Requirement for the Dynein Light Chain km23-1 in a Smad2-dependent Transforming Growth Factor-β Signaling Pathway

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We have identified km23-1 as a novel transforming growth factor-β (TGFβ) receptor (TβR)-interacting protein that is also a light chain of the motor protein dynein (dynein light chain). Herein, we demonstrate by sucrose gradient analyses that, in the presence of TGFβ but not in the absence, km23-1 was present in early endosomes with the TβRs. Further, confocal microscopy studies indicate that endogenous km23-1 was co-localized with endogenous Smad2 at early times after TGFβ treatment, prior to Smad2 translocation to the nucleus. In addition, immunoprecipitation/blot analyses showed that TGFβ regulated the interaction between endogenous km23-1 and endogenous Smad2 in vivo. Blockade of km23-1 using a small interfering RNA approach resulted in a reduction in both total intracellular Smad2 levels and in nuclear levels of phosphorylated Smad2 after TGFβ treatment. This decrease was reversed by lactacystin, a specific inhibitor of the 26 S proteasome, suggesting that knockdown of km23-1 causes proteasomal degradation of phosphorylated (i.e. activated) Smad2. Blockade of km23-1 also resulted in a reduction in TGFβ/Smad2-dependent ARE-Luc transcriptional activity, which was rescued by a km23-1 small interfering RNA-resistant construct. In contrast, a reduction in TGFβ/Smad3-dependent SBE2-Luc transcriptional activity did not occur under similar conditions. Furthermore, overexpression of the dynactin subunit dynamitin, which is known to disrupt dynein-mediated intracellular transport, blocked TGFβ-stimulated nuclear translocation of Smad2. Collectively, our findings indicate for the first time that a dynein light chain is required for a Smad2-dependent TGFβ signaling pathway.

Cytoplasmic dynein is a motor complex that transports membrane vesicles and diverse motor cargoes along microtubules (MTs) in a retrograde manner (1–5). This motor is composed of several subunits, including the dynein intermediate chains (DICs) and the dynein light chains (DLCs) known to be important for cargo binding (1–5). In addition, most cargoes also require the multisubunit complex dynactin for cytoplasmic dynein motor activity, at least in eukaryotes (6–8).

Among the many dynein subunits and associated proteins important for cargo movement within the cell, the DLCs often function as “motor receptors,” providing key links between the motor machinery and the cargo, either directly or through associated proteins (8–11). The three classes of cytoplasmic DLCs that have been identified in mammals to date are LC8 (DYNLL1), Tctex-1/rp3 (DYNLT), and km23/LC7/roadblock (DYNLRB) (1–5, 12–15). In addition to binding to the DIC at distinct regions (16), DLCs have been shown to directly interact with a number of proteins to exert diverse functions. In this regard, we first described the DLC km23-1 as a novel TβR-interacting protein (13, 17), also termed mLC7-1 (13), Robl1 (18), DNL2CA (19), and DYNLRB1 (15). The fact that km23-1 is a DLC, as well as a TβR-interacting protein, suggests that it may function as a motor receptor for the transport of TGFβ signaling components intracellularly (13).

TGFβ is the prototype of a large family of structurally related growth and differentiation factors that initiates its signals from a receptor complex consisting of TGFβ RI (TβRI) and TGFβ RII (TβRII) serine/threonine kinase receptors (20–24). Activated TβRII recruits, phosphorylates, and activates TβRI. Then, the activated receptor complex can phosphorylate Smads 2 and 3, and these receptor-activated Smads (RSmads) then form a complex with Smad4. The TGFβ-activated, heteromeric Smad complexes are translocated to the nucleus, where they induce or repress transcription of defined genes (20, 24–27). Additional data indicate that the interactions among TβRs, Smads, adaptor/scaffolding proteins, and cytoskeletal elements represent important regulatory mechanisms in TGFβ signaling (26, 28).

We have shown that TβRII is absolutely required for phosphorylation of the DLC km23-1, as well as for the recruitment of km23-1 to the rest of the dynein motor through the DIC (13). Further, km23-1 undergoes rapid phosphorylation on serine residues after TβR activation, in keeping with the kinase specificity of the TβRs (13). Moreover, specific mutants of km23-1 block km23-1 binding to the DIC and disrupt TGFβ-mediated transcriptional events (29, 30). In addition, consistent with a...
role for km23-1 in TGFβ signaling, small interfering RNA (siRNA) blockade of km23-1 expression resulted in a decrease in specific TGFβ-mediated cellular responses, including an induction of fibronectin expression and an inhibition of cell cycle progression (31).

Because we have shown that km23-1 is required for mediating specific TGFβ responses, and because it is well established that the Smads are key TGFβ signaling components, here we investigated the role of km23-1 in controlling Smad compartmentalization and transcriptional activation. We provide the first evidence that TGFβ can induce the interaction of endogenous km23-1 with endogenous Smad2. Further, we show that endogenous km23-1 and endogenous Smad2 are co-localized in a TGFβ- and time-dependent manner, prior to Smad2 translocation to the nucleus. Endogenous km23-1 was also localized in early endosomal compartments with the TβRIs after TGFβ treatment. siRNA-specific blockade of km23-1 resulted in a depletion of intracellular Smad2, which was partially blocked by the proteasomal inhibitor lactacystin, suggesting that 26 S proteasomal degradation of Smad2 can occur in the absence of km23-1. In keeping with these results, blockade of km23-1 also reduced TGFβ/Smad2-dependent transcriptional regulation. Finally, we demonstrate for the first time that dynein-dependent intracellular events are required for Smad2 nuclear translocation after TGFβ treatment, because overexpression of dynactin inhibited these effects. Thus, our results demonstrate for the first time that the DLC km23-1, as well as dynein motor activity, are required for a Smad2-dependent TGFβ signaling pathway.

MATERIALS AND METHODS

Reagents—The mouse IgG antibody (Ab) was from Sigma-Aldrich. The anti-DIC monoclonal Ab (MAB1618) was from Chemicon (Temecula, CA). The rabbit IgG, the rabbit TβRII Ab (SC-220), the rabbit TβRI Ab (SC-389), the Lamin A/C Ab (SC-7293), and the proliferating cell nuclear antigen (PC10) Ab (SC-56) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TGFβ1 was purchased from R & D Systems (Minneapolis, MN). The rabbit Smad2 Ab (51–1300) was from Zymed Laboratories Inc. (South San Francisco, CA). The mouse anti-Smad2 (610843) and the mouse EEA1 Ab (610457) were from BD Biosciences Transduction Laboratories (Palo Alto, CA). The FuGENE 6 transfection reagent was from Roche Applied Science. The Dual-Luciferase Reporter Assay System (E1960) was purchased from Promega (Madison, WI). Lactactystin was from Calbiochem (San Diego, CA).

Antibody Production—The rabbit polyclonal km23-1 antiserum used for the immunofluorescence studies was prepared according to the following sequence: MAEVEETLKLQSQK (corresponding to amino acids 1–15 of human km23-1) (AnaSpec, San Jose, CA). The company also provided pre-immune serum. The rabbit km23-1 anti-serum against amino acids 27–43 of human km23-1 (hkm23-1-(27–43)-w) used for Western blotting analysis was prepared as described previously (31).

Cell Culture—Mv1Lu (CCL-64) cells were purchased from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Madin-Darby canine kidney cells (CCL-34) were also obtained from ATCC and were grown in Minimum Essential Medium-α supplemented with 10% fetal bovine serum. Cultures were routinely screened for mycoplasma using Hoechst 33258 staining (13).

Transient Transfections, Immunoprecipitation, and Western Blots—These were performed essentially as described previously (13, 31–35).

Sucrose Gradient Assays—Madin-Darby canine kidney cells were plated at 1.5 × 10⁴ cells/cm² in 10-cm plates. Twenty-four hours after plating, the medium was replaced with serum-free minimum essential-α medium. Thirty minutes after incubation, Madin-Darby canine kidney cells were cultured in the absence or presence of TGFβ (5 ng/ml) for 5 min (ten 10-cm plates each). Early-endosome-containing fractions were then prepared as described previously (36).

siRNAs—km23-1 siRNA and the negative control (NC siRNA) were prepared as described previously (31). The siRNA was designed in a region of km23-1 where the mink and human forms do not differ.

Immunofluorescence Microscopy Analyses—For km23-1 and Smad2 co-localization experiments, Mv1Lu cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature, and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 5 min. Subsequently, these cells were incubated with km23-1 rabbit anti-serum (1:200) and 5 μg/ml anti-Smad2 monoclonal Ab for 1 h, respectively. The bound primary antibodies were visualized with 2 μg/ml Alexa Fluor 488-conjugated goat anti-rabbit IgG (green) and Cy3-conjugated goat anti-mouse IgG (red). Co-localization of km23-1 and Smad2 is indicated by a yellow color (merge). Images were collected with a Leica TCS SP2 AOS confocal microscope. The images in supplemental Fig. S1 were de-convoluted using Huygens Essential software from Scientific Volume Imaging (Exton, PA). Co-localization of km23-1 and Smad2 puncta was quantified using the co-localization function in Image Pro Plus 4.1 software (Media Cybernetics, Inc., Silver Spring, MD). A similar approach has been used to quantify the co-localization of other proteins as described previously (37). At least five cells in each group from each double-labeled experiment were analyzed for co-localization of km23-1 and Smad2. For the immunofluorescence analyses to study the effects of siRNAs on nuclear expression and translocation of Smad2 by TGFβ, the cells were fixed and permeabilized as for the co-localization studies. Subsequently, these cells were incubated with 5 μg/ml anti-Smad2 monoclonal Ab for 2 h, and then the bound Ab was visualized with 2 μg/ml Alexa 594 goat anti-mouse IgG. Immunofluorescence images were captured using a Nikon Diaphot microscope with a Retiga 1300 charge-coupled device camera (BioVision Technologies, Inc., Exton, PA) running IPLab v3.6.3 software (Scanalytics, Inc., Fairfax, VA). One hundred green fluorescence protein (GFP)-positive cells were counted for cultures of both km23-1 siRNA-transfected and NC siRNA-transfected cells. 4',6-Diamidino-2-phenylindole staining designates individual cells. Triplicate fields are shown for each condition. For the immunofluorescence analyses to study the effects of overexpression of dynamin on nuclear translocation of Smad2 by TGFβ, the cells were analyzed as for the studies of the effects of siRNAs on nuclear
expression and translocation of Smad2, except that the cells were co-transfected with GFP and empty vector (EV) or dynamitin.

Cellular Fractionation—The NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (78833, Pierce) was used to fractionate Mv1Lu cells according to the manufacturer’s protocol.

Luciferase Reporter Assays—Mv1Lu cells were plated at 1 × 10^4 cells/cm^2 in 12-well plates. Twenty-four hours after plating, the cells were transfected with the indicated amounts of either km23-1 siRNA or NC siRNA, together with the activin-responsiveness element (ARE)-Luc and FAST-1 (38), or the Smad-binding element (SBE)_2-Luc (39). *Renilla* was used to normalize transfection efficiencies, and pCDNA3.1 was used to normalize the amount of total DNA transfected as described previously (40). Twenty-four hours after transfection, the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium. 1 h after incubation, Mv1Lu cells were cultured in the absence or presence of TGFβ (5 ng/ml) for an additional 18 h. Luciferase activity was measured using Promega’s Dual-luciferase Reporter Assay System following the manufacturer’s instructions. All assays were performed in triplicate. Data are expressed as mean ± S.E.

**km23-1 siRNA-resistant km23-1-FLAG Construct**—The wild-type FLAG-tagged km23-1 plasmid (pCMV-km23-1-FLAG) was constructed by fusing the full-length human km23-1 to the pFLAG-CMV-5a vector (Sigma) (13). To generate the km23-1 siRNA-resistant km23-1-FLAG construct (pCMV-Δkm23-1-FLAG), the pCMV-km23-1-FLAG plasmid was used as a template and was mutated by site-directed mutagenesis PCR (Stratagene) as described by Lassus et al. (41). This modified plasmid contains six silent mutations in the km23-1 siRNA target sequence, corresponding to nucleotides 251–271 of the human km23-1 coding region. The DNA sequences for pCMV-Δkm23-1-FLAG was altered from 5’-AAGACTTATTCCCTGATTGTGA-3’ to 5’-AGGATTACTTATTAATTTGTGA-3’. Compared with pCMV-km23-1-FLAG, there is no change in the amino acid sequence expressed by pCMV-Δkm23-1-FLAG.

**RESULTS**

Previous reports have shown that the early endosome pathway plays a critical role in TβR endocytosis and subsequent TGFβ signal transduction (42–46). Specifically, the TβRs are known to be internalized with Smad2 into early endosomes within minutes of TGFβ addition to cells (42, 45). Further, our previous data suggested that TβRII kinase activity was required for the ability of the DLC km23-1 to bind the dynein motor through the DIC, as well as for TGFβ responses downstream (13). Thus, the DLC km23-1 may recruit early endosomal TGFβ signaling complexes during intracellular transport and downstream effects, following TβR endocytosis. Accordingly, it was of interest to examine whether endogenous km23-1 might be co-localized with the TβRs in early endosomes after TGFβ treatment. To assess this, we performed sucrose flotation gradients to isolate endosomal compartments enriched for early endosome antigen-1 (EEA1) (36), followed by Western blotting with km23-1-specific rabbit anti-serum or TβRII/RII Abs as described under “Materials and Methods.” As expected, in the absence of TGFβ (left panel), the majority of TβRII (top panel) and TβRI (second panel) were present in fractions 6–8. However, upon TGFβ activation (right panel), the amount of TβRII and TβRI present in EEA1-enriched fractions was increased (fractions 4 and 5), consistent with a previous report (45). The bottom panel indicates the localization of EEA1, designating the fractions containing early endosomes. In terms of km23-1 localization (third panel), in the absence of TGFβ (left panel), km23-1 was not present in the early endosomal fractions (fractions 4 and 5). Instead, the majority of km23-1 accumulated in fractions 6–8. However, as early as 5 min after TGFβ addition (right panel), km23-1 was present in the EEA1-enriched early endosomal fractions (fractions 4 and 5). Moreover, even upon a much longer exposure (not shown) of the km23-1 expression data in the absence of TGFβ (Fig. 1, left), no km23-1 was detectable in the EEA1-enriched early endosomal fractions. We also noticed that total km23-1 levels were higher in the presence of TGFβ. However, using EEA1 levels as an expression control to compare two different gradient runs (42), it was clear that the EEA1 levels were also higher in the presence of TGFβ, suggesting that the increase in km23-1 was due to differential loading. Thus, the results in Fig. 1 demonstrate that endogenous km23-1 is present in early endosomes with endogenous TβRs in the presence of TGFβ.

Because Smad2 is a critical intracellular mediator of TGFβ responses (47, 48), co-localized with TβRII in EEA1-positive early endosomes after TβRs endocytosis (42, 45), it is conceivable that km23-1 might be co-localized with Smad2 in a punctate staining pattern, indicative of the co-existence of km23-1 and Smad2 in endosomal compartments. Thus, we performed immunofluorescence studies using confocal microscopy after TGFβ treatment of TGFβ-responsive Mv1Lu cells, using km23-1-specific rabbit anti-serum (left panels) or a Smad2 Ab (middle panels), respectively. As indicated in Fig. 2, in the absence of TGFβ (top panel), Smad2 (middle panel) was concentrated in cytoplasmic punctate vesicular structures, consistent with a previous report (49). Similarly, endogenous km23-1 displayed a punctate staining pattern that was present throughout the cytoplasm in the absence of TGFβ (left, top panel). Co-localization is shown as a yellow color (right, top panel), and was quantified using Image Pro Plus 4.1 software from Media.

**FIGURE 1.** km23-1 is present in the early endosomes after TGFβ treatment. Madin-Darby canine kidney cells were incubated in serum-free medium for 30 min, followed by incubation of the cells in the absence (left panel) or presence of TGFβ (5 ng/ml) for 5 min (right panel). The cells were then harvested for sucrose gradient analysis, followed by Western blot analysis, as described under “Materials and Methods.” EEA1 was used to normalize early endosome/EEA1-enriched fractions. The results shown are representative of two similar experiments.
Cybernetics, Inc. The percentages of co-localization were obtained from multiple images as described under “Materials and Methods.” In the absence of TGFβ, co-localization of km23-1 with Smad2 was ~13%. There was a slight increase in co-localization of km23-1 with Smad2 at 2 min (~18%) after TGFβ treatment. However, TGFβ treatment resulted in greater co-localization of km23-1 and Smad2 at 5 min (~28%) after TGFβ addition to Mv1Lu cells (Fig. 2, third panels). This level of co-localization was similar to that previously reported for quantitation of co-localization of other early endosome proteins (50). In addition, a partial redistribution of km23-1 and Smad2 toward the perinuclear region was observed at 5 min after TGFβ stimulation. However, once Smad2 had translocated to the nucleus by 15 min after TGFβ treatment (bottom panel), km23-1 was still localized in the cytoplasm and was no longer co-localized with Smad2. For all studies, the pre-immune serum and relevant IgG controls were negative (data not shown), confirming the specificity of the km23-1 and Smad2 Abs. High quality TIFF files for the data in Fig. 2 can be found in supplemental Fig. S1. Our immunofluorescence results obtained using confocal microscopy clearly indicate that km23-1 and Smad2 are co-localized intracellularly at early times after TGFβ treatment, prior to entry of Smad2 into the nucleus.

Next, we wished to determine whether endogenous km23-1 and endogenous Smad2 were present in the same complex after TGFβ treatment. To assess this, we performed immunoprecipitation/blot analyses in the absence or presence of TGFβ. As shown in Fig. 3, TGFβ induced a rapid interaction of endogenous km23-1 with endogenous Smad2 (lane 3, top panel). The kinetics were similar to those for km23-1 binding to the DIC (13), with some basal interaction, but with increased association at 5 min after TGFβ addition. In contrast, the association between km23-1 and Smad2 was significantly decreased at 15 min after TGFβ addition to Mv1Lu cells, at a time when Smad2 is translocated to the nucleus in these cells (lane 4, top panel). The results in this figure depict Smad2-km23-1 interactions in total cell lysates, which would include data from cells that have not yet translocated to the nucleus. Therefore, the higher interaction levels at 15 min in this figure, compared with Fig. 2, might be the result of co-localized km23-1 and Smad2 in the cytoplasm. Overall, these results are consistent with those shown in Fig. 2 regarding the kinetics for co-localization of km23-1 and Smad2. As expected, there were no bands in the IgG-negative control (lane 1, top panel). Equal loading and

FIGURE 2. TGFβ induces the co-localization of km23-1 with Smad2 prior to Smad2 nuclear translocation. Mv1Lu cells were cultured in the absence or presence of TGFβ for the indicated times, and then were fixed and permeabilized as described under “Materials and Methods.” Endogenous km23-1 was detected using rabbit km23-1 anti-serum, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (left panel). Smad2 was detected using a mouse monoclonal Smad2 Ab and Cy3-conjugated goat anti-mouse IgG (middle panel). The cells were analyzed using a Leica TCS SP2 AOBS confocal microscope at a magnification of 630 with appropriated filter sets. The merge panel shows potential co-localization of endogenous km23-1 and endogenous Smad2 (right panel). Bar = 10 μm (bottom right panel). Z = 0.35 μm. The results shown are representative of three similar experiments.

FIGURE 3. Endogenous km23-1 interacts with endogenous Smad2 in a TGFβ-dependent manner. Mv1Lu cells were incubated in serum-free medium for 1 h before addition of TGFβ (5 ng/ml) for the indicated times. Top panel, Mv1Lu cells were lysed, and immunoprecipitated (IP) using a polyclonal anti-Smad2 Ab, followed by immunoblot analysis with a hkm23-1-(27–43)-w Ab. Middle panel, the same membrane was re-blotted with a monoclonal Smad2 Ab to show equal protein expression and loading. Western blot analysis with our hkm23-1-(27–43)-w Ab demonstrates equal input km23-1 by autoradiography (third panel). Bottom panel, plot of densitometric scan of results in top panel. The results shown are representative of two similar experiments.
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to the dynein motor for intracellular transport prior to downstream effects (3, 51, 52). Further, our results in Figs. 1–3 support an association of the km23-1 DLC with Smad2-containing early endosomal signaling complexes. Thus, it was of interest to determine whether disruption of dynein motor activity by dynamitin overexpression would block Smad2 nuclear translocation after TGFβ treatment. Thus, we performed immunofluorescence studies after transiently transfecting Mv1Lu cells with dynamitin or EV in the presence of GFP. The presence of the GFP signal designates the cells that were transfected with dynamitin-myc or EV (left panels, Fig. 4). In the absence of TGFβ, both the EV-transfected, GFP-positive cells and the dynamitin-transfected, GFP-positive cells displayed the same pattern of diffuse punctate staining (data not shown). In contrast, as expected, the EV-transfected, GFP-positive cells displayed largely nuclear expression of Smad2 in response to TGFβ (top panel, Fig. 4), indicative of ligand-induced translocation of Smad2 to the nucleus. However, in the dynamitin-transfected, GFP-positive cells, Smad2 displayed a diffuse punctate staining pattern, with reduced nuclear expression of Smad2 (bottom panel, Fig. 4). As expected, untransfected (GFP-negative) cells also responded to TGFβ with significant Smad2 translocation. Our results demonstrate that disruption of the dynein motor complex blocks the ability of Smad2 to reach the nucleus after TGFβ treatment, suggesting that Smad2 intracellular transport requires dynein.

To quantify the effects of dynamitin overexpression on TGFβ-mediated nuclear translocation of Smad2 from Fig. 4, we counted 100 GFP-positive cells in cultures of either EV-transfected or dynamitin-transfected cells treated with TGFβ (5 ng/ml). Of these 100 GFP-positive cells, the cells showing nuclear translocation of Smad2 were counted as described under “Materials and Methods.” In EV-transfected cells, 94% of the GFP-positive cells displayed Smad2 nuclear translocation in response to TGFβ. As expected, 100% of the GFP-negative cells also responded to TGFβ with Smad2 nuclear translocation, whether EV or dynamitin had been co-transfected with GFP. However, only 20% of the dynamitin-transfected, GFP-positive cells still displayed Smad2 nuclear translocation after TGFβ treatment. That is, in the dynamitin-transfected cells, Smad2 displayed a diffuse punctate staining pattern in 80% of the GFP-positive cells. The percentage of disruption of intracellular transport of Smad2 caused by dynamitin overexpression was similar to that observed previously for other dynein-dependent events (53–55). Thus, quantification of our results confirmed what was observed in the immunofluorescence photographs, and suggested that disruption of dynein-dependent functions reduced intracellular transport of Smad2, thereby preventing Smad2 accumulation in the nucleus after TGFβ treatment.

The data in Fig. 4 indicate that disruption of the dynein motor complex blocked the ability of Smad2 to reach the nucleus after TGFβ treatment. If the DLC km23-1 is needed to recruit the TGFβ signaling complexes to the rest of the dynein motor, eventually leading to downstream nuclear events, it might be expected that blockade of endogenous km23-1 would block the transcriptional activation of TGFβ/Smad-dependent target genes in the nucleus. To establish that the km23-1 siRNA could block endogenous km23-1 expression, we transiently transfected Mv1Lu cells with km23-1 siRNA or NC siRNA as described previously. Western blot analysis was then performed as shown in Fig. 5A. Transfection with km23-1 siRNA (lanes 4–6) resulted in a marked decrease in endogenous km23-1 levels compared with controls (lanes 1–3). In addition, we have previously shown that km23-1 siRNA could specifically knock down km23-1 expression in two different epithelial cell lines (31).

Because km23-1 siRNA could specifically knock down endogenous km23-1 expression, we transiently transfected Mv1Lu cells with either km23-1 siRNA or NC siRNA, and then performed ARE-Lux luciferase reporter assays in the absence
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FIGURE 5. siRNA blockade of endogenous km23-1 inhibits Smad2-dependent transcription in TGF-β1 activin reporter assays but has no effect on Smad3-dependent transcriptional activation. A, Mv1Lu cells were transfected with either km23-1 siRNA or NC siRNA, and Western blot analysis of endogenous km23-1 expression was performed to examine the knockdown of endogenous km23-1. Equal loading was confirmed by blotting with an anti-DIC Ab. B, Mv1Lu cells were transfected with increasing amounts of either km23-1 siRNA or NC siRNA (0.100, 0.200, and 0.400 μg) along with 0.2 μg of ARE-lux and 0.2 μg of FAST-1. To normalize transfection efficiencies, 0.2 μg of Renilla was co-transfected as an internal control. Twenty-four hours after transfection, the medium was replaced with serum-free medium for 1 h, followed by incubation of cells in the absence (open bar) and presence (black bar) of TGFβ (5 ng/ml) for an additional 18 h. Luciferase activity was measured using the Dual Luciferase Reporter Assay System. All reporter assays were performed in triplicate. C, Mv1Lu cells were transfected with the indicated forms of either NC siRNA or km23-1 siRNA along with either pCMV-km23-1-FLAG or the km23-1 siRNA-resistant construct pCMV-Δkm23-1-FLAG. D, Mv1Lu cells were transfected with increasing amounts of either km23-1 siRNA or NC siRNA (0.125 and 0.500 μg) along with 0.2 μg of SBE2-Luc. The results shown are representative of two similar experiments.

and presence of TGFβ. The ARE-lux reporter was previously shown to be activated by TGFβ or activin in a Smad2-dependent manner (38). As shown in Fig. 5B, TGFβ induced ARE-lux activity in the EV and NC siRNA cells. In contrast, the cells transfected with km23-1 siRNA displayed a dose-dependent decrease in the fold induction of ARE-lux activity by TGFβ with increasing doses of km23-1 siRNA, relative to NC siRNA. Although 0.1 μg/well of km23-1 siRNA reduced TGFβ-inducible ARE-lux activity to levels that were 67% of control values, higher concentrations of km23-1 siRNA resulted in greater reductions in the fold induction by TGFβ (to levels that were 44 and 23% of NC siRNA values, respectively). Thus, km23-1 is required for TGFβ induction of Smad2-dependent transcriptional activity.

To confirm that km23-1 siRNA knockdown of TGFβ/Smad2-dependent ARE-Lux promoter reporter activity was specifically mediated by the km23-1 siRNA, we designed an siRNA-resistant km23-1 construct (pCMV-Δkm23-1-FLAG) and performed rescue experiments after transiently transfecting Mv1Lu cells with the indicated forms of either NC siRNA or km23-1 siRNA, along with either wild-type pCMV-km23-1-FLAG or pCMV-Δkm23-1-FLAG. The results in Fig. 5C demonstrate that the inhibition of TGFβ/Smad2-dependent ARE-Lux promoter reporter activity by km23-1 knockdown could be rescued by the pCMV-Δkm23-1-FLAG, but not by pCMV-km23-1-FLAG. Therefore, the inhibition of TGFβ/Smad2-dependent transcriptional activity was specifically mediated by the km23-1 siRNA but not by the siRNA off-target effects.

Thus far, our data have focused on the role of km23-1 in mediating Smad2-specific events. It was also of interest to determine whether blockade of km23-1 would affect Smad3-dependent TGFβ transcriptional responses. Thus, we examined the effects of km23-1 siRNA on the Smad3-specific SBE2-Luc luciferase reporter (39) in the absence and presence of TGFβ after transiently transfecting Mv1Lu cells with either km23-1 siRNA or NC siRNAs. The results in Fig. 5D demonstrate that blockade of km23-1 had no effect on Smad3-dependent transcriptional activation, indicating that km23-1 is relatively specific for TGFβ/Smad2-dependent transcriptional activation. These findings further support a specific role for km23-1 in mediating TGFβ- and Smad2-dependent TGFβ signaling events.

Because our results have shown that intracellular transport of Smad2 is dynen-in-dependent, and that blockade of km23-1 specifically inhibited TGFβ/Smad2-dependent transcriptional activity, it was of interest to determine whether blockade of endogenous km23-1 would block Smad2 nuclear translocation after TGFβ treatment. Accordingly, we performed immunofluorescence studies to examine TGFβ-dependent Smad2 translocation to the nucleus in individual cells after siRNA knockdown of km23-1 (Fig. 6). Mv1Lu cells were transiently transfected with either NC siRNA or km23-1 siRNA in the presence of GFP. The presence of the GFP signal designates the cells that were transfected with the relevant siRNAs (left panels, Fig. 6). In the absence of TGFβ, the NC siRNA-transfected cells and the km23-1 siRNA-transfected cells displayed the same pattern of diffuse punctate staining (Fig. 6A). In contrast, as expected, the NC siRNA-transfected, GFP-positive cells displayed largely nuclear expression of Smad2 in response to TGFβ (Fig. 6B), indicative of ligand-induced translocation of Smad2 to the nucleus. However, in the km23-1 siRNA-transfected, GFP-positive cells, Smad2 expression was barely detectable in the nucleus (Fig. 6C). As expected, untransfected (GFP-negative) cells also responded to TGFβ with significant Smad2 translocation. Our results demonstrate that knockdown of km23-1 results in a decrease in nuclear expression of Smad2 after TGFβ treatment.

To quantify the siRNA effects on TGFβ-mediated nuclear
expression of Smad2 from Fig. 6 (A–C), we counted 100 GFP-positive cells in cultures of either km23-1 siRNA-transfected or NC siRNA-transfected cells treated with TGFβ (5 ng/ml). Of these 100 GFP-positive cells, the cells showing nuclear expression of Smad2 were counted as described under “Materials and Methods.” As shown in Table 1, in the NC siRNA-transfected cells, 93% of the GFP-positive cells displayed Smad2 nuclear expression in response to TGFβ. In contrast, in the km23-1 siRNA-transfected cells, Smad2 expression was barely detectable in the nucleus of GFP-positive cells, with only 11% of the km23-1 siRNA, GFP-positive cells still displaying Smad2 nuclear expression. As expected, 100% of the GFP-negative cells responded to TGFβ with increased Smad2 expression, whether the NC or the km23-1 siRNA had been co-transfected with GFP. Thus, the quantitation of our immunofluorescence results confirmed that nuclear Smad2 levels were specifically reduced by km23-1 siRNA in the presence of TGFβ.

As an independent method of verifying whether km23-1 knockdown reduced TGFβ-mediated nuclear expression of Smad2, we performed Western blot analyses after subcellular fractionation of the cells as described under “Materials and Methods.” As shown in Fig. 6D, in mock-transfected cells, there was no detectable phosphorylated Smad2 in the nuclear fraction in the absence of TGFβ (lane 1, top panel). However, levels of phospho-Smad2 in the nucleus were greatly increased after TGFβ treatment for 15 min (lane 2, top panel). Similar results were obtained in the NC siRNA-transfected cells (lanes 3 and 4, top panel). However, in the km23-1 siRNA-transfected cells, phospho-Smad2 levels in the nuclear fraction were significantly decreased after TGFβ treatment (lane 6, top panel). Expression of lamin A/C demonstrate equal loading of nuclear extracts (bottom panel) (56). Thus, the results in Fig. 6D further demonstrate that blockade of km23-1 results in a decrease of phosphorylated Smad2 in the nuclear fraction in the presence of TGFβ, consistent with the results obtained in the immunofluorescence analyses.

It is noteworthy when comparing Fig. 6 (B and C) that a corresponding increase in cytoplasmic Smad2 was not observed, when nuclear Smad2 expression was blocked by the km23-1 siRNA in the presence of TGFβ. This finding would suggest that degradation of Smad2 might occur when km23-1 functions are blocked. This effect was not observed upon overexpression of dynamitin (Fig. 4). Because a previous report has shown that TGFβ-activated Smad2 can be degraded through the ubiquitin-proteasomal-degradation pathway (57), it was of interest to determine whether blockade of this degradation pathway would reverse the km23-1 siRNA-mediated blockade of the TGFβ-dependent nuclear accumulation of Smad2. In addition, previous reports have shown that cells treated with the ubiquitin proteasomal degradation inhibitor lactacystin

## TABLE 1

|          | GFP+ NC siRNA | GFP+ km23-1 siRNA |
|----------|---------------|------------------|
| Cells with nuclear expression of Smad2/GFP positive cells | 93 ± 2          | 11 ± 2           |
| Cells with nuclear expression of Smad2/GFP negative cells  | 100 ± 0         | 99.7 ± 0.3      |

* Nuclear staining of Smad2 after addition of TGFβ (5 ng/ml) for 15 min.
remained fully viable for 8 h after treatment and that, generally, after a 2-h exposure, a 50% inhibition was obtained at 1–10 μM (58). Moreover, pretreatment of Mv1Lu cells with lactacystin (10 μM) for 8 h before cell lysis was shown to cause a significant inhibition of 26 S proteasome degradation (59). Thus, we chose lactacystin (10 μM) pretreatment for 8 h for these experiments.

Mv1Lu cells were transiently transfected either NC siRNA or km23-1 siRNA in the absence or presence of TGFβ (5 ng/ml) and/or lactacystin (10 μM). As expected for the NC siRNA-transfected cells, TGFβ-induced a rapid increase in phosphorylated Smad2 in the nuclear fraction (Fig. 7A, lanes 2–4, left panel). Phosphorylated Smad2 was detectable within 5 min of TGFβ treatment and continued increasing for at least 15 min. After addition of lactacystin to the NC siRNA-transfected cells, the levels at 5–15 min after TGFβ treatment were slightly higher than for TGFβ treatment alone (lanes 6–8, left panel). Consistent with the results in Fig. 6 (C and D), in the km23-1 siRNA-transfected cells, phospho-Smad2 levels in the nuclear fraction were significantly decreased at all time points after TGFβ treatment (lanes 2–4, right panel), with respect to those for the NC siRNA. This decrease was reversed by the proteasomal inhibitor lactacystin (lanes 7 and 8, right panel). To quantify these results, Western blots from two independent experiments were scanned by densitometry, and the results were expressed graphically (Fig. 7B). As shown in this figure, lactacystin treatment resulted in higher levels of phospho-Smad2, especially for the cells receiving km23-1 siRNA. Thus, blockade of ubiquitin proteasomal degradation prevented the loss of phospho-Smad2 that occurs when km23-1 is blocked. Taken together, our results indicate that a proteasomal degradation mechanism is responsible, at least in part, for the reduced levels of TGFβ-activated (i.e. phosphorylated) Smad2 that are observed when km23-1 expression is knocked down.

**DISCUSSION**

km23-1 was previously identified to be both a TβR-interacting protein and a light chain of the motor protein dynein (13). Further, kinase-active TβRs were shown to be required for km23-1 phosphorylation and for recruitment of km23-1 to the dynein motor complex through the DIC (13). In addition, blockade of km23-1 is known to reduce specific TGFβ responses downstream (31). These previous results suggested that, subsequent to TβR activation and endocytosis, TGFβ signaling components such as Smads might represent one type of cargo that could be transported intracellularly by dynein-dependent mechanisms, involving the DLC km23-1. In the current report, we demonstrate for the first time that dynein motor activity is required for TGFβ-dependent Smad2 accumulation in the nucleus. In this regard, we show that overexpression of dynamitin, which is known to disrupt dynein-dynactin functions and to block dynein-dependent intracellular transport, blocked Smad2 nuclear accumulation after TGFβ treatment. We also describe herein the novel findings that after TGFβ treatment, km23-1 is present in early endosomes with TβRs, and it is co-localized with Smad2 prior to Smad2 translocation to the nucleus. Further, we report that km23-1 and Smad2 are present in the same complex after TGFβ treatment and that knockdown of km23-1 reduces both nuclear levels of phospho-Smad2, as well as Smad2-dependent ARE-Lux transcriptional activity. Collectively, our results provide the first evidence that dynein motor activity and the DLC

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**FIGURE 7.** The 26 S proteasome inhibitor lactacystin partially prevents the km23-1 siRNA-mediated depletion of Smad2 after TGFβ treatment. A, Mv1Lu cells were pre-treated with lactacystin (10 μM) for 8 h prior to incubation in serum-free medium for 1 h, followed by addition of TGFβ (5 ng/ml) for the indicated times. Nuclear extracts were obtained as described under “Materials and Methods.” Nuclear phospho-Smad2 expression was examined by Western blot analysis. Proliferating cell nuclear antigen (PCNA) expression was used as a nuclear protein marker and protein quality control. B, densitometric scan results of phospho-Smad2 expression levels from two similar experiments as in A. Expression levels of phospho-Smad2 were normalized to proliferating cell nuclear antigen expression levels. Results plotted are the mean ± range (n = 2).

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km23-1 are required for Smad2-dependent TGFβ signaling events.

Although this is the first report of a positive role for a DLC in a TGFβ- and Smad-dependent signaling pathway, several examples of motor protein light chain regulation of other signaling pathways have been reported previously (9, 10, 60, 61). For example, it has been reported that kinesin light chain 1 is the link between kinesin motor proteins and the c-Jun NH2-terminal kinase (JNK)-interacting proteins, motor receptors known to be important for JNK and p38 mitogen-activated protein kinase signaling (60 – 62). In addition, JNK-associated leucine zipper protein was shown to serve as a link between the kinesin motor proteins and their cargo, namely JNK signaling components (63). Similarly, light chains for the motor protein dynein have been shown to regulate the movement of signaling complexes along MTs (3, 51). For example, the DLC Tctex-1 (DYNLT1) has been shown to associate with the Trk neurotrophin receptors for the transport of neurotrophins during vesicular trafficking, an effect that is thought to result from the direct interaction between the Trk receptors and the dynein motor machinery (64). Further, DLC1 (LC8, DYNLL1) has been shown to have a facilitating role in the nuclear translocation of the estrogen receptor in breast cancer cells (65). In addition, recent evidence suggests that the interaction of DLC1 (DYNLL1) with the RasGRP3 exchange factor for Ras-like small GTPases could play an important role in controlling downstream signaling from diacylglycerol (66). Finally, LC8 (DYNLL1) has been shown to function as a versatile acceptor (i.e. motor receptor) to facilitate dynein-mediated nuclear accumulation of p53 after DNA damage (51).

In addition to the motor light chains themselves, other components of the motor machinery are important for cargo recognition. For example, dynactin has been shown to play a critical role in both cargo binding and regulation of dynein-mediated transport (67, 68). Overexpression of one of the dynactin subunits, termed dynamitin, is known to disrupt dynein-dynactin functions, thereby diminishing dynein motor activities required for the intracellular transport of cargoes. Along these lines, overexpression of dynamitin has been used as an effective tool for examining the requirements of dynein-dependent cargo transport for intracellular signaling events (6, 55, 69, 70). For example, disruption of dynein motor activity by overexpressing dynamitin impaired the accumulation of p53 in the nucleus following DNA damage (71). In addition, overexpression of p50/dynamitin impaired the nuclear accumulation of STAT5β after growth hormone induction (72), as well as the nuclear translocation of the glucocorticoid receptors after ligand stimulation (73). Because our results have shown that overexpression of dynamitin blocked Smad2 nuclear translocation after TGFβ treatment, dynein-dependent intracellular events also appear to be required for TGFβ/Smad2 downstream effects.

As mentioned above, our results are consistent with km23-1 being present in early endosomes after TβR-mediated endocytosis. However, our data suggest, further, that km23-1 may be one of the factors required for the intracellular movement of the endosomal TβR/Smad2 signaling complexes toward the nucleus, based upon the known direction of movement of dynein motors (13). Along these lines, it is well established that TβRs are endocytosed through the clathrin-mediated pathway, which is important for promoting signaling (42 – 46). During clathrin-mediated endocytosis, the TβR complex is targeted to clathrin-coated pits, where it binds to the β2-adaptin subunit of AP2 (45, 74, 75). Dynamin 2ab functions downstream of TβR activation, where it excises the budded vesicle from the plasma membrane (75). After clathrin-mediated endocytosis, TβRs are found for extended periods of time in EEA1-enriched early endosomes (45). The clathrin-mediated endocytic pathway is thought to promote the co-localization of TβRs with downstream signaling components (i.e. Smad2) in early endosomes. In addition, although TβR phosphorylation and association with Smad2 can occur at the plasma membrane, RSmad phosphorylation and downstream signaling only appear to occur after clathrin-dependent endocytosis, requiring an unknown activity or activities downstream of dynamin 2ab function (45, 76). Based upon our current results, km23-1 may participate in the recruitment of Smad2-containing TGFβ signaling endosomes to the rest of the dynein motor for intracellular transport, prior to both nuclear translocation and downstream nuclear events. In this way, km23-1 may represent one of the additional steps, downstream from dynamin 2ab function, that is required for Smad signaling after TβR activation (13).

Based upon our results and those of others, we propose a model for km23-1 action in the recruitment of TGFβ signaling endosomes for intracellular transport along MTs. According to this model, within minutes of ligand binding, activated TβRs are internalized into EEA1/SARA-enriched endosomes, where Smad2 is recruited by SARA (42, 45). Once km23-1 is phosphorylated by TβRII (13) and Smad2 is phosphorylated by TβRI (21, 24, 25), km23-1 selectively interacts with the TβR/Smad2 complex, and recruits the TGFβ signaling endosome to the dynein motor through the DIC-km23-1 interaction. In this regard, our previous results have shown that kinase-active TβRIIs are absolutely required for the interaction of km23-1 with DIC (13). In addition, dynein is known to mediate the association of endosomal membranes with MTs (77, 78). After attachment of the TGFβ signaling endosomes to the rest of the motor, km23-1/dynein transports the TGFβ signaling endosomes along MTs to the next endosomal compartment. The requirement of dynein motor function for intracellular movement of Smad2 was established by our results in Fig. 4 involving dynamitin overexpression. Upon reaching subsequent compartments, the signaling complex may active downstream components or be translocated to the nucleus for transcriptional regulation of target genes.

Although Smads 2 and 3 are highly homologous and share some overlapping activities, they have distinct functions and are regulated differentially (79, 80). For example, previous work has indicated that Smad2 activates ARE-Lux (38), whereas Smad3 activates SBE2-Luc (39). In addition, Smad2 and Smad3 may be phosphorylated in different endocytic locales (81), and this distinct compartmentalization is in keeping with their divergent mechanisms of oligomerization (82), intracellular degradation (83), and regulation of TGFβ cellular effects (84, 85). Our studies have shown that blockade of km23-1 reduced
TGFβ- and Smad2-dependent ARE-Lux transcriptional activity, but not TGFβ- and Smad3-dependent SBE2-Luc activity, suggesting that km23-1’s role in mediating Smad transcriptional activation is somewhat specific for this RSmad. Similarly, we have previously shown that km23-1 is regulated by TGFβ, but not by EGF (31). Further, others have found that receptors for another TGFβ superfamily member (BMPRII) interact with another DLC (TcTeX1, DYNLT1), but not with km23-1.3 Thus, the DLCs also show specificity with regard to the growth factors and receptors that activate them.

Ubiquitin proteasomal-mediated degradation is known to control the levels of Smads transcriptionally and post-translationally (26, 83). Here we have shown that the proteasomal inhibitor lactacystin partially restored TGFβ-stimulated nuclear Smad2 expression, to levels more similar to those observed without km23-1 blockade. Therefore, blockade of km23-1 appears to stimulate a Smad2 ubiquitin proteasomal-mediated degradation pathway, possibly due to the inability of km23-1 to recruit Smad2 to the rest of dynein motor. Smad ubiquitin regulatory factor (Smurf)-mediated ubiquitination pathways have been shown to play critical roles in the degradation of Smads. For example, Smurf1 has been shown to selectively interact with Smads1 and 5, targeting them for degradation (86). In addition, Smurf2 induces ubiquitin-mediated degradation of Smads 1 and 2 (87). Further, a recent report has shown that neural precursor cell-expressed, developmentally down-regulated 4-2 (NEDD4-2), a new member of the Smurf-like E3 ligases, also induces Smad2 degradation via a ubiquitin-dependent degradation pathway. More importantly, it has been suggested previously that some Smad degradation can occur in the cytoplasm through Smurf-mediated ubiquitination pathways (86, 88). Because our data demonstrate that blockade of km23-1 results in a depletion of Smad2 expression in the presence of TGFβ, through a ubiquitin proteasomal degradation pathway, it will be of interest to determine in future studies whether Smurfs are involved in this pathway.

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