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13. ABSTRACT (Maximum 200 Words)
Transforming growth factor β(TGFβ) is a potent growth inhibitor for epithelial cells, often displaying tumor-suppressor activity. As described in our last Progress Report we have identified km23 as a novel TGFβ receptor-interacting protein. Here we show that km23 is ubiquitously expressed in human tissues and that cell-type specific differences in endogenous km23 is TGFβ-dependent. Further, the kinase activity of both TGFβ receptors appears to play a role in TGFβ-mediated phosphorylation of km23. Subcellular fractionation analyses revealed that km23 is a cytoplasmic protein. In addition, immunofluorescence analyses indicate that km23 is colocalized with the TGFβ signaling componenet Smad2, either in the absence of TGFβ treatment, or during early time periods after its addition. km23 also interacted with Smad2 in glutathione-S-transferase (GST) pull-down and immunoprecipitation (IP)/blot assays. Blockade of km23 using small interfering RNA (siRNA) significantly decreased the ability of TGFβ to inhibit thymidine incorporation. Our findings suggest that km23 is necessary, but not sufficient, for TGFβ-mediated inhibition of DNA synthesis. The data also indicate that km23 is co-localized with TGFβ signaling components shortly after TGFβ stimulation, but prior to translocation of the signaling complexes to the nucleus.

14. SUBJECT TERMS
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INTRODUCTION:

We previously demonstrated that TGFß 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 TGFß responsiveness in terms of TGFß's growth inhibitory effects. Since TGFß receptors are serine/threonine kinases, the mechanisms for activation of Ras by TGFß would differ from those observed for tyrosine kinase receptors. Signaling components with SH2 domains would not be expected to directly bind TGFß receptors. The objective of the current proposal was to identify TGFß signaling components that may regulate the activation of Ras/Mapk/JNK pathways by TGFß. These studies are highly relevant to BC, since TGFß proteins are the major endogenous growth inhibitors secreted by human BCCs. More advanced BCCs lose responsiveness to TGFß's growth inhibitory effects. This is largely mediated by alterations in TGFß signaling pathways, such as those under study in this proposal. Elucidation of the diverse signals that are activated by TGFß 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 the last Progress Report, we indicated that we had identified km23 as a TGFß receptor-interacting protein that was also a light chain of the molecular motor protein dynein. We reported that TGFß stimulated not only the phosphorylation of km23, but also the recruitment of km23 to the dynein intermediate chain (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 appeared to function as a motor receptor, linking the dynein motor to specific signaling complexes contained within intracellular vesicular compartments. We also demonstrated that km23 mediated specific TGFß responses, including JNK activation, c-Jun phosphorylation, and growth inhibition. This work is now published in MBC (*Tang et al, 2002*).

Since the binding of km23 to the DIC after TGFß receptor activation is important for specifying the nature of the vesicular contents (i.e., TGFß signaling components) that will be transported along the microtubules (MTs) (*Jin et al, 2003*), any disruption in km23 would be expected to prevent or alter movement of such cargo along MT's. In this way, alterations in km23 result in a mis-localization of these proteins, with a disruption of TGFβ growth inhibitory signals. Accordingly, sequence alterations at specific regions of km23 in human tumors appear to play a role in tumor development or progression (*Ding et al, 2003; Mulder 2003; Ding and Mulder 2004*). During this project period, we have determined that one out of two human breast carcinomas from patients contained km23 alterations (see Table 1). Thus, we have novel data implicating km23 alterations in the development or progression of breast cancer. In addition, we have found several other types of alterations in km23 in human breast tumor sequences in the NCBI database (see Table 2). This indicates that a larger samples size would permit us to detect additional alterations in km23 in human breast cancers from patients. However, since the funds have been expended, we will not be able to proceed with these studies.

Since the last Progress Report, we have also completed studies to address both Objectives 1 and 2. Regarding Objective 1, we have determined that Src does not directly interact with the TGFß receptors (Fig. 1). However, instead, it appears that Src and/or its family members may be in an adaptor complex with other TGFß signaling intermediates. These other signaling components, which include km23, appear to function as adaptors to link the receptors to the direct activators of Ras during endocytosis and early trafficking events. As an example of one of these, we have shown that expression of km23 can induce the interaction of Src with the adaptor kinase Grk2 (Fig. 2). Grk2 has been shown to regulate the Ras/Mapk pathways through its association with Src, the two proteins often linked by an additional adaptor protein (i.e., *Wan et al, 2003*). In addition, previous results have indicated that another DLC, Tctex-1, can interact with the Src family member fyn (*Campbell et al, 1998*). Thus, it seems likely that the DLC we have identified, km23, might also interact with a Src family member. This may occur indirectly through the interaction of Src with Grk2 (Fig. 2), or directly. Future studies will be required to address this possibility. However, our results do suggest that Src may be involved in TGFß activation of the Ras/Mapk pathways, although we did not detect a direct interaction between the receptors and Src. The key is that adaptor complexes containing components such as Grk2, and km23 are required.

With regard to Objective 2, we could not detect a specific, TGFß-inducible change in the guanine nucleotide exchange factors (GEFs) Sos or GRF. However, there are several other GEFs that have been identified since we have submitted this application. It may well be that one of these GEFs plays a more important role in the activation of Ras or Ras family members by TGFß. For example, it has been shown that the TGFß activation of the Ras superfamily member RhoA can involve the GEF NET1 (*Shen et al, 2001*). In addition, a new GEF of the Vav family has been identified (Vav3), and shown to be regulated by TGFß (*Trenkle et al, 2000*). Since the funds have been expended, studies involving these other GEFs are beyond the scope of the current project. However, we feel that the findings we obtained from Objective 1, regarding the important role of these adaptor complexes in Ras activation, as well as the results we obtained regarding the role of km23 alterations in breast cancer development or progression, are highly significant and important outcomes that resulted from the current award.

KEY RESEARCH ACCOMPLISHMENTS:

- We have extensive functional data to demonstrate that km23 is an important component of TGFß 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 TGFß signaling components to their appropriate destinations in the cell.
- Expression of km23 induces the interaction of Src with Grk2, forming an adaptor complex critical for the activation of components such as Ras and Mapks
- km23 alterations occur in human breast cancer at specific sites that are critical

for the cellular functions of km23. The alterations appear to occur at a high frequency in human breast cancer.

- We have validated km23 as a critical anti-cancer therapeutic target using siRNA approaches.

REPORTABLE OUTCOMES:

Tang Q, Staub C, Gao G, Jin Q, Wang Z, Ding W, Aurigemma R, Mulder KM. A novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein. Mol. Biol. Cell 13:4484-4496, 2002.

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Mulder KM. A new target for the development of anti-cancer diagnostics and therapeutics. Amer. Clin. Lab., in preparation, 2003.

Ding W, Charboneau L, Espina V, Liotta LA, Mulder KM. A TGFβ receptor-interacting protein frequently mutated in epithelial ovarian cancer. Cancer Res., in preparation, 2003.

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Mulder KM. km23: A novel target for the development of anti-cancer therapeutics and diagnostics. Protein-Protein Interaction, Newark, NJ, November 4-6, 2002.

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Invited Speaker, Presentation at the University of Texas, San Antonio, TX, 2003.

Invited Speaker, Presentation at the AACR Annual Meeting, Toronto, CA, 2003.

Press Release – *Penn State Newswire* – Study finds new target for development of anti-cancer drugs, September 4, 2002.

Press Release – *Penn State College of Medicine News Release* – Study finds new target for development of anti-cancer drugs: Protein found effective in nearly half of human cancer tissues, September 4, 2002.

Press Release – *Hummelstown Sun* – Anti-Cancer Target, September 11, 2002.

Press Release – *Espicom Business Intelligence* – New target for anticancer therapies identified, September 12, 2002.

Press Release – *Central Penn Business Journal* – Help for Cancer, September 27, 2002.

Funding Received – Based on Work Supported by this Award: Department of Defense, Idea Award, "*Development of km23-based Diagnostics and Therapeutics*", Principal Investigator, Kathleen M. Mulder, $526,875, 04/15/03-04/14/06.

Funding Received – Based on Work Supported by this Award: National Cancer Institute, 1 R01 CA100239-01, "*Role of km23 in Ovarian Cancer*", Principal Investigator, Kathleen M. Mulder, $1,250,000, 04/01/03-03/31/08.

Funding Applied for based on work supported by this award: National Institutes of Health Structural Studies Supplement, "*High-Resolution NMR Structure of km23 Proteins*", Principal Investigator, Kathleen M. Mulder, $40,000, 11/01/03-10/31/04.

Funding Applied for based on work supported by this award: Equipment Supplement, "*Tumor-Host Interactions*", Principal Investigator, Kathleen M. Mulder, $500,000, 09/01/03-08/31/04.

CONCLUSIONS:

1. The TGFβ receptors do not appear to directly interact with Src (Fig. 1).

2. km23 can function as a motor receptor to link TGFβ signaling components (i.e., Src, GEF, Ras, JNKs, Smads) to the motor machinery for their movement along microtubules to their appropriate sites of action (Tang et al, 2002; Mulder,2003; Ding and Mulder, 2004).

3. km23 is co-localized with Smad2 at early time points after TGFβ addition, prior to Smad translocation to the nucleus (Jin et al, 2003).

4. km23 can directly interact with the TGFβ signaling component Smad2 by two different methods (Jin et al, 2003).

5. Blockade of km23 expression using small interfering RNA (siRNA) results in a reduction in the ability of TGFβ to inhibit cell growth and to induce fibronectin expression. Both of these TGFβ responses partially require Ras and Mapks such as JNK (Jin et al, 2003).

6. Alterations in km23 in human breast cancer have been identified in one out of two patients thus far.

7. Alterations in km23 can reduce its ability to interact with the dynein motor complex, thereby limiting the ability of TGFβ signaling components (i.e., Src, GEFs, Ras, JNKs, Smads) to function in the appropriate manner (Jin et al, 2003; Ding et al, 2003).

8. km23 is a ubiquitously expressed, cytoplasmic adaptor protein, which when expressed, can stimulate the interaction between Src and Grk2, thereby facilitating the activation of the Ras/Mapk pathways (Fig. 2).

SO WHAT SECTION:
km23 as a "Drugable" Target: Several Different Therapeutic 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 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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APPENDICES:

Table 1

Table 2

Fig 1

Fig 2

Publication -- Tang Q, Staub CM, Gao G, Jin Q, Wang Z, Ding W, Aurigemma RE, Mulder KM. A novel transforming growth factor-β receptor-interacting protein that is also a light chain of the motor protein dynein. Molecular Biology of the Cell 13:4484-4496, 2002.

Publication -- Jin Q, Staub CM, Tang Q, Ding W, Gao G, Mulder KM. Role of km23 co-localized and interacted with Smad2 in TGFβ-mediated effects. J. Cell Sci., submitted 2003.

Curriculum Vitae -- Kathleen M. Mulder, Ph.D.

Table 1. Detection of Mutations in Breast Cancer Tissues Using Laser Capture Microdissection & Nested-RT-PCR

Case No.	Age (yrs.)	Histology	Stage/Grade	Mutations
1	48	Infiltrating ductal carcinoma	III/ 3	-
2	48	Infiltrating ductal carcinoma	III/ 3	Missing exon 3

Table 2. km23 alterations in human breast tumors in NCBI database

Human EST-accession No.	Tumor Source	Nucleotide alterations	Amino acid residue alterations	Exon
AI910285	Breast	AAC→ACC GAC→AAC	N20T D59N	2nd 3rd
BI861952	Breast adenocarcinoma cell line	56th deletion GGA→GAA	G19E 87aa	2nd
BG030777	Breast	56th deletion GGA→GAA	G19E 87aa	2nd
H45788	Breast	GTG→GTC CGT→GGT 237th deletion	V57V R58G 87aa	3rd
R50440	Breast	GTG→GTC CGT→GGT GAC→GGA	V57V R58G N59G	3rd
BG033914	Mammary adenocarcinoma cell line	56th deletion GGA→GAA	G19E 22aa	2nd
BE077366	Breast	37th, 38th and 47th deletion CAG→CAA AGC→CCA	 S12P 20aa	2nd
BF744548	Breast	ACC→ACG ACC→ATC	T38T T39I	3rd
BF744651	Breast	ACC→ACG ACC→ATC	T38T T39I	3rd

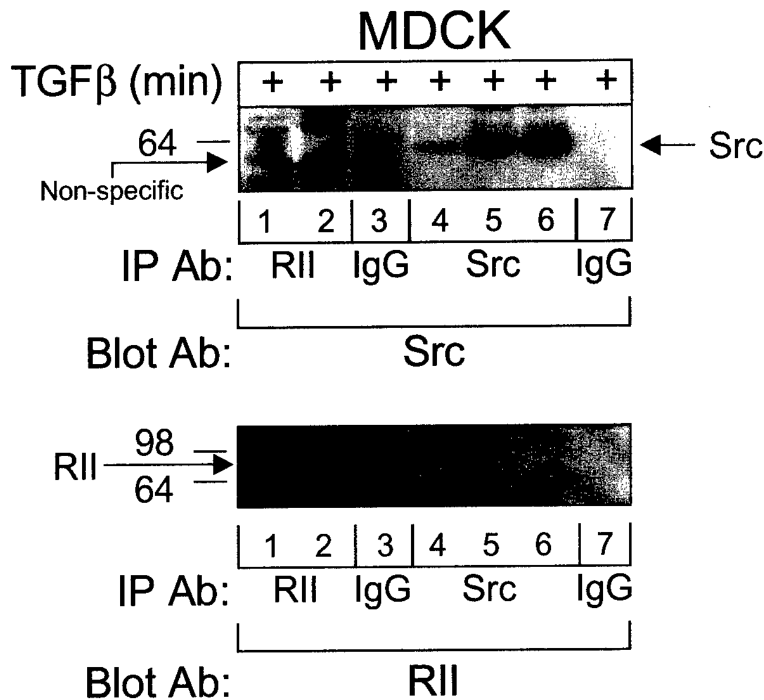

Fig. 1: RII does not directly interact with Src. MDCK cells were treated with TGFβ (5 ng/ml) for the indicated times. Lysates were then immunoprecipitated (IP d) with RII Ab (lanes 1 and 2), Src Ab (lanes 4-6), or IgG (lanes 3 and 7, negative control), analyzed by SDS-PAGE, and transferred to PVDF membrane. Top panel: IP RII/ blot Src (lanes 1 and 2) indicates that RII does not interact with Src. However, IP Src/blot Src indicates Src band at correct position (lanes 4-6). IgG controls had no specific bands (lanes 3 and 7). Bottom panel: IP Src/blot RII (lanes 4-6) indicates that RII does not interact with Src in the opposite direction. However, IP RII/blot RII indicates RII band at correct position (lanes 1 and 2). IgG controls had no specific bands (lanes 3 and 7).

Fig. 2

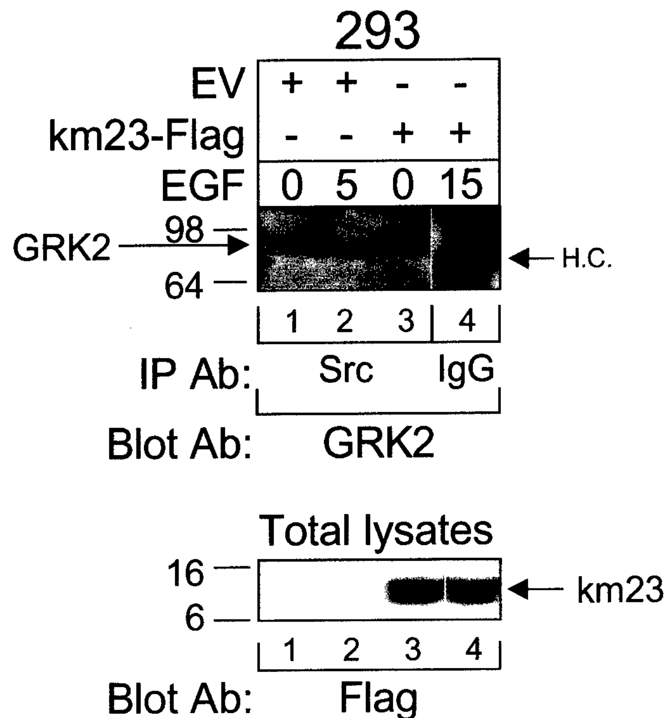

Fig. 2: km23 expression induces an interaction between Src and the adaptor kinase GRK2. 293 cells were transfected with EV of km23-Flag and treated with EGF (50 ng/ml) for the indicated times. Lysates were then immunoprecipitated (IP d) with Src Ab (lanes 1-3), or IgG (lane 4, negative control), analyzed by SDS-PAGE, and transferred to PVDF membrane. Top panel: IP Src/blot GRK2 indicates that treatment with EGF for 5 min stimulates an interaction of GRK2 with Src (lane 2). Transfection of km23 also stimulates the interaction of Src with GRK2 in the absence of EGF stimulation (lane 3). Bottom panel: Equal expression of transfected km23-Flag in the relevant lanes is demonstrated by Western blotting using a Flag Ab (lanes 3 and 4).

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A Novel Transforming Growth Factor-β Receptor-interacting Protein That Is Also a Light Chain of the Motor Protein Dynein

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The phosphorylated, activated cytoplasmic domains of the transforming growth factor-β (TGFβ) receptors were used as probes to screen an expression library that was prepared from a highly TGFβ-responsive intestinal epithelial cell line. One of the TGFβ receptor-interacting proteins isolated was identified to be the mammalian homologue of the LC7 family (mLC7) of dynein light chains (DLCs). This 11-kDa cytoplasmic protein interacts with the TGFβ receptor complex intracellularly and is phosphorylated on serine residues after ligand-receptor engagement. Forced expression of mLC7-1 induces specific TGFβ responses, including an activation of Jun N-terminal kinase (JNK), a phosphorylation of c-Jun, and an inhibition of cell growth. Furthermore, TGFβ induces the recruitment of mLC7-1 to the intermediate chain of dynein. A kinase-deficient form of TGFβ RII prevents both mLC7-1 phosphorylation and interaction with the dynein intermediate chain (DIC). This is the first demonstration of a link between cytoplasmic dynein and a natural growth inhibitory cytokine. Furthermore, our results suggest that TGFβ pathway components may use a motor protein light chain as a receptor for the recruitment and transport of specific cargo along microtublules.

INTRODUCTION

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

TGFβ binds to two types of transmembrane serine/threonine kinase receptors (RI and RII) in a heterotetrameric complex, to activate downstream components (Roberts, 1998; Massague *et al.*, 2000; Sporn and Vilcek, 2000; Yue and Mulder, 2001). The Smad family of signaling intermediates plays an important role in mediating TGFβ responses (Attisano and Wrana, 2000; ten Dijke *et al.*, 2000; Yue and Mulder, 2001). 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 (Hartsough and Mulder, 1995; Frey and Mulder, 1997; Mulder, 2000; Sporn and Vilcek, 2000; Yue and Mulder, 2001). 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). Furthermore, various Smad-interacting proteins have also been identified, including SARA and Dab2, which interact with both Smads and the TGFβ receptors (Tsukazaki *et al.*, 1998; Hocevar *et al.*, 2001; Yue and Mulder, 2001).

Despite advances in our understanding of the mechanisms by which the Smad and Ras/Mapk cascades mediate some TGFβ effects, these pathways seem to regulate primarily transcriptional events (Hocevar *et al.*, 1999; Hu *et al.*, 1999; Sporn and Vilcek, 2000; Yue and Mulder, 2000a, 2001). 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.

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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 (DICs) 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 dynein light chains (DLCs) also seem 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 light chains (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 Jun N-terminal kinase (JNK)-interacting proteins (JIPs) are thought to serve as scaffolding proteins for the JNK signaling pathway (Davis, 2000). These JIP 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). Furthermore, 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). Because 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 that ultimately determine the collective spatial organization of the signaling pathways within the cell.

Herein, we describe a mammalian TGFβ receptor-interacting protein, termed mLC7-1, which is also a DLC. TGFβ stimulates not only the phosphorylation of mLC7-1, but also the recruitment of mLC7-1 to the DIC. Kinase-active TGFβ receptors are required for mLC7-1 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, mLC7-1 seems to function as a motor receptor, linking the dynein motor to specific cargo. We also demonstrate that mLC7-1 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 and mouse IgG were from Sigma-Aldrich (St. Louis, MO). The anti-DIC monoclonal antibody was from Chemicon (Temecula, CA). The anti-V5 antibody (R960 25) was obtained from Invitrogen (Carlsbad, CA) and the anti-hemagglutinin (HA) antibody (1-583-816) was from Roche Applied Science (Indianapolis, IN). The TGFβ RII antibody (SC-220-G or -R), the phospho-c-Jun antibody (KM-1, SC-822), and rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). 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 PerkinElmer Life Sciences (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 American Type Culture Collection (Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum. 293T cells were obtained from T.-W. Wong (Bristol-Myers Squibb, Princeton, NJ) and were maintained as for COS-1 cells. Madin-Darby canine kidney (MDCK) cells (CCL-34) were grown in minimal essential medium-α, supplemented with 10% fetal bovine serum. Cultures were routinely screened for mycoplasma by using Hoechst staining.

Cloning of TGFβ Receptor Targets

Construction of TGFβ Receptor Expression Plasmids. The intracellular domains of TGFβ RII and RI were polymerase chain reaction (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) *Escherichia coli* strains were transformed separately with each of the TGFβ receptor-containing vectors or the corresponding empty vectors (EVs), followed by selection on kanamycin and ampicillin. Expression was induced with isopropyl β-D-thiogalactoside and verified by Western blotting using tag antibodies 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 with 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) by using the Superscript Choice System for cDNA synthesis (Invitrogen). Double-stranded cDNA ligated to *Eco*RI adaptors was size selected, and relevant fractions were pooled and ligated into the TriplEx expression vector (CLONTECH, Palo Alto, CA). 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 base pairs was originally isolated and sequenced (*k*athleen *m*ulder #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). On comparison of our sequence with human expressed sequence tags in the database, the full-length hkm23 gene was obtained. The nucleotide sequences for human (accession no. AY026513) and rat (AY026512) km23 are available at http://www.ncbi.nlm.nih.gov:80/entrez. The protein identifications are AAK18712 and AAK18711, respectively.

Transient Transfections, ^{125}I-TGFβ Cross-Linking, Immunoprecipitation/blot, Westerns, and In Vivo Phosphorylation Assays

These assays were performed essentially as described previously (Hocevar *et al.*, 1999; Yue *et al.*, 1999a; Yue and Mulder, 2000a). To prepare RI-V5, the Alk-5 cDNA was digested with *Not*I and *Xho*I restriction enzymes, followed by subcloning into pcDNA3.1/V5-His (V-810-20; Invitrogen). To prepare km23-FLAG, the coding region of rat or human km23 was PCR amplified with additional suitable flanking restriction enzyme sites for *Bgl*II (5′) and *Sal*I (3′) and inserted into pCMV5-FLAG (Sigma-Aldrich) after digestion with *Bgl*II and *Sal*I restriction enzymes. 293T, MDCK, COS-1, or Mv1Lu cells were transiently transfected using either LipofectAMINE Plus (catalog no. 10964-013; Invitrogen) or LipofectAMINE 2000 (catalog no. 11668-027; Invitrogen), 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/mLC7-1 protein was immunoprecipitated with anti-FLAG, separated by SDS-PAGE, transferred, and visualized by autoradiography. The membrane containing ^{32}P-labeled km23/mLC7-1 was excised, and phosphoamino acid analysis was performed as described previously (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 EV was transfected into Mv1Lu cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection the cells were split at a ratio of 1:5. After another 24 h, 1000 μg/ml G418 was added for a selection period of 11 d, at which time surviving colonies were pooled and maintained in the presence of 1000 μg/ml G418. Expression of km23/mLC7-1 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

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

Growth Assays

The TGFβ responsiveness of cells was verified by [^{3}H]thymidine incorporation assays, performed as described previously (Hartsough and Mulder, 1995). For Figure 5, pools of Mv1Lu cells stably transfected with km23-FLAG or EV were plated at 2×10^3 cells per 96-well dish and were analyzed at several days thereafter using crystal violet (EMScience #1011; Fisher Scientific, Pittsburgh, PA), according to the assay protocol at http://www-ufk.med.uni-rostock.de/lablinks/protocols/e_protocols/cvassay.htm.

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 *Bam*HI (5′) and *Xho*I (3′), and inserted into pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ) after digestion with *Bam*HI and *Xho*I restriction enzymes. The bacterially expressed rkm23-GST was isolated according to the manufacturer's instructions (Amersham Biosciences) and used in the GST pull-downs by standard methods (Current Protocols in Molecular Biology). 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 Figure 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 before screening, as described in MATERIALS AND METHODS. Figure 1B 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. Autophosphorylation of both receptors is clearly visible, as described previously (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). On 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 Figure 1A.

Several positive clones were isolated as described in MATERIALS AND METHODS. Among the clones isolated, km23 was pursued initially because 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 Dpp stimulated transcription of Ubx and that the Ubx protein was necessary

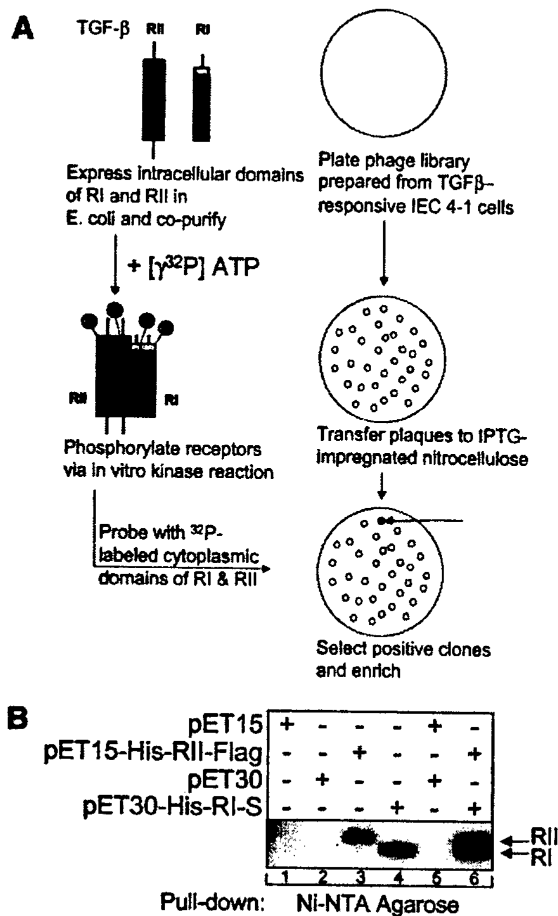

Figure 1. Identification of a novel TGFβ receptor-interacting protein. (A) Method for identifying TGFβ receptor-interacting proteins. The cytoplasmic domains of both receptors were expressed, sequentially isolated, kinase-activated in vitro, and used as probes to screen an expression library. (B) In 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^{2+}-NTA agarose beads before performing an in vitro kinase assay. Bacterial lysates were prepared after expression of either EVs (pET15, pET30, and pET15/pET30), the intracellular domains of RII or RI alone (pET15-His-RII-FLAG and pET30-His-RI-S), or together (pET15-RII-FLAG/pET30-RI-S).

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, because we used 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. Furthermore, 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 before library screening would be expected to prevent binding of Smads to the receptor complex.

The novel TGFβ signaling intermediate we identified, initially termed km23, is a 96-amino acid protein encoded by a 291-base pair open reading frame. It is a ubiquitously expressed, cytoplasmic protein with a predicted molecular mass of 10.667 kDa and a calculated molecular mass of 11 kDa 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 National Center for Biotechnology Information database indicated that km23 is the mammalian homologue of the *Drosophila* protein roadblock (robl), which belongs to the LC7 family of *Chlamydomonas* DLCs (*chl*LC7) (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 percentage of 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 mammalian member of the LC7 family in the National Center for Biotechnology Information database. This form of mLC7 (designated mLC7-2 in Table 1; AA446298) displays 70% homology with the km23/mLC7-1 form we have identified. In contrast, a total of five LC7/robl-like genes have been identified in *Drosophila*, yet *Caenorhabditis elegans* seems to have only a single km23/robl-like gene (National Center for Biotechnology Information database T24H10.6; Bowman *et al.*, 1999). There does not seem 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 (Bowman *et al.*, 1999; King, 2000; Makokha *et al.*, 2002). 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 mLC7-1, respectively. It is conceivable that mLC7-1 also mediates motor complex assembly and connection to the transported cellular cargo.

Because we had identified mLC7-1 by its ability to interact with the cytoplasmic regions of the TGFβ receptors, it was of interest to verify whether mLC7-1 was present in association with the TGFβ receptors intracellularly. Accordingly, affinity cross-linking experiments were performed using ^{125}I-TGFβ (Yue *et al.*, 1999a). Figure 2A indicates that both RI and

Table 1. Comparison of km23/mLC7-1 to some other family members

Homologue	Species	% Homology	% Identity	% Similarity	Amino acids
mLC7-2	*Homo sapiens*	70	77	91	96
robl	*Drosophila melanogaster*	67	71	81	97
ch/LC7	*Chlamydomonas*	59	55	74	105
bxd-like	*C. elegans*	56	47	76	95
bxd	*D. melanogaster*	42	23	51	101

RII are present in km23/mLC7-1 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 (Figure 2A). Furthermore, no receptors were detectable in FLAG IPs after expression of both receptors without km23/mLC7-1 (our unpublished data). The control blots in the lower panels demonstrate that the appropriate constructs were expressed to similar levels. Thus, these results suggest that mLC7-1 is associated with the activated receptor complex.

To determine whether the interaction between the receptors and mLC7-1 occurred rapidly after ligand stimulation,

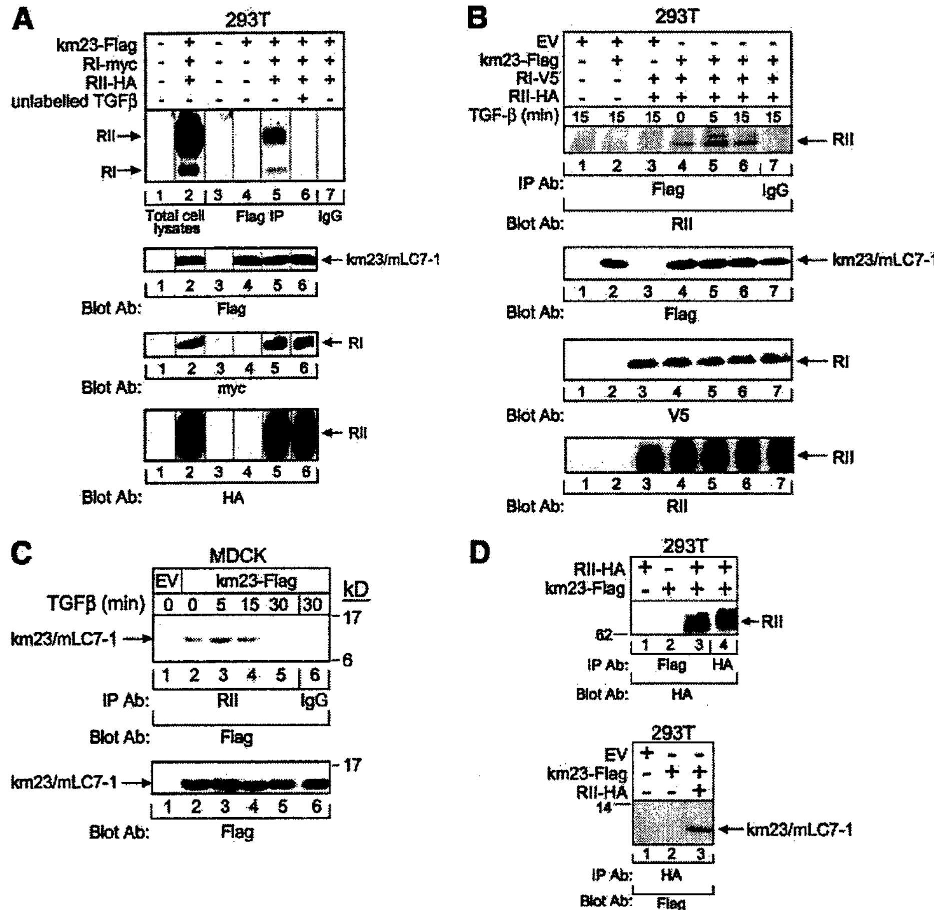

Figure 2. Verification of TGFβ receptor interaction with mLC7-1. (A) RI and RII TGFβ receptors are present in mLC7-1 immunocomplexes. 293T cells were transiently transfected with km23-FLAG, RI-myc, RII-HA, and/or EVs, and affinity labeling was performed. After the 4-h ^{125}I-TGFβ labeling period (4°C), the cross-linking agent disuccinimidyl suberate was added for an additional 15 min. Top, total cell lysates (lanes 1 and 2) or lysates immunoprecipitated with an anti-FLAG M2 antibody (lanes 3–6) or with IgG (lane 7, control) were visualized by SDS-PAGE and autoradiography. No bands were visible in FLAG IPs after transfection of only RI and RII (our unpublished data). Bottom, Western blots for FLAG, myc, and HA demonstrate expression of the relevant constructs (lanes 2 and 4–6 for km23/mLC7-1; lanes 2, 5, and 6 for RI and RII). (B) Interaction between mLC7-1 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 EVs, followed by IP/blot analyses with FLAG as the IP antibody and an RII polyclonal antibody as the blotting antibody (top). Cells were incubated in serum-free medium for 60 min before addition of TGFβ for the indicated times. Bottom, controls for expression and loading of km23/mLC7-1 (FLAG blot), RI (V5 blot), and RII (RII blot). (C) TGFβ induces a rapid association of mLC7-1 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 Figure 2B. (D) mLC7-1 interacts with RII via IP/blot analyses in 293T cells. Cells were transiently transfected with km23-FLAG and RII-HA as indicated. Top, cell lysates were immunoprecipitated with anti-FLAG or HA and blotted with an HA antibody. 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, lysates were immunoprecipitated with anti-HA and blotted with anti-FLAG. The presence of km23/mLC7-1 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.

we performed IP/blot analyses in the presence and absence of TGFβ. Coexpression 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 Figure 2B (lane 4). However, Figure 2B demonstrates not only that km23/mLC7-1 interacts with RII, but also that TGFβ induces this interaction within 5 min of TGFβ addition (lanes 4–6, top). The appearance of the RII band with slightly slower mobility (lanes 5 and 6) suggests that TGFβ also induced the interaction of km23/mLC7-1 with a differentially phosphorylated/modified form of RII. No specific band was apparent after expression of only km23/mLC7-1 or the receptors alone (lanes 2 and 3, top). 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, because an RII antibody was used as the blotting antibody in these experiments, our data indicate that mLC7-1 does associate with RII.

To ensure that the interaction was not the result of overexpression 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 24 h of 10 ng/ml TGFβ addition (our unpublished data). As seen in Figure 2C, TGFβ induced a rapid interaction between km23/mLC7-1 and endogenous TGFβ receptors. The kinetics were similar to those observed for the 293T cells. Thus, TGFβ induces the interaction of mLC7-1 with the TGFβ receptors in two different cell types, and without overexpression of the receptors.

The results in Figure 2, A–C, are consistent with mLC7-1 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). To determine whether both receptors were required for mLC7-1 interaction with the receptor complex, we performed IP/blot analyses after expression of only RII in 293T cells. Figure 2D depicts the interaction of km23/mLC7-1 with RII, either using FLAG as the IP antibody, and the HA antibody as the blotting antibody (top), or by performing the analyses in the reverse direction (bottom). As indicated by the results in either direction, it seems that km23/mLC7-1 can interact with RII alone. In contrast, upon expression of RI alone, no detectable interaction of RI with mLC7-1 was observed (our unpublished data). However, because 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 Figure 2D. Thus, mLC7-1 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 mLC7-1.

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 mLC7-1 is a component of a TGFβ signaling cascade, it is conceivable that the TGFβ receptors could phosphorylate mLC7-1 as a mechanism for activation. To determine whether mLC7-1 was phosphorylated by the TGFβ receptors, we performed in vivo phosphorylation assays (Yue and Mulder, 1999a;b) after transient expression of km23/mLC7-1 and both receptors, each being detectable by distinct tag antibodies, as indicated in Figure 3A. From the results in the top panel, it is clear that the TGFβ receptor complex resulted in phosphorylation of km23/mLC7-1 (lane 5). Expression of km23/mLC7-1 alone did not result in a band at the km23/mLC7-1 position (lane 2), indicating that mLC7-1 is not constitutively phosphorylated when expressed in these cells. The IgG and FLAG binding peptide control lanes (7 and 8) indicate that the band noted is specific for km23/mLC7-1.

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 mLC7-1 is a substrate for the TGFβ receptor kinase activity, phosphorylation of mLC7-1 on serine residues might be expected. To examine whether this was the case, we performed phosphoamino acid analysis of phosphorylated mLC7-1 obtained after coexpression of km23/mLC7-1 and both TGFβ receptors in COS-1 cells, similar to the analyses for Figure 3A in 293T cells. Figure 3B indicates that km23/mLC7-1 is phosphorylated primarily on serine residues in response to TGFβ receptor activation. These findings are consistent with mLC7-1 functioning as a substrate for the kinase activity of the TGFβ receptors. Conversely, mLC7-1 does not seem to stimulate the kinase activity of the receptors (our unpublished data).

Based upon the current model for TGFβ receptor activation, RII mediates the phosphorylation of RI and the activation of downstream TGFβ components and responses (Roberts, 1998; Massague *et al.*, 2000; Sporn and Vilcek, 2000; Yue and Mulder, 2001). Accordingly, if TGFβ activation of the receptor complex is required for phosphorylation of mLC7-1, expression of a kinase-deficient version of RII (KN-RII) would be expected to block mLC7-1 phosphorylation. Figure 3C (top) depicts the results of in vivo phosphorylation of km23/mLC7-1 after coexpression of either wild-type RII (lanes 2 and 3) or KN-RII (lane 4) with wild-type RI. As shown previously, km23/mLC7-1 alone was not constitutively phosphorylated (lane 1), and expression of both TGFβ receptors with km23/mLC7-1 resulted in km23/mLC7-1 phosphorylation (lane 2). Figure 3C indicates, furthermore, that TGFβ treatment for 15 min enhanced km23/mLC7-1 phosphorylation (lane 3). This phosphorylation of km23/mLC7-1 was completely blocked upon expression of the KN-RII (lane 4), thereby demonstrating that the kinase activity of RII is required for mLC7-1 phosphorylation.

To determine whether RI was also required for mLC7-1 phosphorylation, we performed similar in vivo phosphorylation experiments using various kinase-active and kinase-deficient versions of RI. Figure 3D confirmed that expression of both receptors with km23/mLC7-1 induced km23/mLC7-1 phosphorylation (lane 4) and that KN-RII blocked this phosphorylation (lane 7). However, in addition, this figure indicates that km23/mLC7-1 is still phosphorylated after coexpression 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). Because 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 mLC7-1, although it is present in mLC7-1 immunocomplexes with RII by affinity-labeling experiments (Figure 2A). Lane 11 in Figure 3D dem-

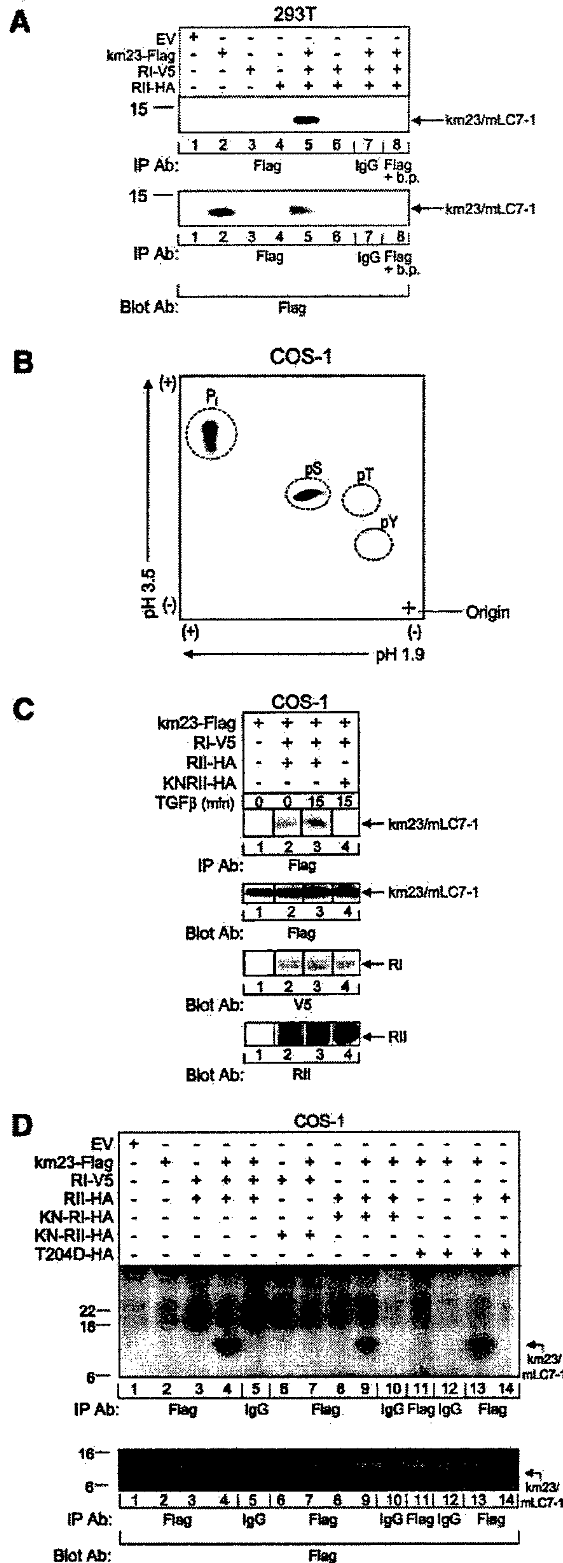

Figure 3. A functional RII TGFβ receptor is required for mLC7-1 phosphorylation. (A) mLC7-1 is phosphorylated upon activation of TGFβ receptors. 293T cells were transiently transfected with RI-V5, RII-HA, and either EV or km23-FLAG. Forty-eight hours after transfection, cells were labeled for 3 h with [$^{32}P_i$], lysed, and immonoprecipitated with an anti-FLAG antibody. Top, in vivo phosphorylation of km23/mLC7-1 was visualized by SDS-PAGE and autoradiography. A blocking peptide (b.p.) for the FLAG antibody was added in lane 8. Bottom, 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 mLC7-1 primarily on serine residues. km23/mLC7-1 was phosphorylated in vivo as for A, and phosphoamino acid analysis was performed. ^{32}P-labeled km23/mLC7-1 was excised from the polyvinylidene difluoride 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 by using Hunter Thin Layer Peptide Mapping Electrophoresis System (CBS Scientific, Del Mar, CA), 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 mLC7-1 when a kinase-deficient RII is expressed with RI. COS-1 cells were transiently transfected as in A, except that the KNRII was coexpressed 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 and 4). Lysates were analyzed as for A. Top, in vivo phosphorylation of km23/mLC7-1. Bottom, expression of transfected km23/mLC7-1, RI, and RII was confirmed by Western blot analysis with FLAG, V5, and a polyclonal RII antibody, respectively, as indicated. Results are representative of two experiments. (D) Kinase activity of RI does not seem to be required for phosphorylation of mLC7-1. In vivo phosphorylation assay and transfection of COS-1 cells was performed as for Figure 3C, except that no TGFβ was added and different receptor mutants were evaluated as indicated. Results are representative of two experiments.

onstrates no detectable phosphorylation of km23/mLC7-1 after expression of a constitutively active RI mutant (T204D). However, when wild-type RII was coexpressed with this mutant, km23/mLC7-1 phosphorylation was observed (lane 13), presumably due to the kinase activity of RII. Collectively, the data suggest that although both receptors may be present in a complex with mLC7-1, the RII kinase is required for mLC7-1 phosphorylation. The data do not rule out the possibility that another kinase is also present in the complex.

The method of isolation of mLC7-1, as well as the results in Figures 2 and 3, suggest that mLC7-1 may function as a signaling intermediate for TGFβ. Thus, it was of interest to examine whether mLC7-1 could mediate any of the known TGFβ signaling events. We have previously shown that TGFβ rapidly activates the JNK family of Mapks (Frey and Mulder, 1997). Furthermore, JNK activation by TGFβ is required for such TGFβ responses as production of TGFβ_1 and induction of fibronectin expression (Hocevar et al., 1999; Yue and Mulder, 2000a). 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 (Derynck *et al.*, 2001; Yue and Mulder, 2001).

To determine the effect of forced expression of wild-type mLC7-1 on JNK activation, we stably expressed a FLAG-tagged version of km23/mLC7-1 in mink lung epithelial cells (Figure 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 Figure 4A, in the EV-expressing cells, TGFβ began activating JNK within 10 min of TGFβ addition; JNK activity increased further by 30 min posttreatment (top, left). These kinetics are similar to those obtained for other cell types (Frey and Mulder, 1997). In contrast, when km23/mLC7-1 was stably expressed in these cells, JNK was superactivated in the absence of TGFβ (top, right). JNK activity was ~15 times greater in the km23/mLC7-1-expressing cells than in the EV-expressing cells during the 2- to 10-min period after TGFβ addition. By 30 min post-TGFβ treatment, JNK activation levels were more similar between the km23/mLC7-1– and EV-expressing cells. These findings suggest that mLC7-1 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). To determine whether this downstream effector of JNK could also be phosphorylated by stable expression of mLC7-1, we performed immunoblot analysis at various times after TGFβ treatment using a phospho-c-Jun–specific antibody. This antibody 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/mLC7-1 that were used for Figure 4A. The results in Figure 4B demonstrate that forced expression of km23/mLC7-1 induced the phosphorylation of c-Jun in the absence of TGFβ (comparing left and right, top). As for JNK activity, c-Jun phosphorylation was superactivated in the absence of TGFβ. Figure 4B, bottom, indicates that c-Jun phosphorylation levels were approximately 10 times greater in the km23/mLC7-1–expressing cells than in the EV-expressing cells. Collectively, the results in Figure 4 suggest that mLC7-1's cellular effects on JNK and c-Jun activation are downstream of TGFβ receptor activation.

In addition, our findings in Figure 4 suggest that overexpression of mLC7-1 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, because one of TGFβ's most prominent biological effects is growth inhibition of epithelial cells, we examined whether overexpression of mLC7-1 in the Mv1Lu-transfected pools could result in growth inhibition in the absence of TGFβ. The results in Figure 5 indicate that, relative to EV-transfected pools, the km23/mLC7-1–expressing cells were growth inhibited by ~50%. These data support the contention that overexpression of mLC7-1 may mediate some TGFβ responses in a constitutive manner. Alternatively, with regard to the growth inhibitory effect observed, the overexpression of mLC7-1 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 above, mLC7-1 is the mammalian homologue of the *chl*LC7 and *Drosophila* robl proteins, which are DLCs (Bowman *et al.*, 1999). Accordingly, it was of interest to determine whether mLC7-1 could interact with the DIC as *chl*LC7/robl does. As shown in Figure 6A, we performed GST pull-down assays after expressing and purifying GST-km23. An anti-DIC antibody was used as the blotting antibody to detect the presence of dynein in the GST-km23 complexes. This anti-

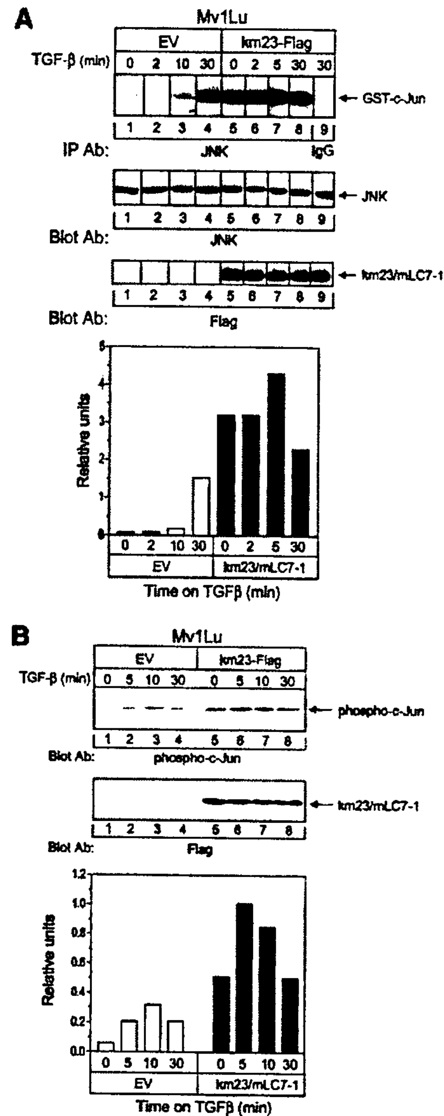

Figure 4. mLC7-1 expression can induce JNK and result in phosphorylation of the downstream target c-Jun. (A) Stable expression of mLC7-1 results in activation of JNK in the absence of TGFβ. Top, Mv1Lu cell pools, stably transfected with either empty vector (lanes 1–4) or km23-FLAG (lanes 5–8), were incubated in serum-free medium for 30 min before addition of 10 ng/ml TGFβ for the indicated times. Cell lysates were immunoprecipitated with anti-JNK (C-17; Santa Cruz Biotechnology) 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, equal JNK and km23/mLC7-1 expression was confirmed by Western blotting. Bottom, plot of densitometric scan of results in top. (B) Stable expression of mLC7-1 results in phosphorylation of c-Jun in the absence of TGFβ. Top, cells were treated with TGFβ and lysates were obtained as for Figure 4A, except that they were analyzed by Western blot analysis with a phospho-c-Jun antibody (KM-1 and SC-822). Middle, Western blot demonstrating equal km23/mLC7-1 expression in the pools stably expressing km23/mLC7-1, but not in the EV-transfected pools. Bottom, plot of densitometric scan of results in top. Results are representative of two experiments for each.

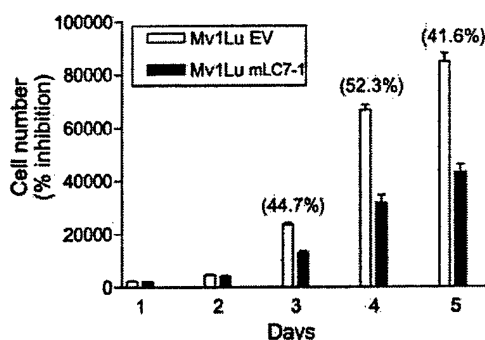

Figure 5. Stable expression of mLC7-1 results in growth inhibition in the absence of TGFβ. Mv1Lu cell pools stably expressing km23/mLC7-1 or EV were plated and analyzed for cell number at several days thereafter as indicated, by using the crystal violet assay described in MATERIALS AND METHODS. The percentage of inhibition of growth is indicated in parentheses on top of the relevant bars.

body detects a protein of ~74 kDa. In Figure 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 mLC7-1 is a dynein-associated protein.

The finding that mLC7-1 associates with and is phosphorylated by activated TGFβ receptors, and that it can activate JNK and c-Jun and inhibit cell growth, suggests that mLC7-1 may function in a TGFβ signaling pathway. Furthermore, because 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 and cytokines) 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 mLC7-1 to the DIC. For these studies, we performed IP/blot analyses by using anti-DIC as the IP antibody and anti-FLAG as the blotting antibody. Figure 6B, top, demonstrates that km23/mLC7-1 does interact with cytoplasmic DIC by IP/blot analyses. In addition, as shown in lanes 3–5 and 8–10 of this figure, 10 ng/ml TGFβ induced a rapid recruitment of km23/mLC7-1 to the DIC. Although a basal level of interaction between km23/mLC7-1 and DIC was detectable in some cases (lane 2), a threefold increase in this association was visible within 15 min of TGFβ addition to the TGFβ-responsive MDCK cells. This increase in the interaction between km23/mLC7-1 and DIC began as early as 2 min after TGFβ addition (top right) and seemed to remain relatively constant for at least 60 min (lanes 4 and 5, top). The bottom panels demonstrate roughly equal expression and loading. Thus, TGFβ rapidly induced the recruitment of the mLC7-1 to the DIC.

The results in Figure 6B indicate that TGFβ can stimulate the recruitment of mLC7-1 to the DIC, suggesting a connection between TGFβ signaling and DLC recruitment. To provide definitive evidence that TGFβ receptor activation is required for the mLC7-1–DIC interaction, we examined the interaction between mLC7-1 and DIC in the absence and presence of a kinase-deficient form of TGFβ RII. This receptor mutant can function in a dominant negative manner to block the kinase activity of endogenous RII when overexpressed in cells (Wieser *et al., 1993*). Furthermore, we have shown in Figure 3D that expression of KN RII with wild-type RI does not permit mLC7-1 phosphorylation. Figure 6C indicates that the TGFβ-induced interaction between km23/mLC7-1 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/mLC7-1 and KN RII in the relevant lanes was also confirmed (middle and bottom panels). Thus, mLC7-1 phosphorylation by kinase-active TGFβ receptors is necessary for the recruitment of mLC7-1 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. Furthermore, we have verified the success of this method with the isolation of a unique TGFβ receptor-interacting protein, termed mLC7-1. The mLC7-1 interaction with the TGFβ receptors was confirmed by ^{125}I-TGFβ affinity labeling and by IP/blot analysis. Furthermore, TGFβ induced the interaction of mLC7-1 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, mLC7-1 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 mLC7-1 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). Although this could suggest that mLC7-1 is a direct substrate of the TGFβ receptor kinase activity, it is also possible that another kinase is associated with the mLC7-1/TGFβ receptor complex. There are consensus phosphorylation sites for protein kinase C and casein kinase II within the mLC7-1 coding region. Perhaps, these or other serine kinases are the immediate activators of mLC7-1. However, it is clear that TGFβ does stimulate the interaction of mLC7-1 with the receptors, and that TGFβ receptor activation leads to mLC7-1 phosphorylation and recruitment of mLC7-1 to DIC.

Our results indicate, furthermore, that the kinase activity of the RII receptor is required for mLC7-1 phosphorylation and interaction with DIC, because a kinase-deficient version of RII blocked TGFβ induction of both events. TGFβ RI did not seem to be required for mLC7-1 phosphorylation, although RI was present in mLC7-1 immunoprecipitates in affinity-labeling experiments. Several pieces of evidence support the conclusion that RII is the activating receptor for mLC7-1. First, coexpression of RII with a kinase-deficient version of RI induced mLC7-1 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/mLC7-1 phosphorylation compared with expression of only RII. Finally, constitutively active RI alone did not result in phosphorylation of mLC7-1, as it does for the RSmads. Similarly, previous studies have described TGFβ signaling molecules that were regulated specifically by the RII receptors. For

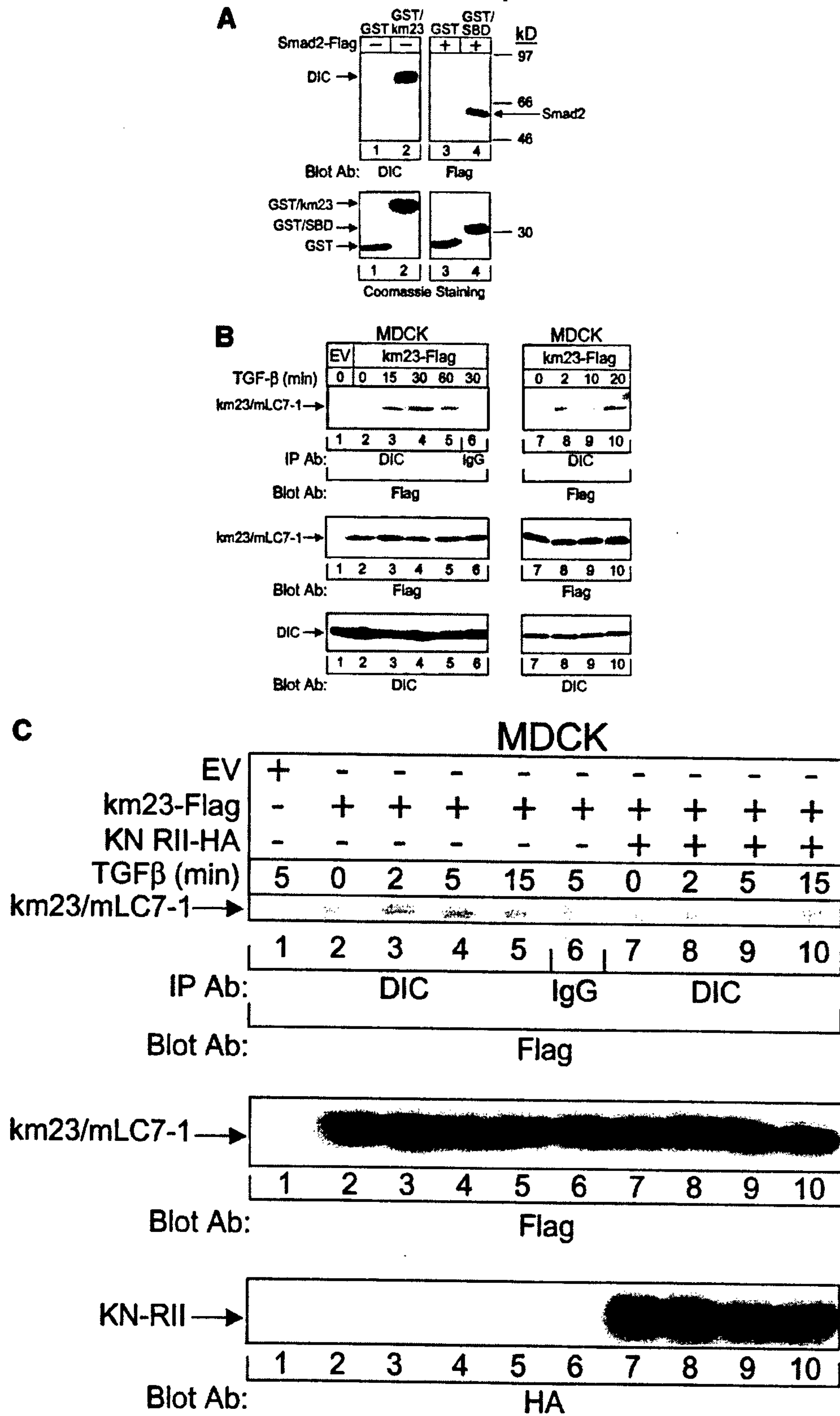

Figure 6. Interaction between the mLC7-1 and the DIC is regulated by TGFβ and requires RII kinase activity. (A) mLC7-1 interacts with DIC via GST pull-down assays. Top, MDCK cell lysates were incubated with Sepharose-bound bacterially expressed GST alone, GST-km23, or GST-SBD (positive control). GST-bound proteins were analyzed by SDS-PAGE (10%) and were immunoblotted with an anti-DIC antibody. Proteins were detected by enhanced chemiluminescence. 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, 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 mLC7-1 to the DIC within 2 min of TGFβ treatment. MDCK cells were transiently transfected with either empty vector or km23-FLAG. Thirty-six hours after transfection, cells were incubated in serum-free medium for 60 min before addition of 10 ng/ml TGFβ for the indicated times. Cell lysates were subjected to IP by using a monoclonal anti-DIC antibody, followed by immunoblot analysis with an anti-FLAG antibody (top). Western blot analysis with anti-FLAG (middle) or anti-DIC (bottom) 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 mLC7-1 is required for recruitment of mLC7-1 to the DIC. Cell treatments and IP/blot analyses were performed in MDCK cells as for B, except that cells were transfected with km23-FLAG in the absence (left, top) or presence (right, top) of KN RII. Western blot controls for expression of km23/mLC7-1 and KN RII are shown in the middle and bottom panels, respectively.

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 cloning of the *Drosophila* robl protein and the *chl*LC7 (Bowman *et al.*, 1999), km23/mLC7-1 is the mammalian homologue of the DLC/LC7/robl. We have shown that TGFβ leads to the recruitment of mLC7-1 to the DIC in a rapid, TGFβ-inducible manner. This interaction, however, occurred within a slightly different time frame than the interaction of mLC7-1 with the TGFβ receptors. This finding suggests that the receptors themselves may not be the cargo that dynein will transport via mLC7-1. That is, the mLC7-1-receptor interaction peaks at 5 min, and seems to begin declining by 15 min after TGFβ addition (Figure 2, B and C), consistent with the receptors being released once mLC7-1 has been phosphorylated. In contrast, it is clear from Figure 6, B and C, that the interaction between mLC7-1 and DIC begins as early as 2 min after TGFβ addition, yet mLC7-1 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 mLC7-1 with DIC.

Although evidence indicates that Smads 2/3/4 may be distributed along the MT network, the MTs seemed to sequester the Smads from the receptor before cellular stimulation by TGFβ (Dong *et al.*, 2000). Perhaps this occurs because a motor protein light chain such as mLC7-1 is in an inactive, unphosphorylated state until TGFβ receptor activation occurs. Phosphorylation of the DLC may affect a conformational change in this protein, followed by its recruitment to a motor complex for transport of TGFβ signaling components (i.e., Smads and JNKs) 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 mLC7-1 recruitment to the DIC, and dynein motoring of TGFβ signaling components along the MTs, represent at least some of these activities.

Because 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 mLC7-1 in both TGFβ signaling and dynein-mediated transport along MTs. It is likely that the binding of mLC7-1 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 mLC7-1 could prevent or alter movement of specific cargo along MT's. In this way, alterations in mLC7-1 might result in a mislocalization 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 mLC7-1 in human tumors might play a role in tumor development or progression. Future studies will address this possibility.

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Role of km23 co-localized and interacted with Smad2 in TGFβ-mediated effects

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SUMMARY

Transforming growth factor β (TGFβ) is a potent growth inhibitor for epithelial cells, often displaying tumor-suppressor activity. We have identified km23 as a novel TGFβ receptor-interacting protein. Here we show that km23 is ubiquitously expressed in human tissues and that cell-type specific differences in endogenous km23 protein expression exist. In addition, we demonstrate that the phosphorylation of km23 was TGFβ-dependent. Further, the kinase activity of both TGFβ receptors appeared to play a role in the TGFβ-mediated phosphorylation of km23. Subcellular fractionation analyses revealed that km23 is a cytoplasmic protein. In addition, immunofluorescence analyses indicate that km23 is colocalized with the TGFβ signaling component Smad2, either in the absence of TGFβ treatment, or during early time periods after its addition. km23 also interacted with Smad2 in glutathione-*S*-transferase (GST) pull-down and immunoprecipitation (IP)/blot assays. Blockade of km23 using small interfering RNA (siRNA) significantly decreased the ability of TGFβ to inhibit thymidine incorporation. Our findings suggest that km23 is necessary, but not sufficient, for TGFβ-mediated inhibition of DNA synthesis.

INTRODUCTION

TGFβ is growth inhibitory for normal cells of endothelial, hematopoietic, neuronal, and epithelial origin (Piez and Sporn, 1993; Akhurst and Derynck, 2001; Yue and Mulder, 2001). However, cancers are often refractory to this growth inhibitory effect, due to genetic loss of TGFβ receptors or, more commonly, perturbation of TGFβ signaling pathways (Akhurst and Derynck, 2001; Yue and Mulder, 2001). In fact, most of the components of TGFβ signal transduction pathways have been shown to be involved in a tumor suppressor pathway (Sporn and Vilcek, 2000; Akhurst and Derynck, 2001; Attisano and Wrana, 2002).

Resistance to growth inhibition by TGFβ due to a lack of TGFβ receptor expression has been reported for a variety of different human cancer cell lines, including gastric and colon cancer cells (Hartsough and Mulder, 1997; Kim et al., 2000; Massague et al., 2000). In a number of colon cancers, resistance to TGFβ-mediated growth inhibition is associated with mutations in either RII or in the signal transducers Smad4 and Smad2 (Hartsough and Mulder, 1997; Fink et al., 2001). Defects in TGFβ receptors and in Smad2 have been reported in ovarian cancer (Wang et al., 2000). Mutations in RI have been reported in head and neck cancer metastases, metastatic breast cancer, and ovarian cancer (Chen et al., 1998; Chen et al., 2001a; b). In addition, alterations in Smad4 have been reported for pancreatic carcinomas and colon carcinomas (Massague et al., 2000; Derynck et al., 2001; Yue and Mulder, 2001). However, such alterations only account for TGFβ resistance in a small percentage of

tumors (Akhurst and Derynck, 2001; Yue and Mulder, 2001). Thus, alterations in novel TGFβ signaling components are likely to be found.

In addition to cancer, aberrant TGFβ signaling results in developmental disorders, including hereditary chondrodysplasia and persistent mullerian duct syndrome, as well as others (Wall and Hogan, 1994; Massague et al., 2000; Yue and Mulder, 2001; Attisano and Wrana, 2002). TGFβ superfamily members play a role in almost every cellular process, including early embryonic development, cell growth, differentiation, cell motility, and apoptosis (Hartsough and Mulder, 1997; Massague, 1998; Roberts, 1998). Since TGFβ is an important factor in the development of such a large number of human diseases, any component that can be shown to play a role in TGFβ-mediated cellular events is of interest to explore.

TGFβ initiates its signals by producing an active tetrameric receptor complex consisting of RI and RII serine/threonine kinase receptors. After TGFβ binds to RII, it transphosphorylates, and thereby activates RI. The active receptor complex then propagates signals to downstream cellular components and regulatory proteins (Feng and Derynck, 1997; Attisano and Wrana, 1998; Massague et al., 2000; Miyazono, 2000; Sporn and Vilcek, 2000; ten Dijke et al., 2000). Two primary signaling cascades downstream of the TGFβ receptors have been elucidated: the Smads and the Ras/mitogen-activated protein kinase (MAPK) pathways (Yue and Mulder, 2001). In addition, several TGFβ receptor-interacting factors (Yue and Mulder, 2001) and Smad-interacting factors (ten Dijke et al., 2000) have been reported. Further, NF-κB, SnoN/Ski oncoproteins, and others have been shown to act as negative regulators of

TGFβ signaling, often by regulating the Smad proteins (Massague et al., 2000; Derynck et al., 2001; Yue and Mulder, 2001). However, the mechanisms by which TGFβ elicits its numerous biological effects have not nearly been defined.

We have previously identified a novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein (Tang et al., 2002). This TGFβ signaling intermediate, termed km23, interacts with the receptor complex through TGFβ RII and it is phosphorylated after TGFβ receptor activation. Here we show that km23 is a ubiquitously expressed cytoplasmic protein. Further, TGFβ mediated a rapid increase in km23 phosphorylation, and the kinase activity of both receptors appeared to play some role in this phosphorylation event. Immunofluorescence studies revealed that km23 is colocalized with Smad2 before Smad2 is translocated to the nucleus. In addition, km23 interacted with Smad2 in GST pull-down and IP/blot assays. Blockade of km23 using siRNA decreased the ability of TGFβ to inhibit DNA synthesis in Madin Darby canine kidney (MDCK) epithelial cells. Our findings suggest that km23 plays a role in mediating the inhibitory effect of TGFβ, and that Smad2 is likely involved in this pathway.

MATERIALS AND METHODS

Reagents--The anti-Flag M2 (F3165) antibody (Ab) and mouse IgG were from Sigma-Aldrich (St. Louis, MO). The anti-dynein intermediate chain (DIC) monoclonal Ab (MAB1618) and anti-Lamin A/C Ab (MAB3211) were from Chemicon (Temecula, CA). The anti-hemagglutinin (HA) Ab (1-583-816) was from Roche Applied Science (Indianapolis, IN). The TGFβ RII Ab (SC-220-R), K-Ras Ab (SC-30), Sp1 Ab (SC-59) and rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Smad2 Ab (51-1300) was from Zymed Laboratories (South San Francisco, CA). Protein A or G agarose were purchased from Invitrogen (Carlsbad, CA). ^{32}P-orthophosphate (NEX-053), γ-^{32}P-ATP (BLU002H), and [^{3}H]thymidine (NET-027X) were from PerkinElmer Life Sciences (Boston, MA). TGFβ_1 was purchased from R & D Systems (Minneapolis, MN). The human tissue mRNA blot was purchased from BD Biosciences Clontech (Palo Alto, CA). Alexa 594 goat anti-rabbit IgG and Alexa 488 goat anti-mouse IgG (H+L) were purchase from Molecular probes (Eugene, OR). Anti-Smad2/3 mAb was (610843) from BD Biosciences transduction laboratories (Palo Alto, CA). Anti-Myc (06-549) was from Upstate Biotechnology (Waltham, MA).

Antibody production--The rabbit km23 anti-serum used for Western blotting studies was prepared against the following sequence: GIPIKSTMDNPTTTQYA (corresponding to amino acids 27-43) of hkm23 by either Strategic BioSolutions (Newark, DE) or Covance Reseach Products, Inc. (Denver, PA). Preimmune serum was also provided by each company. The rabbit km23 anti-serum used for the immunofluorescence

studies was prepared against the following sequence: MAEVEETLKRIQS (corresponding to amino acids 1-13 of hkm23) by Strategic BioSolutions.

Cell culture--Mv1Lu (CCL-64), 293 (CRL-1573), and COS-1 (CRL-1650) cells were purchased from American Type Culture Collection (Manassas, VA) and were grown in DMEM supplemented with 10% FBS. MDCK cells (CCL-34) and HepG2 cells (HB-8065) were also obtained from ATCC and were grown in MEM-α supplemented with 10% FBS. 293T cells were obtained from T-W. Wong (Bristol-Myers Squibb, Princeton, NJ) and were maintained as for 293 cells. OVCA 433 cells were obtained from R.C. Bast Jr. (M.D. Anderson Cancer Center, Houston, TX) and were maintained in MEM supplemented with 10% FBS. FET cells were maintained as described previously (Zipfel et al., 1993). Cultures were routinely screened for mycoplasma using Hoechst 33258 staining.

Transient transfections, IP/blot, Westerns, and In vivo phosphorylation assays were performed essentially as described previously (Hocevar et al., 1999; Yue et al., 1999a; b; Yue and Mulder, 2000; Tang et al; 2002).

siRNA expression--siRNA was purchased from Dharmacon Research (Lafayette, CO) and interference was performed according to the manufacturer's recommendations. The double-stranded siRNA corresponded to nucleotides 77-97 of the hkm23 coding region (5'-AAGGCATTCCCATCAAGAGCA-3'). siRNA was transfected using Oligofectamine (12252-011; Invitrogen).

Fluorescence microscopy--Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 minutes at room temperature, and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 5 minutes. Subsequently, these cells

were incubated successively with km23 rabbit anti-serum (1:200) or 5 μg/ml anti-Smad2/3 mAb for one hour. The bound primary antibodies were visualized with 5 μg/ml Alexa 594 goat anti-rabbit IgG or Alexa 488 goat anti-mouse IgG, respectively. Finally, immunofluorescence images were captured using a Nikon Diaphot microscope with a Retiga 1300 CCD camera (BioVision Technologies, Inc., Exton, PA) running IPLab v3.6.3 software (Scanalytics, Inc., Fairfax, VA).

Cellular fractionation--The NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (78833; Pierce, Rockford, IL) was used to fractionate Mv1Lu cells according to the manufacturer's protocol.

[^{3}H]thymidine incorporation assays were performed as described (Hartsough and Mulder, 1995).

Statistical analyses--A Student's t-test was used to determine the level of statistical significance of the decrease in [^{3}H]thymidine incorporation by TGFβ between siRNA-treated and mock-treated MDCK cells.

GST pull-downs were performed as described (Tang et al., 2002).

RESULTS

km23 is a novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein (Tang et al., 2002). As indicated in Fig. 1, hkm23 is very similar to the *Danio Rerio* (Zebrafish) ZFIN (80% identity or 93% similarity), *Drosophila* robl (68% identity or 82% similarity), and *Chlamydomonas* LC7 (55% identity or 81% similarity) sequences. In addition, a counterpart of km23 in *Caenorhabditis elegans* (T24H10.6) displays 56% homology to hkm23, and the predicted protein would be 76% similar. In contrast, hkm23 displays 71% similarity to *Spermatozopsis* B15 (Dole et al., 2000), 54% similarity to *Drosophila* bithoraxoid (bxd) and only 33% homology (predicted protein would be 32% similar) to *Leishmania* LMAJFV1 (Akopyants et al., 2001). The single-celled flagellar parasite *Leishmania* is an animal-like, Protozoan form of Protista, the Kingdom that also includes plant-like forms of green algae, such as *Chlamydomonas* and *Spermatozopsis.* Thus, km23 displays a considerable degree of conservation across different Kingdoms and Phyla.

There also appears to be another mammalian km23 form, designated km23-2 in Table 1. Table 1 lists the % homologies, identities, and similarities of hkm23 compared with the rat and mouse sequences for the two forms. As indicated, rkm23-1 (96% identity or 98% similarity) and mkm23-1 (95% identity or 98% similarity) are highly conserved when compared with hkm23. In addition, hkm23-2 displays 70% homology and 91% similarity with hkm23-1. Similarly, the rat and mouse forms of km23-2 display 71% homology and 91% similarity with hkm23-1. We have also sequenced the cadnine km23-1 from a.a ? to ?, and this region, it displays /% identity

Thus, the mammalian forms of both km23-1 and km23-2 have very similar sequence , although they may be ? distinct.

In order to determine the tissue distribution of hkm23, we performed Northern blot analysis using a human tissue blot obtained from BD Biosciences Clontech. The coding region of hkm23-1 was used as the probe (Fig. 2, top). The blots were stripped and reprobed with β-actin cDNA as a control for equal loading of RNA (Fig. 2, middle). Both blots were then scanned by densitometry and the km23 values were corrected for differences in RNA loading using the β-actin levels (Fig. 2, bottom). As indicated in Fig. 2, km23 was expressed at a high level in brain, kidney, and placenta. Intermediate levels of expression were observed in the heart, skeletal muscle, and liver. Low levels were detectable in the colon, spleen, small intestine, and lung. In longer exposures, barely detectable levels were observed in the thymus and in peripheral blood leukocytes. The calculated size of the hkm23-1 mRNA from the blot was approximately 0.7 kb.

The cellular effects of TGFβ are dependent upon cell type, stage of differentiation, growth conditions, concentration of ligands, and the presence of other growth factors (Roberts and Sporn, 1990; Yue and Mulder, 2001). Since km23 was identified as a TGFβ receptor-interacting protein, it was of interest to determine whether TGFβ could modulate the expression of the endogenous km23 protein. In order to perform these studies, we developed polyclonal rabbit km23 anti-serum against amino acids 27-43 of hkm23 and performed Western blot analysis in MDCK epithelial cells to determine whether the km23 anti-serum was specific. As indicated

in Fig. 3A (left panel), the rabbit km23 anti-serum specifically recognized a single band of 11 kDa by Western blot analysis (left panel, lanes 1-4). This band was not present after blotting with preimmune serum (right panel, lane 2). The results of Fig. 3 clearly indicate that TGFβ does not induce the expression of endogenous km23 (left panel, lanes 1-4).

In order to determine whether cell-type specific differences in km23 protein expression existed, we examined the relative expression levels of endogenous km23 in various mammalian cell types. Due to the high level of similarity (98%) among the mammalian km23-1 forms from amino acids 27-43, the polyclonal km23 anti-serum could detect endogenous km23 levels equally well in the different species. As shown in Fig. 3B, km23 is highly expressed in HepG2 human hepatoma cells (lane 4, top panel) and in MDCK cells (lane 6, top panel), while Mv1Lu mink lung epithelial cells (lane 5, top panel) displayed reduced expression levels. We also found that the expression level of km23 in the TGFβ–resistant, human ovarian cancer cell line OVCA 433 (lane 3, top panel) was lower than that in either the MDCK or HepG2 cells. km23 was expressed at intermediate levels in 293 human embryonic kidney cells (lane 1, top panel) and in TGFβ–responsive FET human colon cancer cells (lane 2, top panel). Equal loading was confirmed by blotting with a DIC Ab (bottom panel), since we have previously determined that DIC levels are relatively constant among such cell lines. Thus, cell-type specific differences in km23 protein expression do exist.

In order to examine the subcellular localization of km23, we performed Western blot analysis after subcellular fractionation of cells incubated in the presence or absence of TGFβ. As indicated in Fig. 4, when km23 was expressed in the absence of

TGFβ (lanes 1-6, left panel), both endogenous and expressed km23 were easily detectable in the cytoplasmic fraction (lane 3, top panel). In contrast, as expected, endogenous Lamins A and C were not detectable in the cytoplasmic fraction (lanes 1-3, bottom panel). Further, both endogenous and expressed km23 were absent in the nuclear fraction (lanes 4-6, top panel), whereas Lamins A and C were easily detectable in this compartment (lanes 4-6, bottom panel) (Georgatos et al., 1994). The results indicate that km23 is a cytoplasmic protein. A similar result was observed after the cells were exposed to TGFβ (lanes 7-12, right panel), indicating that TGFβ treatment does not affect a nuclear translocation of km23. As expected, no change in the localization of Lamins A and C was detected after TGFβ treatment.

The serine/threonine kinase activity of the TGFβ receptors mediates phosphorylation of downstream molecules to affect TGFβ responses. We have previously shown that km23 was phosphorylated by the TGFβ receptor complex in human embryonic kidney 293T cells (Tang et al, 2002). However, we did not examine the ligand-dependent role of TGFβ in km23 phosphorylation in those cells. Here, we performed in vivo phosphorylation assays (Yue et al., 1999a; b) after transient expression of km23-flag, RI-HA, and RII-HA in the absence and presence of TGFβ. As shown in Fig. 5 (left panel), km23 was phosphorylated upon co-expression of both types of TGFβ receptors (lane 2). A ligand-dependent increase in km23 phosphorylation was observed at 5 and 15 minutes after TGFβ treatment (lanes 3 and 4). As shown previously, km23 was not auto-phosphorylated (lane 1), nor was there

phosphorylation in the IgG control lane (lane 5). These results indicate that km23 phosphorylation by TGFβ is ligand-dependent.

Since TGFβ stimulation increased phosphorylation levels of km23, it was of interest to see whether another growth factor ligand produced a similar effect. Epidermal growth factor (EGF) is known to phosphorylate many downstream targets after activation of its receptor (Jorissen et al, 2003). For example, Shc (Sakaguchi et al, 1998), Src (Mao et al, 1997), STATs (David et al, 1996), PLC-γ (Wahl et al, 1990), and PPARγ (Camp and Tafuri, 1997) have all been shown to be phosphorylated within 30 minutes of EGF treatment. In order to determine whether km23 could be phosphorylated by EGF, we performed in vivo phosphorylation assays similar to those for TGFβ. As shown in Fig. 5 (right panel), EGF did not stimulate km23 phosphorylation during a treatment period of 0-30 minutes (lanes 7-12). EGF has been shown to phosphorylate other substrates during this time period in this cell line (Camp and Tafuri, 1997). As expected, no km23 phosphorylation was observed in the negative control (lanes 6-13). Collectively, our results suggest that km23 phosphorylation may be mediated specifically by TGFβ.

We have also previously shown that overexpression of either the activated TGFβ receptor complex or RII alone could phosphorylate km23 in COS-1 cells (Tang et al, 2002). However, it was unclear whether co-expression of RI with RII altered km23 phosphorylation by RII. As shown in Fig. 6 the level of ligand-dependent phosphorylation of km23 by RII alone (lanes 7 and 8) was similar to that observed after co-expression of both receptors (lanes 2 and 3). As previously shown, km23

phosphorylation was completely blocked upon expression of KN RII, even after treatment with TGFβ (lanes 11 and 12). As expected, co-expression of RI and RII without km23 resulted in no detectable phosphorylation (lane 1). The IgG control (lane 4) was also negative. Thus, phosphorylation of km23 by TGFβ can occur in the absence of RI, and the presence of wild-type RI does not alter the ability of RII to phosphorylate km23. These results also confirm the ligand-dependent phosphorylation of km23 in another cell line, COS-1 (Fig. 6, lanes 2 and 3).

In order to address the role of the kinase activity of RI in the TGFβ-mediated phosphorylation of km23, in vivo phosphorylation assays were performed using various mutant forms of RI and RII alone or in combination. Here, we examined whether a kinase deficient version of RI (KN RI) could block ligand-dependent phosphorylation of km23. By comparison of the bands in lanes 7 and 9, in the absence of TGFβ km23 was phosphorylated to a reduced extent after co-expression of RII with KN RI, as opposed to RII alone. In contrast, in the presence of TGFβ, KN RI reduced km23 phosphorylation by approximately 3-fold, relative to that observed after expression of RII alone (lanes 8 and 10). As shown in lanes 5 and 6, km23 was not phosphorylated when RI alone was co-expressed with km23. Thus, phosphorylation of km23 by TGFβ can occur without the kinase activity of RI, and the presence of wild-type RI does not alter the ability of RII to phosphorylate km23. However, the kinase activity of RI appears to contribute to km23 phosphorylation in some manner.

Our previous data suggested that km23 might function as a "motor receptor" to recruit TGFβ signaling complexes to the dynein motor for intracellular transport along MT toward the nucleus (Tang et al, 2002). Since Smad2 is a critical intracellular mediator of TGFβ signaling (Macias-Silva et al., 1996; Heldin et al., 1997; Attisano and Wrana, 1998, Massague 1998), and it is co-localized with RII in specific endosome compartments after receptor endocytosis (Hayes et al., 2002). It was conceivable that Smad2 might function as one of the cargo that km23 might recruit. If this was the case, we would expect Smad2 to be co-localized with km23. In order to determine whether this was the case, we performed immunofluorescence studies in TGFβ-responsive MDCK cells, using the km23 rabbit antiserum prepared against amino acids 1-13. As indicated in Fig. 7A, in the absence of TGFβ treatment, endogenous km23 was present in perinuclear puncta and was partially co-localized with endogenous Smad2. TGFβ treatment resulted in a greater co-localized of Smad2 with km23 at both 2(B) and 5(C) minutes after its addition to MDCK cells. In contrast, once Smad2 had translocated to the nucleus by 15 minutes (D), km23 was still localized in the cytoplasm and no longer co-localized. Smad2 would be expected to execute its functions in the transcriptional regular of gene expression, whereas km23 is likely to be transported to acidic vesicular compartments for degradation. Thus, our results indicate that km23 and Smad2 are co-localized either in the absence of TGFβ, or following TGFβ treatment for 2 or 5 min, prior to the entry of Smad2 into the nucleus.

Since km23 and Smad2 appeared to be co-localized in the absence of TGFβ, but were then separated into distinct cell locales after TGFβ treatment, it was of interest

to determine whether km23 could directly interact with Smad2. We performed GST pull-down assays after transfection of Smad2-flag into 293T cells. Cell lysates were incubated with sepharose-bound, bacterially-expressed GST alone or GST-rkm23. An anti-flag Ab was used as the blotting Ab to detect Smad2-flag in the GST-km23 complex. As shown in Fig. 8A, Smad2 was visible in the GST-km23 immunoprecipitates (lane 4), but not in the immunoprecipitates from GST only (lane 3). EV control lanes were also negative (lanes 1, 2). The results indicate that km23 interacts with Smad2 in vitro.

In order to determine whether km23 and Smad2 interacted in vivo, we performed IP/blot analysis after transfection of either EV or km23-Flag. As shown in Fig. 8B, immunoprecipitation of the Smad2 protein using an anti-Smad2 Ab was able to co-IP km23 (lane 2, left panel). In contrast, the EV control was negative (lane 1, left panel). The interaction between Smad2 and Sp1 (Feng et al., 2000, Pardali et al., 2000) is shown as a positive control for comparison (lane 3, right panel). Further, we did not find a direct interaction between Smad2 and DIC (lane 4, right panel), yet the positive control (IP DIC, blot DIC, lane 5, right panel) did reveal the appropriate location of the DIC band. The results clearly demonstrate that km23 interacts with Smad2 under physiological conditions, but that it cannot directly interact with the dynein motor complex.

A noted biological effect of TGFβ in epithelial cells is growth inhibition (Piez and Sporn, 1993; Akhurst and Derynck, 2001; Yue and Mulder, 2001). Further, inactivation of Smad2 has been shown to effectively eliminate TGFβ-induced growth inhibition, without an apparent effect on the ability of TGFβ to induce expression of other genes

(Feng et al., 2002). Since we have shown that km23 interacts with and co-localized with Smad2, it was of interest to determine whether blockade of km23 would lead to a change in the ability of TGFβ to inhibit the cell cycle in late G1, as assessed by a change in DNA synthesis. The MDCK cells used for these studies are naturally responsive to the growth inhibitory effects of TGFβ, and display an inhibition of [^{3}H]thymidine incorporation of approximately 90%. Therefore, we transfected MDCK cells with siRNA to disrupt the expression of endogenous km23 using Oligofectamine as described in the "Materials and Methods." Western blot analysis was then performed to determine whether km23 expression was blocked. As shown in Fig. 9A, treatment with either 0.10 μM or 0.12 μM siRNA induced a marked decrease (compared to mock-treated cells) in km23 levels at both 24 hours (lanes 1-3) and 48 hours (lanes 4-6) after addition of the siRNA. The decrease in endogenous km23 was maximal at 24 hours after transfection of siRNA (lanes 2 and 3, top panel), with a reduction of km23 expression by approximately 65%. The silencing effect was apparent for at least an additional 24 hours, yet this effect was not enhanced when the siRNA concentration was increased from 0.10 μM to 0.12 μM (lanes 2, 3, 5, and 6, top panel). After 72 hours, there was no difference in endogenous km23 levels between the mock-treated and siRNA-treated cells (lanes 7-9, top panel). The bottom panel of Fig. 9A depicts the results of densitometric scans of the top panel, with the percent of control values provided above the relevant bars.

Since 0.1 μM siRNA resulted in maximal silencing at 24 hours after addition to MDCK cultures, we examined TGFβ-mediated inhibition of DNA synthesis of MDCK

cells in the presence and absence of 0.1 μM siRNA at this time point. The [^{3}H]thymidine incorporation analyses in Fig. 9B indicate that blockade of endogenous km23 led to a reduction in the ability of TGFβ to inhibit DNA synthesis. While TGFβ inhibited the DNA synthesis of the mock-treated cells by 92%, cells transfected with km23 siRNA displayed only a 73% inhibition of DNA synthesis by TGFβ. Statistical analyses using the Student's t-test revealed that this difference was statistically significant ($p<0.01$). Together, these results demonstrate that km23 plays a role in TGFβ-mediated inhibition of DNA synthesis.

DISCUSSION

We have isolated km23 via its ability to interact with the intracellular portions of the TGFβ receptors. Further, we have demonstrated that this TGFβ receptor-interacting protein is associated with the TGFβ receptor complex through RII (Tang et al., 2002). Here we show that TGFβ induced a ligand-dependent increase in km23 phosphorylation, mediated by preferentially by the kinase activity of RII. However, co-expression of a kinase-deficient version of RI with RII decreased km23 phosphorylation relative to that observed after expression of only RII. Thus, the kinase activity of RI also appears to play a role in phosphorylation of km23.

Of greater significance, km23 appeared to be partially required for TGFβ-mediated inhibition of thymidine incorporation in MDCK cells. A complete blockade of thymidine incorporation by the km23 siRNA would not be expected, however for two reasons. First, the siRNA blockad of km23 expression was not complete (only 65%). Second, other pathway components are known to be involved in mediating this complex biological response with regard to the latter point, TGFβ activation of Ras, extracellular signal-regulated kinases (Erks) and of stress-activated protein kinases/Jun N-terminal kinases (Sapk/JNKs) is known to be required for TGFβ-mediated growth inhibition (Mulder and Morris, 1992; Hartsough et al., 1996; Frey and Mulder, 1997; Yue et al., 1998; Yue et al., 1999b; Mulder, 2000). Additional evidence indicate that over-expression of the p65 subunit of the NF-κB/Rel family in Hs578T cells can abrogate the ability of TGFβ to inhibit cell growth (Sovak et al., 1999),

suggesting that TGFβ regulation of NF-κB/Rel activity is also associated with TGFβ-mediated growth inhibition. Thus, several TGFβ-regulated components appear to be required to mediate the growth inhibitory response to TGFβ. km23 may represent a novel member of this group of signaling components.

Subcellular localization of a signaling molecule plays an important role in its ability to function during the transduction of cellular signals. It is essential for a signaling molecule to be in the appropriate cell location at the appropriate time for efficient activation (Whitmarsh and Davis, 2001). Our data indicate that km23 is a TGFβ receptor-interacting protein that directly interacts with Smad2 in the cytoplasm under both in vivo and in vitro conditions. Further, km23 is co-localizes with Smad2 either in the absence of TGFβ, or at early time periods after TGFβ addition, suggesting that Smad2 may be one of the cellular cargo that km23 recruits to the dynein complex for intracellular transport along MTs. After 15 minutes of TGFβ treatment, however, Smad2 translocates to the nucleus, while km23 remains in the cytosol.

Smads2/3/4 have been shown to be distributed along the MT network (Dong et al. 2000). However, little is known about how Smads transition from the MTs to an activated state. We have shown that km23 is the light chain of dynein, and TGFβ leads to the recruitment of km23 to the DIC in a rapid, TGFβ-inducible manner (Tang et al. 2002). Based upon our finding that km23 may control the cell localization of TGFβ signaling components such as Smad2 in the cytoplasm, it is possible that km23 can transport Smad2 toward the nucleus along the MTs, prior to Smad2's effects on nuclear transcription events.

It has been shown recently via genetic studies in *Drosophila* that a trio of membrane-associated proteins act in a common pathway to regulate epithelial cell polarity and cell growth (Bilder et al., 2000). Since TGFβ and its signaling components can function as tumor suppressors, similar to this trio of protein, perhaps km23 contributes to appropriate trafficking of provides TGFβ signaling components such as Smad2, thereby maintain cell polarity in the signaling pathway. In this regard, we have provided evidence to suggest that km23 is important for maintain the appropriate localization of TGFβ signaling components such as Smad2. It is conceivable that an alteration of km23 might result in a mis-localization of such signaling intermediators, with a concomitant disruption of TGFβ growth inhibitory signals. Indeed, an alteration in the homologue to km23 (rob1) resulted in accumulation of cargoes and an increase in mitotic index (Bowman et al; 1999). Our results using km23-specific siRNA suggest that km23 may, indeed, play an important role in TGFβ-mediated growth suppression.

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FIGURE LEGENDS

Fig. 1. Alignment of amino acid sequences comparing km23 family members. The coding region for the following km23 family members are aligned to illustrate the relative identities: hkm23-1 (accession # AY026513), ZFIN (accession # AAH46084; 74% homology to hkm23-1), robl (accession # AF141920; 67% homology to hkm23-1), LC7 (accession # AF140239; 59% homology to hkm23-1), T24H10.6 (accession #Z54216; 56% homology to hkm23-1), B15 (accession # AJ243446; 56% homology to hkm23-1), bxd (accession # M27999; 40% homology to hkm23-1), and LMAJFV1 (accession # AQ850960; 33% homology to hkm23-1).

Fig. 2. Northern blot analysis of km23 mRNA expression in several human tissues. Top panel, a human tissue mRNA blot was obtained from BD Bioscience Clontech and was probed with the ^{32}P-labeled cDNA for hkm23. Middle panel, the membrane was reprobed with β-actin as a control. As expected, the primary β-actin band was 2 kb, whereas an additional species of 1.7 kb was observed in heart and skeletal muscle. Bottom panel, plot of densitometric scan of results from top panel after normalization of km23 expression levels against β-actin control levels.

Fig. 3. km23 protein expression was detectable by Western blot analysis using a rabbit polyclonal km23 anti-serum. **A:** TGFβ does not modulate the expression of endogenous km23. Left panel, MDCK cells lysates were incubated in serum-free α-

MEM medium for 1 hour prior to treatment with (lanes 2-4) or without (lane 1) TGFβ (10 ng/ml). Lysates were analyzed by SDS-PAGE (15%) and transferred to a PVDF membrane. The membrane was then incubated with rabbit polyclonal km23 anti-serum, followed by incubation with an anti-rabbit IgG secondary antibody (1:7500). Right panel, MDCK cells lysates were analyzed as in left panel, except that one lane was incubated with preimmune rabbit serum as a control (lane 2) to demonstrate the specificity of the Ab. **B:** The rabbit km23 polyclonal anti-serum could detect km23 in various species. Top panel, protein lysates (75 μg) were prepared from 293, FET, OVCAR 433, HepG2, Mv1Lu, and MDCK cells, and analyzed as in A. Equal loading was confirmed by blotting with a DIC Ab (Chemicon).

Fig. 4. TGFβ does not cause a translocation of km23 to the nucleus. Mv1Lu cells were either mock transfected or transiently transfected with EV or km23-flag. Twenty-four hours later, cells were treated without (lanes 1-6) or with (lanes 7-12) TGFβ (10 ng/ml) for 30 minutes. Cells were then fractionated as described in "Materials and Methods." Top panel, both cytoplasmic and nuclear fractions were subjected to SDS-PAGE (15%), transferred to a PVDF membrane, and blotted with anti-flag. Bottom panel, the membrane was then reprobed with anti-lamin A/C Ab as a nuclear marker.

Fig. 5. Specificity of TGFβ-dependent phosphorylation of km23. 293T cells were transiently transfected with either EV, km23-flag, and/or RI-HA and RII-HA as indicated. Twenty-four hours after transfection, cells were labeled for 3 hours with [$^{32}P_i$] in the

absence or presence of TGFβ (5 μg/ml) (left) or EGF (100 ng/ml) (right) for the indicated times. Thereafter, cells were lysed and IP'd with anti-Flag. In vivo phosphorylation of km23-flag was visualized by SDS-PAGE (15%) and autoradiography.

Fig. 6. Role of RI and RII in ligand-dependent phosphorylation of km23. COS-1 cells were transiently transfected with either EV, km23-flag, and/or wild-type or mutant RI and RII as indicated. Twenty-four hours after transfection, cells were labeled for 3 hours with [$^{32}P_i$] in the absence or presence of TGFβ (10 μg/ml, 10 minutes), followed by cell lysis and IP with an anti-Flag Ab. Top panel, in vivo phosphorylation of km23-flag was visualized by SDS-PAGE (15%) and autoradiography. Bottom panel, plot of densitometric scans of km23 phosphorylation. km23 expression values from a flag immunoblot (not shown) were used to normalize loading and expression among the lanes.

Fig. 7. km23 colocalizes with Smad2 in TGFβ dependant maner. **A:** MDCK cells were fixed, permeabilized, and stained with rabbit km23 anti-serum (left, top row) and mouse anti-Smad2 (left, second row). The overlap and a region from this panel magnified are shown in third row. No signal was detected in the control (right panel). **B:** MDCK cells were fixed, permeabilized, and stained with rabbit km23 anti-serum (left, top row) and mouse anti-Smad2 (left, second row). The overlap and a region from this panel magnified are shown in third row. No signal was detected in the control (right

panel). **B:** MDCK Cells were incubated with TGFβ (2.5ng/ml) for 2 minutes, and then cells were fixed, permeabilized, and stained with rabbit km23 anti-serum (left, top row) and mouse anti-Smad2 (left, second row). The overlap and a region from this panel magnified are shown in third row. No signal was detected in the control (right panel). **C:** MDCK Cells were incubated with TGFβ (2.5ng/ml) for 5 minutes, and then cells were fixed, permeabilized, and stained with rabbit km23 anti-serum (left, top row) and mouse anti-Smad2 (left, second row). The overlap and a region from this panel magnified are shown in third row. No signal was detected in the control (right panel). **D:** MDCK Cells were incubated with TGFβ (2.5ng/ml) for 15 minutes, and then cells were fixed, permeabilized, and stained with rabbit km23 anti-serum (left, top row) and mouse anti-Smad2 (left, second row). The overlap and a region from this panel magnified are shown in third row. No signal was detected in the control (right panel).

Fig. 8. km23 interacts with Smad2 via GST pull-down and IP/blot assays. **A:** Top panel, 293T cells were transfected with either Flag-tagged Smad2 or EV, and lysates were incubated with sepharose bound, bacterially-expressed GST alone or GST-rkm23. GST- bound proteins were analyzed by SDS-PAGE and were immunoblotted with an anti-flag Ab. Smad2 interacts with GST-km23 (lane 4), but not with GST alone (lane 3). EV control lanes (lanes 1, 2) were also negative. Lower panel, Western blot analysis of total cell lysates to confirm expression of Smad2 in the relevant samples prior to assay (lanes 3, 4). **B:** IP/blot analysis of Smad2 interaction with km23. Left panel, Mv1Lu cells were transfected with EV or km23-Flag. Following IP with a Smad2 Ab, lysates were subjected to SDS-PAGE (15%), transferred to a PVDF membrane, and

blotted with an anti-flag Ab. Right panel, Mv1Lu lysates were analyzed by IP/blot, as indicated, to verify the interaction between Smad2 and Sp1 (positive control), but not between Smad2 and DIC (negative control).

Fig. 9. siRNA blockade of km23 expression reduces TGFβ-mediated inhibition of DNA synthesis in MDCK cells. **A:** Blockade of endogenous km23 protein expression using siRNA. Top panel, MDCK cells were either mock-transfected or transfected with 0.10 μM or 0.12 μM double-stranded siRNA corresponding to nucleotides 77 to 97 of the hkm23 coding region (5'-AAGGCATTCCCATCAAGAGCA-3') as described in "Materials and Methods." After incubation for 24-72 hours, expression levels of endogenous km23 were analyzed via Western blot analysis using rabbit km23 anti-serum (1:500) as described for Fig. 3B. Bottom panel, plot of densitometric results from top panel. km23 levels are shown as a percent of control in parentheses above the relevant bars relative to the corresponding mock-treated values. **B:** The TGFβ-mediated repression of [^{3}H]thymidine incorporation was partially reversed following blockade of endogenous km23 by siRNA. MDCK cells were either mock-transfected or transfected with 0.1 μM siRNA as described in A. Twenty-four hours after transfection, [^{3}H]thymidine incorporation analyses were performed as described in "Materials and Methods." The asterisk indicates a statistically significant difference (Student's t-test, $p<0.01$) in [^{3}H]thymidine incorporation by TGFβ between siRNA-treated and mock-treated MDCK cells.

Fig. 1

```
hkm23-1    1                  M A E V E E T L K R L Q S - Q K G V Q G  19
ZFIN       1                  M A E V E E T I K R I Q S - Q K G V Q G  19
robl       1                M S Q E V E E T L K R I Q S - H K G V V G  20
LC7        1            M V D I A A V D E T F K R L Q S - H K G V L G  22
T24H10.6   1                  M S D F E E T I R R L Q S - E K G V V G  19
B15        1                M A E A I Q A T L E R I Q K - H K G V L G  20
bxd        1  M N R I L E K V I H Q N G T I V D R I L S - E H T I G Y  27
LMAJFV1    1            T T F A Y L H H S D Q R N R N R A P P P A P  22

hkm23-1   20  I I V V N T E G I P I K S T M D N P T T T Q Y A S L M H  47
ZFIN      20  I I I V N A E G I P I K S T L D N T S T V Q Y A A N I H  47
robl      21  T I V V N N E G I P V K S T L D N T T T V Q Y A G L M S  48
LC7       23  I I V I N A E G I A I R T T F D N D L T V Q Y A A L V S  50
T24H10.6  20  I I V V D S A G R V I H S T I D S D A T Q S H T A F L Q  47
B15       21  T I I I D H N G V T L H S T L D D K T T A E Y T E L I P  48
bxd       28  V V S D N T A N A V A E T S F D N T S A Q A I L K H L H  55
LMAJFV1   23  L H S T H T H T H T K R E E V R T P C T H F L D S A L S  50

hkm23-1   48  S F I L K A R S - T V R D I D P Q N D L T F L R I R S K  74
ZFIN      48  Q L L M K A R G - I V R D I D P Q N D L T F L R V R S K  74
robl      49  Q L A D R S - - - V V R D L D P S N D M T F L R V R S K  73
LC7       51  H F T V K A R S - A V R K L D G D N D L K F L R I R S K  77
T24H10.6  48  Q L C E K T K T - S I R E L D S S N D L T F L R L R T K  74
B15       49  A L S M L A K N - L V R D V D P Q N D L D F L R V R S L  75
bxd       56  G L L V S T C Q S V V R D I D P S N K L C F M R L G T R  83
LMAJFV1   51  M S A S W M K K N M Y L A I L W P H D L V L I G A Q P N  78

hkm23-1   75  K N E I M V A P D K D - - - - - Y F L I V I Q N P T E      96
ZFIN      75  K N E I M I A P D K D - - - - - Y F L I V I Q N P T E      96
robl      74  K H E I M V A P D K D - - - - - F I L I V I Q N P T D K A  97
LC7       78  K H E I M I A P E F E R S H E Y Y L V V V Q D P S R E A  105
T24H10.6  75  K N E I M I A P D K D - - - - - H V I M V I K D L S        95
B15       76  K H E I M V A P K E E - - - - - F L L I V I Q D P A A V    98
bxd       84  K F E Y L V A P E E Y - - - - - F T I T V V Q              101
LMAJFV1   79  E L E L I V A V D L L Q H - - C L G A H S Q R L E K C      103
```

Fig. 2

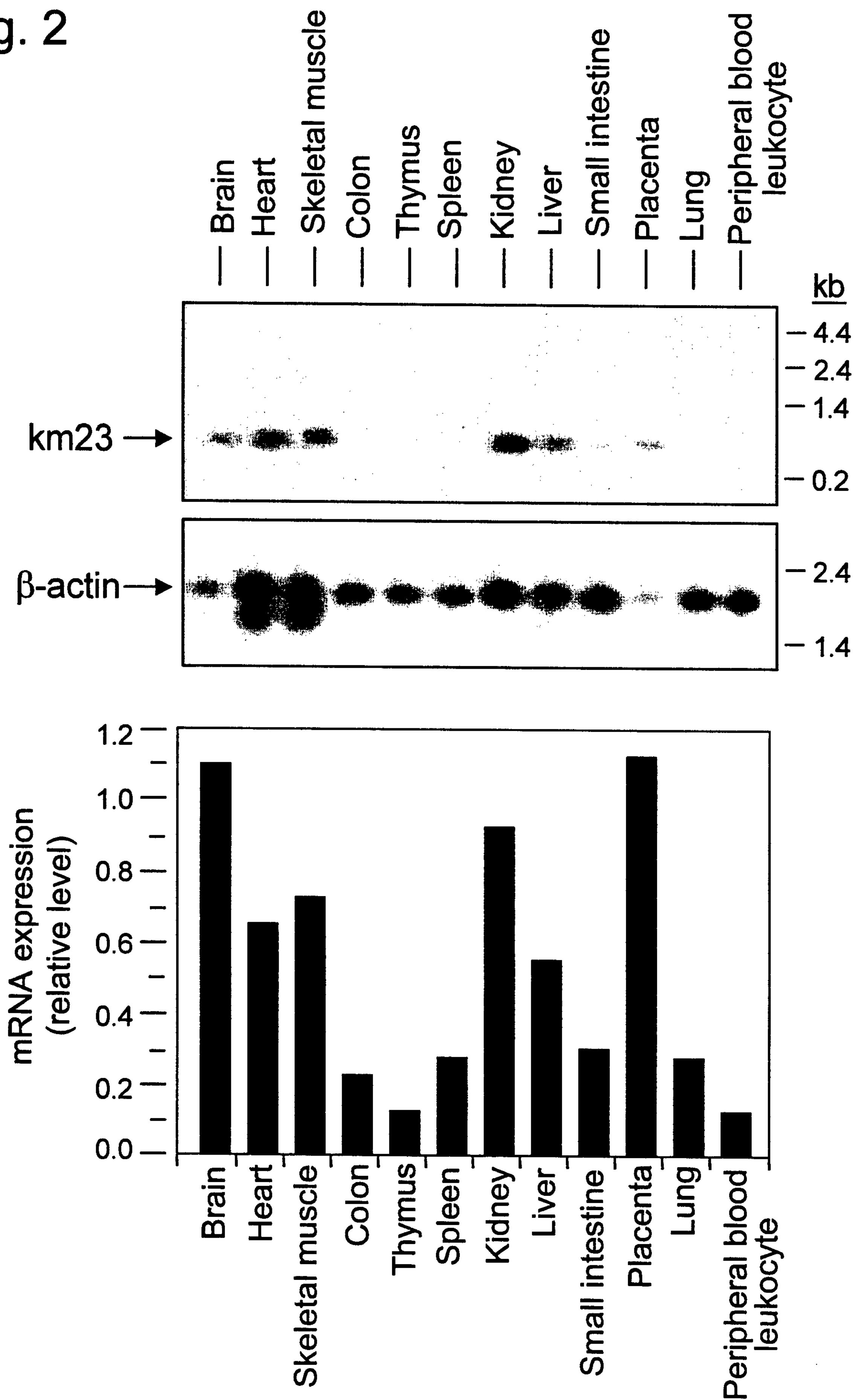
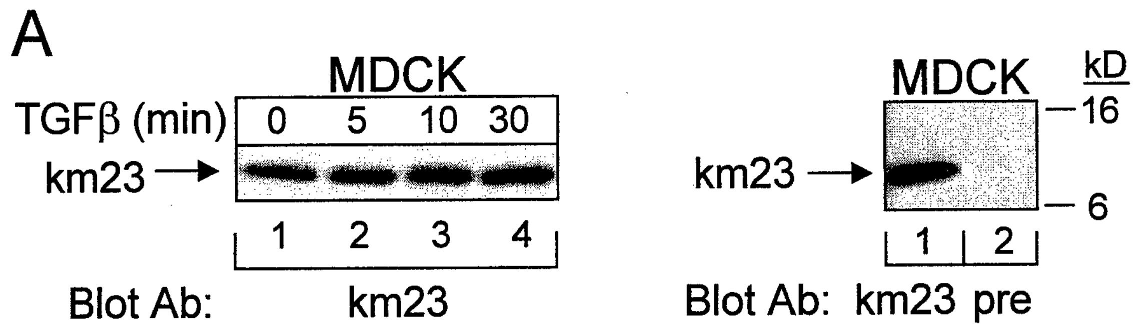


Fig. 3



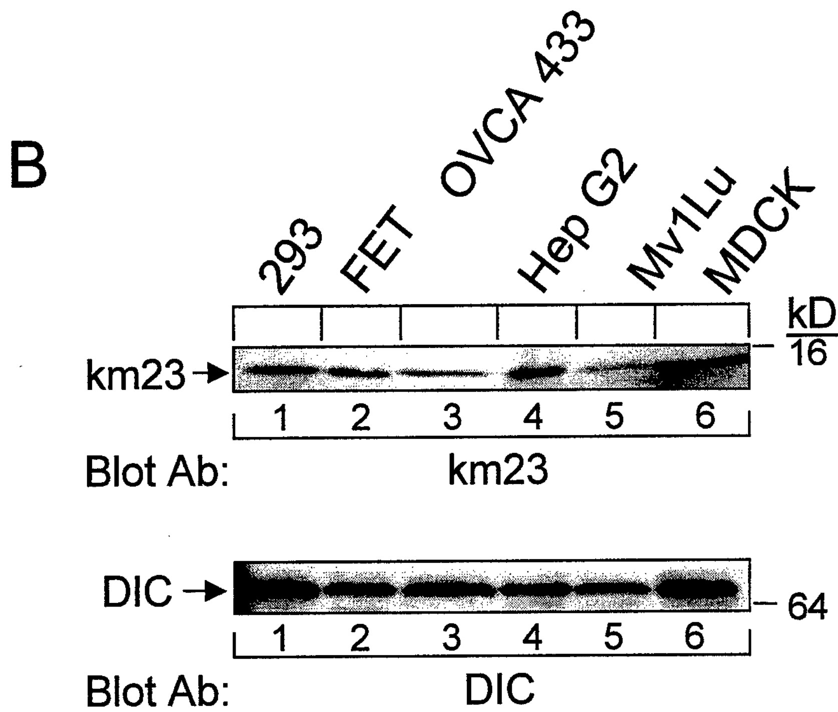


Fig. 4

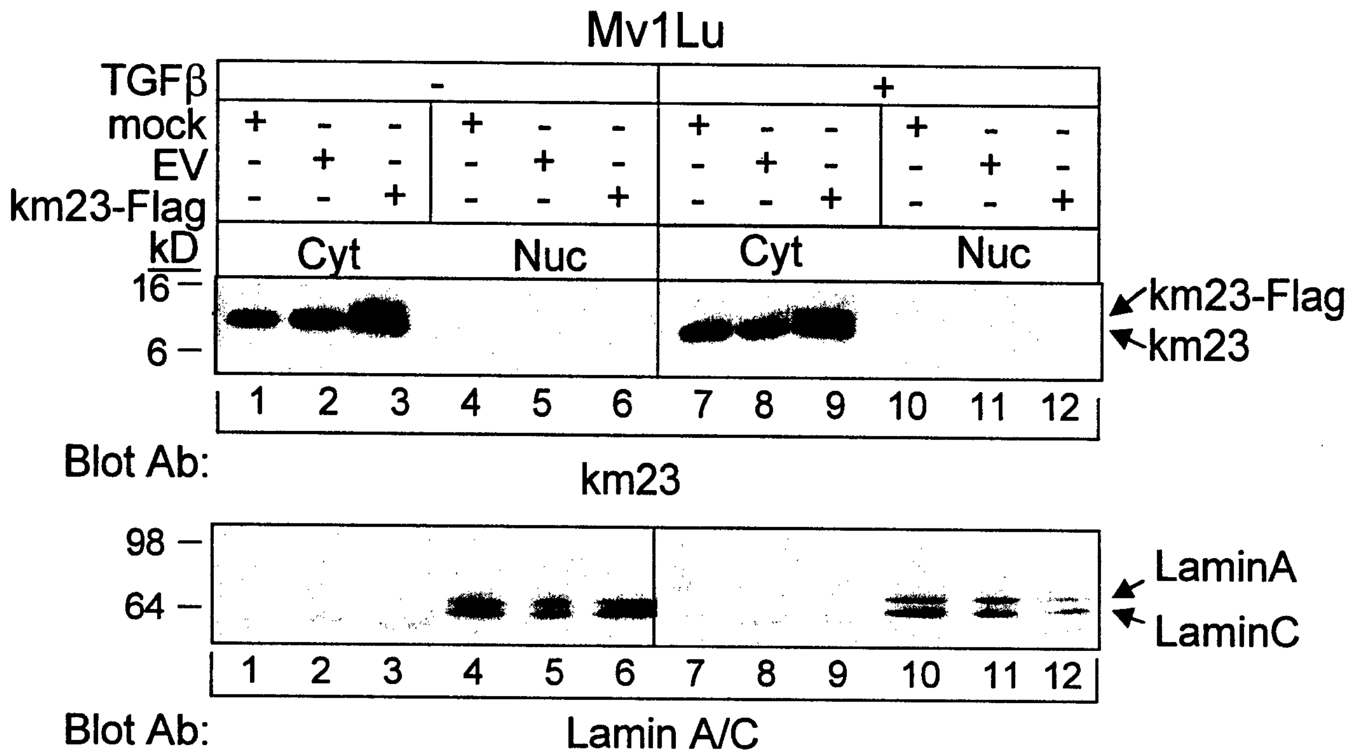


Fig. 5

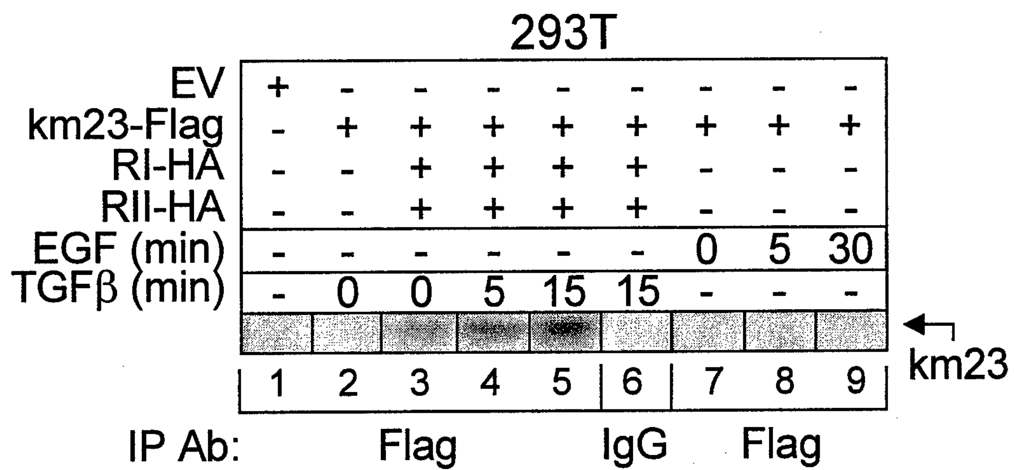
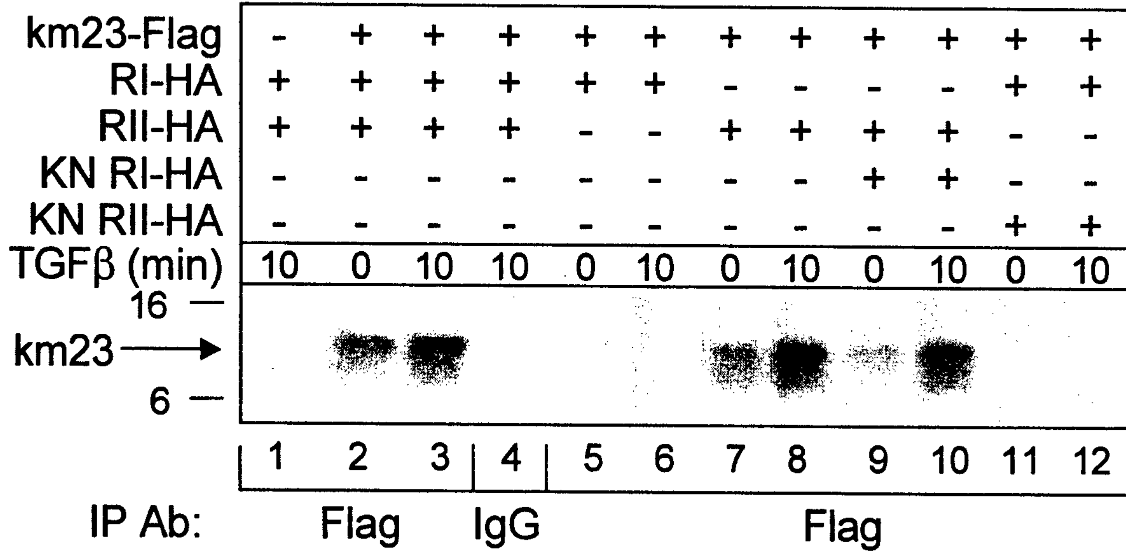


Fig. 6



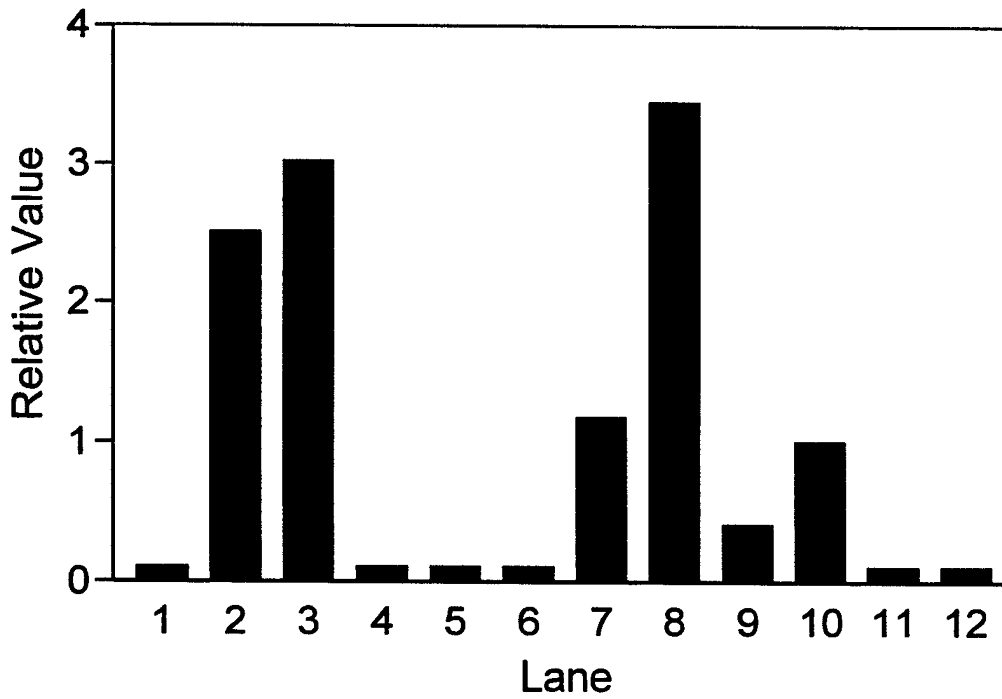
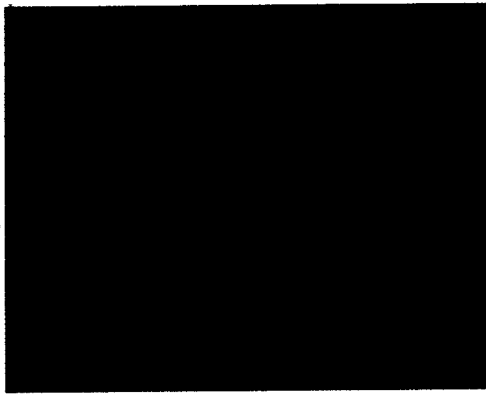
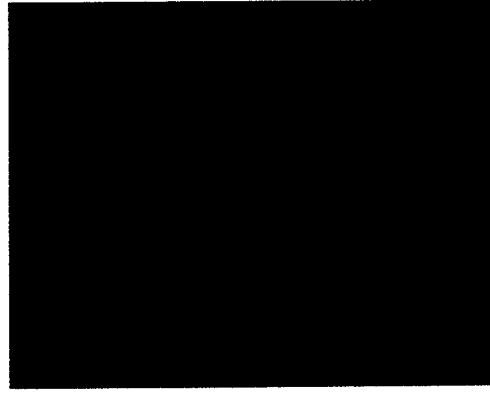


Fig.7

A. -TGFβ

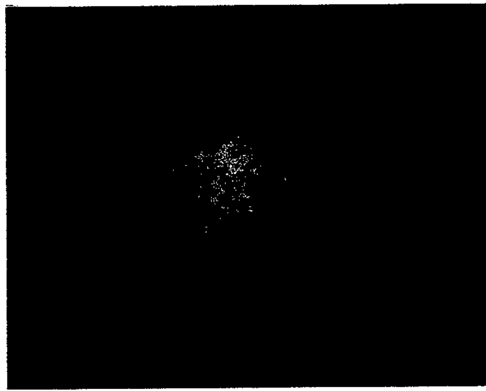
km23

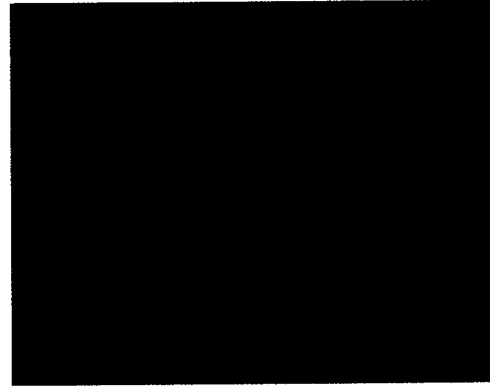




Preimmuno serum

Smad2





IgG

merge

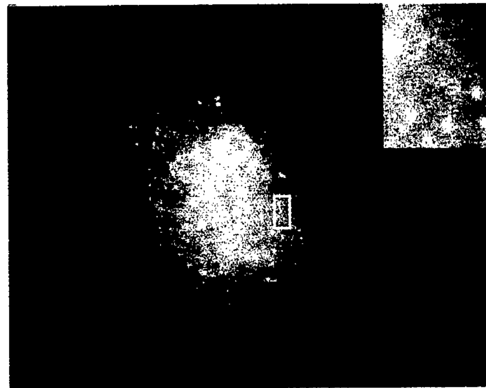


Fig.7

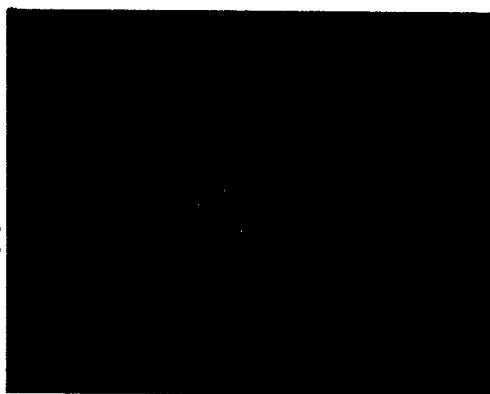
B.

+TGFβ 2min

km23

Preimmuno serum

Smad2



IgG

merge

Fig.7

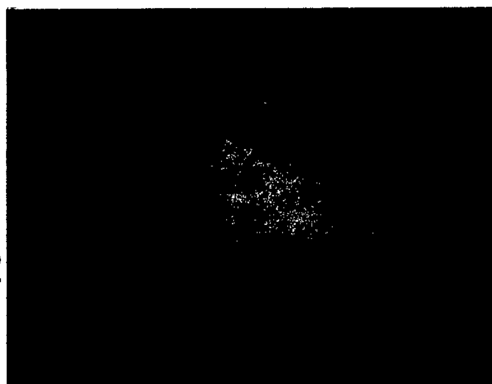
C.

+TGFβ 5min

km23

Preimmuno serum

Smad2



IgG

merge

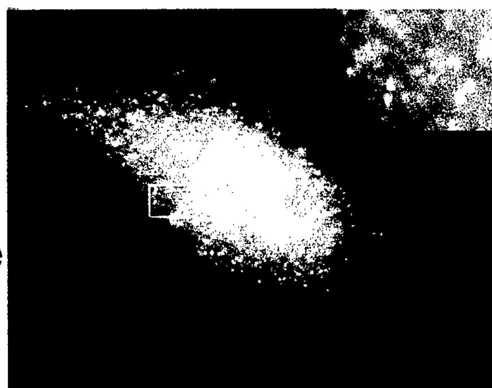
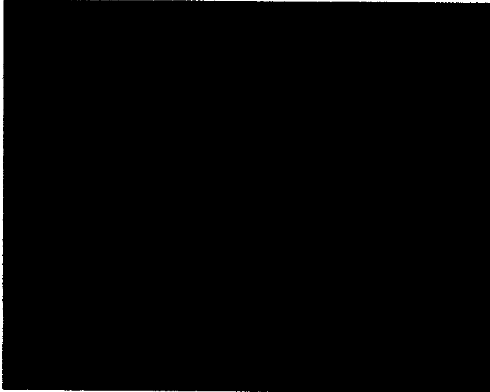


Fig.7

D.

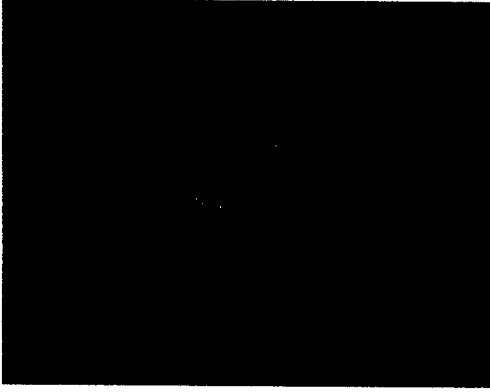
+TGFβ15min

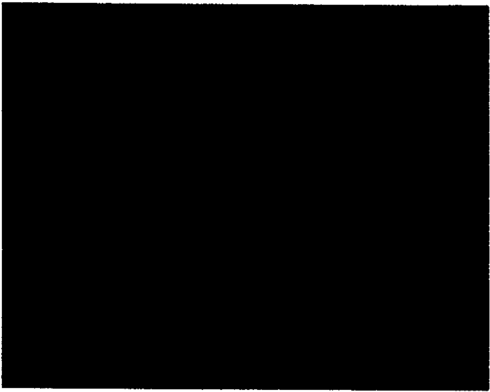
km23



Preimmuno serum

Smad2





IgG

merge

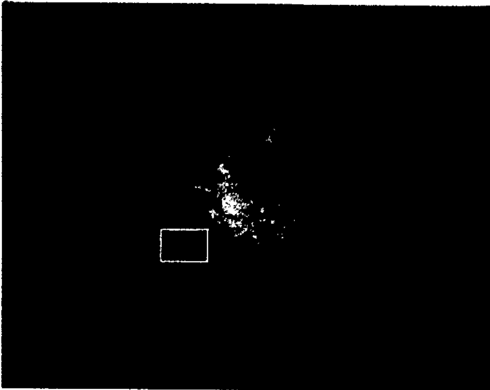
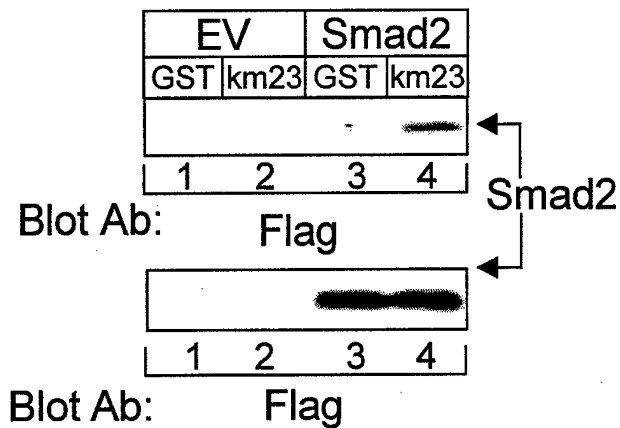


Fig. 8

A



B

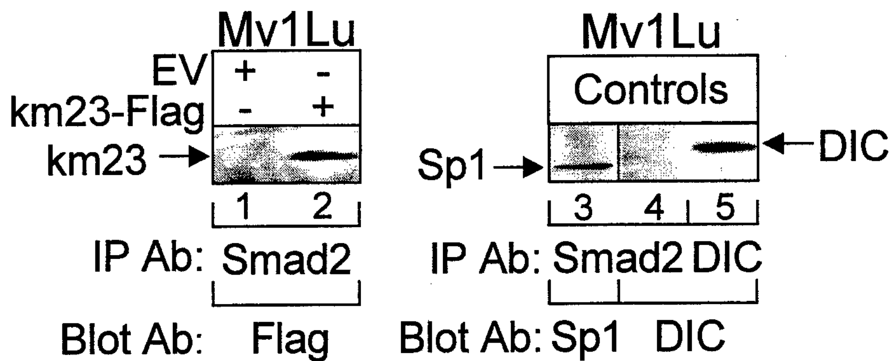
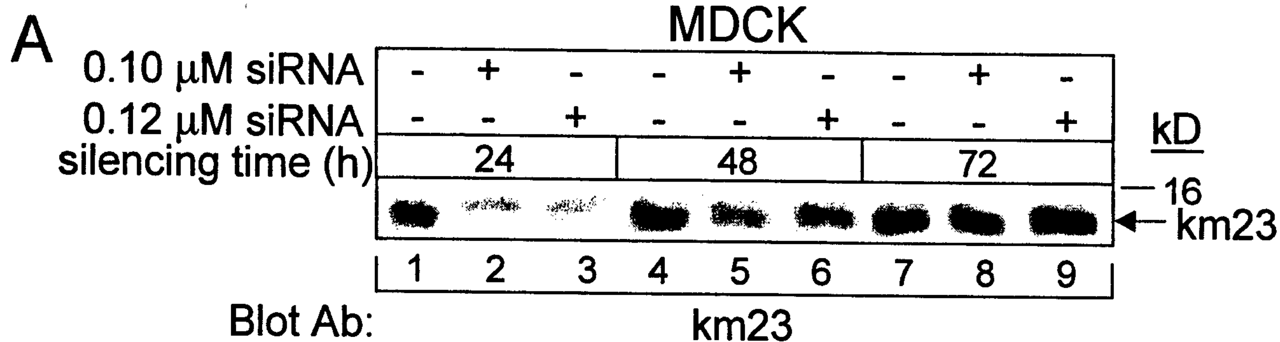
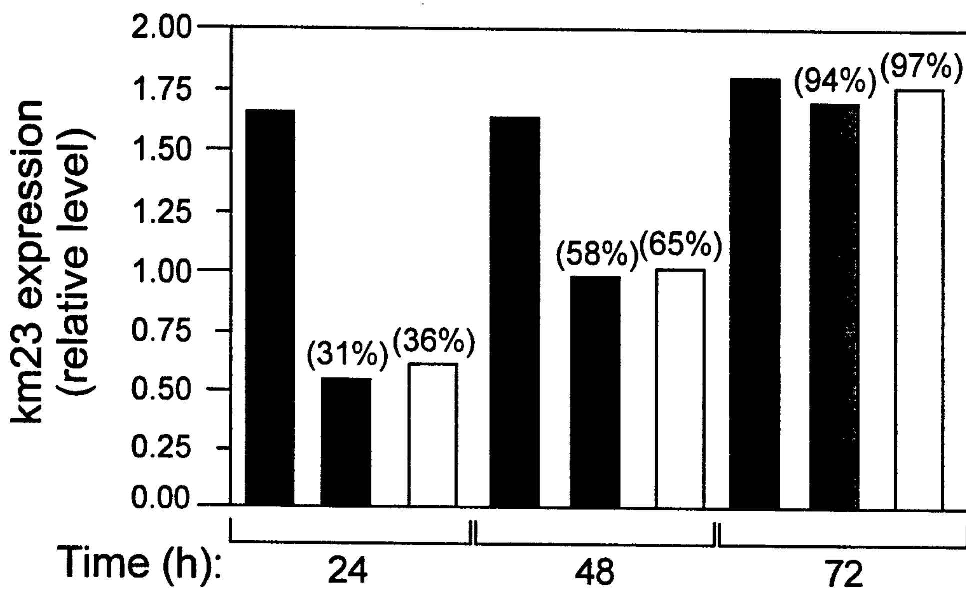


Fig. 9





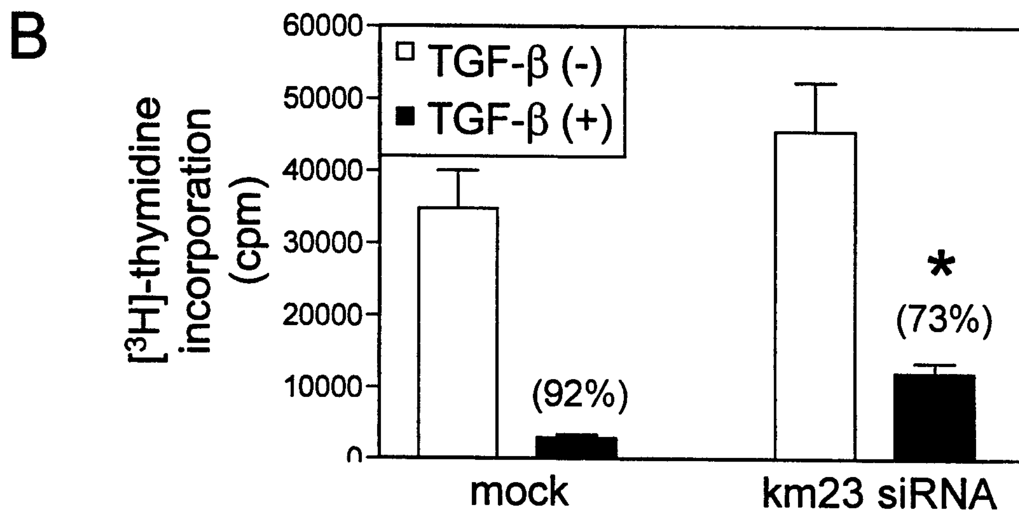


Table I: Comparison of hkm23-1 to closely related sequences

Homologue	Species	% homology	% identity	% similarity
rkm23-1	*Rattus norvegicus*	91	96	98
mkm23-1	*Mus musculus*	90	95	98
hkm23-2	*Homo sapiens*	70	77	91
rkm23-2	*Rattus norvegicus*	71	76	91
mkm23-2	*Mus musculus*	71	76	91

CURRICULUM VITAE

NAME: **KATHLEEN M. MULDER, Ph.D.** **TITLE:** Professor with tenure

Website:
http://www.hmc.psu.edu/depts/cgibin/faculty.pl?name=mulder.html&folder=faculty&code=pharmacologydept

ADDRESS:

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Work: Dept. of Pharmacology – MC H078
Penn State Univ. College of Medicine
500 University Drive
Hershey, PA 17033
(717) 531-6789
(717) 531-5013 (FAX)

Email: kmm15@psu.edu

EDUCATION:

Muhlenberg College, Allentown, PA	B.S.	1979	Natural Sciences/Math Psychology
State University of NY at Buffalo, NY	Ph.D.	1985	Pharmacology

APPOINTMENTS:

- 1980-1984 Graduate Research Assistant, SUNY AB, Buffalo, NY
- 1985-1988 Research Associate, Department of Pharmacology, Baylor College of Medicine, Houston, TX
- 1988-1991 Assistant Professor (tenure-track), Department of Pharmacology, Baylor College of Medicine, Houston, TX
- 1990-1991 Consultant, Bristol-Myers Squibb, Wallingford, CT
- 1991-1993 Assistant Professor (tenure-track), Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA
- 1993-1998 Tenured Associate Professor, Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA
- 1998-Pres Tenured Professor, Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA

AWARDS:

- 1980-1983 NIH Pre-doctoral Training Grant recipient
- 1984 First Prize - Sigma XI Research Award Competition
- 1989-1994 NIH PHS First Independent Research Support and Transition Award: 5.2 percentile
- 1993-1998 NIH Research Career Development Award (NCI)

CURRENT RESEARCH GRANT SUPPORT (P.I. on all):

R01 CA90765-02; Mechanisms of TGFβ Production in Human Cancer Cells;
$1,200,000 (total direct costs); 04/01/01-03/31/06; 1.8 percentile

R01 CA92889-01; Role of TGFβ in Microtubule Dynamics;
$1,125,000 (total direct costs); 07/01/01-06/30/06

RO1 CA 100239-01; Role of km23 in ovarian cancer; $1,250,000 (total direct costs);
04/01/03-03/31/08; 1.7 percentile

DOD Ovarian cancer, Development of km23-based diagnostics and therapeutics;
Recommended for funding 12/16/02 (98 percentile; 1.3 priority score); $526,875
04/15/03-04/14/06

CURRENT RESEARCH GRANT SUPPORT (Consultant)

R01 HD33852-05 (McAllister, PI); 17α-Hydroxylase Expression in Human Ovarian Cells;
$175,000 (total direct costs); 07/01/01-06/30/06

PENDING GRANTS (P.I. on all)

Equipment Supplement: Tumor-Host Interactions; $500,000 (total direct costs); 09/03-09/04

NIH Structural Studies Supplement: High-Resolution NMR Structure of km23 Proteins;
$40,000 (total direct costs); 11/03-10/04

Grants Completed (P. I. on all):

BC 996476 (DOD Concept Award); Mechanism of Ras Activation by TGFβ;
$50,000 (total direct costs); 05/01-06/03.

R01 CA51452-12; Mechanisms of TGFβ Signaling;
$627,683 (total direct costs – last 3 years only); 04/89-03/03.

R01 CA68444 Intracellular Mediators of TGFβ Effects;
$545,880 (total direct costs); 04/96-04/01.

R01 CA54816 Role of MAPK's in TGFβ-Mediated Cellular Responses;
$632,878 (total direct costs- 3 years only); 07/92 - 06/01.

5 K04 CA59552 Mediators of Growth Factor Independence in Colon Cancer.
$345,573 (total direct costs); 05/93-04/98.
Research Career Development Award, National Cancer Institute

PATENT PENDING: Control of TGFβ signaling by km23 superfamily members

PEER REVIEW SERVICE

Grants:

NCDDG Grant Review - 10/89
NIH Program Grant Site Visits- 6/90, 3/94, 9/95
NIH R13 Grant Review - 12/90, 12/96
VA Merit Review Applic. - 3/92, 9/92, 8/95
Institutional Research Grant Review Committee - 3/93-present
Israel Science Foundation - 5/93
National Cancer Institute of Canada, Program Project Site Visit - 10/93
American Cancer Society, Local Chapter - 10/94-present
Active Member, NIH Special Study Section SSS2 - 11/94-11/96
NIH R03/RFA Study Section - 3/95
USAMRMC Breast Cancer Reviews - 11/95, 9/96, 9/97, 9/98, 8/99
North Carolina Biotech Center - 4/96
Ad Hoc Member, Metabolic Pathology Study Section, NIH - 6/96-3/97
National Science Foundation - 9/97
Regular Member, Metabolic Pathology Study Section, NIH - 6/97-7/01
Reviewer, NCI Phased Application Awards/SBIR-STTR Initiative,
  Cancer Prognosis & Prediction, 10/02
Consultant, for NCI Phased Application Awards, SBIR-STTR Initiative,
  Cancer Prognosis & Prediction, 10/02
Reviewer, Tobacco Settlement Research Funds RFA, PSCOM, 4/03

Manuscripts:

Reviewer for:
American Journal of Pathology
American Journal of Physiology
Cancer Chemotherapy and Pharmacology
Cancer Communications/Oncology Research
Cancer Detection and Prevention
Cancer Research
Cell Growth and Differentiation
EMBO Journal
Experimental Cell Research
Gastroenterology
International Journal of Cancer
Journal of Biological Chemistry
Journal of Cellular Physiology
Journal of Cell Science
Journal of Clinical Endocrinology and Metabolism
Molecular Biology of the Cell
Molecular Pharmacology
Oncogene

Editorial Board Member: **The Journal of Biological Chemistry**, 07/01/01-06/30/06

PROFESSIONAL AFFILIATIONS

American Association for Cancer Research
American Association for the Advancement of Science
Women in Cancer Research
Federation of American Societies for Experimental Biology
American Society for Biochemistry and Molecular Biology
American Society for Cell Biology

INVITED SPEAKER

Albert Einstein School of Medicine, Bronx, NY	1988
National Institute of Environmental Health Sciences, Chapel Hill, NC	1988
Bristol-Myers Co., Wallingford, CT	1988
University of Maryland, Baltimore, MD	1989
University of Tennessee, Memphis, TN	1989
Bristol-Myers Co., Wallingford, CT	1989
Northwestern University School of Medicine, Chicago, IL	1990
M.D. Anderson Cancer Center, Houston, TX	1990
University of Connecticut Health Center, Farmington, CT	1990
University of Alabama College of Medicine, Birmingham, AL	1990
Triton Biosciences, Oakland, CA	1990
Univ. of North Dakota School of Medicine, Grand Forks, ND	1990
Dartmouth Medical School, Hanover, NH	1990
Cell and Molecular Biology Program, Penn State College of Medicine, Hershey, PA	1991
Medical College of Wisconsin Cancer Center, Milwaukee, WI	1992
Case Western Reserve University, Cleveland, OH	1992
Michigan Cancer Foundation, Detroit, MI	1992
Medical College of Ohio, Toledo, OH	1992
Chicago Medical School, North Chicago, IL	1992
Cellular and Molecular Physiology, Penn State College of Medicine, Hershey, PA	1992
Division of Endocrinology, Department of Medicine, Penn State College of Medicine, Hershey, PA	1992
Instituto Nationale per la ricerca Sul Cancro, Genova, Italy	1992
Bloomsburg University, Bloomsburg, PA	1992
Roswell Park Cancer Institute, Buffalo, NY	1993
Symposium of the American Gastroenterological Assoc., Vail, CO	1994
Bristol Myers Squibb, Princeton, NJ	1994
Fifth International Congress on Anti-Cancer Chemotherapy, Paris, France	1995
Division of Endocrinology, Department of Medicine, Penn State College of Medicine, Hershey, PA	1995
Department of Biological Chemistry and Molecular Biology, Penn State College of Medicine, Hershey, PA	1995
Cancer Research Center of Hawaii, Honolulu, HI	1995
Bowman Gray Medical School of Wake Forest Univ., Winston-Salem, NC	1995

University of Miami Medical Center, Miami, FL	1995
Division of Endocrinology, Department of Medicine, Penn State College of Medicine, Hershey, PA	1996
Department of Cellular and Molecular Physiology, Penn State College of Medicine, Hershey, PA	1996
AACR Special Conference: Cell Signaling and Cancer Treatment, Telfs-Buchen, Austria	1997
Penn State University Cancer Center, Hershey, PA	1997
National Cancer Institute, Biological Carcinogenesis and Development Program, Frederick, MD	1997
Onyx Pharmaceuticals, Inc., Richmond, CA	1997
Indiana University Cancer Center, Indianapolis, IN	1997
Creative BioMolecules, Inc, Boston, MA	1998
Baylor College of Medicine, Houston, TX	1998
Department of Microbiology & Immunology, Penn State College of Medicine, Hershey, PA	1998
University of Arizona at Tucson, Tucson, AZ	1998
Division of Endocrinology, Department of Medicine, Pennsylvania State University College of Medicine, Hershey, PA	1999
Laboratory of Cell Regulation and Carcinogenesis, NIH, Bethesda, MD	1999
Lombardi Cancer Center, Georgetown University Medical Center, Washington, D.C.	1999
TGFβ: Biological Mechanisms and Clinical Applications; 3rd Inter. Conf., National Institutes of Health, Bethesda, MD	1999
Session Chair, AACR Mini-symposium, Cell Signaling thru the Cytoplasm	2000
Program in Genetics, Pennsylvania State University, University Park, PA	2000
MD Anderson Cancer Center, Houston, TX	2000
Division of Surgery, Department of Medicine, Penn State College of Medicine, Hershey, PA	2000
MCP - Hahnemann School of Medicine, Philadelphia, PA	2001
Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA	2001
11th International Conference on Second Messengers and Phosphoproteins: 2nd Messengers, Melbourne, Australia	2001
Southern Research Institute, Birmingham, AL	2001
Virginia Commonwealth University, Richmond, VA	2001
Medical College of Ohio, Toledo, OH	2001
University of Alabama at Birmingham Cancer Center	2001
FASEB Summer Research Conference, Tucson, AZ	2001
MD Anderson Cancer Center, Houston, TX	2001
Duke University School of Medicine, Durham, NC	2001
Penn State University, University Park, PA	2002
Penn State Cancer Institute, Hershey, PA	2002
University of Texas, San Antonio, TX	2003
AACR Annual Meeting, Toronto, CA	2003

TEACHING EXPERIENCE

Graduate Courses - *Division of Molecular Biology, Department of Pharmacology Baylor College of Medicine, 1988-1990*

Polypeptide Growth Factors and Their Receptors: Transforming Growth Factor-beta (TGF-β)-Structure and Cellular Effects, Receptor/Ligand Interaction and Associated Events

Oncogenes and Growth Control: Nuclear Oncogenes and Regulatory Factors, Regulation of c-myc Expression in Normal and Transformed Cells

Graduate Courses - *Penn State University College of Medicine*

Pharmacology 501: Tyrosine Kinase Receptor Signaling, Serine/Threonine Kinase Receptor Signaling

Pharmacology 502: Growth Factors, Oncogenes, and Cell Cycle in Cancer

Molecular Pharmacology Techniques

Reproductive Hormones: Inhibins, Activins, and Follistatins: Endocrine/Paracrine/Autocrine Regulators of the Reproductive System

The Biology of Neoplasia: Growth Factor/Cytokine Signal Transduction Pathways

Cell Communication: Growth Factors, Receptors, and Signal Transduction

Core Cell Biology, CMBIO 540: Actin Microfilaments
Microtubules
Intermediate Filaments
Serine/Threonine Receptor Kinase Signaling

Medical Pharmacology Course

Teaching Committee, Baylor College of Medicine, 1989-1991
Lectures: Adrenergic Drugs, Penn State Univ. College of Medicine, 1991-1993

INSTITUTIONAL COMMITTEE SERVICE

Student Affairs Committee, Baylor College of Medicine, 1988-1991
Medical Pharmacology Teaching Committee, Baylor College of Medicine, 1989-1991
Summer Medical and Research Training Program, Baylor College of Medicine, 1989-1991
Graduate Faculty, Penn State University College of Medicine, 1991-present
Library Advisory Committee, Penn State University College of Medicine, 1991-present
Medical Student Selection Committee, Penn State University College of Medicine, 1992-1995
Pharmacology Faculty Search Committee, Penn State University College of Medicine, 1993-present
Medical Student Curriculum Committee, Penn State University College of Medicine, 1995-1998
Summer Symposium in Molecular Biology, Penn State University, University Park, 1996-present
Faculty Member, Life Sciences Consortium, Graduate Program in Integrative Biosciences, Penn State University, College of Medicine, 1996-present
Faculty Member, Penn State University Graduate Programs:
Pharmacology, 1991-present
Cell and Molecular Biology, 1992-present
MD/PhD Program, 1995-present
Molecular Medicine, 1996-present

Chemical Biology, 1999-present
Cellular and Molecular Mechanisms of Toxicity/Molecular Toxicology, 1999-present
Genetics, 1999-present

Chair - Appointment, Promotion, Tenure Committee, Dept. of Pharmacology, PSU College of Medicine, 2001- present
Co-Chair, Seminar Committee, Dept. of Pharmacology, PSCOM, 2001-present
Member, Education Committee, PSCOM Cancer Institute, 2001-present
Member, Search Committee for Director, PSCOM Cancer Institute, 2002-2003
Member, Search Committee for Director, Gittlen Cancer Research Institute, 2003-present

NEWS RELEASES ABOUT RESEARCH FROM THE MULDER LAB

Date	Source	Title
09/04/2002	Penn State Newswire	Study finds new target for development of anti-cancer drugs
09/04/2002	Penn State College of Medicine News Release	Study finds new target for development of anti-cancer drugs: Protein found effective in nearly half of human cancer tissues
09/11/2002	Hummelstown Sun	Anti-Cancer Target
09/12/2002	Espicom Business Intelligence	New target for anticancer therapies identified
09/27/2002	Central Penn Business Journal	Help for Cancer

PUBLICATIONS

1. Roth, J.A., Eddy, B.J., Pearce, B., and **Mulder, K.M.**: Phenylhydrazine: Selective inhibition of human brain type B monoamine oxidase. *Biochem. Pharmacol.,* 30:945-950, 1981.
2. **Mulder, K.M.**, and Kostyniak, P.J.: Stabilization of glutathione in urine and plasma: Relevance to urinary metal excretion studies. *J. Anal. Toxicol.,* 9:31-35, 1985.
3. **Mulder, K.M.**, and Kostyniak, P.J.: Involvement of glutathione in the enhanced renal excretion of methyl mercury in CFW Swiss mice. *Tox. Appl. Pharmacol.,* 78:451-457, 1985.
4. **Mulder, K.M.**, and Kostyniak, P.J.: Effect of L-(alpha-S,5S)-alpha-amino-3-dichloro-4,5-dihydro-5-isoxazoleacetic acid on urinary excretion of methyl mercury in the mouse. J. *Pharmacol. Expt. Ther.,* 234:156-160, 1985.
5. **Mulder, K.M.**, Levine, A.E., Hernandez, X., McKnight, M.K., Brattain, D.E., and Brattain, M.G.: Modulation of c-myc by transforming growth factor-β in human colon carcinoma cells. *Biochem. Biophys. Res. Commun.,* 150:711-716, 1988.

6. **Mulder, K.M.**, and Brattain, M.G.: Alterations in c-myc expression in relation to maturational status of human colon carcinoma cells. *Int. J. Cancer,* 42:64-70, 1988.
7. **Mulder, K.M.**, McKnight, M.K., Hoosein, N.M., Levine, A.E., Hernandez, X., Brattain, D.E., and Brattain, M.G.: Characterization of TGF-β-resistant subclones isolated from a TGF-β-sensitive human colon carcinoma cell line. *Cancer Res.,* 48:7120-7125, 1988.
8. **Mulder, K.M.**, and Brattain, M.G.: Continuous maintenance of transformed fibroblasts under reduced serum conditions: Utility as a model system for investigating growth factor-specific effects in non-quiescent cells. *J. Cell. Physiol.,* 138:450-458, 1989.
9. Hoosein, N.M., McKnight, M.K., Levine, A.E., **Mulder, K.M.**, Childress, K.E., Brattain, D.E., and Brattain, M.G.: Differential sensitivity of subclasses of human colon carcinoma cell lines to the growth inhibitory effects of transforming growth factor-β. *Exp. Cell Res.,* 181:442-453, 1989.
10. **Mulder, K.M.**, and Brattain, M.G.: Growth factor expression and response in human colon carcinoma cells: In: *The Cell and Molecular Biology of Colon Cancer* (Augenlicht, L., ed.), CRC Press: Boca Raton, FL, pp. 45-67, 1989.
11. **Mulder, K.M.**, Levine, A.E., and Hinshaw, X.H.: Up-regulation of c-myc in a transformed cell line approaching stationary phase growth in culture. *Cancer Res.,* 49:2320-2326, 1989.
12. **Mulder, K.M.**, and Brattain, M.G.: Effects of growth stimulatory factors on mitogenicity and c-myc expression in poorly-differentiated and well-differentiated human colon carcinoma cells. *Mol. Endocrinol.,* 3:1215-1222, 1989.
13. **Mulder, K.M.** and Childress-Fields, K.E.: Characterization of a serum-free culture system comparing growth factor requirements of transformed and untransformed cells. Exp. Cell Res., 188:254-261, 1990.
14. **Mulder, K.M.**, Humphrey, L.E., Choi, H.-G., Childress-Fields, K.E., and Brattain, M.G.: Evidence for c-myc in the signaling pathway for TGF-β in well-differentiated human colon carcinoma cells. *J. Cell. Physiol.,* 145:501-507, 1990.
15. **Mulder, K.M.**, Zhong, W., Choi, H.-G., Humphrey, L.E., and Brattain, M.G.: Inhibitory effects of transforming growth factor-β_1 on mitogenic response, transforming growth factor-α, and c-myc in well-differentiated human colon carcinoma cells. *Cancer Res.,* 50:7581-7586, 1990.
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