997). Smads share extensive sequence homology in two distinct regions, an N-terminal domain (MH1) and a C-terminal domain (MH2), while the proline-rich linker regions are poorly conserved (Fig. 4). The MH1 domain is highly conserved among RSmads and co-Smads, but not in the inhibitory Smads. In the inactive state, the MH1 domain binds to the MH2 domain to inhibit the transcriptional activity of the MH2 domain. In the active state, the MH1 domain is the DNA-binding domain; it is required for the transcriptional activity of the Smad complex.

The MH2 domain is required for Smad homodimerization. In the active state, the MH2 domain can transiently associate with TβRI. It is also responsible for the association of RSmads with both Smad4 and other transcription factors. These associations are required for the transcriptional activity of RSmads.

The active TGF-β receptor complex results in phosphorylation of the RSmad at the sequence SSXS in the C-terminal tail of the MH2 domain. This phosphorylation event is required for the activation of RSmads. Smad4 does not have this SSXS motif, and Smad4 is not phosphorylated. Structural analysis suggests that the inability of Smad4 to bind to RI is due to a lack of a basic surface at the MH2 domain of Smad4 (Wu et al., 2000). In the linker region of RSmads, there are several consensus MAPK phosphorylation sites that are responsible for crosstalk between the Smad and MAPK pathways (Mulder, 2000; Yue et al., 1999a, 1999b; X. J. Liu & K. M. Mulder, submitted for publication).

5.3. Functions of Smads

Targeted mutagenesis studies have provided strong evidence that Smads play a vital role in embryogenesis and TGF-β signaling. *Smad4-/-* mice were embryonic lethal at day 6.5, without signs of gastrulation (Sirard et al., 1998). When rescued by a wild-type Smad4, the *Smad4+/-* mutant mice exhibited severe anterior truncation (Sirard et al., 1998). Similar phenotypes have also been observed for *Smad2-/-* mice. *Smad2* null mice died at embryonic day 9.5 due to a failure to form the anterior-posterior axis; this indicates that Smad2 also plays an important role during development (Nomura & Li, 1998; Heyer et al., 1999). In contrast, the *Smad3-/-* mice only died between 1 and 8 months after birth due to defects in immune functions and forelimb development (Yang et al., 1999; Datto et al., 1999). The *Smad3* null mice also showed accelerated wound healing and an impaired local inflammatory response (Ashcroft et al., 1999). One group reported that *Smad3-/-* mice developed invasive colonic adenocarcinomas (Zhu et al.,

1998). *Smad6* mutant mice developed multiple cardiovascular abnormalities, indicating that Smad6 plays a role in the regulation of the development and homeostasis of the cardiovascular system (Galvin et al., 2000). The mutations in *Smad4*, and less so for *Smad2*, have been found in many pancreatic, breast, and colon cancers (Hahn et al., 1996; Schutte et al., 1996; Prunier et al., 1999; Riggins et al., 1996, 1997; Uchida et al., 1996; Eppert et al., 1996).

5.4. Smad signaling

The general mechanisms mediating TGF-β receptor activation of Smads have been investigated (Fig. 5). Binding of the ligand to the TGF-β receptor complex induces the phosphorylation of RSmads. Generally, Smads 1, 5, and 8 can be phosphorylated by BMPs, while TGF-β and activin can phosphorylate Smad2 and Smad3. We have demonstrated that TGF-β can also phosphorylate Smad1 in Hs578T human breast cancer cells and IEC 4-1 cells (Liu et al., 1998; Yue et al., 1999a, 1999b; X. J. Liu & K. M. Mulder, submitted for publication). In addition, it has been shown that TGF-β can phosphorylate Smad5 in human hematopoietic cells (Bruno et al., 1998).

The phosphorylation of SSXS at the C-terminus of RSmads causes a conformational change in RSmad, which opens up the MH2 domain. Thereafter, the phosphorylated RSmads dissociate from the receptor complex and form a heteromeric complex with co-Smads (i.e., Smad4). According to the current working model, the Smad complex is then translocated to the nucleus. Once in the nucleus, the Smad complex either can directly bind to DNA or can associate with other transcription factors to induce target gene transcription (reviewed in Massagué, 1998; Piek et al., 1999; Heldin et al., 1997; Derynck et al., 1998). Moreover, Smad signaling can be modulated by many Smad-associated proteins and by other signaling pathways, including the Ras/MAPK pathways (reviewed in Mulder, 2000; Zhang & Derynck, 1999). The inhibitory Smads, 6 and 7, appear to block the activation of RSmads by TGF-β receptors (reviewed in Wrana, 2000).

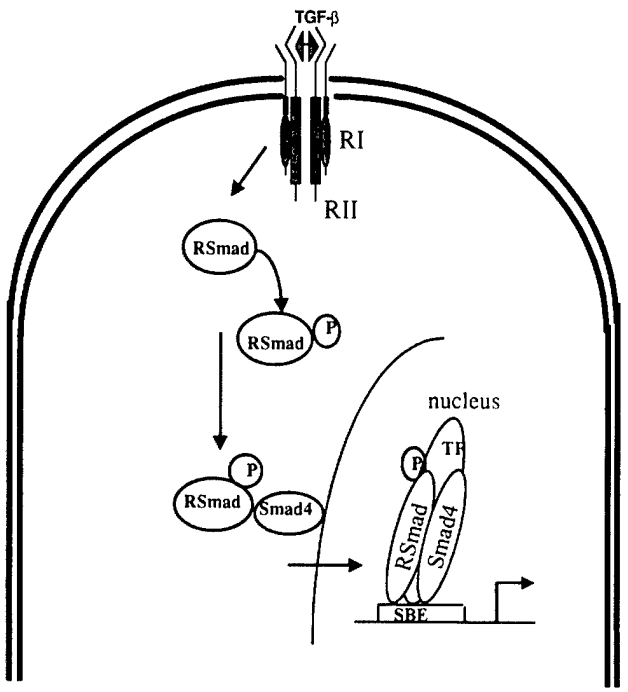

Fig. 5. Model for TGF-β regulation of transcription through Smad signaling proteins. P, phosphorylation; TF, transcription factor.

5.5. The function of the Smad anchor for receptor activation

After ligand binding, RSmads can transiently associate with Type I receptors with the help of the Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998). The SARA protein is a FYVE domain protein, which attaches to membrane lipids by binding to phosphatidylinositol-3-phosphate. SARA functions to recruit both Smad2 and Smad3 to the receptor complexes by controlling the subcellular localization of Smad2 and Smad3 (Tsukazaki et al., 1998).

Recently, the crystal structure of the Smad2 MH2 domain bound to a SARA Smad-binding domain (SBD) was determined (Wu et al., 2000). The SARA SBD exists as an extended conformation consisting sequentially of a rigid coil, a helix, and a β-strand. The SARA SBD interacts with the β-sheet and the three-helix bundle of the Smad2 MH2 domain. Recognition specificity between Smad2 and SARA is determined by the interaction between the SARA rigid coil and the strands B8, B9, B5, and B6 of the Smad2 β-sheet. The binding between the β-strand of SARA and the three-helix bundle of Smad2 contributes to binding affinity (Wu et al., 2000). Five residues in the Smad2 β-sheet, which determine binding specificity, are conserved in Smad3, but differ in Smads 1, 5, and 8. Thus, SARA can interact with Smad2 and Smad3, but not other RSmads (Wu et al., 2000).

5.6. Other features of Smad signaling

As shown in the crystal structure of the Smad4 cytoplasmic domain (MH2), 17 highly conserved amino acids, termed the L3 loop, protrude from the conserved MH2 core. The L3 loop is responsible for the association of Smad4 with RSmads (Shi et al., 1998). For RSmads, this L3 -L45 loop interaction on the Type I receptor plays a role in the specificity of RSmad for Type I receptors (Chen et al., 1998; Lo et al., 1998). Swapping two different amino acids in the L3 loop of Smad1 and Smad2 switched the activation of these two Smads by TGF-β or BMP, respectively (Lo et al., 1998). In addition, the α-helix 1 in the MH2 domain of Smads is important for the interaction between receptor I and RSmads. Along these lines, ALK1, ALK2, and *Drosophila* Saxophone can all phosphorylate Smad1; they have different L3 loop sequences, but a similar α-helix 1 (Chen & Massagué, 1999; Macias-Silva et al., 1998).

Given the pleiotropic nature of the TGF-βs, it is possible that the specificity between TGF-β receptors and RSmads may be controlled by multiple domains other than those previously described. We have cloned a rat homologue of Smad1 by screening an IEC cDNA library. Using an in vitro kinase assay with Smad1 as the substrate, we have demonstrated that the TGF-β-receptor complex can directly phosphorylate Smad1. Both TGF-β and BMP can also directly phosphorylate Smad1 in vivo in Hs578T cells and in untransformed IECs (Liu et al., 1998; Yue et al., 1999b; X. J. Liu & K. M. Mulder, submitted for publication). The RI and RII TGF-β receptors were detectable in Smad1 immunocomplexes (Yue et al., 1999b). TGF-β could also increase Smad1-Smad4 hetero-oligomerization and Smad1 nuclear translocation, as did BMP2 (Liu et al., 1998). In addition, the combination of transient expression of Smad1 and TGF-β treatment had a synergistic effect on induction of the TGF-β-responsive reporters 3TP-Lux and Smad-binding element (SBE)-Luc (Liu et al., 1998; X. J. Liu & K. M. Mulder, submitted for publication). The first of these reporters contains 3 AP-1 sites and 400 bp of the PAI-1 promoter (Wrana et al., 1992). The latter is a Smad-specific reporter containing four repeats of the SBE (Zawel et al., 1998). Collectively, our results clearly demonstrate that TGF-β can regulate Smad1, at least in some cell types. Future studies will verify the functional receptor types in these cells and will determine the cellular targets of TGF-β regulation of Smad1 in these cells.

5.7. Smad-binding elements

It has been shown that the MH1 domain of *Drosophila* Mad can directly bind to a GC-rich sequence in the Dpp-inducible vestigial promoter (Kim et al., 1997). In further studies, the MH1 domains of Smads 1, 3, and 4 have been shown to have DNA-binding affinity (Zawel et al., 1998; Yingling et al., 1997; Jonk et al., 1998; Dennler et al., 1998; Zhang et al., 1998; Shi et al., 1998). Although Smad2 cannot bind to DNA because of the additional insertion in its MH1 domain, Smad2 can associate with Smad4 and other transcription factors, including Fast-1 or Fast-2, to induce Mix2 or goosecoid gene expression, respectively, in *Xenopus* (Chen, X. et al., 1996, 1997; Labbe et al., 1998; Zhou et al., 1998). It has been shown that the Smad3/4 complex can directly bind to a conserved DNA sequence, AGAC, or its complementary sequence, GTCT, to induce gene transcription (Zawel et al., 1998; Jonk et al., 1998; Dennler et al., 1998; Shi et al., 1998; Zhang et al., 1998). Such a sequence has been found in the promoters of PAI-1, JunB, and collagenase I genes (Jonk et al., 1998; Dennler et al., 1998; Wong et al., 1999).

Although some TGF-β target genes contain one or more SBEs, other target genes, such as $p21^{Cip1}$ and $p15^{InkB}$, do not appear to contain these optimal SBE sites in their promoters. Thus, it is possible that TGF-β may also utilize other signaling pathways to regulate gene expression, or Smads may bind to sites other than the SBE to induce gene transcription. Along these lines, it has been shown that Smad1, like *Drosophila* Mad, can bind to a consensus GCCGnCGC motif; BMP, but not TGF-β or activin, can regulate the GCCG-Lux reporter containing multiple repeats of GCCG (Kusanagi et al., 2000).

5.8. Smad-associated transcription factors

Smads have been shown to associate with other transcription factors to induce target gene transcription. As mentioned in the previous section, the Smad2/4 complex can associate with Fast-1 or Fast-2 for Mix2 or goosecoid gene transcription (Chen, X. et al., 1996, 1997; Labbe et al., 1998; Zhou et al., 1998). Smad3/4 can associate with c-Jun and c-Fos to bind to 12-O-tetradecanoylphorbol-13-acetate response element (TRE) sites (Zhang et al., 1998). For PAI-1 expression, Smad3/4 association with the transcription factor μE3 is required for TGF-β activation of PAI-1 expression (Hua et al., 1998).

Two closely related cAMP-responsive element-binding proteins and p300 proteins can interact with Smad complexes to bring the sequence-specific activators within proximity of the general transcriptional machinery, and to modify the chromatin structure through histone acetylation (Feng et al., 1999; Janknecht et al., 1998; Shen et al., 1998; Topper et al., 1998). The transcription factor MSG1 has been shown to increase the functional link between Smads and p300/CBP by interacting with both Smad proteins and p300/CBP through N- and C-terminal regions of MSG1, respectively (Shioda et al., 1998; Yahata et al., 2000). Moreover, p300 can promote the interaction between Smad1 and signal transducer and activator of transcription (STAT)3, thereby leading to the synergistic actions of BMP2 and the leukemia inhibitory factor on astrocyte differentiation (Nakashima et al., 1999).

In addition, TGF-β can activate Smad3 to form a complex with a member of the steroid receptor coactivator-1 protein family in the nucleus, which can potentiate the transcriptional activity of the vitamin D receptor (Yanagisawa et al., 1999). Smad3 and Smad4 can also associate with the acute myeloid leukemia family of transcription factors to synergistically regulate the expression of human germ-line IgA genes (Pardali et al., 2000). Further, Smad2 can associate with paired-liked homeodomain transcription factors of the Mix family, Mixer and Milk; the Mixer/Milk/Smad2/Smad4 complex then binds to the Mixer/Milk binding site to mediate activin/TGF-β-induced transcription (Germain et al., 2000).

More recently, Smad1 has been shown to interact with the homeobox DNA-binding protein Hoxc-8. The interaction between Smad1 and Hoxc-8 dislodges Hoxc-8 binding from its element, thereby resulting in initiation of gene transcription (Shi et al., 1999). In response to BMP2, Smad1 can also associate with an Olf-1/EBF-associated 30-zinc finger protein as a DNA-binding factor to transcriptionally

activate Xvent2, a homeobox regulator of *Xenopus* mesoderm and neural development (Hata et al., 2000).

5.9. Negative regulation of Smad signaling

TGF-β, activins, and BMPs can induce the expression of Smad6 and Smad7 to establish a negative-feedback loop. Smad6 and Smad7 bind to TGF-β family member receptors and inhibit the activation of RSmads by these receptors (Hayashi et al., 1997; Nakao et al., 1997; Hata et al., 1998). Moreover, Smad6 and Smad7 may inhibit RSmad association with Smad4 by competitively binding to Smad4 (Hata et al., 1998). Normally, Smad6 selectively inhibits BMP signaling, while Smad7 mainly suppresses TGF-β and activin signaling (Hata et al., 1998; Nakao et al., 1997; Hayashi et al., 1997; Ishisaki et al., 1999). Smad6 can also act as a transcriptional corepressor by interacting with Hoxc-8 and Hoxc-9 and binding to DNA. This Smad6/Hoxc-8 complex can prevent Smad1 from interacting with Hoxc-8, thereby inhibiting Smad1-mediated transcription and BMP signaling (Bai et al., 2000). Whether Smad7 can act as a corepressor remains to be determined.

In addition, it has been shown that epidermal growth factor and phorbol ester may activate the MAPKs to increase Smad6 and Smad7 expression (Afrakhte et al., 1998). Interferon-γ can induce the expression of Smad7, which, in turn, can inhibit TGF-β activation of Smad3 and its downstream responses (Ulloa et al., 1999). Tumor necrosis factor-α can induce Smad7 expression to inhibit TGF-β/Smad signaling via nuclear factor (NF)-κB/RelA (Bitzer et al., 2000).

It has been shown that TGF-β can increase the expression of two oncoproteins, Ski and SnoN, which then recruit the corepressor N-CoR to terminate Smad-mediated gene transcription (Luo et al., 1999; Nishihara et al., 1999; Stroschein et al., 1999; Sun et al., 1999a, 1999b). In addition, oncoprotein Evi-1 can suppress Smad3 transcriptional activities by competitively binding the MH2 domain of Smad3 and by inhibiting the binding of Smad3 to DNA (Kurokawa et al., 1998). TGF-β can induce Smad2 association with 5'TG3' interacting factor, a ubiquitously expressed homeodomain protein. 5'TG3' interacting factor then recruits HDACs to a Smad target promoter and represses transcription (Wotton et al., 1999a, 1999b). Similarly, Smad-interacting protein-1, a novel Zinc finger/homeodomain repressor, can interact with receptor-activated RSmads to bind to a target promoter region and repress the transcription of targeted genes, at least in activin-dependent signaling (Verschueren et al., 1999).

TGF-β can also activate multi-ubiquitination of phosphorylated nuclear Smad2, leading to its degradation and, subsequently, to the termination of Smad signaling (Lo & Massagué, 1999). Similarly, a Smad ubiquitination regulatory factor-1 can selectively interact with Smad1 and Smad5 to trigger their ubiquitination and degradation, leading to the inactivation of BMP signaling. However, the activation of Smad ubiquitination regulatory factor-1 occurs independently of BMP receptor activation (Zhu et al., 1999).

5.10. Other Smad-associated proteins

Smads can associate with other cytoplasmic proteins. As mentioned in Section 5.5, Smad2 and Smad3 are associated with SARA (Tsukazaki et al., 1998). Although it was thought that SARA only functions to anchor Smads to their receptors, the interaction between Smads and SARA induced by TGF-β may also activate other signaling pathways (Tsukazaki et al., 1998). Moreover, it is possible that a similar anchor protein exists for Smads 1, 5, and 8.

Smads have also been shown to interact with calmodulin, which may affect Ca^{2+} signaling (Zimmerman et al., 1998). More recently, Smads 2, 3, and 4 have been shown to associate with microtubules (Dong et al., 2000). Activation of Smad2 results in its dissociation from β-tubulin. Disrupting microtubules causes phosphorylation of Smad2 in the absence of ligand and Smad-dependent transcriptional activity. These data suggest that microtubules may negatively regulate Smad activity (Dong et al., 2000). On the other hand, the dissociation of Smads from microtubules induced by TGF-β treatment may also initiate other signaling besides Smad activation.

6. Mitogen-activated protein kinases

Since our first demonstration that TGF-β could activate the Ras/MAPK pathways in untransformed epithelial cells, additional data have accumulated to indicate that TGF-β activation of the Ras/MAPK pathways plays a critical role in TGF-β signaling (Hartsough & Mulder, 1997). These components are important mediators of TGF-β biological responses (Mulder, 2000).

6.1. Types of mitogen-activated protein kinases

The MAPKs are proline-directed, serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a wide array of extracellular stimuli (reviewed in Davis, 1998; Kyriakis, 1998). Three distinct groups of MAPKs have been identified in mammalian cells, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), and p38 (reviewed in Davis, 1998; Kyriakis, 1998). These three groups of MAPKs are mediators of signal transduction from the cell surface to the nucleus. Although these three types of MAPKs are activated by dual phosphorylation on Thr and Tyr within protein kinase subdomain VIII, the activation motif for these three MAPKs is different: the TEY motif for ERKs, the TPY motif for JNK/SAPK, and the TGY motif for p38 (Cano & Mahadevan, 1995; Derijard et al., 1994; Han et al., 1994; Kyriakis et al., 1994; Payne et al., 1991).

MAPKs can detect a minimum consensus target sequence of Ser/Thr Pro (Whitmarsh & Davis, 1996). The ERK phosphorylation motif, PXS/TP, is more stringent than other MAPKs (Gonzalez et al., 1991; Pearson & Kemp, 1991). Although the MAPKs can phosphorylate many targets, some of the primary targets of the MAPKs are transcription factors, which, upon phosphorylation by MAPKs, bind to the promoter region of the target gene to induce gene transcription (Whitmarsh & Davis, 1996).

Three distinct MAPK signal transduction pathways have been identified in mammalian cells; these lead to activation of ERKs, SAPKs/JNKs, and p38 (Fig. 6). These MAPK pathways are highly evolutionarily conserved. In these signaling cascades, MAPKs can be activated by the dual-specificity MAPK kinases (MAPKKs) upon being phosphorylated on Thr and Tyr. MAPKKs are, in turn, activated by Ser/Thr kinases, termed MAPKK kinases (MAPKKKs). Ras and other small molecular weight G-proteins, including Rac and RhoA, can activate the MAPKKKs (Kyriakis, 1998).

The p42 MAPK, also known as ERK2, was the first mammalian MAPK identified (Hoshi et al., 1988; Ray & Sturgill, 1987). Subsequently, p44 MAPK, now referred to as ERK1, was cloned and shares 96% homology with ERK2 (Boulton et al., 1991a; Charest et al., 1993). The homology between mammalian ERK1 and ERK2 and the corresponding yeast MAPKs FUS3 and KSS1 is as high as 50% (Courchesne et al., 1989; Elion et al., 1990). ERK1 and ERK2 play a central role in the Ras-regulated, mitogenic signal transduction pathways (Cano & Mahadevan, 1995). Two other ERK subfamily members, ERK5 and ERK3, remain poorly understood with regard to their targets and regulation (Boulton et al., 1991b). The transcription factors activated by the ERKs include Elk1 and other ternary complex factors (TCFs), c-myc, and Ca^{2+}/cAMP response element-binding protein (CREB) (reviewed in Whitmarsh & Davis, 1996). The phosphorylation of TCFs by ERKs results in either increased binding of TCFs to the serum response factor-serum response element (SRE) complex or increased activation of TCFs already bound to the serum response factor-SRE, thereby leading to the induction of gene transcription (reviewed in Foletta, 1996). Moreover, ERKs can phosphorylate a variety of other cytoplasmic targets, such as myelin basic protein, Tau, epidermal growth factor receptors, phospholipase (PL)A_2, and S6 kinase (Lenormand et al., 1993). The upstream MAPKKs for ERKs are MEK1/2, which, in turn, can be activated by Raf, a MAPKKK. In most cases, the ERK cascade is initiated by Ras, a small monomeric G-protein and upstream effector of Raf activation (Kyriakis, 1998). Protein kinase C (PKC) and cAMP have also been reported to activate ERKs (Burgering et al., 1993; Marquardt et al., 1994).

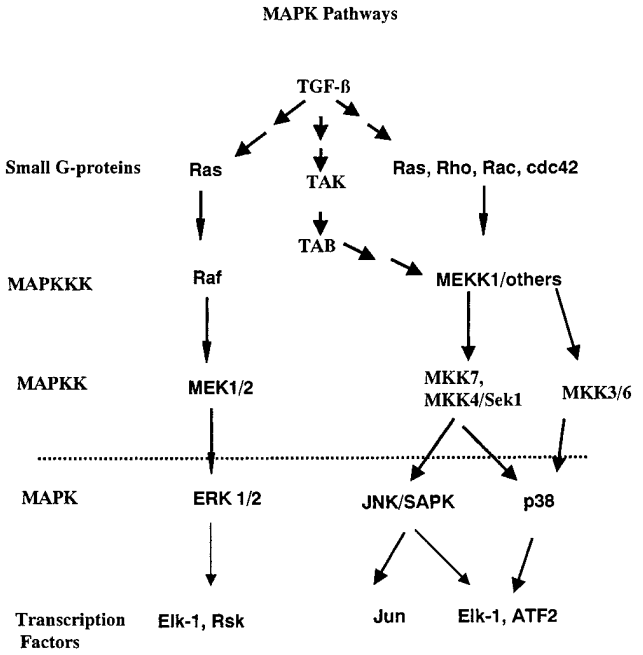

Fig. 6. MAPK signaling cascades activated by TGF-β.

The SAPKs/JNKs are the main MAPKs that respond to cytokines and stress in order to regulate many processes, including proliferation and apoptosis (reviewed in Davis, 1998). Thus far, three SAPKs/JNKs (SAPKα/JNK2, SAPKβ/JNK3, and SAPKγ/JNK1) have been identified. These three share 40–50% homology with ERKs (Davis, 1998). JNKs only poorly phosphorylate myelin basic protein, but can efficiently phosphorylate c-Jun at Ser63/73 to activate this AP-1 protein (reviewed in Whitmarsh & Davis, 1996). Activated c-Jun can either form a homodimer or a heterodimer with a Fos member, which, in turn, can bind to AP-1 sites to induce gene transcription (Rahmsdorf, 1996). JNKs can also phosphorylate ATF-2 and CREB, which, in turn, bind to the CRE site to induce gene transcription (reviewed in Whitmarsh & Davis, 1996). The upstream MAPKK for SAPKs/JNKs is MKK4 (SEK1) or MKK7 (Davis, 1998). MEKK1 is the MAPKKK for MKK4 and MKK7. Ras and other low-molecular weight GTPases (i.e., Rac, Cdc42, and Rho), have been shown to activate MEKK1 or other MEKKs (Fig. 6) (Davis, 1998; Kyriakis, 1998).

Another stress-activated MAPK, p38, is a mammalian homologue of the yeast HOG1 kinase. This kinase can also be activated by stress, most notably inflammatory cytokines and irradiation (Davis, 1998). p38 can also phosphorylate transcription factors, such as Elk1, ATF-2, and Max, to induce gene transcription. The upstream kinases for p38 include MKK3, 4, or 6, and MEKK1 (Fig. 6) (Davis, 1998; Kyriakis, 1998).

6.2. Activator protein-1 proteins

The AP-1 transcription factors are primary targets of the MAPK pathways. AP-1 proteins are composed of members of the Jun and Fos families, and belong to the bZIP group of DNA-binding proteins (Fig. 7) (reviewed in Rahmsdorf,

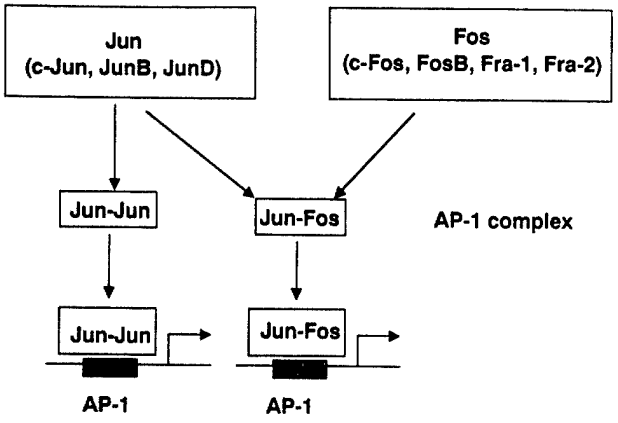

Fig. 7. AP-1 family members. The AP-1 family includes members of the Jun and Fos transcription factors.

1996; Foletta, 1996). Fos and Jun family members can associate with each other in various combinations and with other transcription factors, including CREB/ATF, NF of activated T-cells, ETS, NF-κB, and Smad. AP-1 proteins bind to the TRE, with a consensus binding sequence of TGACTCA. Related, atypical TREs also bind AP-1 proteins (i.e., TGTCTCA or TGAGTCA). The binding affinity of a particular TRE is dependent upon the composition of the AP-1 complex. TREs are present in the promoters of many immediate early genes. MAPK signaling pathways can increase AP-1 activity by both increasing the abundance of AP-1 components and by directly stimulating their transcriptional activity (Rahmsdorf, 1996; Foletta, 1996).

The first two members of the AP-1 family to be identified, and subsequently to be well-characterized, were the proto-oncogenes *c-Jun* and *c-Fos*. *c-Jun* is the cellular homologue of *v-jun*, carried by the avian sarcoma virus 17 (Maki et al., 1987). A region in the C-terminal domain of c-Jun is identical to the DNA-binding domain of the yeast transcription factor GCN4 (Vogt et al., 1987). Later, two more Jun-related genes, *junB* and *junD*, were identified. These Jun family members share 75% amino acid homology in the DNA-binding and leucine zipper regions (Schutte et al., 1989; Hirai et al., 1989). The Jun family members can form either homodimeric or a heterodimeric complexes with a Fos family member in order to bind at the TREs of the promoters of their target genes (Rahmsdorf, 1996).

c-fos is the cellular homologue of the viral *v-fos* oncogene (Finkel & Biskis, 1966; Curran et al., 1987). Other Fos members include *FosB*, *Fra-1*, and *Fra-2* (Cohen & Curran, 1988; Nishina et al., 1990; Zerial et al., 1989). Similar to the Jun family members, the amino acid sequence among the Fos family members is highly conserved in both the DNA-binding domain and the leucine zipper region (Foletta, 1996). Unlike the Jun family, Fos members cannot form homodimers and cannot bind to the TRE alone. They must associate with a Jun member in order to form a functional AP-1 complex (Cohen et al., 1989).

The MAPKs pathways can regulate AP-1 activity in several different ways. First, activated ERK pathways can phosphorylate TCF/Elk1 transcription factors, which, in turn, can bind to the SRE site in the c-fos promoter region to induce c-Fos expression. An SRE site has also been found in the Fra-2 promoter region (Foletta, 1996). Second, JNK pathways can lead directly to phosphorylation of c-Jun and JunD. Phosphorylated c-Jun and JunD have higher DNA-binding affinities than their non-phosphorylated counterparts (Whitmarsh & Davis, 1996). Moreover, activated c-Jun or JunD can form either homodimers or heterodimers with Fos family members, which, in turn, can bind to the AP-1 site in the *c-Jun* promoter to induce c-Jun expression (Foletta, 1996). In addition, ERKs can directly phosphorylate Fra-2 to enhance the transcriptional activity of Fra-2/c-Jun heterodimers (Murakami et al., 1999).

6.3. Transforming growth factor-β activation of the Ras/mitogen-activated protein kinase pathways

Given that many extracellular stimuli, including cytokines, can activate Ras, it is not surprising that TGF-β, a pleiotropic cytokine, can also stimulate Ras activity. We have demonstrated that TGF-β treatment of untransformed epithelial cells results in a rapid activation of Ras (within 3-10 min); the activation of Ras by TGF-β does not occur in TGF-β-resistant cells (Mulder & Morris, 1992). In accord with our studies, TGF-β activation of Ras was observed in human colon cancer cells (Yan et al., 1994). We have since demonstrated that TGF-β can activate ERKs within 5-30 min. The activation of ERK1 by TGF-β is not transient, but lasts at least 90 min after TGF-β addition (Hartsough & Mulder, 1995; Frey & Mulder, 1997a, 1997b). This effect was not observed in TGF-β-resistant cells (Hartsough & Mulder, 1995; Frey & Mulder, 1997a, 1997b). Moreover, we demonstrated that overexpression of a dominant-negative mutant of Ras (RasN17), or addition of a specific MEK1 inhibitor PD98059, blocks the ability of TGF-β to activate ERK1 (Hartsough et al., 1996; Yue et al., 1998). The activation of ERKs by TGF-β has since been observed in many other cell types (Kivinen & Laiho, 1999; Hu et al., 1999; Reimann et al., 1997; Mucsi et al., 1996; Han et al., 2000; Hayashida et al., 1999; Bellis et al., 1999; Chin et al., 1999). The activation of Raf/ERKs by BMP has been observed in *Xenopus* as well (Xu et al., 1996).

TGF-β can also activate SAPKs/JNKs in a wide variety of cell types (reviewed in Hartsough & Mulder, 1997; Mulder, 2000). We have demonstrated that TGF-β activates JNK in Hs578T cells, but not in TGF-β-resistant cancer cell lines (Frey & Mulder, 1997a, 1997b). Moreover, expression of a dominant-negative mutant of TβRII abrogates the ability of TGF-β to activate JNK in Hs578T cells (J. B. Yue & K. M. Mulder, submitted for publication). TGF-β only weakly modulates JNK1 activity in GEO human colon cancer cells, which express low levels of RI. Forced expression of RI in GEO cells potentiates the ability of

TGF-β to activate JNK1 (J. B. Yue & K. M. Mulder, submitted for publication). Accordingly, these results indicate that the TGF-β receptors are required for TGF-β activation of JNK1.

In addition, we have observed that TGF-β activates JNK1 in untransformed epithelial cells; expression of RasN17 or a dominant-negative mutant of MKK4 (DNMKK4) blocks TGF-β activation of JNK1 (J. B. Yue & K. M. Mulder, submitted for publication). Thus, Ras and MKK4 are two upstream components for JNK1 activation by TGF-β. The activation of SAPKs/JNKs has also been observed by other groups in their systems (Hocevar et al., 1999; Atfi et al., 1997; Zhou et al., 1999; Engel et al., 1999). Taken together, these data indicate that the activation of JNKs by TGF-β is a common event in TGF-β signaling.

In addition to ERKs and JNKs, TGF-β has also been shown to activate p38 in several cell types (Ravanti et al., 1999; Adachi-Yamada et al., 1999; Hanafusa et al., 1999). A novel kinase, TGF-β-activated kinase-1 (TAK1), and its binding protein (TAB1) have been identified (Yamaguchi et al., 1995; Shibuya et al., 1996b). TGF-β can activate TAK1 in osteoblast cells and in 293T cells, which, in turn, activates MKK4, and p38 or JNKs (Yamaguchi et al., 1995; Shibuya et al., 1996a, 1996b; Zhou et al, 1999). Activated TAK1 can increase PAI-1 gene transcription (Yamaguchi et al., 1995; Shibuya et al., 1996b). However, the activation of JNK through TAK1 in 293T cells is delayed, and whether TAK1 is a common downstream target for TGF-β signaling is still not clear. TAK1 and TAB1 can also function in the BMP signal transduction pathway in early *Xenopus* development (Shibuya et al., 1996a). The human X-chromosome-linked inhibitor of apoptosis protein was identified as an adaptor protein to link the BMP receptors to TAB1-TAK1 in BMP signaling (Yamaguchi et al., 1999).

6.4. Cellular targets of transforming growth factor-β activation of the Ras/mitogen-activated protein kinase pathways

A considerable number of studies have been performed to determine the cellular targets of TGF-β activation of the Ras/MAPK pathways. We have demonstrated that Ras was partially required for TGF-β-mediated growth inhibition (Hartsough et al., 1996). That is, expression of RasN17 caused a 50% reversal of the inhibition of Cdk2 activity, a 78% reversal of the down-regulation of cyclin A protein expression, and a 21% reversal of the inhibition of DNA synthesis by TGF-β (Hartsough et al., 1996). Moreover, we have demonstrated that expression of RasN17 completely blocked TGF-β up-regulation of both $p21^{Cip1}$ expression and new synthesis of $p27^{Kip1}$ (Yue et al., 1998).

The requirement of the Ras/ERK pathways for TGF-β up-regulation of $p21^{Cip1}$ has been confirmed by two other groups (Kivinen & Laiho, 1999; Hu et al., 1999). Moreover, we have also shown that expression of DN MKK4 in IECs blocked TGF-β up-regulation of new synthesis of $p27^{Kip1}$ (J. Y. Yue and K. M. Mulder, unpublished data). The requirement of the Ras/MAPKs pathways for TGF-β up-regulation of $p21^{Cip1}$ and $p27^{Kip1}$ is similar to that in budding yeast. That is, pheromones can result in a growth arrest through the yeast MAPK pathways to up-regulate yeast CKIs (Peter & Herskowitz, 1994; Herskowitz, 1995).

It was shown previously that TGF-β regulates expression of many of its target genes through the AP-1 sites in their promoter regions (Eickelberg et al., 1999; Chung et al., 1996; Mauviel et al, 1996; Virolle et al., 1998; Uria et al., 1998). As mentioned in Section 6.2, one of the targets of the Ras/MAPKs pathways are the AP-1 proteins. Thus, it was conceivable that TGF-β could regulate target gene expression through the Ras/MAPK pathways via the AP-1 site.

We have examined the role of the Ras/MAPK pathways in TGF-β3-induced TGF-β1 production (Yue & Mulder, 2000). Our results indicate that expression of RasN17 or DNMKK4, or addition of the MEK1 inhibitor PD98059, inhibits TGF-β3-induced TGF-β1 production, as determined by RNase protection assays. We have also demonstrated that the AP-1 site in the TGF-β1 promoter is essential for TGF-β to transactivate the TGF-β1 promoter and that TGF-β inducible AP-1 complex formation preceded TGF-β stimulation of TGF-β1 mRNA expression. The expression of RasN17 or DNMKK4, or addition of PD98059, blocked the TGF-β-inducible AP-1 complex at the TGF-β1 promoter. The primary components present in this TGF-β-inducible AP-1 complex were JunD and Fra-2. Thus, our results indicate that TGF-β3-induced TGF-β1 production is mediated through the Ras/MAPK signaling cascades, including Ras, MAPKKs (MEK1, MKK4), MAPKs (ERKs and SAPKs), and AP-1 proteins (JunD, Fra-2). Our results also indicate that both Smad3 and Smad4 are required for TGF-β3-stimulated TGF-β1 production in CCL64 cells, although they did not appear to be present in the AP-1 complex (Yue & Mulder, 2000).

We have also examined the role of SAPK/JNKs in TGF-β regulation of the urokinase receptor (uPAR). Our results indicate that TGF-β can potently up-regulate uPAR protein expression. Expression of DN MKK4 blocked the ability of TGF-β to up-regulate uPAR. Moreover, we demonstrated that TGF-β utilized the distal, not the proximal, AP-1 site in the uPAR promoter for uPAR regulation. The primary components present in the TGF-β-inducible AP 1 complexes were JunD and Fra-2 (J. B. Yue & K. M. Mulder, submitted for publication). In addition, it has been shown that TGF-β activation of SAPK/JNK is required for fibronectin synthesis, possibly through the CRE/ATF site in the fibronectin promoter (Hocevar et al., 1999). Additional cellular targets activated by TGF-β stimulation of the Ras/MAPK pathways are likely to be elucidated in the future.

6.5. Cross-talk between Ras/mitogen-activated protein kinases and Smads

In the linker regions of the RSmads there are several MAPK consensus phosphorylation sites (Yue et al., 1999a, 1999b; Liu et al., 1998; Kretzschmar et al., 1997, 1999; X. J. Liu & K. M. Mulder, submitted for publication). For Smad1, there are four consensus ERK phosphorylation sites. We have provided evidence that expression of RasN17, or addition of the MEK1 inhibitor PD98059, inhibited the ability of both TGF-β and BMP to phosphorylate endogenous Smad1 in untransformed IECs (Yue et al., 1999a). We have also demonstrated that blockade of TGF-β activation of Ras or ERKs inhibited the ability of TGF-β to stimulate 3TP-Lux activity. Accordingly, our results indicate that the Ras/MEK/ERK pathway is partially required for the ability of TGF-β to activate Smad1 (Yue et al., 1999a, 1999b).

In follow up studies, we demonstrated that Smad1 could potentiate the ability of TGF-β to activate a Smad-specific reporter SBE-Luc. Addition of PD98059 partially inhibited TGF-β stimulation of SBE-Luc activation. Mutation of anywhere from 1 to 4 of the ERK consensus phosphorylation sites (PXSP/AP) in the linker region of Smad1 decreased the ability of TGF-β to activate SBE-Luc to the same extent as PD98059 did (X. J. Liu & K. M. Mulder, submitted for publication). Taken together, our results clearly indicate that TGF-β can activate the Ras/MEK/ERK pathways to positively modulate Smad1 activity.

Smad2 can also be positively regulated by MEKK1, but not by MEK1 (Brown, J. D. et al., 1999). A constitutively active form of MEKK1 can selectively phosphorylate Smad, possibly through the linker region of Smad2, but not through the C-terminal SSXP motif. The phosphorylation of Smad2 by MEKK1 enhanced the Smad2-Smad4 interaction, and subsequently, the nuclear translocation, thereby leading to increased Smad2 transcriptional activity (Brown, J. D. et al., 1999). Similarly, de Caestecker and co-workers have reported that hepatocyte growth factor induces both phosphorylation and nuclear translocation of Smad2. Thus, Smad2 can act as a positive effector for both hepatocyte growth factor tyrosine kinase signaling and TGF-β serine threonine kinase signaling (de Caestecker et al., 1998).

In contrast, Kretzschmar and co-workers (1997) have reported that Smad1 is phosphorylated by mitogenic growth factors and that this ERK-mediated phosphorylation of Smad1 inhibits BMP effects on Smad1. However, their data cannot be completely explained by the model they propose. For example, they propose that ERK-mediated phosphorylation of Smad1 inhibits Smad1 nuclear translocation. Their data demonstrate that ERK site-mutated Smad1 is predominantly located in the nucleus, even in the absence of BMP. However, BMP still induced the phosphorylation of the ERK site-mutated Smad1 to the same extent, or to an even greater extent, than the wild-type Smad1. This finding is not consistent with the fact that most of the ERK site-mutated Smad1 would already be localized in the nucleus and would not be accessible to BMP regulation through BMP receptor activation. Thus, it would appear that phosphorylation of Smad1 at the ERK consensus phosphorylation sites cannot simply be interpreted as an inhibitory effect on Smad1 function.

The same group has also reported that oncogenic Ras negatively regulates TGF-β signaling by inhibiting TGF-β-induced nuclear translocation of Smad2 and Smad3 and Smad-dependent transcription (Kretzschmar et al., 1999). However, overexpression of oncogenic Ras generally leads to resistance to the growth inhibitory effects of TGF-β, and may also cause cell transformation (Filmus et al., 1992; Longstreet et al., 1992; Coppa et al., 1997; Huggett et al., 1990; Oft et al., 1996). Thus, the inability of TGF-β to activate Smad2 and Smad3 in these oncogenic Ras-expressing cells may be at least partly due to the fact that these cells are less sensitive to TGF-β than are the control cells. Additionally, constitutive activation of Ras would be expected to prevent ligand-stimulated Ras activation. Thus, TGF-β would not be expected to regulate the Smads under these conditions.

We were the first to demonstrate that TGF-β could also activate the SAPK/JNK type of MAPKs (Frey & Mulder, 1997a, 1997b). More recently, TGF-β has been shown to activate SAPKs/JNKs in both Smad-independent and -dependent pathways. This TGF-β activation of SAPK/JNK can lead to phosphorylation of Smad3 outside its SSXS motif. Moreover, the phosphorylation of Smad3 by JNK can increase Smad3 nuclear translocation and transcriptional activities (Engel et al., 1999). Two groups have also reported that TGF-β can directly phosphorylate ATF-2 via the TAK1 and p38 pathways. This phosphorylated ATF-2 can then associate with Smad3-Smad4 complexes to induce gene transcription (Sano et al., 1999; Hanafusa et al., 1999).

7. Transforming growth factor-β regulation of other cytoplasmic signaling proteins

In addition to Smads and MAPKs, TGF-β can regulate other cytoplasmic signaling proteins in different cell types, such as PKC, NF-κB, protein kinase A (PKA), PLC, and protein phosphatase 1. With regard to PKA, it has been shown that TGF-β can rapidly increase PKA activity in murine mesangial cells. Addition of a PKA inhibitory peptide completely blocked the ability of TGF-β to activate PKA (Wang et al., 1998). In addition, expression of PKA inhibitory peptide abrogated the ability of TGF-β to directly phosphorylate cAMP response element-binding protein and to increase fibronectin expression (Wang et al., 1998). However, it is still not clear whether TGF-β can regulate PKA in other cell types and whether TGF-β stimulation of PKA plays an important role in TGF-β signaling.

It has also been reported that TGF-β can either increase or decrease NF-κB activity in different cell types. For

example, TGF-β can inhibit NF-κB activity by induction of IκBα expression in human salivary gland cells (Azuma et al., 1999). TGF-β-mediated inhibition of NF-κB/Rel activity is also associated with TGF-β-mediated growth inhibition in two human breast cancer cells, Hs578T and MCF7. Overexpression of the p65 subunit in Hs578T cells abrogated the ability of TGF-β to inhibit cell growth (Sovak et al., 1999). TGF-β can also decrease the activity of NF-κB by increasing IκBα expression to induce apoptosis in WEHI231 immature B-cells (Arsura et al., 1996). In contrast, TGF-β can increase NF-κB activity to stimulate the human immunodeficiency virus-1 enhancer in human keratinocytes HaCaT (Li et al., 1998). TGF-β appears to increase NF-κB activity through a non-classical NF-κB activation mechanism in HaCaT cells. That is, TGF-β treatment of HaCaT cells did not change either the expression levels of IκBα or IκBβ or the expression levels and the DNA binding activity of p50 or p65 subunits (Li et al., 1998). It is possible that TGF-β increases the transcriptional activity of NF-κB through other kinases, such as MAPKs.

Although it has been reported by several groups that TGF-β can regulate PKC, PLC, and protein phosphatase 1, evidence regarding the role and the activation of these signaling proteins in TGF-β biology is incomplete (reviewed in Hartsough & Mulder, 1997). Additional studies will be required to delineate the activation mechanisms and significance of these signaling components in TGF-β-mediated events. Other known and unknown cytoplasmic signaling proteins also probably play a role in TGF-β signaling.

8. Role of transforming growth factor-β in cancer

Alterations in the expression of TGF-β family members and their signaling pathways have been associated with multiple human diseases (reviewed in Roberts & Sporn, 1990; Hartsough & Mulder, 1997). Low expression levels of TGF-β have been associated with impaired wound healing and diabetes in the elderly (Sporn & Roberts, 1993; Beck et al., 1993). A lack of TGF-β has also been associated with autoimmune diseases (Letterio & Roberts, 1998). In contrast, pathological overexpression of TGF-β appears to contribute to many human fibrotic diseases in the kidney, lung, liver, heart, skin, and bone marrow (Border & Noble, 1994). A frameshift mutation of the *GDF5/CDMP1* gene has been identified in patients with recessive chondrodysplasia syndrome, a Hunter Thompson-type acromedomelic chondrodysplasia. These patients have short and dislocated limbs (Thomas et al., 1996). As mentioned in Section 4.1, mutations of the Type I receptor ALK1 or of the Type III receptor endoglin have been suggested to be the cause of the human hereditary hemorrhagic telangiectasia. Such patients develop epithelial vascular dysplasia and have a high propensity to hemorrhage in the nasal and gastrointestinal mucosa (Johnson et al., 1996; McAllister et al., 1994). In addition, mutations in MIS and its receptors can cause Müllerian duct syndrome (Knebelmann et al., 1991; Imbeaud et al., 1995; Faure et al., 1996).

Among the extensive index of pathologies associated with TGF-β, the role of TGF-β in cancer has been studied most widely. Over the years, it was found that TGF-β is a potent inhibitor for many tumor cells of epithelial origin. However, mutations in either TGF-β receptors or downstream signaling components can cause tumor cells to become refractory to the growth inhibitory effects of TGF-β (reviewed in Markowitz & Roberts, 1996).

8.1. The autocrine and paracrine actions of transforming growth factor-β in cancer

TGF-β can regulate the growth of cancer cells in an autocrine or a paracrine fashion (Roberts & Sporn, 1990; Hartsough & Mulder, 1997). For TGF-β-sensitive tumor cells, autocrine TGF-β inhibits the growth of these cells and diminishes their tumorigenic potential (Roberts & Sporn, 1990; Hartsough & Mulder, 1997). TGF-β autocrine-negative effects have been demonstrated by the ability of TGF-β neutralizing antibodies to stimulate the growth of several cell lines (Hafez et al., 1990; Arteaga et al., 1990). Also, antisense TGF-β1-transfected human colon cancer cells that expressed less endogenous TGF-β1 displayed a higher tumorigenicity than control cells, which were still sensitive to exogenous TGF-β (Wu et al., 1992). Moreover, transfection of RII into RII-deficient cancer cells diminished the tumorigenic potential of these tumor cells (Sun et al., 1994; Wang et al., 1995). In addition, in transgenic mice with high expression levels of TGF-β1, the ability of TGF-α and 7,12-dimethylbenzanthracene to induce mammary tumors was significantly inhibited (Pierce et al., 1995).

One of the most convincing pieces of evidence for TGF-β as a tumor suppressor gene came from heterozygous *TGF-β1-/+* mice. These mice only expressed 10–30% of the TGF-β1 compared with that in the wild-type *TGF-β1* mice. Treatment of these mice with chemical carcinogens resulted in enhanced tumorigenesis compared with wild-type *TGF-β1* mice. These data indicate that TGF-β1 is haplo-insufficient for tumor suppression (Tang et al., 1998). Taken together, these data clearly show that TGF-β autocrine-negative effects are important for maintaining a growth equilibrium in cancer cells.

Conversely, for TGF-β-resistant tumor cells that still secrete high amounts of TGF-β, TGF-β acts in a paracrine fashion to enhance the tumorigenesis of these cells by building a positive environment for tumor growth and survival (Chang et al., 1993; Steiner & Barrack, 1992; Arrick et al., 1992; Cui et al., 1996). For example, TGF-β can enhance tumorigenesis by stimulating proliferation of mesenchymal cells, increasing ECM production, enhancing migration of tumor cells, inducing immunosuppression, and increasing angiogenesis (Roberts & Sporn, 1990; Hartsough & Mulder, 1997).

8.2. Mutations in transforming growth factor-β receptors in cancer

Both TβRI and TβRII and Smads have been found to be mutated in some human cancer cells (reviewed in Massagué, 1998; Markowitz & Roberts, 1996). Mutation of TβRII receptors was first found in certain human colon cancers that were refractory to the growth inhibitory effects of TGF-β (Markowitz et al., 1995). In these tumor cells, the Type II receptor mutations were associated with microsatellite instability. Specifically, one or two additional adenosines were added or deleted within a 10-bp polyadenine repeat (bp 709–718 at the coding regions of the extracellular domain). This frame-shift mutation introduced early stop condons and resulted in truncated, inactive TβRII receptors. These truncated forms of TβRII are comprised of 125 and 128 amino acids, and are lacking transmembrane and cytoplasmic domains (Markowitz et al., 1995).

Microsatellite instability is common to many sporadic human colon and gastric cancers. The repetitive DNA sequence is prone to frame-shift mutations, and such mutations are well correlated with defects in DNA repair enzymes (Bronner et al., 1994; Papadopoulos et al., 1994). Also, microsatellite instability is characteristic of cancers from individuals with hereditary non-polyposis colon cancer, a familial cancer syndrome characterized by a high incidence of colon, endometrial, and gastric cancers (Eshelman & Markowitz, 1995; Aaltonen et al., 1993; Thibodeau et al., 1993). *TβRII* frameshift mutations are found exclusively in these sporadic human colon and gastric cancers and in colon and gastric tumors from hereditary non-polyposis colon cancer, but are rare in somatic or hereditary cancers of the endometrium, breast, pancreas, and liver (Myeroff et al., 1995; Parsons et al., 1995). These results indicate that *TGβRII* frameshift mutations are specifically selected for in colon and gastric cancers.

The inactivation of *TβRII* by insertion of a GT in a GTGT sequence in the kinase domain, or by missense mutations in this domain, has also been observed in head and neck squamous carcinomas (Garrigue-Antar et al., 1995). Other types of *TβRII* mutations have also been observed in T-cell lymphoma and gastric cancers (Myeroff et al., 1995; Knaus et al., 1996; Park et al., 1994).

In addition to *TβRII* mutations in human cancers, *TβRI* mutations in human cancers have also been identified. In LnCaP human prostate cancer cells, *TβRI* is mutated. Transfection of *TβRI* back into LnCaP cells restores the TGF-β responsiveness (Kim et al., 1996). Also, a deletion in the *TβRI* gene has been reported in a cutaneous T-cell lymphoma, which abolished both the expression of surface Type I receptors and the growth inhibitory effects of TGF-β (Schiemann et al., 1999).

Diminished TGF-β growth inhibitory effects in B-cell chronic lymphocytic leukemia, colon, gastric, and pancreatic cancer cells can also be associated with decreased expression of *TβRI* in these cells (Kang et al., 1999a). In human gastric cancer, aberrant methylation of CpG islands in the 5′ region of the *TβRI* gene resulted in the development of TGF-β resistance. Transient transfection of *TβRI* into TGF-β-resistant SNU-601 human gastric cancer cells restored TGF-β responsiveness (Kang et al., 1999a). Stable transfection of *TβRI* into GEO human colon cancer cells, which have low expression levels of endogenous *TβRI*, enhanced the ability of TGF-β to inhibit the tumorigenic potential of these cells (Wang et al., 1995). Accordingly, these results indicate that *TβRI* is also a target for alterations in human cancer cells.

Recently, the *TβRI* gene was mapped to 9q22 and exhibited a polymorphism, TβRI(6A), in which 3 alanines from a 9-alanine stretch were deleted (Pasche et al., 1998). The frequency of *TβRI*(6A) homozygotes and heterozygotes was higher in tumor than that in nontumor (Pasche et al., 1998, 1999). Moreover, it was found that *TβRI*(6A) was an impaired mediator of growth inhibition by TGF-β (Pasche et al., 1999). These results suggest that *TβRI*(6A) may be a candidate tumor susceptibility allele.

8.3. Mutations of Smads in cancer

Mutation of Smads can also lead to TGF-β resistance in some tumor cells (reviewed in Massagué, 1998). Smad4/DPC4 initially was identified as a candidate tumor suppressor in pancreatic carcinomas since the Smad4 gene on chromosome 18q21 was deleted in 50% of these tumors (Hahn et al., 1996; Schutte et al., 1996). Smad4 mutations also occurred in many colorectal carcinomas and less frequently in ovarian, breast, head, neck, prostatic, esophageal, and gastric cancers (Schutte et al., 1996). *Smad2* mutations have also been found in some colon cancers (Riggins et al., 1996, 1997). Zhu and coworkers (1998) have shown that *Smad3* null mice develop colon cancers.

The mutations of *Smad4* or *Smad2* in cancers have been caused by either complete deletion of their chromosome locus, small deletions, frame-shift mutations, missense mutations, or nonsense mutations (Massagué, 1998). More recently, a novel *Smad4* gene mutation has been identified in seminoma germ cell tumors, in that a single thymine was inserted between 1521 bp and 1522 bp of the *Smad4* C-terminal domain (Bouras et al., 2000). This insertional mutation created a new stop codon and resulted in a truncated Smad4 protein (Bouras et al., 2000). This mutation may block the signaling mediated by TGF-β and other TGF-β members, and may result in TGF-β resistance.

9. Summary

In summary, TGF-β can initiate at least two prominent signaling cascades, the Smad and the Ras/MAPK pathways, in order to regulate a wide variety of biological responses (Fig. 8). Moreover, there is cross-talk between the Ras/

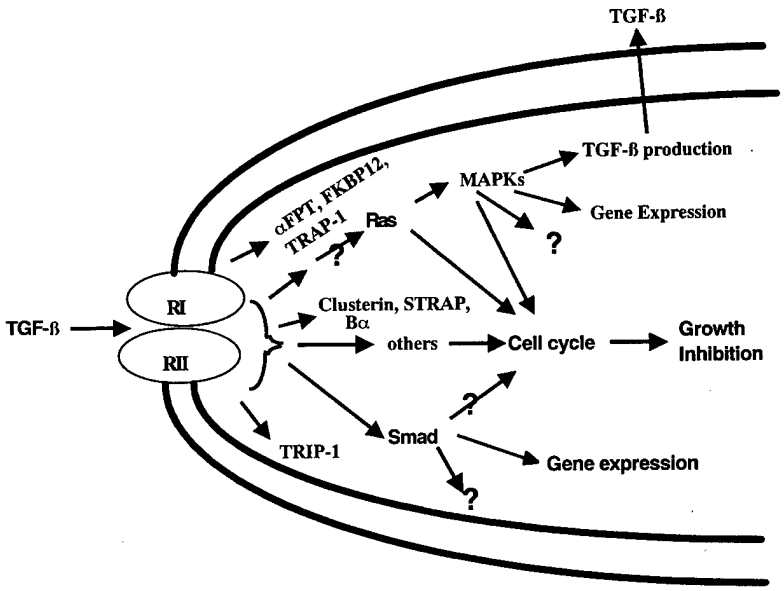

Fig. 8. Summary of TGF-β signaling pathways in epithelial cells. αFPT, α subunit of farnesyltransferase; STRAP: serine threonine kinase receptor-associated protein; Trap-1, TGF-β receptor interacting protein; TRIP-1: TβRI-associated protein-1.

MAPK and Smad pathways. Smad pathways also intersect with other signaling components, such as the JAK/STAT pathways (Nakashima et al., 1999). Crosstalk between other signaling pathways and Smads or Ras/MAPK pathways may also exist. The sum total of the distinct, yet intersecting, signaling pathways that are activated simultaneously after TGF-β receptor activation, plays a significant role in both normal growth and development and in a variety of critical pathologic conditions. Accordingly, identification of additional TGF-β signaling components and pathways will greatly advance our overall understanding of intracellular targets that dictate the fate of the organism.

Acknowledgments

This work was supported by National Institution of Health Grants CA51452, CA54816, CA68444, and CA90765 awarded to K.M.M.

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Mol. Biol. of the Cell, In Press, 2002

km23: A novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein

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Running title: TGFβ receptors bind a dynein light chain

Key words: signal transduction, robl, microtubule, bithoraxoid, growth factor

ABSTRACT

The phosphorylated, activated cytoplasmic domains of the TGFβ receptors were used as probes to screen an expression library that was prepared from a highly TGFβ-responsive intestinal epithelial cell line. The receptor-interacting protein km23 was isolated and identified as a light chain of the motor protein dynein (DLC). This 11-kD cytoplasmic protein interacts with the TGFβ receptor complex intracellularly and is phosphorylated on serine residues after ligand-receptor engagement. Forced expression of km23 induces specific TGFβ responses, including an activation of JNK, a phosphorylation of c-Jun, and an inhibition of cell growth. Further, TGFβ induces the recruitment of km23 to the intermediate chain of dynein (DIC). A kinase-deficient form of TGFβ RII prevents both km23 phosphorylation and interaction with DIC. This is the first demonstration of a link between cytoplasmic dynein and a natural, growth inhibitory cytokine. Further, our results suggest that TGFβ pathway components may utilize a motor protein light chain as a receptor for the recruitment and transport of specific cargo along MTs.

INTRODUCTION

TGFβ is the prototype for the TGFβ superfamily of highly conserved growth regulatory polypeptides that also includes the activins, inhibins, bone morphogenetic proteins (BMPs), decapentaplegic (Dpp), nodal, Lefty and others (*Yue and Mulder, 2001; Roberts, 1998; Sporn and Vilcek, 2000*). Alterations in the TGFβ signaling components and pathways have been implicated in a vast array of human pathologies, including cancer (*Sporn and Vilcek, 2000; Massague et al, 2000, Derynck et al, 2001*).

TGFβ binds to two types of transmembrane serine/threonine kinase receptors (RI, RII) in a heterotetrameric complex, to activate downstream components (*Yue and Mulder, 2001; Roberts, 1998; Sporn and Vilcek, 2000; Massague et al, 2000*). The Smad family of signaling intermediates plays an important role in mediating TGFβ responses (*Yue and Mulder, 2001; Attisano and Wrana, 2000; ten Dijke et al, 2000*). Moreover, TGFβ has been shown to regulate Ras (*Mulder and Morris, 1992; Hartsough et al, 1996; Yue et al, 1998)* and several components of the mitogen-activated protein kinase (Mapk) pathways (*Mulder, 2000; Yue and Mulder, 2001; Sporn and Vilcek, 2000; Hartsough and Mulder, 1995; Frey and Mulder, 1997*). In addition to the Ras/Mapk and Smad pathways, several proteins have been identified based upon their interaction with the TGFβ receptors (*Yue and Mulder, 2001*). Further, various Smad-interacting proteins have also been identified, including SARA and Dab2, which

interact with both Smads and the TGFβ receptors (*Yue and Mulder, 2001; Tsukazaki et al, 1998; Hocevar et al, 2001*).

Despite advances in our understanding of the mechanisms by which the Smad and Ras/Mapk cascades mediate some TGFβ effects, these pathways appear to regulate primarily transcriptional events (*Yue and Mulder, 2001; Sporn and Vilcek, 2000; Yue and Mulder, 2000a; Hu et al, 1999; Hocevar et al, 1999*). However, TGFβ is multifunctional and its biological responses are diverse. Thus, identification of additional TGFβ signaling pathways and components will assist in our understanding of the mechanisms by which alterations in these pathways contribute to human disease.

Dynein is a molecular motor protein that mediates intracellular transport by conveying cargo along polarized microtubules (MTs) toward the minus ends (*Hirokawa, 1998).* Cytoplasmic dynein superfamily members control various cell functions and are important for establishing epithelial polarity (*Tai et al, 2001*). Several different subunits of cytoplasmic dynein can bind to a variety of cargoes (*Kamal and Goldstein, 2002; Karcher et al, 2002*). However, little is known about the regulation of the movement that dynein motors drive. Two dynein intermediate chains (DIC) are known to be important for cargo binding. In addition, most cargoes interact with dynein through dynactin, which binds to DIC (*Kamal and Goldstein, 2002; Karcher et al, 2002*). Four light intermediate chains (LICs) and several light chains (DLCs) also appear to be involved in imparting proper cargo selection. Finally, a variety of receptor systems and transporters have been shown to bind to molecular motors,

either directly through the LCs, or through motor receptors or adaptor proteins (*Klopfenstein et al, 2000; Kamal and Goldstein, 2002; Karcher et al, 2002*).

Motor protein binding and transport of cargoes intracellularly sometimes utilizes a set of proteins involved in cell signaling (*Bowman et al, 2000; Goldstein, 2001*). For example, the JNK-interacting proteins (JIPs) are thought to serve as scaffolding proteins for the JNK signaling pathway (*Davis, 2000*). These proteins also bind with high affinity and specificity to the motor protein kinesin (*Verhey et al, 2001*). It is thought that kinesin carries the JIP scaffolding proteins, preloaded with cytoplasmic and transmembrane signaling molecules. Similarly, dynein-dependent movement of signaling molecules along MTs has been reported. For example, p53 was found to be localized to the MTs and physically associated with tubulin (*Giannakakou et al, 2000*). The transport of p53 along MTs was dynein-dependent, suggesting that the interaction of p53 with dynein facilitated its accumulation in the nucleus after DNA damage (*Giannakakou et al, 2000*). Further, a receptor-DLC interaction has been reported for the photoreceptor rhodopsin (*Tai et al, 1999*). The interaction between rhodopsin and Tctex-1 is thought to represent a novel mode of dynein-cargo interaction in which a dynein subunit directly binds to an integral membrane protein cargo molecule that serves as a dynein receptor.

Activation of a motor may occur by posttranslational modifications, local changes in the cellular environment, or chaperone binding (*Hollenbeck, 2001*). Since growth factors and cytokines are known to regulate such events, the receptors and signaling pathways for these polypeptides are potential mediators of motor protein

activation and organelle trafficking, events which ultimately determine the collective spatial organization of the signaling pathways within the cell.

Here we describe a TGFβ receptor-interacting protein, termed km23, which is also a DLC. TGFβ stimulates not only the phosphorylation of km23, but also the recruitment of km23 to the DIC. Kinase-active TGFβ receptors are required for km23 phosphorylation and interaction with DIC. Recruitment of DLCs to the dynein complex is important not only for specifying the cargo that will bind (*Vaughan and Vallee, 1995*), but also for the regulation of intracellular transport itself (*Karcher et al, 2002*). Thus, km23 appears to function as a motor receptor, linking the dynein motor to specific cargo. We also demonstrate that km23 can mediate specific TGFβ responses, including JNK activation, c-Jun phosphorylation, and growth inhibition.

MATERIALS AND METHODS

Reagents--The anti-Flag M2 (F3165) and anti-c-myc (M5546) antibodies (Abs) and mouse IgG were from Sigma. The anti-DIC monoclonal Ab was from Chemicon (Temecula, CA). The anti-V5 Ab (R960 25) was obtained from Invitrogen and the anti-HA Ab (1-583-816) was from Boehringer Manheim. The TGFβ RII Ab (SC-220-G or -R), the phospho-c-Jun Ab (KM-1, SC-822), and rabbit IgG were from Santa Cruz Biotech. Protein A or G agarose were purchased from Invitrogen. ^{125}I-TGFβ (NEX-267), ^{32}P-orthophosphate (NEX-053), γ-^{32}P-ATP (BLU002H), and ^{3}H-thymidine (NET-027X) were from Perkin Elmer (Boston, MA). TGFβ$_1$ was purchased from R & D Systems (Minneapolis, MN).

Cell Culture--COS-1 cells (CRL-1650) and Mv1Lu cells (CCL-64) were obtained from ATCC (Rockville, MD) and were grown in DMEM supplemented with 10% FBS. 293T cells were obtained from T-W. Wong (Bristol-Myers Squibb) and were maintained as for COS-1 cells. Madin-Darby canine kidney (MDCK) cells (CCL-34) were grown in MEM-α supplemented with 10% FBS. Cultures were routinely screened for mycoplasma using Hoechst staining.

Cloning of TGFβ receptor targets--Construction of TGFβ receptor expression plasmids: The intracellular domains of TGFβ RII and RI were PCR amplified using the full-length human cDNA's for TGFβ RII (*Lin et al, 1992*) or TGFβ RI (*Franzen et al, 1993*), respectively, as templates. These domains were inserted into the pET15b-mod (containing N-terminal His and Flag tags) or pET30c (containing N-terminal His

and S tags) expression constructs, respectively, and the correct DNA sequences were confirmed.

Expression and activation of intracellular domains: The BLR (DE3) or HMS174 (Novagen) E. coli strains were transformed separately with each of the TGFβ receptor-containing vectors or the corresponding empty vectors (EV), followed by selection on kanamycin and ampicillin. Expression was induced with IPTG and verified by Western blotting using tag antibodies (Ab's) that differed for each receptor cytoplasmic domain. Recombinant receptor domains were affinity purified sequentially to isolate heteromeric receptors enriched for the activated complex. In vitro kinase assays (*Bassing et al, 1994*) were performed to phosphorylate the intracellular domains. Phosphorylation of both RI and RII was confirmed by SDS-PAGE. The higher degree of RI phosphorylation in kinase reactions performed with both receptors, as opposed to only RI, suggested that transphosphorylation of RI by RII had occurred. Supernatants derived from kinase assays utilizing cold ATP were used to approximate the specific activity of ^{32}P-labeled proteins.

Preparation and screening of expression library from IEC 4-1 cells. An expression library was prepared from the rat 4-1 IEC line (*Mulder et al, 1993*) using the Superscript Choice System for cDNA synthesis (Gibco BRL). Double-stranded cDNA ligated to Eco RI adaptors was size selected, and relevant fractions were pooled and ligated into the TripIEx expression vector (Clontech). The ligated DNA was incorporated into phage particles (Gigapack II gold, Stratagene, La Jolla, CA) and titered by infection of E. coli strain XL1-Blue, according to the manufacturer's instructions (Clontech). Recombinant phage were screened using a modified CORT

protocol (*Skolnik et al, 1991*). Briefly, the activated intracellular domains of both TGFβ receptors (prepared as described above) were incubated with filters, and interactions between phosphorylated receptors and library-expressed proteins were detected by autoradiography. Positive plaques were picked and enriched. Numerous positive clones were identified using this method, of which one will be described in detail herein. A partial cDNA of approx. 463 bp was originally isolated and sequenced (kathleen mulder #23 in the series, km23). This partial cDNA was then used to obtain the full-length rat km23 gene, including the 5' and 3' regions. A human placental cDNA library (Clontech) was screened to isolate human km23 (hkm23). Upon comparison of our sequence with human ESTs in the database, the full-length hkm23 gene was obtained. The nucleotide sequences for human (accession # AY026513) and rat (AY026512) km23 are available at http://www.ncbi.nlm.nih.gov:80/entrez. The protein id's are AAK18712 and AAK18711, respectively.

Transient transfections, ^{125}I-TGFβ crosslinking, immunoprecipitation/blot, Westerns, and in vivo phosphorylation assays were performed essentially as described previously (*Yue and Mulder, 2000a; Yue et al, 1999a; Hocevar et al, 1999*). To prepare RI-V5, the Alk-5 cDNA was digested with NotI and XhoI restriction enzymes, followed by subcloning into pcDNA3.1/V5-His (Invitrogen V-810-20). To prepare km23-Flag, the coding region of rat or human km23 was PCR amplified with additional suitable flanking restriction enzyme sites for BglII (5') and SalI (3'), and inserted into pCMV5-Flag (Sigma) after digestion with BglII and SalI restriction enzymes. 293T, MDCK, COS-1, or Mv1Lu cells were transiently transfected using

either Lipofectamine Plus (Invitrogen, Cat. No. 10964-013) or Lipofectamine 2000 (Invitrogen, Cat. No. 11668-027), according to the manufacturer's instructions.

Phosphoamino acid analysis--COS-1 cells were transfected and labeled as for in vivo phosphorylation assays. After the cell lysates were normalized for radioactivity, labeled km23 protein was immunoprecipitated with anti-Flag, separated by SDS-PAGE, transferred, and visualized by autoradiography. The membrane containing ^{32}P-labeled km23 was excised, and phosphoamino acid analysis was performed as previously described (*Boyle et al, 1991*).

Stable transfections--hkm23-flag was inserted into a pEGFP-C1 plasmid (Clontech) to create an N-terminal GFP tag. The resulting construct or the equivalent empty vector (EV) was transfected into Mv1Lu cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. 24 hrs after transfection the cells were split at a ratio of 1:5. After another 24 h, G418 (1000 ug/ml) was added for a selection period of 11 d, at which time surviving colonies were pooled and maintained in the presence of G418 (1000 ug/ml). Expression of km23 was verified by Western blot analysis, and stably transfected pools of km23-flag or EV transfected pools were used for JNK, c-Jun, and growth assays.

JNK in vitro kinase assays--were performed as described previously (*Frey and Mulder, 1997; Yue and Mulder, 2000b*), except that anti-JNK (C-17, Santa Cruz) was used for the IPs and GST-c-JUN (1-79) (Santa Cruz) was the substrate.

Growth assays—The TGFβ responsiveness of cells was verified by ^{3}H-thymidine incorporation assays, performed as described (*Hartsough and Mulder, 1995*). For Fig. 5, pools of Mv1Lu cells stably transfected with km23-flag or EV were plated at 2 x

10^3 cells per 96-well dish, and were analyzed at several days thereafter using the Crystal Violet Assay (EMScience #1011, Fisher), according to the manufacturer's instructions.

GST pull-downs— To prepare GST-km23, the coding region of rat or human km23 was PCR amplified with additional suitable flanking restriction enzyme sites for BamHI (5') and XhoI (3'), and inserted into pGEX-4T-1 (Amersham Pharmacia Biotech) after digestion with BamHI and XhoI restriction enzymes. The bacterially expressed rkm23-GST was isolated according to the manufacturer's instructions (Amersham Pharmacia Biotech) and used in the GST pulldowns by standard methods (Curr Protoc Molec Biol). The products were analyzed by SDS-PAGE or immunoblotting/Coomassie staining.

RESULTS

We have developed a novel method for the identification of TGFβ receptor-interacting proteins, as depicted in Fig. 1A. The phosphorylated, activated cytoplasmic domains of the TGFβ receptors were used as probes to screen an expression library that was prepared from a highly TGFβ-responsive IEC line (*Mulder et al, 1993*). The cytoplasmic regions of both receptors were phosphorylated in vitro using a kinase assay prior to screening, as described in the "Materials and Methods." Fig. 1 B illustrates the results of an vitro kinase assay performed using the cytoplasmic regions of the receptors. Lanes 3 and 4 depict the phosphorylated receptor proteins after expression of either RII or RI alone, as indicated. Auto-phosphorylation of both receptors is clearly visible, as previously described (*Lin et al, 1992; Bassing et al, 1994; Chen and Weinberg, 1995*). No phosphorylation is visible after expression of only empty vectors (pET 15/30). Upon expression of both receptor domains (lane 6), there is an increase in the phosphorylation level of both receptors, indicating that trans-phosphorylation was also occurring. These data indicate that the cytoplasmic domains of RI and RII can interact and become catalytically activated in vitro. These phosphorylated receptor domains were used to screen the expression library as illustrated in Fig. 1A.

Several positive clones were isolated as described in the "Materials and Methods." Among the clones isolated, km23 was pursued initially, since early database searches identified the Drosophila bithoraxoid (bxd) region of the bithorax complex (BX-C) as being most closely related. The BX-C is a cluster of homeotic

genes that transcribe positional information into segmental identity for specific parasegments (*Morata and Kerridge, 1981; Martin et al, 1995).* bxd is a 40-kb region of BX-C, immediately upstream from the Ultrabithorax (Ubx) unit, and capable of exerting cis regulatory control over expression of this unit (*Lipshitz et al., 1987*). It had already been shown that the TGFβ superfamily member Decapentaplegic (Dpp) stimulated transcription of Ubx, and that the Ubx protein was necessary but not sufficient for full activation of dpp expression (*Mathies et al, 1994; Sun et al, 1995; Eresh et al., 1997*). Thus, it was conceivable that a homologue of the regulatory region of Ubx might be important in TGFβ signaling. In addition, the TGFβ superfamily of secreted polypeptides is known to convey critical signals during the control of development in various contexts, and BX-C is also important in development.

Several other clones were obtained in our screen, including a previously recognized TGFβ RI-interacting protein, the alpha subunit of farnesyl protein transferase (*Kawabata et al, 1995; Ventura et al, 1996*). The other clones identified in our screen will be the subjects of future investigations. We would not have expected to identify Smads in our screen, since we utilized catalytically active TGFβ receptors as the probes. It has been proposed that activation of RSmads by RI releases them from the complex, to mediate downstream signaling. For example, Macias-Silva *et al* (1996) have demonstrated that the interaction between the TGFβ receptor complex and Smad2 was increased when RI was made inactive by mutation of the kinase domain. Further, Lo *et al* (1998) have shown that removal of the C-terminal domain of Smad2 increased its interaction with RI, suggesting that docking was inhibited when

the C-tail was phosphorylated. Therefore, in our screen, the in vitro kinase assay performed on the receptors prior to library screening would be expected to prevent binding of Smads to the receptor complex.

The novel TGFβ signaling intermediate we identified, termed km23, is a 96-amino acid protein encoded by a 291-bp open reading frame. It is a ubiquitously expressed, cytoplasmic protein with a predicted molecular weight (MW) of 10.667 kD and a calculated MW of 11 kD on Western blots. The rat and human km23 amino acid sequences differ by only three amino acids and are 98% similar. Additional alignments of km23 with sequences in the NCBI database indicated that km23 is the mammalian homologue of the Drosophila protein roadblock (robl) (*Bowman et al, 1999*). robl is a light chain of the motor protein dynein that interacts with the DIC. It is involved in mitosis and axonal transport. Mutants lacking this gene display defects in intracellular transport, and an accumulation of cargoes, as well as an increase in the mitotic index.

Table 1 lists the % homologies, identities, and similarities of some of the DLCs of the km23/robl/LC7 family. Differences in the number of amino acids are also shown. As indicated, there is a second member of the mammalian km23 DLCs in the NCBI database. This form of km23 (designated km23-2 in Table 1; AA446298) displays 70% homology with the km23 form we have identified. In contrast, a total of five robl/robl-like genes have been identified in Drosophila, yet C. elegans appears to have only a single km23/robl-like gene (NCBI database T24H10.6, *Bowman et al, 1999*). There does not appear to be a family member in Saccharomyces cerevisiae. There are also other DLC families that bind to DIC, including Tctex-1/LC14, Tctex-

2/LC2, LC6, and LC8/PIN (*Makokha et al, 2002; Bowman et al 1999; King 2000*). Of these other DLCs that bind to the DIC, Tctex-1 and LC8 have been shown to function as motor receptors to link cargo to the motor machinery (*Almenar-Queralt and Goldstein, 2001*). Although Tctex-1 and LC8 share limited sequence identity, both bind a number of unrelated cargo in a similar manner (*Mok et al, 2001; Makokha et al, 2002*). Similarly, these DLCs are only 8% and 14% identical to km23, respectively. It is conceivable that km23 also mediates motor complex assembly and connection to the transported cellular cargo.

Since we had identified km23 by its ability to interact with the cytoplasmic regions of the TGFβ receptors, it was of interest to verify that km23 was present in association with the TGFβ receptors intracellularly. Accordingly, affinity crosslinking experiments were performed using ^{125}I-TGFβ (*Yue et al, 1999a*). Fig. 2A indicates that both RI and RII are present in km23-flag immunocomplexes (lane 5) from cell lysates of 293T cells, which had been transiently transfected with both TGFβ receptors and km23-flag. The positions of RI and RII were confirmed by analysis of total cell lysates (lane 2). Unlabeled TGFβ completely competed for binding to both receptors as shown in lane 6 (Fig. 2A). Further, no receptors were detectable in flag IP's after expression of both receptors without km23 (not shown). The control blots in the lower panels demonstrate that the appropriate constructs were expressed to similar levels. Thus, these results suggest that km23 is associated with the activated receptor complex.

In order to determine whether the interaction between the receptors and km23 occurred rapidly after ligand stimulation, we performed immunoprecipitation (IP)/blot analyses in the presence and absence of TGFβ. Co-expression of both TGFβ receptors is known to result in heteromeric complex formation and receptor activation in the absence of ligand (*Ventura et al, 1994*), as shown in Fig. 2B (lane 4). However, Fig. 2B demonstrates not only that km23 interacts with RII, but also that TGFβ induces this interaction within 5 min of TGFβ addition (lanes 4-6, top panel). The appearance of the RII band with slightly slower mobility (lanes 5, 6) suggests that TGFβ induced the interaction of km23 with either a differentially phosphorylated/modified form of RII. No specific band was apparent after expression of only km23-flag or the receptors alone (lanes 2,3, top panel). We were unable to assess whether RI was also present in the complex using this assay, due to the interference of the IgG bands at the RI position on such blots. However, since an RII Ab was used as the blotting Ab in these experiments, our data indicate that km23 does associate with RII.

In order to ensure that the interaction was not the result of over-expression of the TGFβ receptors, we performed similar IP/blot analyses in MDCK cells expressing endogenous TGFβ receptors. These cells are TGFβ responsive as revealed by a 70% inhibition of cell growth within 24h of TGFβ (10 ng/ml) addition (data not shown). As you can see in Fig. 2C, TGFβ induced a rapid interaction between km23 and endogenous TGFβ receptors. The kinetics were similar to those observed for the 293T cells. Thus, km23 interacts with TGFβ receptors in two different cell types, and without over-expression of the receptors.

The results in Figs. 2A-C are consistent with km23 interacting with both receptors in the complex simultaneously or with RII alone, due to the fact that RII interacts with and controls ligand binding to the complex (*Wrana et al, 1992*). In order to determine whether both receptors were required for km23 interaction with the receptor complex, we performed IP/blot analyses after expression of only RII in 293T cells. Fig. 2D depicts the interaction of km23 with RII, either using flag as the IP Ab, and the HA Ab as the blotting Ab (top panel), or by performing the analyses in the reverse direction (bottom panel). As indicated by the results in either direction, it appears that km23 can interact with RII alone. In contrast, upon expression of RI alone, no detectable interaction of RI with km23 was observed (data not shown). However, since 293T cells do express a low level of endogenous RI receptors, overexpression of RII could cause an interaction of RII with the endogenous RI receptors. It is possible, then, that some RI is still present in the receptor complex in Fig. 2D. Thus, km23 may interact with the receptor complex through the RII receptor, and RI may not be a direct binding partner. In contrast, expression of RII alone may be sufficient for TGFβ regulation of km23.

TGFβ receptors have serine/threonine kinase activity, which can mediate the phosphorylation of intracellular proteins as one mechanism for initiating TGFβ signaling events and responses. Thus, if km23 is a component of a TGFβ signaling cascade, it is conceivable that the TGFβ receptors could phosphorylate km23 as a mechanism for activation. In order to determine whether km23 was phosphorylated by the TGFβ receptors, we performed in vivo phosphorylation assays (*Yue and Mulder,*

1999a; b) after transient expression of km23 and both receptors, each being detectable by distinct tag Abs, as indicated in Fig. 3A. From the results in the top panel, it is clear that the TGFβ receptor complex resulted in phosphorylation of km23 (lane 5). Expression of km23 alone did not result in a band at the km23 position (lane 2), indicating that km23 is not constitutively phosphorylated when expressed in these cells. The IgG and flag binding peptide control lanes (7, 8) indicate that the band indicated is specific for km23.

After complex formation, the TGFβ receptors are known to become phosphorylated on specific serine and threonine residues (*Souchelnytskyi et al, 1996*). Moreover, TGFβ receptor activation affects the phosphorylation of specific serine residues in RSmads, which are required for TGFβ signaling (*Souchelnytskyi et al, 1997)*. Thus, if km23 is a substrate for the TGFβ receptor kinase activity, phosphorylation of km23 on serine residues might be expected. In order to examine whether this was the case, we performed phosphoamino acid analysis of phosphorylated km23 obtained after co-expression of km23 and both TGFβ receptors in COS-1 cells, similar to the analyses for Fig. 3A in 293T cells. Fig. 3B indicates that km23 is phosphorylated primarily on serine residues in response to TGFβ receptor activation. These findings are consistent with km23 functioning as a substrate for the kinase activity of the TGFβ receptors. Conversely, km23 does not appear to stimulate the kinase activity of the receptors (data not shown).

Based upon the current model for TGFβ receptor activation, RII mediates the phosphorylation of RI and the activation of downstream TGFβ components and

responses (*Yue and Mulder, 2001; Roberts, 1998; Sporn and Vilcek, 2000; Massague et al, 2000*). Accordingly, if TGFβ activation of the receptor complex is required for phosphorylation of km23, expression of a kinase-deficient version of RII (KN-RII) would be expected to block km23 phosphorylation. Fig. 3C (top panel) depicts the results of in vivo phosphorylation of km23 after co-expression of either wild-type RII (lanes 2, 3) or KN-RII (lane 4) with wild-type RI. As shown previously, km23 alone was not constitutively phosphorylated (lane 1), and expression of both TGFβ receptors with km23 resulted in km23 phosphorylation (lane 2). Fig. 3C indicates, further, that TGFβ treatment for 15 min enhanced km23 phosphorylation (lane 3). This phosphorylation of km23 was completely blocked upon expression of the KN-RII (lane 4), thereby demonstrating that the kinase activity of RII is required for km23 phosphorylation.

In order to determine whether RI was also required for km23 phosphorylation, we performed similar in vivo phosphorylation experiments using various kinase-active and kinase-deficient versions of RI. Fig. 3D confirmed that expression of both receptors with km23 induced km23 phosphorylation (lane 4), and that KN-RII blocked this phosphorylation (lane 7). However, in addition, this figure indicates that km23 is still phosphorylated after co-expression of RII with KN-RI (lane 9). Only limited phosphorylation of Smad2 has been reported to occur under such conditions (*Macias-Silva et al, 1996*). Since the KN-RI would be expected to abrogate any residual activity from endogenous RI receptors present in COS-1 cells, these data suggest that the RI kinase is not required for phosphorylation of km23, although it is present in km23 immunocomplexes with RII by affinity labeling experiments (Fig. 2A).

Lane 11 in Fig. 3D demonstrates no detectable phosphorylation of km23 after expression of a constitutively active RI mutant (T204D). However, when wild-type RII was co-expressed with this mutant, km23 phosphorylation was observed (lane 13), presumably due to the kinase activity of RII. Collectively, the data suggest that while both receptors may be present in a complex with km23, the RII kinase is required for km23 phosphorylation. The data do not rule out the possibility that another kinase is also present in the complex.

The method of isolation of km23, as well as the results in Figs. 2 and 3, suggest that km23 may function as a signaling intermediate for TGFβ. Thus, it was of interest to examine whether km23 could mediate any of the known TGFβ signaling events. We have previously shown that TGFβ rapidly activates the Jun N-terminal kinase (JNK) family of Mapks (*Frey and Mulder, 1997*). Further, JNK activation by TGFβ is required for such TGFβ responses as production of $TGF\beta_1$ and induction of fibronectin expression *(Yue and Mulder, 2000a; Hocevar et al, 1999*). JNK activation by TGFβ may also play a role in TGFβ-mediated growth inhibition, either through the amplification of TGFβ production, via cross-talk with the Smads, and/or by regulation of cell cycle inhibitors (*Yue and Mulder, 2001; Derynck et al, 2001*).

In order to determine the effect of forced expression of wild-type km23 on JNK activation, we stably expressed a flag-tagged version of km23 in mink lung epithelial cells (Fig. 4A, third panel), and performed in vitro kinase assays to determine the ability of JNK to phosphorylate GST-c-Jun in the absence and presence of TGFβ. As shown in Fig. 4A, in the EV expressing cells, TGFβ began activating JNK within 10 min

of TGFβ addition; JNK activity increased further by 30 min post-treatment (top, left panel). These kinetics are similar to those obtained for other cell types (*Frey and Mulder, 1997*). In contrast, when km23 was stably expressed in these cells, JNK was super-activated in the absence of TGFβ (top, right panel). JNK activity was approximately 15 times greater in the km23-expressing cells than in the EV-expressing cells during the 2-10 min period after TGFβ addition. By 30 min post-TGFβ treatment, JNK activation levels were more similar between the km23- and EV-expressing cells. These findings suggest that km23 may function as a signaling intermediate for the activation of JNK by TGFβ.

Previous results have indicated that c-Jun, a downstream effector of JNK, can be phosphorylated by TGFβ (*Huang et al 2000*). In order to determine whether this downstream effector of JNK could also be phosphorylated by stable expression of km23, we performed immunoblot analysis at various times after TGFβ treatment using a phospho-c-Jun specific Ab. This Ab is specific for c-Jun phosphorylated at serine-63, and does not cross-react with unphosphorylated c-Jun or with the phosphorylated forms of Jun B or Jun D. These studies were performed in the same Mv1Lu cells stably expressing km23 that were used for Fig. 4A. The results in Fig. 4B demonstrate that forced expression of km23 induced the phosphorylation of c-Jun in the absence of TGFβ (comparing left and right top panels). As for JNK activity, c-Jun phosphorylation was super-activated in the absence of TGFβ. The lower panel in Fig. 4B indicates that c-Jun phosphorylation levels were approximately ten times greater in the km23-expressing cells, than in the EV-expressing cells. Collectively, the results in

Fig. 4 suggest that km23's cellular effects on JNK and c-Jun activation are downstream of TGFβ receptor activation.

In addition, our findings in Fig. 4 suggest that over-expression of km23 may result in the constitutive activation of specific TGFβ signaling components and pathways. These intermediates may, in turn, be involved in mediating specific TGFβ responses in the absence of ligand activation of receptors. Accordingly, since one of TGFβ's most prominent biological effects is growth inhibition of epithelial cells, we examined whether over-expression of km23 in the Mv1Lu transfected pools could result in growth inhibition in the absence of TGFβ. The results in Fig. 5 indicate that, relative to EV-transfected pools, the km23-expressing cells were growth inhibited by approximately 50%. These data support the contention that over-expression of km23 may mediate some TGFβ responses in a constitutive manner. Alternatively, with regard to the growth inhibitory effect observed, the overexpression of km23 may have disrupted the interaction of cytoplasmic dynein with the kinetochore, thereby reducing growth. Similar results have been reported upon overexpression of dynamitin, a dynactin subunit that can disrupt the dynein/dynactin interaction (*Echeverri et al, 1996*).

As mentioned earlier, km23 is the mammalian homologue of the Drosophila protein robl, which is a DLC (*Bowman et al, 1999*). Accordingly, it was of interest to determine whether km23 could interact with the DIC as robl does. As shown in Fig. 6A, we performed GST pull-down assays after expressing and purifying GST-km23. An anti-DIC Ab was used as the blotting Ab to detect the presence of dynein in the

GST-km23 complexes. This Ab detects a protein of approximately 74 kD. In Fig. 6A, it is clear that dynein is visible in GST-km23 immunoprecipitates (lane 2), but not in immunoprecipitates from GST alone (lane 1). The interaction between the Smad binding domain (SBD) of SARA and Smad2-flag (*Tsukazaki et al, 1998*) is shown as a positive control for comparison (lane 4). The results clearly demonstrate that km23 is a dynein-associated protein.

The finding that km23 associates with and is phosphorylated by activated TGFβ receptors, and that it can activate JNK and c-Jun and inhibit cell growth, suggests that km23 may function in a TGFβ signaling pathway. Further, since it is thought that DLCs may be important for specifying the nature of the cargo that will be carried by the motor (*Klopfenstein et al, 2000; Kamal and Goldstein, 2002*), it is likely that extracellular factors (such as growth factors, cytokines, etc) might be able to select the particular DLCs that are recruited to the motor in specific cellular contexts. Accordingly, it was of interest to determine whether TGFβ could mediate the recruitment of km23 to the DIC. For these studies, we performed immunoprecipitation (IP)/blot analyses using anti-DIC as the IP Ab and anti-flag as the blotting Ab. Fig. 6B (top panel) demonstrates that km23 does interact with cytoplasmic DIC by IP/blot analyses. In addition, as shown in lanes 3-5 and 8-10 of this figure, TGFβ (10 ng/ml) induced a rapid recruitment of km23 to the DIC. Although a basal level of km23-DIC association was detectable in some cases (lane 2), a 3-fold increase in this association was visible within 15 min of TGFβ addition to the TGFβ-responsive MDCK cells. This increase in the DIC-km23 interaction began as

early as 2 min after TGFβ addition (top right panel) and appeared to remain relatively constant for at least 60 min (lanes 4, 5, top panel). The lower panels demonstrate roughly equal expression and loading. Thus, TGFβ rapidly induced the recruitment of the km23 DLC to the DIC.

The results in Fig. 6B indicate that TGFβ can stimulate the recruitment of km23 to the DLC, suggesting a connection between TGFβ signaling and DLC recruitment. In order to provide definitive evidence that TGFβ receptor activation is required for the km23-DLC interaction, we examined the km23-DIC interaction in the absence and presence of a kinase-deficient form of TGFβ RII. This receptor mutant can function in a dominant-negative fashion to block the kinase activity of endogenous RII when over-expressed in cells (*Wieser et al, 1993*). Further, we have shown in Fig. 3D that expression of KN RII with wild-type RI does not permit km23 phosphorylation. Fig. 6C indicates that the TGFβ-induced interaction between km23 and DIC (lanes 3-5) was blocked when KN RII was expressed (lanes 7-10). No specific band was detectable in EV and IgG control lanes. Expression of km23 and KN RII in the relevant lanes was also confirmed (middle and lower panels). Thus, km23 phosphorylation by kinase-active TGFβ receptors is necessary for the recruitment of km23 to the DIC.

DISCUSSION

Our results provide a novel method for the identification of TGFβ signaling components, based upon their ability to bind to the phosphorylated intracellular domains of the TGFβ receptors. Further, we have verified the success of this method with the isolation of a unique TGFβ receptor-interacting protein. The km23 interaction with the TGFβ receptors was confirmed by ^{125}I-TGFβ affinity labeling and by IP/blot analysis. Further, TGFβ induced the interaction of km23 with endogenous TGFβ receptors within 5 min of ligand addition in MDCK cells, and a similar kinetic profile was observed in at least one other cell type. Finally, km23 was able to transduce specific TGFβ signaling events, including an activation of JNK, a phosphorylation of c-Jun, and an inhibition of cell growth.

We have also shown that TGFβ receptor activation results in the phosphorylation of km23 primarily on serine residues, consistent with the kinase specificity for the receptors. For example, the RSmads are activated by serine phosphorylation at a C-terminal SSxS motif (*Souchelnytskyi et al, 1997).* While this could suggest that km23 is a direct substrate of the TGFβ receptor kinase activity, it is also possible that another kinase is associated with the km23/TGFβ receptor complex. There are consensus phosphorylation sites for protein kinase C and casein kinase II within the km23 coding region. Perhaps, these or other serine kinases are the immediate activators of km23. However, it is clear that TGFβ does stimulate the

interaction of km23 with the receptors, and that TGFβ receptor activation leads to km23 phosphorylation and recruitment of km23 to DIC.

Our results indicate, further, that the kinase activity of the RII receptor is required for km23 phosphorylation and interaction with DIC, since a kinase-deficient version of RII blocked TGFβ induction of both events. TGFβ RI did not appear to be required for km23 phosphorylation, although RI was present in km23 immunoprecipitates in affinity labeling experiments. Several pieces of evidence support the conclusion that RII is the activating receptor for km23. First, co-expression of RII with a kinase-deficient version of RI induced km23 phosphorylation to an extent equivalent to that which occurred by expression of RII alone. Second, expression of both TGFβ receptors resulted in no additional increase in km23 phosphorylation compared to expression of only RII. Finally, constitutively active RI alone did not result in phosphorylation of km23, as it does for the RSmads. Similarly, previous studies have described TGFβ signaling molecules that were regulated specifically by the RII receptors. For example, the Daxx adaptor protein has been proposed to mediate TGFβ-induced apoptosis through its interaction with RII (*Perlman et al, 2001*).

Based upon the report describing the Drosophila robl protein (*Bowman et al, 1999*), km23 is the human homologue of the dynein light chain/LC7/robl. We have shown that TGFβ leads to the recruitment of km23 to the DIC in a rapid, TGFβ-inducible manner. This interaction, however, occurred within a slightly different time frame than the interaction of km23 with the TGFβ receptors. This finding suggests

that the receptors themselves may not be the cargo that dynein will transport via km23. That is, the km23-receptor interaction peaks at 5 min, and appears to begin declining by 15 min after TGFβ addition (Figs. 2B, C), consistent with the receptors being released once km23 has been phosphorylated. In contrast, it is clear from Figs. 6B and C that the km23-DIC interaction begins as early as 2 min after TGFβ addition, yet km23 is still bound to DIC at 60 min after TGFβ addition. Previous studies have indicated that the transport of p53 along MTs was dynein-dependent, suggesting that the interaction of p53 with dynein facilitated its accumulation in the nucleus after DNA damage (*Giannakakou et al, 2000*). Similarly, subsequent to receptor activation, TGFβ signaling components may be transported along the MTs through the interaction of km23 with DIC.

Although evidence indicates that Smads 2/3/4 may be distributed along the MT network, the MTs appeared to sequester the Smads from the receptor prior to cellular stimulation by TGFβ (*Dong et al, 2000*). Perhaps this occurs because a motor protein light chain such as km23 is in an inactive, unphosphorylated state until TGFβ receptor activation occurs. LC phosphorylation may affect a conformational change in the DLC, followed by its recruitment to a motor complex for transport of TGFβ signaling components (ie, Smads, JNKs, etc) along the MTs.

A link between TGFβ receptor signaling and the minus-end MT motor protein dynein has not been demonstrated previously. However, a receptor-DLC interaction has been reported for the photoreceptor rhodopsin (*Tai et al, 1999*). In addition, the Trk neurotrophin receptors have been shown to associate with the DLC Tctex-1,

suggesting that transport of neurotrophins during vesicular trafficking may occur through this direct interaction between the Trk receptor and the dynein motor machinery (*Yano et al, 2001*). It has been shown that nerve growth factor remains bound to TrkA after endocytosis, thereby allowing the receptor to continue to activate signaling proteins (*Grimes et al, 1996*). In the case of TGFβ, however, the receptor location for either initiation or transmission of TGFβ signaling activities has not been clearly defined. It has been shown that heteromeric TGFβ receptors are internalized and down-regulated after TGFβ activation via a clathrin-dependent mechanism (*Anders et al, 1997; Doré et al, 1998*), and that the kinase activity of RII is required for these processes to occur optimally (*Anders et al, 1998*). A more recent report has indicated that Smad phosphorylation does not occur until the GTPase dynamin 2ab excises the budded vesicle from the plasma membrane to form an endocytic vesicle (*Penheiter et al, 2002*). This report also demonstrated that the formation and activation of the receptor complex was not sufficient for Smad signaling, and that an activity or activities downstream of dynamin 2ab function was/were required. It is possible that km23 recruitment to the DIC, and dynein motoring of TGFβ signaling components along the MTs, represent at least some of these activities.

Since vesicles derived from a donor compartment fuse with specific acceptor membranes to directionally transfer cargo molecules during trafficking (*Gonzalez and Scheller, 1999*), it is likely that distinct events occur in different cell compartments during TGFβ signaling. Thus, the fate of the TGFβ-receptor complex and specific signaling complexes may differ. With regard to the Drosophila TGFβ superfamily

member Dpp, the rates of endocytic trafficking and degradation determine Dpp signaling range (*Entchev et al, 2000*). A similar situation may exist for TGFβ in mammalian cells. However, further investigation will be required for a complete understanding of how TGFβ receptor endocytosis, intracellular trafficking, and cell signaling events are integrated.

Collectively, our data are consistent with a role for km23 in both TGFβ signaling and dynein-mediated transport along MT's. It is likely that the binding of km23 to the DIC after TGFβ receptor activation is important for specifying the nature of the cargo that will be transported along the MTs. Any disruption in km23 could prevent or alter movement of specific cargo along MT's. In this way, alterations in km23 might result in a mis-localization of these proteins, with a disruption of TGFβ growth inhibitory signals. Along these lines, protein traffic direction is required for the maintenance of cell polarity, which, if lost, can result in tumor formation (*Peifer, 2000; Bilder et al, 2000*). Accordingly, sequence alterations at specific regions of km23 in human tumors might play a role in tumor development or progression. Future studies will address this possibility.

ACKNOWLEDGMENTS

We wish to thank L. Liotta (NCI) and T. W. Wong (Bristol-Myers Squibb) for helpful discussions. We appreciate the assistance of S. R. Hann and M. A. Gregory (Vanderbilt) with the phosphoamino acid analysis procedure. We also thank J. Massague (Sloan-Kettering) for KNRI-HA and KNRII-HA, J. Wrana (Toronto, Canada) for pCMV5-HA-TGFβRII, Smad2-flag, and GST-SBD, R. Derynck (UCSF) for RI-myc, K. Miyazono (Tokyo, Japan) for the Alk-5 RI cDNA, Q. Chen (Penn State COM, Hershey, PA) for the pcDNA3.1/V5-His vector, H. Lodish (Cambridge, MA) for the RII cDNA, H. Moses (Vanderbilt) for the T204D RI, and T. W. Wong for pFlag-CMV5 and for preparing the RI-pFlag-CMV5 expression construct. This work was supported by National Institutes of Health Grants CA51452, CA54816, CA68444, CA90765, and CA92889, and DOD award DAMD 17-01-1-0592 to K.M.M.

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FIGURE LEGENDS

Fig. 1. Identification of a novel TGFβ receptor-interacting protein. **A**: Method for identifying TGFβ receptor-interacting proteins. The cytoplasmic domains of both receptors were expressed, sequentially isolated, kinase-activated in vitro, and used as probes to screen an expression library. **B:** In vitro kinase activation of the cytoplasmic regions of TGFβ RI and RII result in both auto- and trans-phosphorylation. Bacterially expressed TGFβ receptor proteins were precipitated with Ni-NTA agarose beads prior to performing an in vitro kinase assay. Bacterial lysates were prepared after expression of either EVs (pET15, pET30, pET15/pET30), the intracellular domains of RII or RI alone (pET15-His-RII-flag, pET30-His-RI-S), or together (pET15-RII-flag/pET30-RI-S).

Fig. 2. Verification of TGFβ receptor interaction with km23. **A:** RI and RII TGFβ receptors are present in km23 immunocomplexes. 293T cells were transiently transfected with km23-flag, RI-myc, RII-HA, and/or EV's, and affinity labeling was performed. After the 4-h ^{125}I-TGFβ labeling period (4°C), the cross-linking agent DSS was added for an additional 15 min. Top panel, total cell lysates (lanes 1-2), or lysates immunoprecipitated (IP'd) with an anti-flag M2 Ab (Lanes 3-6) or with IgG (lane 7, control) were visualized by SDS-PAGE and autoradiography. No bands were visible in Flag IP's after transfection of only RI and RII (not shown). Lower panels, Western blots for Flag, myc, and HA demonstrate expression of the relevant constructs (lanes

2, 4-6 for km32; lanes 2,5,6 for RI and RII). **B**: The interaction between km23 and the TGFβ receptors occurs within 5 min of TGFβ addition. 293T cells were transiently transfected with km23-flag, RI-V5, RII-HA, and/or EV's, followed by IP/blot analyses using Flag as the IP Ab and an RII polyclonal Ab as the blotting Ab (top panel). Cells were incubated in serum-free medium for 60 min prior to addition of TGFβ for the indicated times. Lower panels, controls for expression and loading of km23 (Flag blot), RI (V5 blot), and RII (RII blot). **C**: TGFβ induces a rapid association of km23 with endogenous TGFβ receptors in MDCK cells. EV or km23-flag constructs were expressed in MDCK cells, and TGFβ treatments and IP/blot analyses were performed as for Fig. 2B. **D**: km23 interacts with RII via IP/blot analyses in 293T cells. Cells were transiently transfected with km23 flag and RII-HA as indicated. Top panel, cell lysates were IP'd with anti-flag or HA and blotted with an HA Ab. The presence of RII in lanes 3 and 4, but not in lanes 1 and 2, demonstrates an interaction between km23-flag and RII-HA. Bottom panel, lysates were IP'd with anti-HA and blotted with anti-flag. The presence of km23 in only lane 3 indicates that an interaction between km23-flag and RII-HA is detectable in this direction as well. Results are representative of two experiments for each.

Fig. 3. A functional RII TGFβ receptor is required for km23 phosphorylation **A:** km23 is phosphorylated upon activation of TGFβ receptors. 293T cells were transiently transfected with RI-V5, RII-HA, and either empty vector (EV) or km23-flag. 48h after transfection, cells were labeled for 3h with [$^{32}P_i$], lysed, and IP'd with an anti-flag Ab.

Top panel, In vivo phosphorylation of km23 was visualized by SDS-PAGE and autoradiography. A blocking peptide (b.p.) for the Flag Ab was added in lane 8. Lower panel, expression of transfected km23-flag was confirmed by immunoblot analysis. Results are representative of three experiments. **B**: Activation of the TGFβ receptors results in phosphorylation of km23 primarily on serine residues. km23 was phosphorylated in vivo as for A, and phosphoamino acid analysis was performed. ^{32}P-labeled km23 was excised from the PVDF membrane, and subjected to acid hydrolysis (6 M HCl, 1 h, 110 °C). Phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) were separated in two dimensions using HTLE (C.B.S. Scientific), together with phosphoamino acid standards. Labeled and standard phosphoamino acids were visualized by ninhydrin spray (0.25% in acetone). ^{32}P-labeled phosphorylated amino acids were visualized by autoradiography. **C:** TGFβ cannot phosphorylate km23 when a kinase-deficient RII is expressed with RI. COS-1 cells were transiently transfected as in A, except that the KNRII was co-expressed with wild-type RI in lane 4. Cells were incubated in serum-free, phosphate-free medium for 30 min and TGFβ was added during the last 15 min of the labeling period (lanes 3, 4). Lysates were analyzed as for A. Top panel, In vivo phosphorylation of km23. Lower panels, expression of transfected km23, RI, and RII was confirmed by Western blot analysis with flag, V5, and a polyclonal RII Ab, respectively, as indicated. Results are representative of two experiments. **D:** The kinase activity of RI does not appear to be required for phosphorylation of km23. In vivo phosphorylation assay and transfection of COS-1 cells was performed as for Fig. 3C, except that no TGFβ was

added and different receptor mutants were evaluated as indicated. Results are representative of two experiments.

Fig. 4. km23 expression can induce JNK and result in phosphorylation of the downstream target c-Jun. **A:** Stable expression of km23 results in activation of JNK in the absence of TGFβ. Top panel, Mv1Lu cell pools, stably transfected with either empty vector (lanes 1-4) or km23-flag (lanes 5-8), were incubated in serum-free medium for 30 min prior to addition of TGFβ (10 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-JNK (C-17, Santa Cruz), and subjected to in vitro kinase assays using GST-c-JUN (1-79) as the substrate. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. Normal rabbit IgG was used as the negative control. Middle panels, equal JNK and km23 expression was confirmed by Western blotting. Lower panel, plot of densitometric scan of results in top panel. **B**: Stable expression of km23 results in phosphorylation of c-Jun in the absence of TGFβ. Top panel, cells were treated with TGFβ and lysates were obtained as for Fig. 4A, except that they were analyzed by Western blot analysis using a phospho-c-Jun Ab (KM-1, SC-822). Middle panel, Western blot demonstrating equal km23 expression in the pools stably expressing km23, but not in the EV-transfected pools. Bottom panel, plot of densitometric scan of results in top panel. Results are representative of two experiments for each.

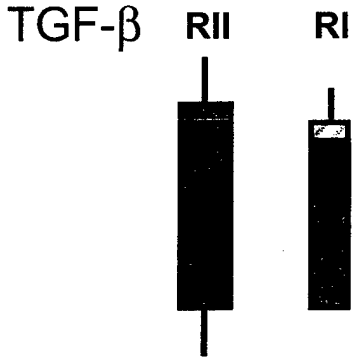
Fig. 5. Stable expression of km23 results in growth inhibition in the absence of TGFβ. Mv1Lu cell pools stably expressing km23 or EV were plated and analyzed for cell

number at several days thereafter as indicated, using the crystal violet assay described in "Materials and Methods." The percent inhibition of growth is indicated in parentheses on top of the relevant bars.

Fig. 6. The interaction between the DLC km23 and the DIC is regulated by TGFβ and requires RII kinase activity. **A:** km23 interacts with DIC via GST pull-down assays. Top panel, MDCK cell lysates were incubated with sepharose-bound bacterially expressed GST alone, GST-rkm23, or GST-SBD (Smad-binding domain of SARA, positive control). GST- bound proteins were analyzed by SDS-PAGE (10%) and were immunoblotted with an anti-DIC Ab. Proteins were detected by ECL. Dynein interacts with GST-km23 (lane 2), but not with GST alone (lane 1). The interaction between Flag-tagged Smad2 and GST-SBD (*Tsukazaki et al, 1998*) was confirmed as a positive control (lane 4). Bottom panel, Coomassie staining of gel in top panel demonstrating the presence of GST and GST fusion proteins in the relevant lanes. The sizes are as expected for the different fusion proteins (approx. 37 kDa for GST-km23, approx. 35 kDa for GST-SBD) or GST alone (approx. 27 kDa). **B**: TGFβ stimulates the recruitment of the km23 DLC to the DIC within 2 min of TGFβ treatment. MDCK cells were transiently transfected with either empty vector or km23-flag. 36h after transfection, cells were incubated in serum-free medium for 60 min prior to addition of TGFβ (10 ng/ml) for the indicated times. Cell lysates were subjected to IP using a monoclonal anti-DIC Ab, followed by immunoblot analysis using an anti-flag Ab (top panel). Western blot analysis with anti-flag (middle panel) or anti-DIC (bottom panel) demonstrates equal protein expression and loading. The right side shows the

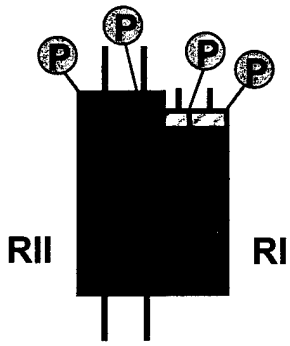
results at earlier time points. Results are representative of three experiments. **C**: Phosphorylation of km23 is required for recruitment of km23 to the DIC. Cell treatments and IP/blot analyses were performed in MDCK cells as for Fig. 6B, except that cells were transfected with km23-flag in the absence (left side, top) or presence (right side, top) of KN RII. Western blot controls for expression of km23-flag and KN RII are shown in the middle and lower panels, respectively.

Fig. 1A



Express intracellular domains of RI and RII in E. coli and co-purify

+ [γ^{32}P] ATP



Phosphorylate receptors via in vitro kinase reaction

Probe with ^{32}P-labeled cytoplasmic domains of RI & RII

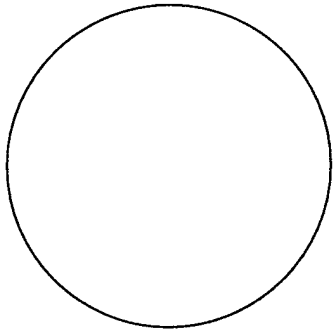
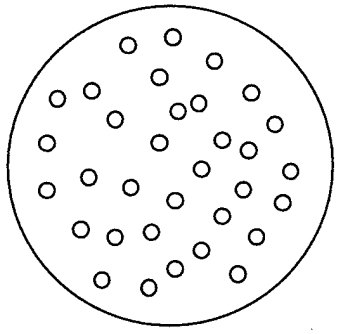
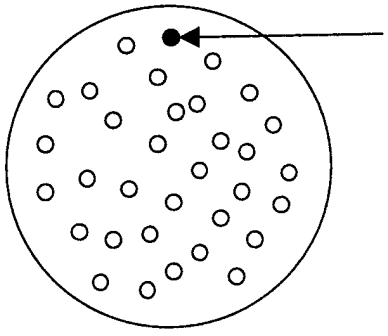


Plate phage library prepared from TGFβ–responsive IEC 4-1 cells

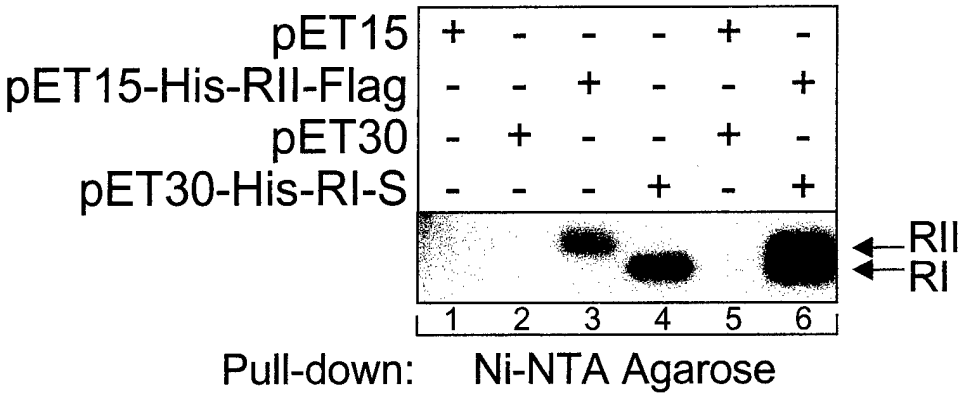


Transfer plaques to IPTG-impregnated nitrocellulose



Select positive clones and enrich

Fig. 1B


pET15 + - - - + -
pET15-His-RII-Flag - - + - - +
pET30 - + - - + -
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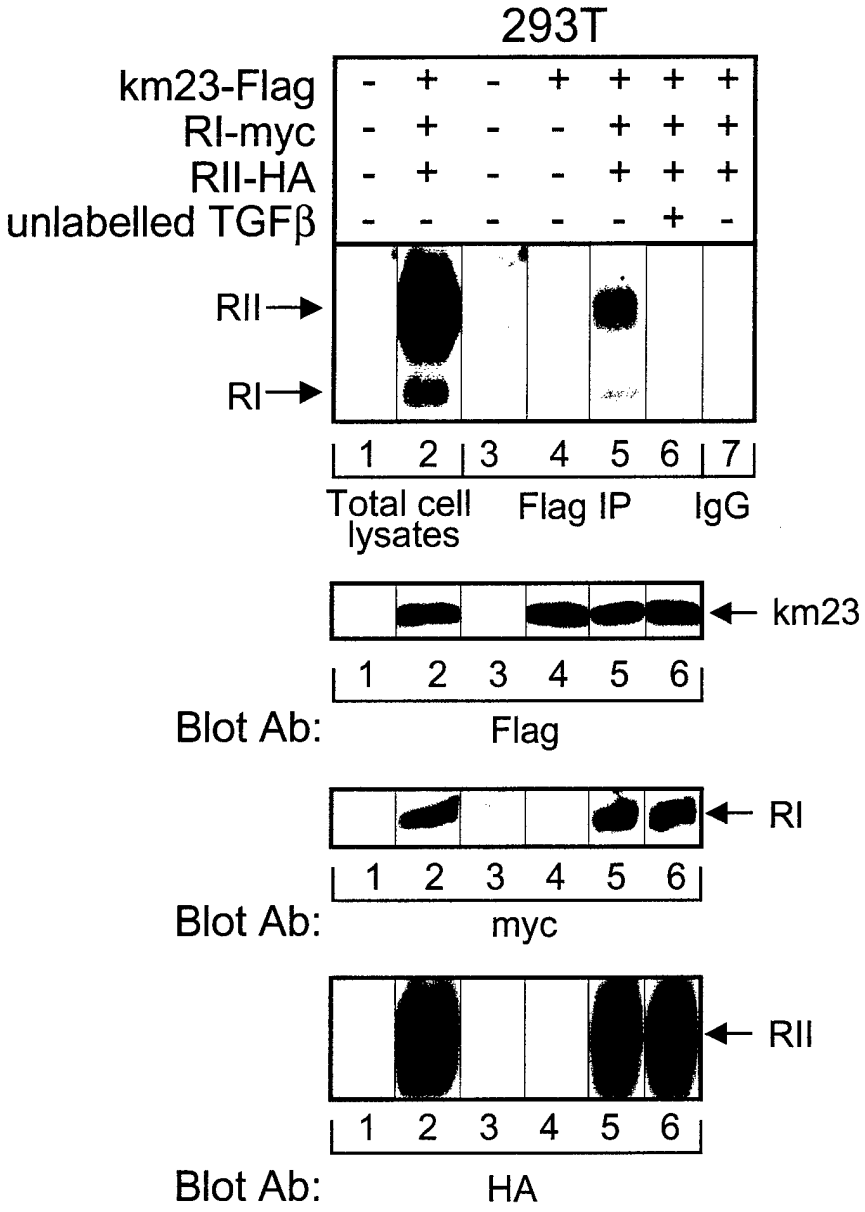
RII
RI
1 2 3 4 5 6
Pull-down: Ni-NTA Agarose

Fig. 2A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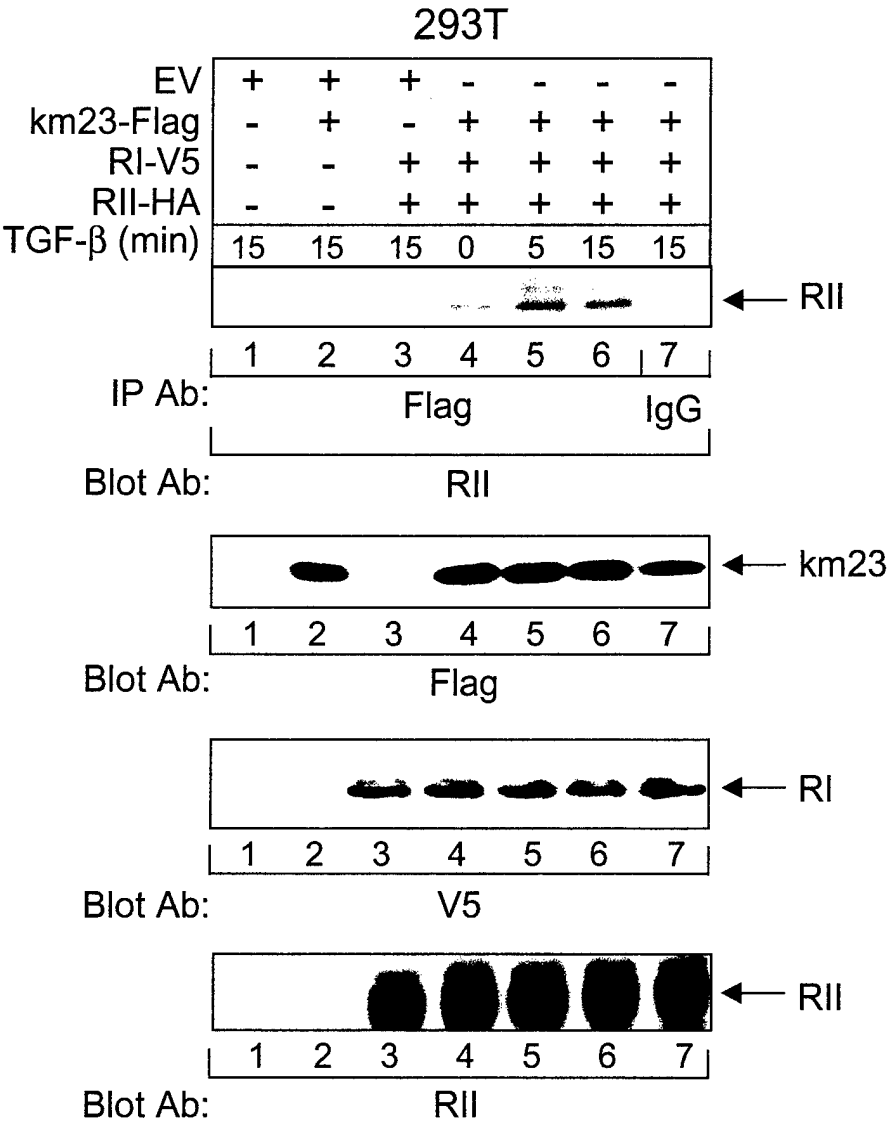
km23-Flag - + - + + + +
RI-myc - + - - + + +
RII-HA - + - - + + +
unlabelled TGFβ - - - - - + -
RII
RI
1 2 3 4 5 6 7
Total cell lysates
Flag IP
IgG
km23
1 2 3 4 5 6
Blot Ab: Flag
RI
1 2 3 4 5 6
Blot Ab: myc
RII
1 2 3 4 5 6
Blot Ab: HA

Fig. 2B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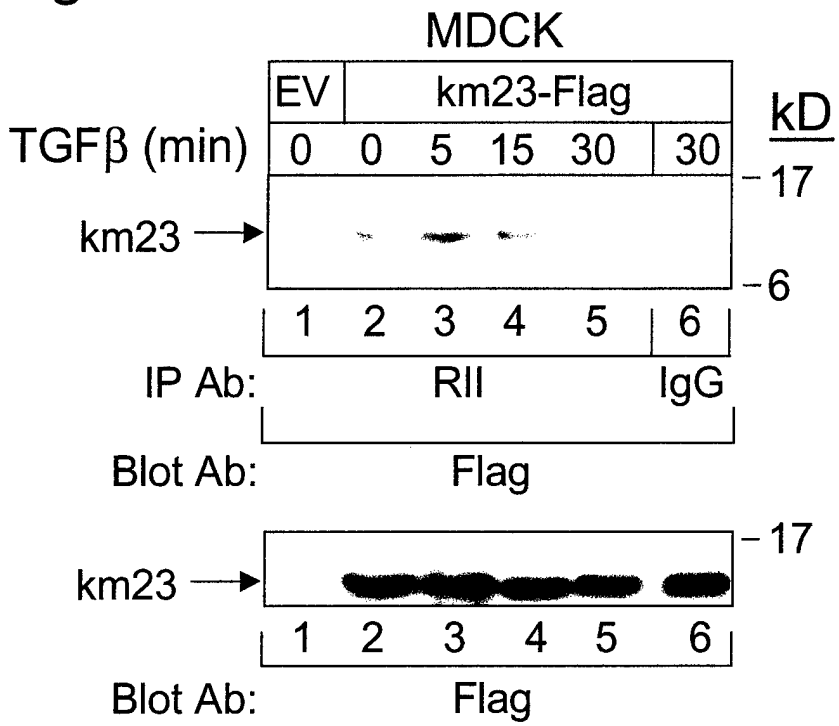
EV + + + - - - -
km23-Flag - + - + + + +
RI-V5 - - + + + + +
RII-HA - - + + + + +
TGF-β (min) 15 15 15 0 5 15 15
RII
1 2 3 4 5 6 7
IP Ab: Flag IgG
Blot Ab: RII
km23
1 2 3 4 5 6 7
Blot Ab: Flag
RI
1 2 3 4 5 6 7
Blot Ab: V5
RII
1 2 3 4 5 6 7
Blot Ab: RII

Fig. 2C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km23-Flag
kD
TGFβ (min)
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17
km23
6
1 2 3 4 5 6
IP Ab:
RII
IgG
Blot Ab:
Flag
17
km23
1 2 3 4 5 6
Blot Ab:
Flag

Fig. 2D

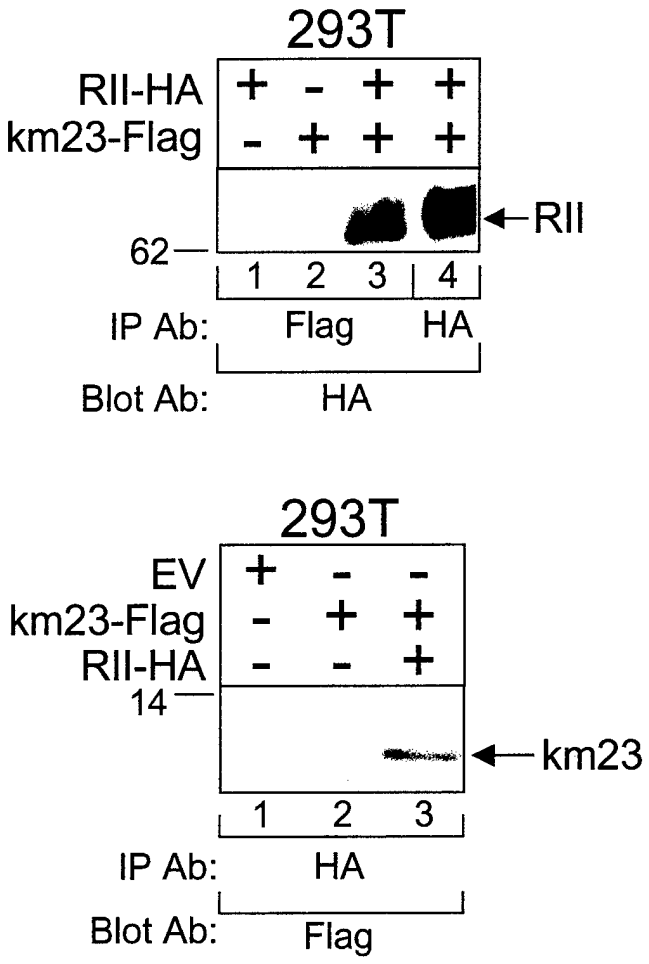

293T
RII-HA
km23-Flag
+ - + +
- + + +
RII
62
1 2 3 4
IP Ab: Flag HA
Blot Ab: HA
293T
EV
km23-Flag
RII-HA
+ - -
- + +
- - +
14
km23
1 2 3
IP Ab: HA
Blot Ab: Flag

Fig. 3A

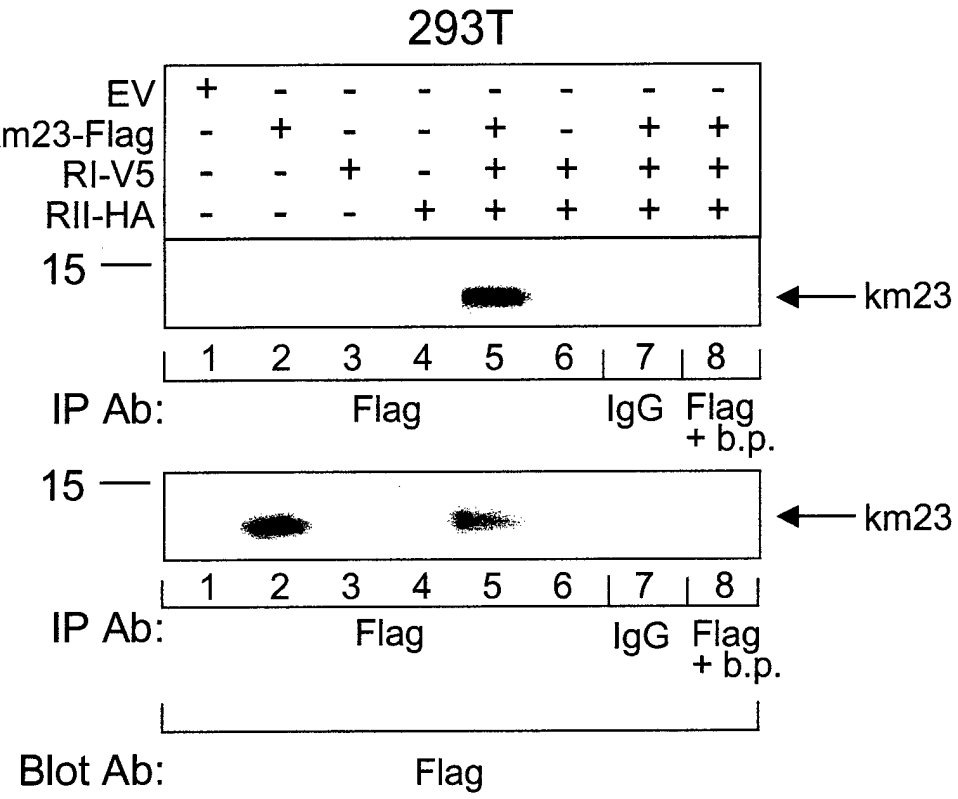

293T
EV + - - - - - - -
km23-Flag - + - - + - + +
RI-V5 - - + - + + + +
RII-HA - - - + + + + +
15
km23
1 2 3 4 5 6 7 8
IP Ab: Flag IgG Flag + b.p.
15
km23
1 2 3 4 5 6 7 8
IP Ab: Flag IgG Flag + b.p.
Blot Ab: Flag

Fig. 3B

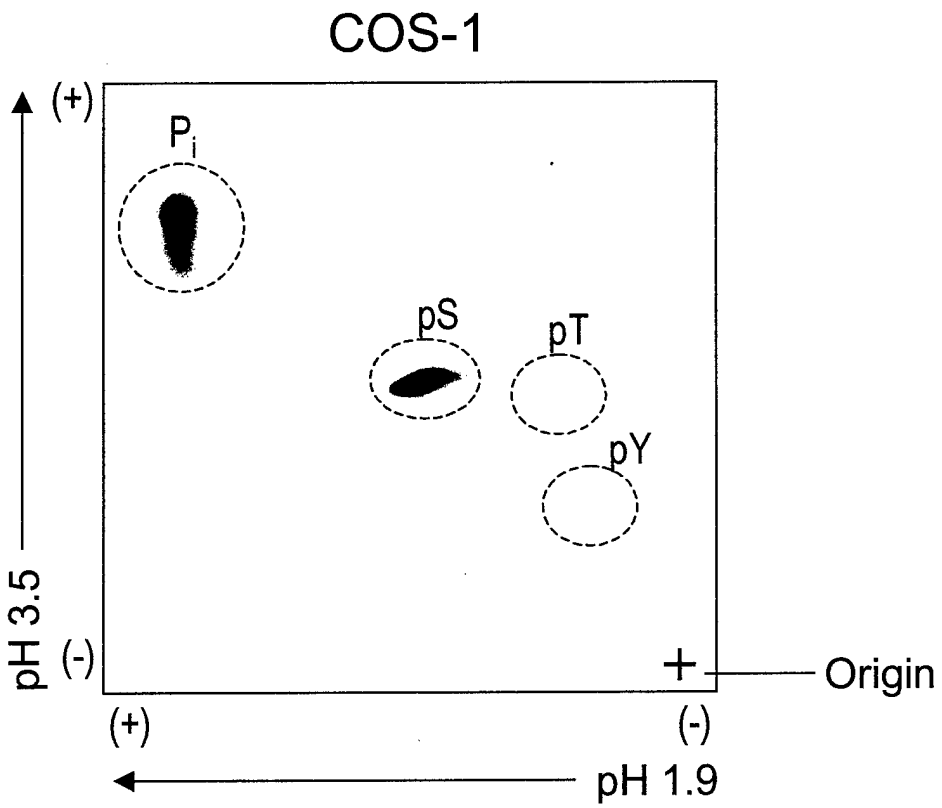
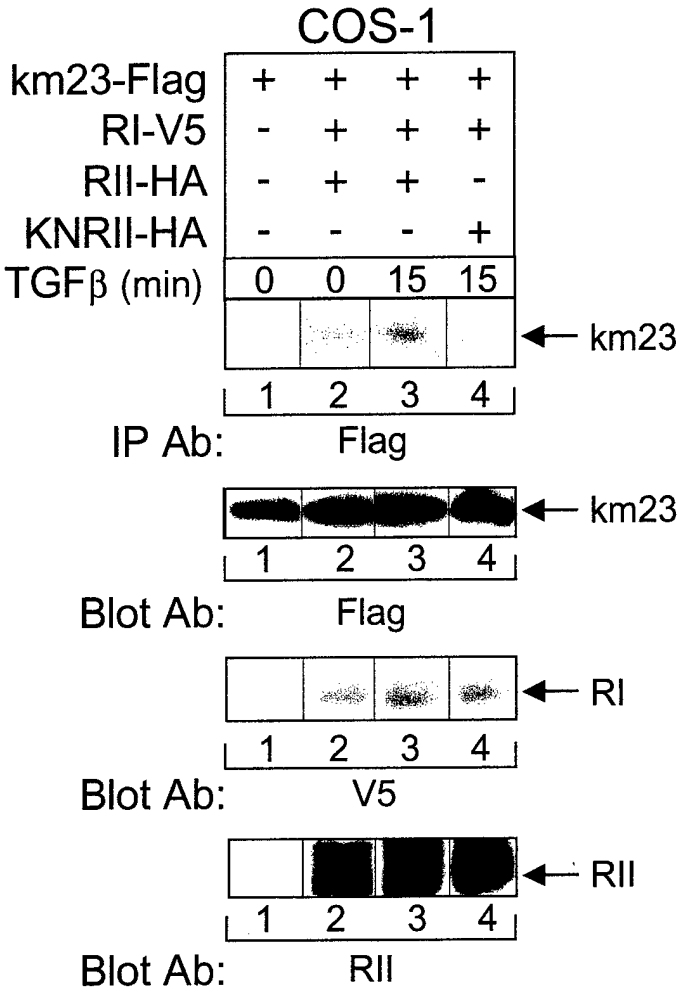


Fig. 3C


COS-1
km23-Flag + + + +
RI-V5 - + + +
RII-HA - + + -
KNRII-HA - - - +
TGFβ (min) 0 0 15 15
km23
1 2 3 4
IP Ab: Flag
km23
1 2 3 4
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Blot Ab: V5
RII
1 2 3 4
Blot Ab: RII

Fig. 3D

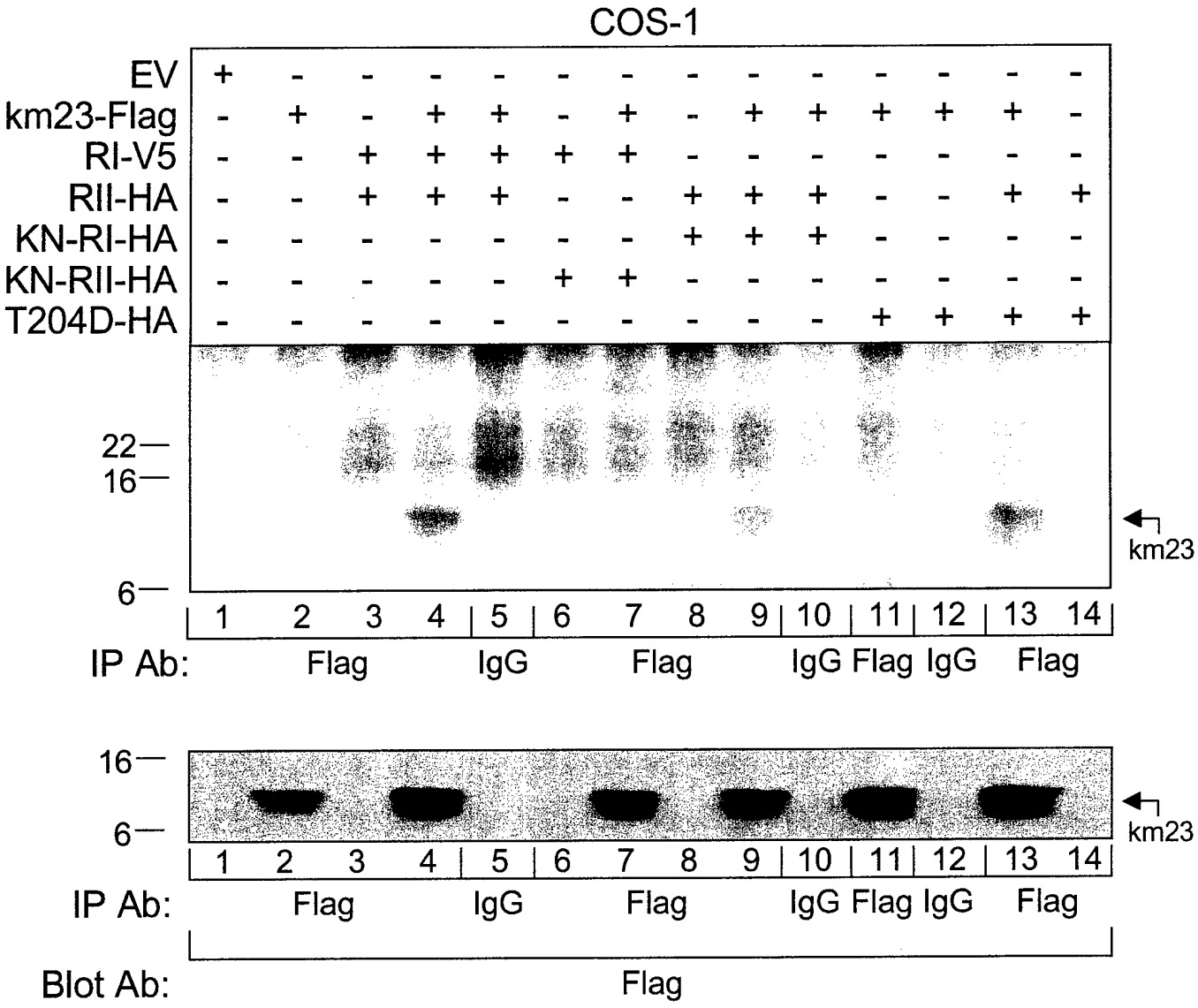
COS-1
EV + - - - - - - - - - - - - -
km23-Flag - + - + + - + - + + + + + -
RI-V5 - - + + + + + - - - - - - -
RII-HA - - + + + - - + + + - - + +
KN-RI-HA - - - - - - - + + + - - - -
KN-RII-HA - - - - - + + - - - - - - -
T204D-HA - - - - - - - - - - + + + +
22—
16—
6—
km23
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP Ab: Flag IgG Flag IgG Flag IgG Flag
16—
6—
km23
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP Ab: Flag IgG Flag IgG Flag IgG Flag
Blot Ab: Flag

Fig. 4A

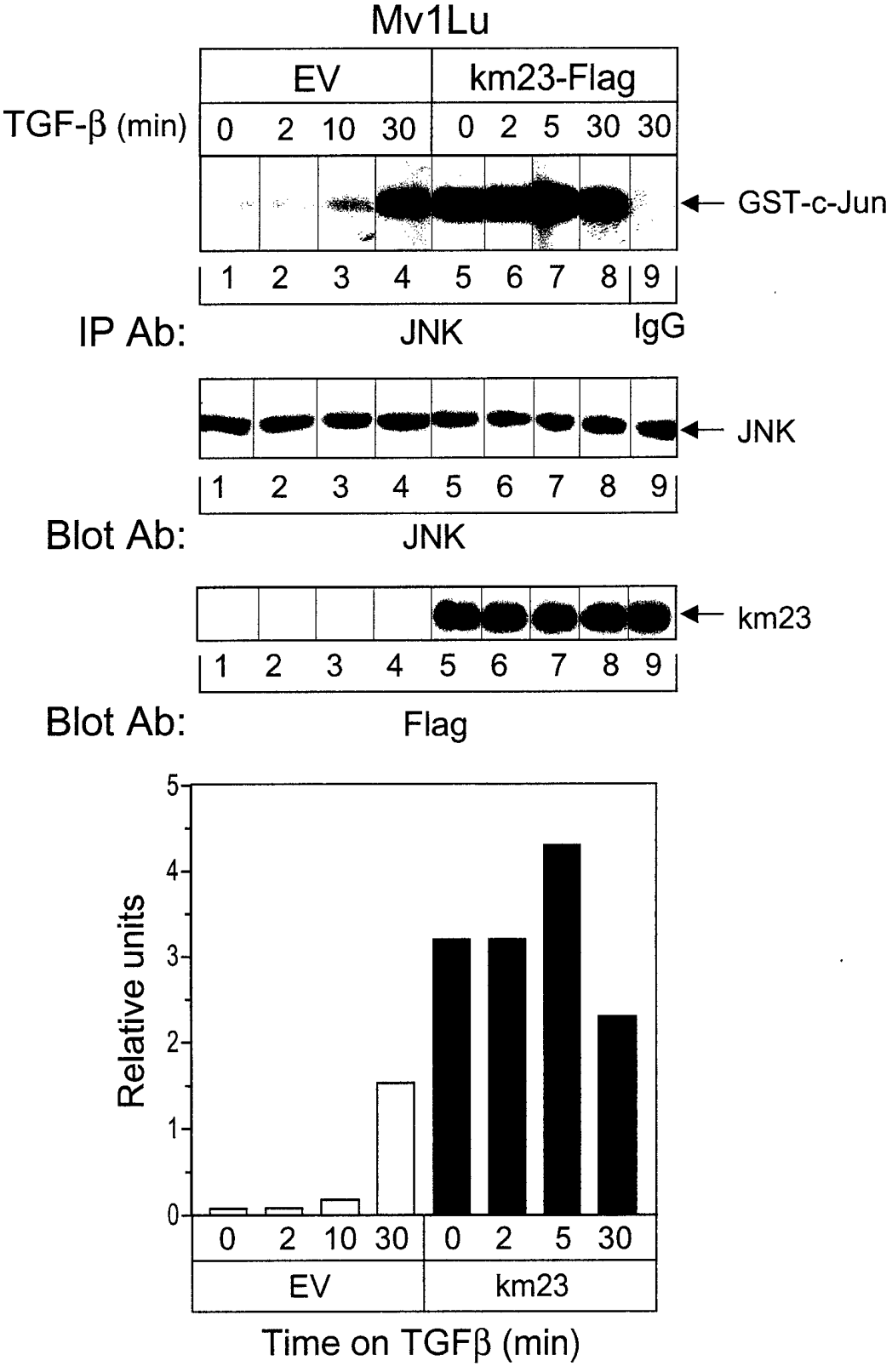
Mv1Lu
EV
km23-Flag
TGF-β (min)
0 2 10 30 0 2 5 30 30
GST-c-Jun
1 2 3 4 5 6 7 8 9
IP Ab:
JNK
IgG
JNK
1 2 3 4 5 6 7 8 9
Blot Ab:
JNK
km23
1 2 3 4 5 6 7 8 9
Blot Ab:
Flag
Relative units
0
1
2
3
4
5
0 2 10 30 0 2 5 30
EV
km23
Time on TGFβ (min)

Fig. 4B

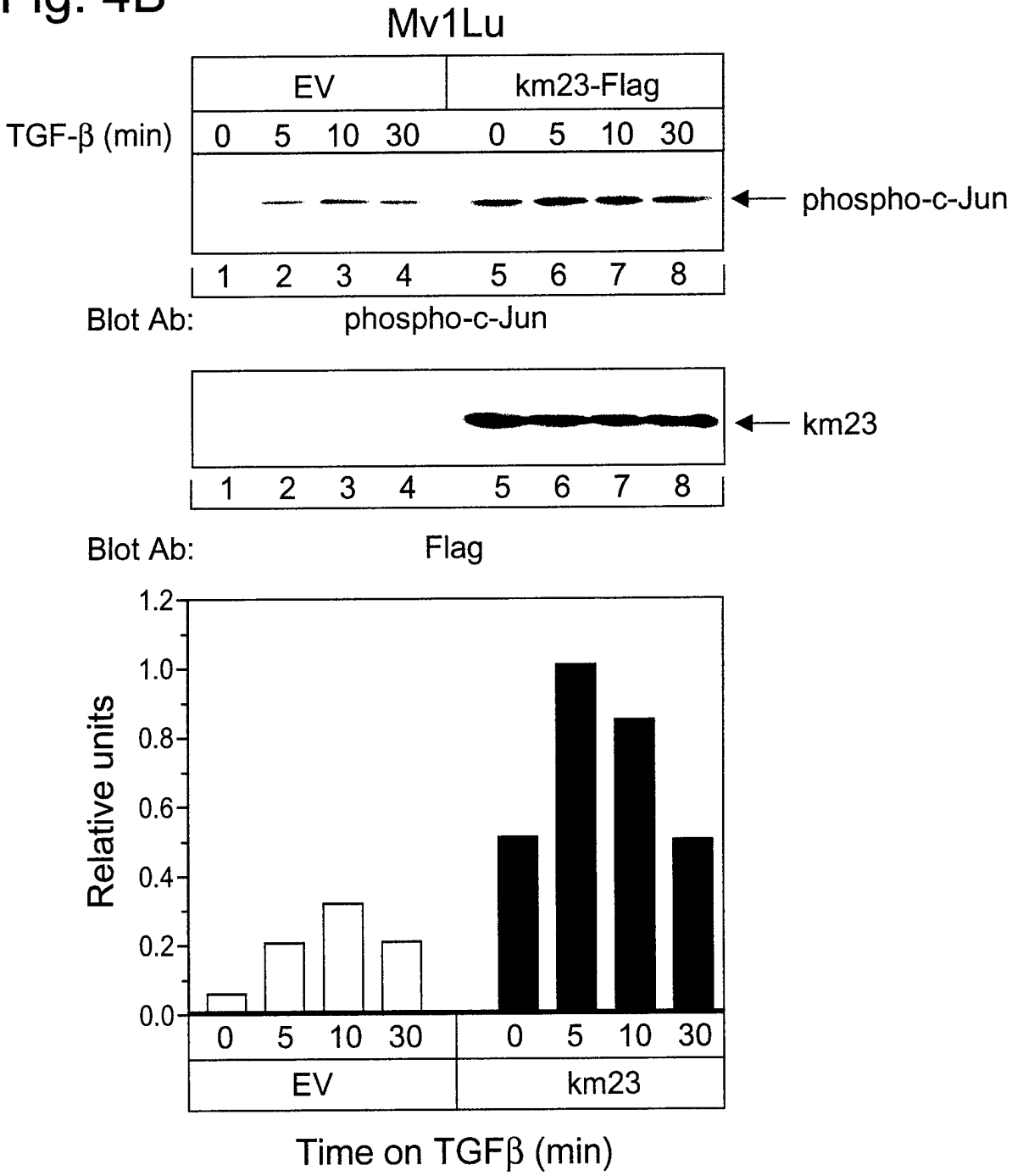
Mv1Lu
EV
km23-Flag
TGF-β (min)
0 5 10 30 0 5 10 30
phospho-c-Jun
1 2 3 4 5 6 7 8
Blot Ab:
phospho-c-Jun
km23
1 2 3 4 5 6 7 8
Blot Ab:
Flag
Relative units
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 5 10 30 0 5 10 30
EV
km23
Time on TGFβ (min)

Fig. 5

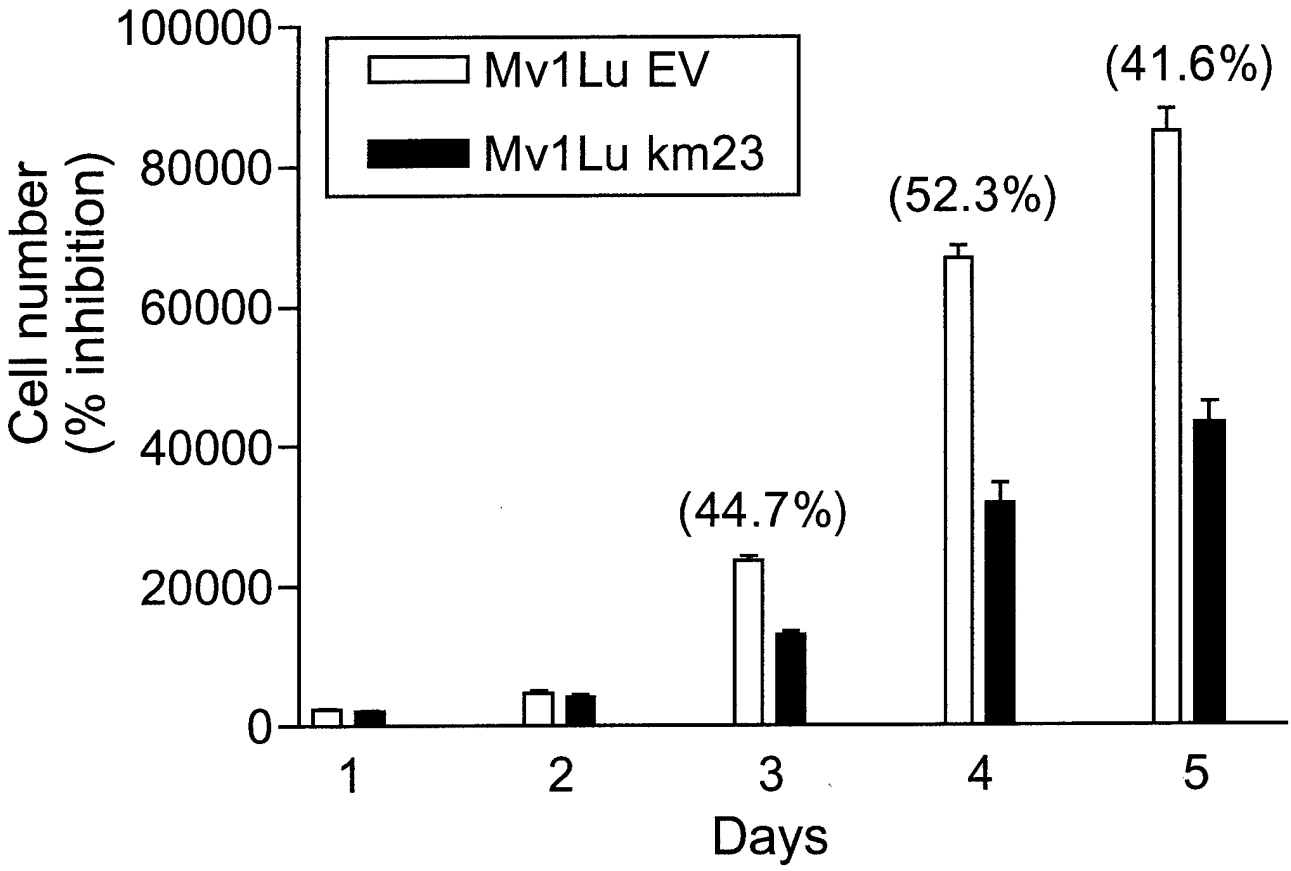
100000
80000
60000
40000
20000
0
Cell number
(% inhibition)
Mv1Lu EV
Mv1Lu km23
(44.7%)
(52.3%)
(41.6%)
1
2
3
4
5
Days

Fig. 6A

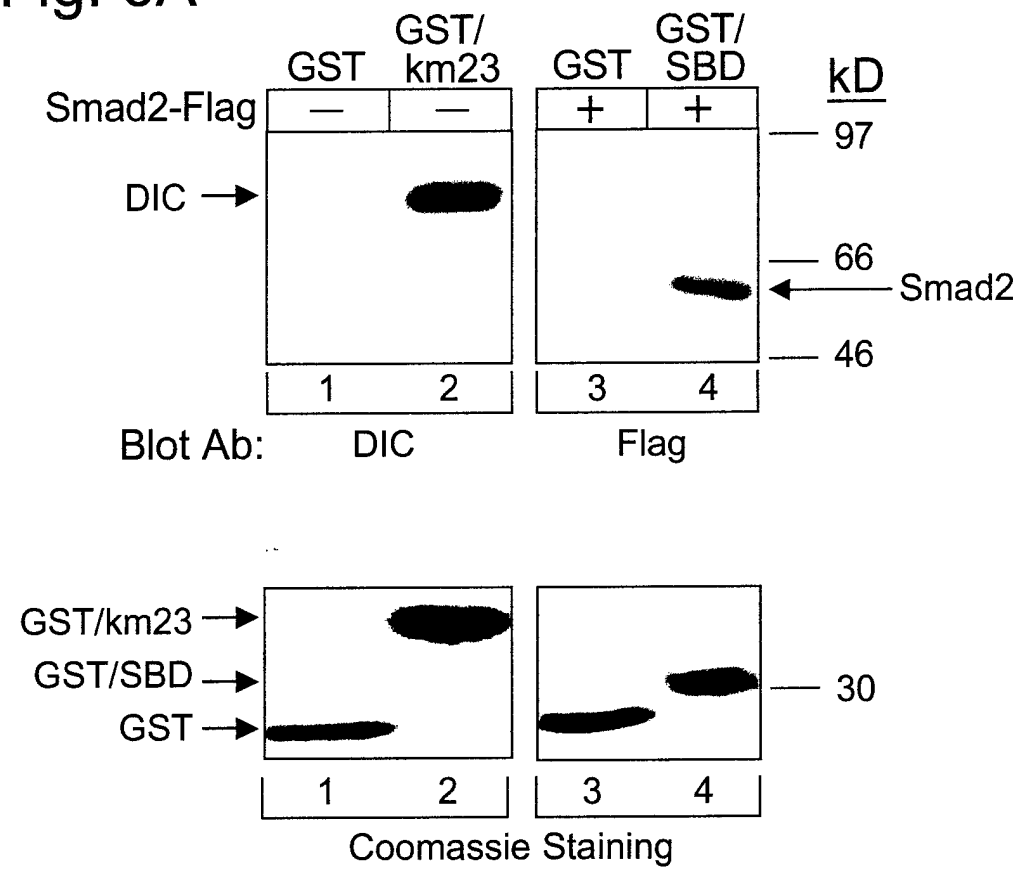
GST
GST/ km23
GST
GST/ SBD
kD
Smad2-Flag
—
—
+
+
DIC
97
66
Smad2
46
1
2
3
4
Blot Ab:
DIC
Flag
GST/km23
GST/SBD
GST
30
1
2
3
4
Coomassie Staining

Fig. 6B

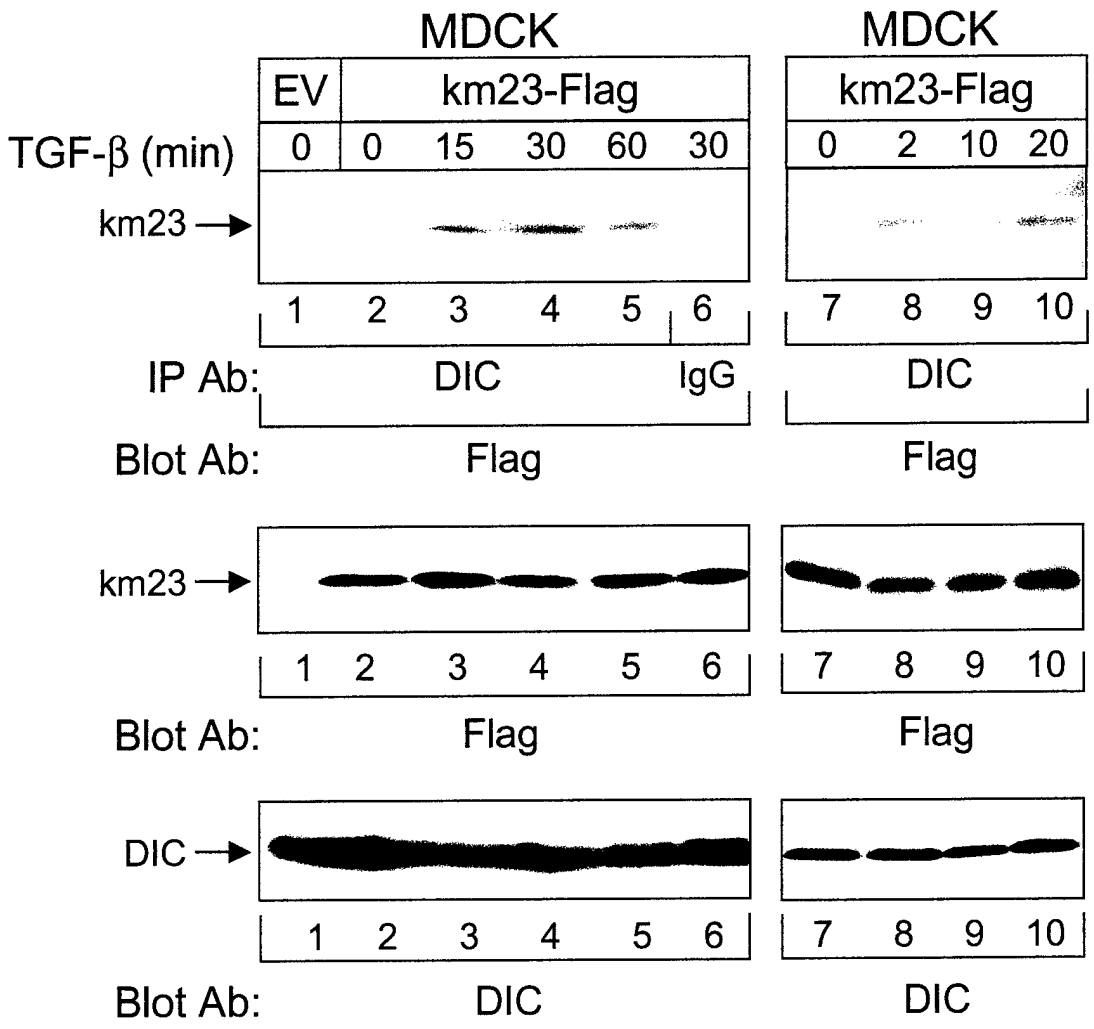
MDCK
MDCK
EV
km23-Flag
km23-Flag
TGF-β (min)
0
0 15 30 60 30
0 2 10 20
km23
1 2 3 4 5 6
7 8 9 10
IP Ab:
DIC
IgG
DIC
Blot Ab:
Flag
Flag
km23
1 2 3 4 5 6
7 8 9 10
Blot Ab:
Flag
Flag
DIC
1 2 3 4 5 6
7 8 9 10
Blot Ab:
DIC
DIC

Fig. 6C

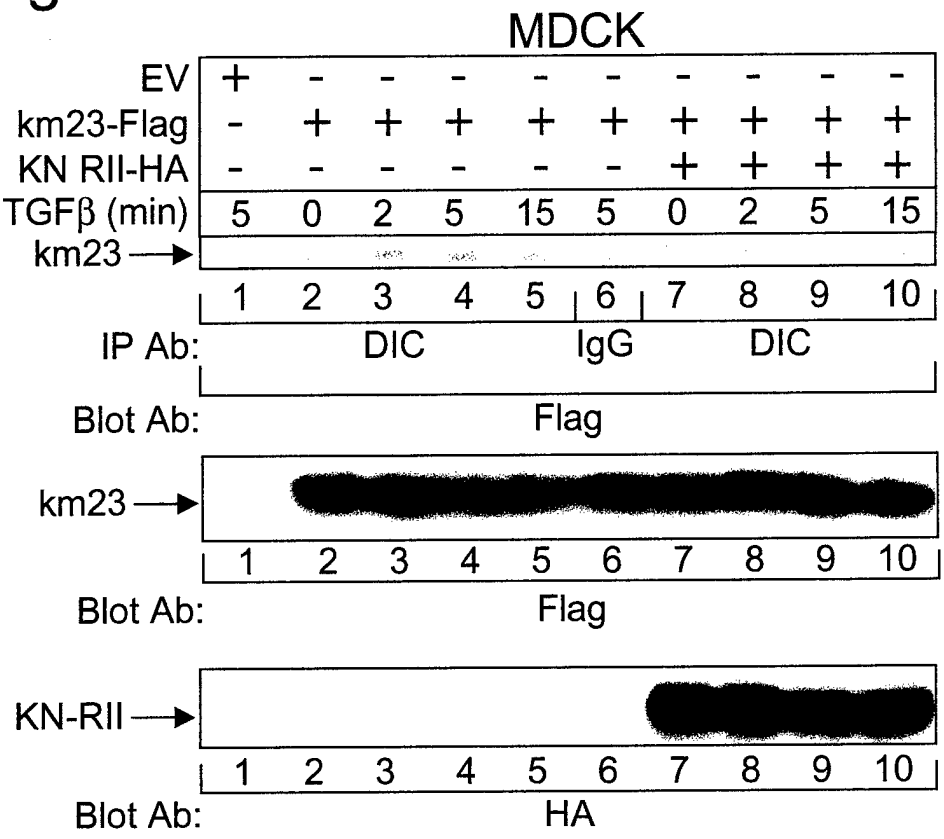
MDCK
EV + - - - - - - - - -
km23-Flag - + + + + + + + + +
KN RII-HA - - - - - - + + + +
TGFβ (min) 5 0 2 5 15 5 0 2 5 15
km23
1 2 3 4 5 6 7 8 9 10
IP Ab: DIC IgG DIC
Blot Ab: Flag
km23
1 2 3 4 5 6 7 8 9 10
Blot Ab: Flag
KN-RII
1 2 3 4 5 6 7 8 9 10
Blot Ab: HA

Table I: Comparison of km23 to some other family members

Homologue	Species	% homology	% identity	% similarity	Amino acids
km23-2	*Homo sapiens*	70	77	91	96
robl	*Drosophila melanogaster*	67	71	81	97
LC7	*Chlamydomonas*	59	55	74	105
bxd-like	*Caenorhabditis elegans*	56	47	76	95
bxd	*Drosophila melanogaster*	42	23	51	101

FASEB Summer Research Conference: The TGFβ Superfamily: Signaling and Development, July 7-12, 2001.

Mulder, K.M. km23: A TGFβ signaling intermediate that interacts with the molecular motor dynein.

The identification of specific cytoplasmic signaling components and pathways which mediate the diverse cellular responses of TGFβ has been difficult. The majority of those identified mediate transcriptional responses. Here we describe the identification of a novel TGFβ receptor-interacting protein, termed km23. This 11-kD cytoplasmic protein is phosphorylated by the TGFβ receptors and synergizes with them to transcriptionally activate the cAMP-responsive element (CRE) in reporter assays. However, of greater interest, km23 belongs to a superfamily of dynein-associated proteins. We have shown, further, that km23 interaction with cytoplasmic dynein is inducible by TGFβ in epithelial cells. Since cytoplasmic dynein is a molecular motor which facilitates the transport of cargo along polarized microtubules (MT's) toward the minus ends, our results suggest that km23, and therefore TGFβ, may regulate specific dynein functions. In addition, we report that a large percentage of laser capture microdissected human tumor specimens display mutations in the km23 sequence. Although MT dynamics are known to be altered in cancer cells and are the target of some cancer chemotherapeutic agents, this is the first demonstration of a link between control of dynein MT dynamics and a natural growth inhibitor such as TGFβ. km23 may be useful as a target against which to design novel therapeutics for cancer.

Molecular Motors Meeting Sept 6-8, 2001 in Woods Hole, MA:

km23: A TGFß Signaling Intermediate that Interacts with Dynein

QIAN TANG, CORY M. STAUB, and KATHLEEN M. MULDER, Department of Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania

TGFß is a natural growth inhibitor for epithelial cells and components of TGFß signaling pathways can function as tumor suppressors. However, the identification of specific cytoplasmic signaling components and pathways which mediate the diverse cellular responses of TGFß has been difficult. The majority of those identified mediate transcriptional responses. Here we describe the identification of a novel component of the TGFß receptor signaling pathway, termed km23. This 11 kD cytoplasmic protein interacts with the TGFß receptors intracellularly and is phosphorylated by them. Stable expression of km23 results in a cellular phenotype which is similar to TGFß treatment of epithelial cells. We demonstrate that km23 interacts with cytoplasmic dynein and that this interaction is rapidly induced by TGFß in epithelial cells. In fact, km23 belongs to a superfamily of dynein-associated proteins that includes the Drosophila roadblock and bithoraxoid genes. Since cytoplasmic dynein is a molecular motor which facilitates the transport of cargo along polarized microtubules toward the minus ends, our results suggest that km23, and therefore TGFß, may regulate specific dynein functions important for epithelial cell polarity. This is the first demonstration of a link between cytoplasmic dynein microtubule motors and a natural growth inhibitor. Further, we have identified amino acid alterations in km23 in human cancer cells within specific functional domains.

Penn State University Dinner and Poster Session, Penn State Univ., University Park, PA, April 2002

Tang, Q., Staub, C.M., Ding, W., Jin, Q., Wang, Z., and **Mulder, K.M.** km23: A novel TGFβ signaling component that is truncated in human cancers.

The identification of specific cytoplasmic signaling components and pathways which mediate the diverse cellular responses of TGFβ has been difficult. The majority of those identified mediate transcriptional responses. Here we describe the identification of a novel TGFβ receptor-interacting protein, termed km23. This 11-kD cytoplasmic protein is phosphorylated by the TGFβ receptors and synergizes with them to transcriptionally activate the cAMP-responsive element (CRE) in reporter assays. However, of greater interest, km23 belongs to a superfamily of dynein-associated proteins. We have shown, further, that km23 interaction with cytoplasmic dynein is inducible by TGFβ in epithelial cells. Since cytoplasmic dynein is a molecular motor which facilitates the transport of cargo along polarized microtubules (MT's) toward the minus ends, our results suggest that km23, and therefore TGFβ, may regulate specific dynein functions. In addition, we report that a large percentage of laser capture microdissected human tumor specimens display mutations in the km23 sequence. Although MT dynamics are known to be altered in cancer cells and are the target of some cancer chemotherapeutic agents, this is the first demonstration of a link between control of dynein MT dynamics and a natural growth inhibitor such as TGFβ. km23 may be useful as a target against which to design novel therapeutics for cancer.

Anti-Cancer Drug Discovery and Development Summit, Princeton, N.J., June 17-19, 2002

Tang, Q., Staub, C.M., Gao, G., Jin, Q., Wang, Z., Ding, W., Aurigemma, R., and **Mulder, K.M.** km23: A novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein.

The phosphorylated, activated cytoplasmic domains of the TGFβ receptors were used as probes to screen an expression library that was prepared from a highly TGFβ-responsive epithelial cell line. The TGFβ receptor-interacting protein km23 was isolated and identified to be a light chain of the motor protein dynein. This 11-kD cytoplasmic protein is associated with the TGFβ receptor complex intracellularly, and it is phosphorylated on serine residues after ligand-receptor engagement. A kinase-deficient form of TGFβ RII prevents km23 from being phosphorylated after TGFβ treatment. Cellular expression of km23 can mimic specific TGFβ responses, namely the activation of JNK, the phosphorylation of c-Jun, and the inhibition of cell growth. TGFβ induces the recruitment of km23 to the intermediate chain of dynein, and the kinase activity of the TGFβ receptors is required for this interaction. km23 specifies the signaling intermediates that are transported along microtubules by dynein after TGFβ receptor activation. This is the first demonstration of a link between cytoplasmic dynein and a natural, growth inhibitory cytokine. Altered forms of km23 have been identified in a large percentage of cancer patient specimens, as well as in a number of human cancer cell lines. Certain alterations occur at "hot spots" in km23, and are not found in normal tissues.

Drug Discovery Technology, IBC Life Sciences, Boston, MA, August 3-8, 2002

km23 -- A Novel TGF-beta-Related Target that is Altered in Human Cancers*

Kathleen M. Mulder, PhD, Professor, Penn State College of Medicine, Dept. of Pharmacology, 500 University Drive, Hershey, PA 17033.

TGFβ is a potent growth inhibitor for epithelial cells. However, as tumor epithelial cells progress, they lose responsiveness to the tumor suppressive action of TGFß. Alterations in signaling pathways play a significant role in the development of this TGFß resistance. We have developed a novel method for the identification of TGFß signaling components, which led to the isolation of a unique TGFβ receptor-interacting protein, termed km23. Cellular expression of km23 results in growth inhibition and activation of known TGFß signaling components. Of additional significance, km23 is a light chain of the motor protein dynein, consistent with a role for km23 in both TGFβ signaling and dynein-mediated transport along microtubules (MTs). This is the first demonstration of a link between TGFβ receptor signaling and the minus-end motor protein dynein. Phosphorylation of km23 after TGFß receptor activation is required for recruitment of km23 to the intermediate chain of dynein. It appears that this interaction of km23 with the dynein intermediate chain is important for specifying the nature of the cargo (ie, TGFß signaling components) that will be transported along the MTs.

We have identified alterations in km23 in TGFß-resistant human cancer cell lines and in 45% of cancer patient specimens. In addition, it appears that, in some cases, the alterations in km23 may occur at an early stage of disease. Our results suggest that various TGFß responses may be lost in human cancers expressing the altered forms of km23, and that restoration or blockade (depending upon the precise TGFß response) of these endpoints is critical to tumor progression. km23 has been validated as an important anti-cancer target in vitro. Further, we are setting up pharmacological screens using km23 as the target to identify novel agents to block the defects caused by the km23 alterations in human tumors. Finally, we have developed a diagnostic assay that we are adapting for use in patients, based upon the km23 mutants we have found in human cancer.