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Differing contributions of LIMK and ROCK to TGFβ-induced transcription, motility and invasion

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ABSTRACT

The ability of transforming growth factor β (TGFβ) to induce epithelial–mesenchymal transition (EMT) is mediated by SMAD-dependent and SMAD-independent pathways such as the activation of Rho GTPase signalling. Upon activation, GTP-bound Rho stimulates the ROCK kinases, which in turn phosphorylate numerous substrates including the LIM kinases (LIMK). The net result of ROCK activation is increased actin–myosin contractile force generation, with a contribution from LIMK-induced actin filament stabilisation. In this study, we made use of siRNA-mediated knockdown and selective inhibitors to determine the contributions of ROCK and LIMK to TGFβ-induced responses. We find that both ROCK and LIMK are required for TGFβ stimulation of serum-response factor (SRF) transcriptional activity and actin stress fibre formation during EMT. In contrast, although LIMK inhibition had little effect on cell motility in scratch wound and Transwell migration assays, ROCK inhibition actually promoted TGFβ-induced cell motility by helping individual cells to break free from the epithelial sheet. Furthermore, we demonstrate that selective inhibition of LIMK, but not ROCK, effectively blocked TGFβ driven invasion through a layer of matrigel extracellular matrix protein. These results indicate that the roles of LIMK and ROCK in the Rho signalling pathway downstream of TGFβ are not identical and suggest that LIMK represents an attractive therapeutic target in TGFβ driven organ fibrosis and metastatic cancer spread.

Introduction

The epithelial–mesenchymal transition (EMT) involves numerous cellular responses that lead to reduced cell–cell adhesion and the acquisition of migratory and invasive abilities (Thiery et al., 2009). The conversion from epithelial to mesenchymal is important for the formation and differentiation of various tissues and organs during development. EMT also contributes to tissue repair, but in pathological conditions may promote organ fibrosis and facilitate metastasis. EMT induction requires transcriptional responses mediated by several pathways and transcription factors, as well as direct effects on regulators of the actin-cytoskeleton.

One of the major EMT inducers is transforming growth factor β (TGFβ), which activates signalling by initiating the assembly of a tetrameric complex composed of type I and type II serine/threonine kinase receptors (Moustakas and Heldin, 2009). Receptor activation leads to Smad2 and Smad3 phosphorylation, which then associate with Smad4 and accumulate in the nucleus to regulate transcription of genes that contribute to EMT. TGFβ receptor activation also leads to increased signalling through the Rho GTPase pathway (Dimitris et al., 2009). When Rho is activated, the exchange of GDP for GTP promotes association/activation of effector proteins, with the ROCK1 and ROCK2 serine/threonine kinases being amongst the most important (Wickman et al., 2010). The ROCK kinases phosphorylate a variety of substrates, which collectively lead to increased myosin regulatory light chain phosphorylation, increased myosin ATPase activity, filamentous actin (F-actin) stabilisation and association of actin–myosin bundles with membrane-linked protein anchors; the output of these events being increased actin–myosin contractile force generation (Olson and Sahai, 2009). The consequences of actin–myosin force generation are dependent upon localisation and intensity, but in the case of TGFβ-induced EMT typically result in actin cytoskeleton reorganisation, morphological changes and altered cell motility and invasion.

The LIM kinases 1 and 2 (collectively LIMK) are activated downstream of the Rho-ROCK pathway (Scott and Olson, 2007). When active, LIMK phosphorylates and inactivates the filamentous-actin severing functions of cofilin proteins, resulting in F-actin stabilisation. Spatially and temporally regulated cycles of cofilin inactivation and activation, regulated principally by LIMK-mediated phosphorylation and phosphatase-mediated dephosphorylation, initiate dynamic alterations to the actin cytoskeleton (Van Troys et al., 2008). Since cofilin selectively targets aged portions of actin

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filaments in which ATP has been hydrolysed to ADP (Carlier et al., 1997), the effect of LIMK inhibition is to reduce levels of long actin filaments and inhibit the generation of actin barbed ends for sites of polymerisation (Olson and Sahai, 2009). The net effect of ROCK and LIMK activation is to reduce actin "treadmilling" and decrease the cellular pool of free monomeric globular actin (G-actin).

Although ROCK and LIMK lie in a linear pathway downstream of Rho, their roles in actin cytoskeleton regulation are not identical. LIMK is only one among many ROCK-regulated pathways. In this study, we aimed to discover which TGFβ responses were mediated by ROCK via LIMK activation using siRNA-mediated knockdown and a novel first-in-class LIMK inhibitor. We also aimed to determine whether there were differences in the responses to selective ROCK or LIMK inhibitors during TGFβ-induced EMT. The results indicate that there is a ROCK/LIMK pathway leading to the regulation of gene expression. In addition, ROCK and LIMK contribute in a similar, although not identical, manner to TGFβ-induced morphological changes. However, the cell motility and invasion responses to TGFβ were differently affected by ROCK or LIMK inhibition, indicating that their contributions to these processes are not identical. These results also may have implications for the potential use of ROCK or LIMK inhibitors as therapeutic agents to reduce tissue/organ fibrosis and the metastatic cancer spread.

Materials and methods

Plasmids and cell lines

NMuMG mouse mammary epithelial cells were maintained in DMEM medium containing 10% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Swiss 3T3 mouse fibroblast cells conditionally expressing V14RhoA have been described previously (Morin et al., 2009). Cells were treated with TGFβ at 5 ng/ml for the indicated times. Y27632 (Sigma) and LIMKi were used at 10 μM. Firefly luciferase gene expression vector 3D.ALuc regulated by SRF binding sites has been described (Copeland and Treisman, 2002). Renilla luciferase gene expression vector plasmid (Promega) was used to normalise Firefly luciferase values.

Transient transfection and luciferase assays

0.5×10^5 NMuMG cells were seeded in 6 wells dishes. After 24 h, they were transfected with a pair of siRNA duplexes against LIMK1 and LIMK2 or a non targeting siRNA sequence using DharmaFect3 reagent according to the manufacturer's instructions. 24 h later, cells were transfected with luciferase expression vectors using Effectene (QIAGEN) according to the manufacturer's instructions in serum free medium. 24 h later, cells were stimulated with TGFβ for 8 h. Alternatively, cells were only transfected with luciferase expression vectors and then stimulated with TGFβ in the presence of vehicle (DMSO), Y27632 or LIMKi. Cells lysates were analysed with dual luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Western blotting

0.5×10^6 cells were seeded in 10 cm diameter dishes and serum-starved for 24 h. Cells were subsequently stimulated with TGFβ for the indicated times, with or without Y27632, or with or without LIMKi. Cells lysates were Western blotted as detailed previously (Coleman et al., 2001) with: anti-phospho-ERK1/2 (Thr202/Tyr24) (9106), anti-phospho-SMAD3 (Ser423/425) (9514), anti-phospho-cofilin (Ser3) (3313), anti-LIMK1 (3842) and anti-cofilin (3312) from Cell Signalling; anti-LIMK2 (sc5577) from Santa Cruz; anti-ERK2 from C.J. Marshall, Institute of Cancer Research, London, UK. Membranes were probed with appropriate Alexa Fluor or Horseradish peroxidase conjugated secondary antibodies for 1 h, and immunoreactivity visualised either with a LI-COR Odyssey instrument or with photographic films using SuperSignal West Femto substrate (Thermo Scientific) according to the manufacturer's instructions.

Real time PCR

NMuMG cells (0.5×10^6) in 10 cm dishes were stimulated with TGFβ in the presence or absence of Y27632 (10 μM) or LIMKi (10 μM) for the indicated times prior to trypsinisation. Cells were pelleted by centrifugation at $1000 \times g$ for 15 min. Total RNA was prepared using the Qiagen RNeasy Kit according to the manufacturer's instructions. RNA (5 μg) was reverse-transcribed using Superscript (Invitrogen) and oligo-dT. cDNAs were amplified by real-time (RT)-PCR using iTaq SYBR Green Supermix (Bio-Rad) and the following primers: MuAlphaSMA-1S ACTGGGACGACATGGAAAAG, MuAlphaSMA-2A CATCTCCAGAGTCCAGCAC, MuSnail-2A CAGCGTGTGGCTTCGGATG, MuSnail-1S ACCTTCCAGCAGCCCTACG, MuGAPDH ACCACAGTCCATGCCATCA, MuGAPDH-2A TCCACCACCCTGTTGCTGTA, MuCaldesmon-1S CCTATGAAGCCAGCAGCATC, MuCaldesmon-2A GCGAGATGGCTTGCAGTTGGRT. PCR was performed with the Chromo4™ RT-PCR Detector (Bio-Rad). Each condition was done in triplicate and the experiment repeated with 2 independent mRNA preparations. The relative expression differences between conditions were expressed as mean ± standard error.

Immunofluorescence studies

Cells were fixed and stained as detailed previously (Coleman et al., 2001) with: E-cadherin antibodies (610182; BD Transduction Lab) for NMuMG cells or with pan cadherin antibodies (C1821, Sigma) for Swiss 3T3 cells. After 1 h, cells were washed twice with PBS before incubation with Cy5 or Texas-red-conjugated secondary antibody diluted in 3% BSA/PBS for 1 h. F-actin was stained with Oregon green phalloidin (O-7465; Molecular Probes). Cells were washed twice with PBS and once with water before mounting in Vectashield medium containing DAPI (Vector Laboratories) under glass coverslips. Cells were visualised with a Zeiss Axioplan2 microscope.

Wound healing assays

Cells were seeded into 6-well plates and then transfected with a pair of siRNA duplexes against LIMK1 and LIMK2 or a non-targeting siRNA. 24 h later, cells were stimulated with TGFβ for 24 h. Alternatively, cells were treated with TGFβ in the presence of vehicle, Y27632 or LIMKi. Cells were then scratched with a 20 μl pipette tip. Pictures of the monolayer wounds were taken every 10 min for 24 h using a motorised Zeiss time-lapse microscope equipped with a temperature and CO_2 regulated environmental chamber. Images were then processed using Metamorph and ImageJ software.

Cell adhesion/spreading assay

Kinetics of cell adhesion and spreading were assayed using the xCELLigence Real-Time Cell Analyser (RTCA) DP. E-plate 16 wells were incubated with 100 μl of 25 μg/ml fibronectin for 1 h at 37 °C, then 10,000 cells were plated in each well with DMSO vehicle, Y27632 or LIMKi. Cells were allowed to settle for 30 min at room temperature, before being placed in the RTCA DP in a humidified incubator at 37 °C with 5% CO_2. Readings were taken every 15 min for 12 h and plotted curves represent the averages from 4 independent wells.