Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Distinct role of endocytosis for Smad and non-Smad TGF-β signaling regulation in hepatocytes

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Background & Aims: In injured liver, TGF-β affects all hepatic cell types and participates in wound healing and fibrogenesis. TGF-β downstream signaling is highly complex and cell type dependent, involving Smad and non-Smad signaling cascades thus requiring tight regulation. Endocytosis has gained relevance as important mechanism to control signaling initiation and termination. In this study, we investigated endocytic mechanisms for TGF-β mediated Smad and non-Smad signaling in hepatocytes.

Methods: Endocytosis in hepatocytes was elucidated using chemical inhibitors, RNAi, viral gene transfer and caveolin-1−/− mice. TGF-β signaling was monitored by Western blot, reporter assays and gene expression analysis.

Results: In hepatocytes, Smad activation is to a large degree accomplished AP-2 complex dependent on the hepatocyte surface without the necessity of clathrin coated pit formation or an endocytic step. In contrast, non-Smad/AKT pathway activation required functional dynamin mediated endocytosis and the presence of caveolin-1, an essential protein for caveolae formation. Furthermore, these two TGF-β signaling initiation platforms discriminate distinct signaling routes that integrate at the transcriptional level as shown for TGF-β target genes, Id1, Smad7, and CTGF. Endocytosis inhibition increased canonical Smad signaling and culminated in a superinduction of Id1 and Smad7 expression, whereas caveolin-1 mediated AKT pathway activation was required for maximal CTGF induction.

Conclusions: Endocytosis is critical for TGF-β signaling regulation in hepatocytes and determines gene expression signature and (patho)physiological outcome.

Introduction

Transforming growth factor-β (TGF-β) is a key regulator of developmental processes and tissue homeostasis mediating cell growth, migration, differentiation, and apoptosis. Abnormal and disturbed signaling associates with severe diseases like tissue fibrosis and cancer [1,2]. Initiation of TGF-β signaling is launched upon binding of TGF-β to its cognate receptors type I (TβRI) and type II (TβRII). This allows the association and phosphorylation of receptor Smads (R-Smads), Smad2 and Smad3. Activated R-Smads then heteromerize with cytoplasmic Smad4 and in complex, they shuttle to the nucleus to regulate gene expression [3,4]. Besides the canonical Smad pathway, TGF-β can induce activation of other signaling cascades in a cell context dependent manner, including ERK1/2, p38, c-Jun N-terminal kinase JNK and AKT [5].

In liver, TGF-β is the most potent inducer of fibrosis [6] and affects all resident liver cells, including hepatocytes. Besides its capacity to induce apoptosis of this cell type, TGF-β mediates epithelial to mesenchymal transition (EMT) of a tremendous fraction of hepatocytes *in vitro* and *in vivo* [7,8], although hepatocyte EMT *in vivo* was recently challenged [9]. Transitioned hepatocytes may considerably contribute to matrix remodeling and scar formation by expressing collagens and connective tissue growth factor (CTGF) [10]. The induction of CTGF is important as it amplifies profibrogenic actions of TGF-β and has already been associated with the severity of fibrosis [11–13].

Harboring elementary functions in tissue homeostasis and development, TGF-β signaling is tightly regulated at several stages of the cascade. One mechanism is regulating the availability of TGF-β receptors by subcellular trafficking. This includes distribution on the cell surface in specialized compartments like cholesterol-rich lipid rafts or domains of clathrin-coated pits, as well as internalization into different cellular compartments like early endosomes, lysosomes or recycling vesicles [14].

Molecular and Cell Biology

Keywords: Caveolin-1; Clathrin; CTGF; Dynamin; Fibrosis; Liver.
Received 18 May 2010; received in revised form 10 October 2010; accepted 2 November 2010; available online 22 December 2010

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Abbreviations: AP-2, adaptor protein-2; CP, chlorpromazine; CTGF, connective tissue growth factor; EMT, epithelial to mesenchymal transition; EPS15, EGFR pathway substrate #15; Id1, inhibitor of DNA binding 1; TβR, TGF-β receptor; TGF-β1, transforming growth factor beta 1.

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Internalization of the TGF-β/receptor complex may occur via a clathrin-dependent route facilitating signaling and a caveolae dependent pathway leading to signaling termination [15]. The promoting internalization route transports the active receptor complex to early endosomes and thereby increases its proximity to R-Smads and their adaptor proteins, e.g. SARA [16,17]. Abrogation of signaling is mediated by TβRI ubiquitination with subsequent degradation via Smad7/Smurf association in caveolae [18,19].

Further investigations aimed to clarify the necessity of endocytosis for TGF-β mediated signal transduction have generated controversial results. Some cell types require intact endocytosis for R-Smad phosphorylation, e.g. Cos7, HeLa, and Mv1Lu cell lines [15,20,21]. In primary mesangial cells and NMuMG cells, phosphorylation of Smads takes place in the absence of endocytosis, whereas nuclear translocation is affected [22]. Moreover, clathrin-coated pit formation was described as required for TGF-β signaling initiation [23].

In hepatocytes, TGF-β induced Smad signaling does neither depend on endocytosis nor on clathrin-coated pit assembly, but requires an intact adaptor protein-2 (AP-2) complex formation. In contrast, non-Smad TGF-β signaling leading to AKT phosphorylation demands caveolae dependent endocytosis, indicating that a complete hepatocyte specific TGF-β response is reliant on the existence of two distinct TGF-β signaling initiation platforms.

Materials and methods

Reagents

Recombinant TGF-β1, Peprotech (Hamburg, Germany), used at a final concentration of 5 ng/ml or as indicated; dynamin inhibitors Dynasore and MiTMAB, Merck (Darmstadt, Germany); chlorpromazine, Sigma (Munich, Germany). Transfection reagent (RNAiMAX), Invitrogen (Karlsruhe, Germany); siRNA, Dharmacon (siGenome SMARTpool) and Qiagen (Hilden, Germany); AdK44A dynamin was described previously [24]; AdLacZ was used as control. An MOI of 100 was used for infection with AdK44A dynamin and LacZ or as indicated. The control lentivirus (pHR'SIN-IRES-EGFP with EGFP as transgene) and the lentiviral vector containing the human caveolin-1 in combination with IRES and EGFP (pHR'SIN-CAV1) have been described elsewhere [25]. Virus production, titration and transduction were performed as described [26]. Antibodies used: Smad3 (Epitomics), Smad4, Smad2 (Zymed), AKT, pAKT, pSmad2, pSmad3, dynamin, GAPDH (Cell Signaling), β-actin (Sigma), TβRI, caveolin-1, Histone H1 (Santa Cruz). Horseradish peroxidase-linked secondary antibodies, anti mouse and rabbit were from Santa Cruz. Alexa Fluor 555 goat anti-rabbit was from Invitrogen, Molecular Probes.

Cell culture

Hepatocytes were isolated from C57Bl/6 and hepatocytes lacking caveolin-1 from caveolin-1 knockout mice ($Cav1^{tm1Mls}$/J, Jackson Laboratory, stock number 004585) by collagenase perfusion [27], and cultured as described on collagen coated plates [28]. Mv1Lu cells were cultured in DMEM with 10% FCS and glutamine; for starvation, FCS was removed the day before starting the experiment.

Surface protein biotinylation

Surface biotinylation was adapted from [29]. Briefly, cells were washed with HBSS and subsequently incubated with 1 ml of 0.25 mg/ml EZ-link-Sulfo-NHS-SS-Biotin (Pierce) in HBSS for 30 min on ice. After washing, cells were rewarmed to 37 °C and stimulated with TGF-β1 for 30 min to allow internalization of the biotinylated surface proteins. Cells were either lysed with 1 ml RIPA buffer to obtain total biotinylated cell surface proteins (membrane bound and internalized) or returned to 4 °C. Remaining biotin on the cell surface was removed by glutathione treatment (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10 mM EDTA and 1% BSA) at 4 °C which yielded a fraction solely containing internalized biotinylated proteins. Protein lysates were incubated with avidin beads (Pierce) and SDS–PAGE was performed with immunoblotting against TβRI. Densitometrical analyses were performed with Aida (2.11) software.

Nuclear-cytoplasmic fractionation

Hepatocytes were washed and resuspended in lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, protease inhibitors; Roche) and incubated for 15 min on ice. After addition of 10% NP40, lysates were vortexed and subsequently, proteins were centrifuged for 1 min full speed (4 °C, 14,000 rpm); supernatant containing cytoplasmic proteins was removed and pelleted nuclei were lysed with 20 mM Hepes pH 7.9, 25% Glycerin, 0.4 M NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0 (including proteinase and phosphatase inhibitors). Nuclei in lysis buffer were incubated 15 min on ice, while vortexing every 2 min. Then, lysates were centrifuged for 5 min at 4 °C full speed (14,000 rpm); supernatant contains nuclear proteins.

Potassium depletion

Cells were shocked with hypotonic media for 5 min in Williams E/water (1:1) for hepatocytes or DMEM/water for Mv1Lu and HeLa and thereafter incubated for 30 min in minimal media (20 mM Hepes at pH 7.5, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM magnesium sulphate, 5.5 mM glucose and 0.5% BSA); controls were incubated in buffer additionally containing 10 mM KCl.

Immunoprecipitation

Smad2 was immunoprecipitated using exactacruz kit (Santa Cruz) from RIPA lysates. Briefly, 2.5 μg antibody was preincubated at 4 °C, rotating o/n with 40 μl of matrix slurry, followed by three washing steps with ice cold RIPA and subsequent incubation at 4 °C, rotating with 500 μg protein lysate. After washing the precipitate with ice cold RIPA and addition of SDS loading buffer, samples were boiled at 95 °C for 5 min and dissolved on 10% SDS/PAGE with subsequent immunoblotting.

Western blotting

Thirty micrograms RIPA protein lysates were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Pierce). Non-specific binding was prevented by blocking in 5% milk in TBST. Every figure shown is representative for at least three independent experiments.

For SDS–PAGE preparation of secreted proteins, 400 μl cell supernatant was added to 1600 μl acetone (−80 °C), stored for 5 min at −80 °C and then incubated at −20 °C for 1 h. Subsequently, precipitated proteins were centrifuged with 13,000 rpm for 10 min at 4 °C. Supernatant was carefully aspirated and precipitate was dissolved in SDS loading buffer, denatured and separated by gel electrophoresis.

Immunofluorescence staining

Immunostainings were performed as described previously [8]. Draq5 was used to stain nuclei. Confocal microscopy and image acquisition was carried out using the confocal microscope Leica TCS SP2.

EGF uptake

Inhibition of dynamin dependent endocytosis was tested via EGF uptake. The uptake of EGF follows a clathrin- and dynamin-dependent route [30] and is therefore suitable to test the inhibitory tools in hepatocytes. Hence, cells were treated for 30 min with 50 μM MiTMAB or 80 μM Dynasore (or vehicle control) and treated with labeled EGF 647 (Molecular Probes, Invitrogen, E-35351) for 30 min (4 μg/ml). Cells were fixed and prepared for confocal microscopy. Nuclei were stained with Sytox green (Invitrogen). Blockage via dominant negative dynamin was tested by infection with AdLacZ as control or AdDynamin K44A (MOI 100) and treatment with EGF the next day. Clathrin-dependent endocytosis inhibition was tested by performing potassium depletion and subsequent treatment with labeled EGF.

Reporter gene assay

The adenovirus $Ad(CAGA)_9$-MLP-Luc was used as luciferase reporter system. Luciferase substrate was from Promega (Mannheim, Germany). Luminometric measurements were performed using either Tecan infinite M200 or Thermo