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Requirement of km23 for TGFβ-mediated growth inhibition and induction of fibronectin expression

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Abstract

We previously 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 is TGFβ-dependent, in that EGF was unable to phosphorylate km23. Further, the kinase activity of both TGFβ receptors appears to play a role in the TGFβ-mediated phosphorylation of km23, although TGFβ RII kinase activity is absolutely required for km23 phosphorylation. Blockade of km23 using small interfering RNAs significantly decreased key TGFβ responses, including induction of fibronectin expression and inhibition of cell growth. Thus, our results demonstrate that km23 is required for TGFβ induction of fibronectin expression and is necessary, but not sufficient, for TGFβ-mediated growth inhibition.
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Keywords: TGFβ; Signal transduction; siRNA; Growth; Fibronectin; km23; EGF; Dynein

1. Introduction

Transforming growth factor β (TGFβ) is the prototype for a family of highly conserved ubiquitous peptides that show a remarkable diversity in the biological actions they mediate. These biological responses include effects on cell growth, cell death, cell differentiation, and the extracellular matrix (ECM) [1–3]. TGFβ is growth inhibitory for normal cells of endothelial, hematopoietic, neuronal, and epithelial origin [1,4,5]. However, cancers are often refractory to this growth inhibitory effect, due to genetic loss of TGFβ signaling components or, more commonly, perturbation of TGFβ signaling pathways [1,4].

TGFβ initiates its signals by producing an active tetrameric receptor complex consisting of TGFβ RI (TβRI) and TGFβRII (TβRII) serine/threonine kinase receptors. After TGFβ binds to TβRII, it transphosphorylates, and thereby activates TβRI. The active receptor complex then propagates signals to downstream cellular components and regulatory proteins [2,3,6]. Two primary signaling cascades downstream of the TGFβ receptors have been elucidated: the Smads and the Ras/mitogen-activated protein kinase (MAPK) pathways [1,2,7]. In addition, several TGFβ receptor-interacting factors [1] and Smad-interacting factors [8] have been reported. However, additional TGFβ signaling components and pathways are likely required to mediate the diverse biological responses of this polypeptide factor.

Abbreviations: TGFβ, Transforming growth factor β; ECM, Extracellular matrix; TβRI, TGFβ RI receptor; TβRII, TGFβ RII receptor; JNK, Jun N-terminal kinases; siRNA, Small interfering RNA; NC siRNA, Negative control siRNA; MDCK, Madin Darby canine kidney; Ab, Antibody; DIC, Dynein intermediate chain; DIC Ab, Dynein intermediate chain antibody; EGF, Epidermal growth factor; hkm23, Human km23; FACS; Fluorescence-activated cell sorting.

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Fibronectin, a major component of the ECM, plays important roles in cell adhesion, migration, growth, and differentiation [9,10]. TGFβ is one of the most potent stimulators of the ECM, and it has been shown to play a significant role in the accumulation of specific ECM components such as fibronectin and collagen [1,11,12]. Despite the suggestion that Smads play a critical role in TGFβ-mediated responses, the signaling mechanisms leading to TGFβ-mediated accumulation of ECM proteins are unclear. For example, Hocevar et al. [13] have shown that TGFβ can induce fibronectin synthesis through a Jun N-terminal kinase (JNK)-dependent pathway, but that Smad4 was not involved. In addition, Gooch et al. [14] reported that calcineurin was involved in TGFβ-mediated regulation of ECM accumulation. It is likely that other novel TGFβ signaling intermediates are required for mediating the diverse effects of TGFβ on such a large repertoire of biological responses, which include the effects on ECM.

We have previously identified a novel TGFβ receptor-interacting protein that is also a light chain of the motor protein dynein [15]. This TGFβ signaling intermediate, termed km23, interacts with the receptor complex through TβRII and is phosphorylated after activation of the TGFβ receptor complex. 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 a role in this phosphorylation event. Blockade of km23 using small interfering RNAs (siRNAs) decreased the ability of TGFβ to both inhibit cell growth and induce fibronectin expression in Madin Darby canine kidney (MDCK) epithelial cells. Our findings indicate that km23 is a mediator of the growth inhibitory effects of TGFβ and is also required for the induction of fibronectin expression by TGFβ.

2. Materials and methods

2.1. 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 rabbit IgG was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-fibronectin Ab (610078) was from BD Biosciences Transduction Laboratories (Palo Alto, CA). Protein A or G agarose were purchased from Invitrogen (Carlsbad, CA). ^{32}P-orthophosphate (NEX-053) was from PerkinElmer Life Sciences (Boston, MA). TGFβ_1 was purchased from R & D Systems (Minneapolis, MN). Epidermal growth factor (EGF) was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Lipofectamine 2000 (11668-019) was from Invitrogen (Carlsbad, CA). Propidium iodide (537059) was from Calbiochem (San Diego, CA). Ribonuclease A (R4875) and Sodium citrate (C7254) were from Sigma Aldrich (St. Louis, MO). FuGENE 6 Transfection Reagent (11814443001) was from Roche Diagnostics (Indianapolis, IN).

2.2. Antibody production

The rabbit km23 anti-serum was prepared against the following sequence: GIPIKSTMDNPTTTQYA (corresponding to amino acids 27–43) of human km23 (hkm23) (Strategic BioSolutions, Newark, DE, or Covance Reseach Products, Denver, PA). Each company also provided pre-immune serum.

2.3. 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 [16]. Cultures were routinely screened for mycoplasma using Hoechst 33258 staining.

2.4. Transient transfections, IP/blot, Westerns, and In vivo phosphorylation assays

Transient transfections, IP/blot, Westerns, and In vivo phosphorylation assays were performed essentially as described previously [13,15,17–19].

2.5. siRNAs

The km23 siRNAs used for the [^{3}H]thymidine incorporation assays were purchased from Dharmacon Research (Lafayette). The double-stranded km23 siRNA corresponded to nucleotides 77–97 of the hkm23 coding region (5′-AAGGCATTCCCATCAAGAGCA-3′). The siRNAs were transfected using Oligofectamine (12252-011; Invitrogen). For the FACS and fibronectin experiments (Figs. 5 and 6), siRNA plasmids were constructed as follows. The sense strand of the hairpin km23 siRNA-1 corresponded to nucleotides 230–250 of the km23 coding region (5′-AAATTATGGTTGCACCAGATA-3′). The sense strand of hairpin km23 siRNA-2 corresponded to nucleotides 130–150 of the km23 coding region (5′-AACCTCATGCACAACTT-CATC-3′). km23 siRNA-1 and km23 siRNA-2 plasmids were transfected using Lipofectamine 2000 reagent for the fibronectin experiments. The sense strand of the hairpin km23 siRNA-3 corresponded to nucleotides 251–271 of the

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