Proteomics-based identification of proteins interacting with Smad3: SREBP-2 forms a complex with Smad3 and inhibits its transcriptional activity

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Abstract **Smad3 is an important component of transforming growth factor-β (TGFβ) intracellular signalling. To identify novel interacting proteins of Smad3, we performed pull-down assays with Smad3 constructs fused to glutathione-*S*-transferase. Proteins which formed complexes with these constructs were analyzed by two-dimensional gel electrophoresis, and were identified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. We identified 14 proteins interacting with the Smad3 construct lacking the N-terminal Mad homology domain 1 (MH1), and 12 proteins interacting with the construct lacking the C-terminal MH2 domain. Proteins involved in signalling processes, in metabolism regulation, novel proteins, and components of cytoskeleton form four groups of interacting proteins. Interactions of AGP7, sex-determining region Y protein, actin β and sterol regulatory element binding protein-2 (SREBP-2) proteins with Smad3 constructs were confirmed by immunoblotting with specific antibodies. Interaction of Smad3 with SREBP-2 was also confirmed by co-immunoprecipitation of myc-Smad3 and Flag-SREBP-2 upon expression in mammalian cells. We found that SREBP-2 inhibited the transcriptional activity of Smad3 in luciferase reporter assays.**

Keywords: Proteomics; Smad3; Sterol regulatory element binding protein-2; Transforming growth factor-β

1. Introduction

Smad proteins are crucial components of the intracellular transforming growth factor-β (TGFβ)-dependent signalling. In mammals, 8 Smads, divided in three groups, have been described [1–3]. The first group contains receptor-regulated Smads, with Smad2 and Smad3 being directly phosphorylated by TGFβ and activin receptors, and Smad1, Smad5 and Smad8 being activated by bone morphogenic protein receptors. The second group has only one member in mammals, Smad4, which is a common mediator. Smad4 forms complexes with receptor-regulated Smads and participates in the regulation of gene transcription. The third group contains Smad6 and Smad7 which, despite their structural homology to other Smads, are negative regulators of TGFβ signalling [1–3].

In Smad proteins, three structural regions have been identified. The N-terminal and the C-terminal parts, which contain the Mad homology-1 (MH1), and the MH2 domains, respectively [4]. These two domains are connected by a linker region which probably does not have a domain structure. The MH1 domain of certain Smads, including Smad3, binds directly to DNA, while the MH2 domain mediates homo- and hetero-oligomerization between Smad proteins. The linker region was claimed to regulate Smad localization in cells and regulates stability of Smads. All three parts of Smad3 have been shown to interact with a number of proteins [1–4]. Interacting proteins significantly affect functions of Smad proteins, forming various complexes depending on an activation status of Smads. For instance, TGFβ-induced interaction with the transcriptional co-activator CBP/p300 strongly promotes Smad-dependent gene expression [5,6], and an interaction with the transcriptional co-repressors Ski/Sno suppresses the transcriptional activity of Smad3 [7].

Proteomics technologies have proven to be efficient tools in studies of protein complexes [8–13]. An analysis of proteins interacting with 32 known components of the TNFα signalling pathway unveiled novel modulators of the signalling [13]. In an application to the TGFβ family signalling, we have described a number of novel interacting proteins of bone morphogenetic protein receptor-II [12]. Here, we performed a screen for interacting proteins using a pull-down assay with two constructs of Smad3, the first encompassing the N-terminal MH1 domain and the linker region, and the second containing the C-terminal domain and the linker region, fused to glutathione-*S*-transferase (GST). Interacting proteins were resolved by two-dimensional gel electrophoresis (2D-GE) and were identified by peptide mass fingerprinting by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI TOF MS). We identified 25 proteins in 26 protein

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Abbreviations: TGFβ, transforming growth factor-β; 2D-GE, two-dimensional gel electrophoresis; MALDI TOF MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; SREBP, sterol regulatory element binding protein; GST, glutathione-*S*-transferase; MH, Mad homology domain; SRY, sex-determining region Y protein

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spots, which formed complexes with Smad3 constructs, and explored the effect of one of the interacting proteins, sterol regulatory element binding protein (SREBP-2), on the transcriptional activity of Smad3.

2. Materials and methods

2.1. GST fusion constructs of Smad3

GST fusion constructs used in this study were generated by cloning the Smad3 MH1 domain and the linker (Met1-Glu321; GST-Smad3-MH1L), and the linker region and the MH2 domain (Pro135-Ser424; GST-Smad3-LMH2) into a pGEX-4T-1 vector. The proteins were expressed in DH5α cells and were purified according to standard protocols using glutathione–Sepharose (Amersham Biosciences, Uppsala, Sweden).

2.2. Sample preparation

Mv1Lu, 293T, COS7 and HepG2 cell lines were obtained from ATCC and were maintained in DMEM containing 10% FBS. For GST pull-down assays Mv1Lu mink lung epithelial cells were used. Labelling was performed for 4 h or for 6 h in a methionine/cysteine-free medium with 20 μCi/ml of labelling mix (ProMix, Amersham Biosciences, Sweden). The proteins were extracted with a lysis buffer containing 1% Triton X-100, 20 mM Tris, pH 7.5, 150 mM NaCl, 10 μg/ml aprotinin and 1 mM PMSF. Equal amounts of GST-fusion-proteins were added to the cell lysate (lysate from 6×10^6 cells per pull down) and incubated overnight at 4 °C. After 3 washes with ice-cold lysis buffer, the samples were washed once with 20 mM Tris, pH 7.4, and resuspended in a sample buffer for isoelectric focusing (8 M urea, 4% CHAPS, 0.5% dithiothreitol (DTT), and IPG buffer, pH 3–10).

2.3. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described earlier [12]. Briefly, samples were subjected to isoelectric focusing using IPGDry strips with immobilized pH gradient, pH range 3–10, 18 cm, linear (Amersham Biosciences, Sweden). 2D-GE was performed according to the protocol described by Hassel et al. [12]. SDS–PAGE was performed in 10% or in 12% polyacrylamide gels. Two gels per each running and ^{35}S labelling condition were generated. After the electrophoresis, gels were fixed in 10% acetic acid and 20% methanol for 10–12 h. Proteins were detected by silver staining, as described earlier [14]. Gels were dried and scanned in a FujiX2000 phosphorimager (Fuji, Japan). The pH gradient of the first-dimension electrophoresis was evaluated as proposed by the manufacturer of the strips. Totally, 15 gels with samples from three experiments were prepared and subjected to analysis.

2.4. Gel analysis

Silver stained gels were scanned in an ImageScanner with the MagicScan32 software and analyzed by the ImageMaster 2D Elite software (Amersham Biosciences, Sweden). Gels were exposed in a FujiX2000 phosphorimager and scanned using the AIDA software (IMG GmbH, Germany). Images of silver stained gels and images of ^{35}S labelled proteins in the same gels were compared. As ^{35}S labelled proteins originate from Mv1Lu cells, this comparison allows distinguishing on silver stained gels between co-precipitated Mv1Lu proteins and non-labelled proteins which originate from bacteria, including GST fusion protein fragments. Cellular proteins, which specifically co-precipitated with Smad3 constructs and not with GST alone, were selected for identification.

2.5. Protein identification

Protein spots were excised from the gels, destained and subjected to in-gel digestion with trypsin (modified, sequence grade porcine, Promega, Madison, WI, USA), as described earlier [14,15]. Tryptic peptides were concentrated and desalted on a "nano-column" [16]. Peptides were eluted with about 65% acetonitrile, containing the matrix α-cyano-4-hydroxycinnamic acid, and applied directly onto the metal target and analyzed by MALDI TOF MS on a Bruker Biflex (Bruker Daltonics, Bremen, Germany). Peptide spectra were internally calibrated using autolytic peptides from trypsin. To identify proteins, we performed searches in the NCBInr sequence database using the ProFound (http://65.219.84.5/service/prowl/profound.html) search engine. One miscut, alkylation, and partial oxidation of methionine were allowed. Significance of the identification was evaluated according to the probability value, "*Z*" value, and sequence coverage.

2.6. Immunoblotting

GST pull-down assay was performed as described in sample preparation (2.2), using mink Mv1Lu and human HeLa cells. Proteins were subjected to SDS–PAGE, transferred to nitrocellulose membrane, and subjected to immunoblotting with antibodies specific to AGP7 (K-17; Santa Cruz), sex-determining region Y protein (SRY; C-17; Santa Cruz), actin β (C-11; Santa Cruz), and SREBP-2 (gift from Maria Teresa Bengoechea Alonso and Johan Ericsson) as described earlier [12].

2.7. Co-precipitation

COS7 cells were transiently transfected with myc-Smad3, Flag-SREBP-2 and constitutively active TβR-I, as indicated in Fig. 4. Constitutively active TβR-I was used to initiate TGFβ signalling. Twenty-four hours after transfection, cells were treated with TGFβ1 (10 ng/ml), as indicated in Fig. 4. Then, cells were harvested and the extract was clarified by centrifugation. Immunoprecipitation was performed with anti-Flag antibodies (M2, Sigma, USA) to precipitate Flag-SREBP-2, immunoprecipitate was resolved by SDS–PAGE and proteins were transferred onto a nitrocellulose membrane. Co-precipitated myc-tagged Smad3 was detected by immunoblotting with anti-myc antibodies (9E10, Santa Cruz, USA). Expression of myc-Smad3 and Flag-SREBP-2 was evaluated by immunoblotting of whole cell extracts with anti-myc and anti-Flag antibodies.

2.8. Luciferase reporter assay

Luciferase reporter assay was performed as described earlier [17]. Briefly, HepG2 cells were transiently transfected with plasmids encoding for myc-Smad3, Flag-SREBP-2, β-galactosidase (pCH110), and CAGA(12)-luc or Gal4(TK)-luc reporter plasmids. Twenty-four hours after transfection, cells were treated for the next 24 h with TGFβ1 (10 ng/ml), as indicated in Fig. 5. Cells were harvested, and luciferase and β-galactosidase activities were measured. Transfection efficiency was normalized to expression of β-galactosidase. Significance of changes was evaluated by the Student's *t* test.

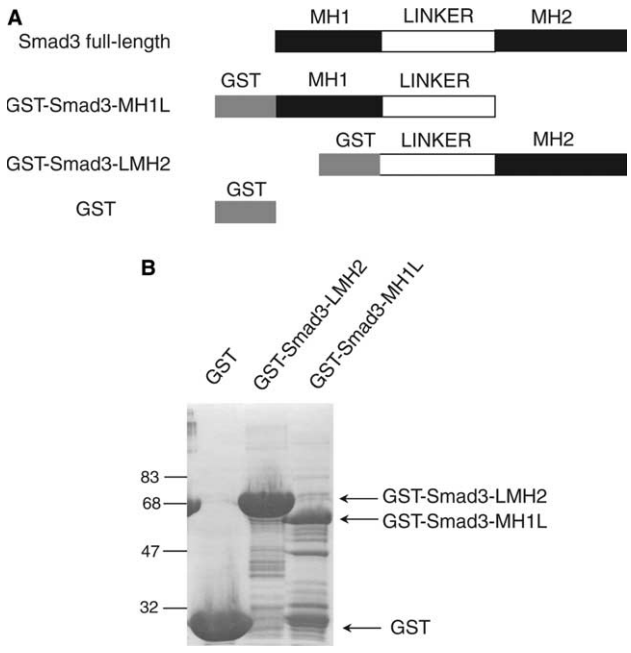

Fig. 1. Constructs of Smad3 used in this study. (A) Schematic presentation of GST-fusion constructs used in this study, compared to the full-length Smad3 protein. The MH1 and MH2 domains and the linker region are indicated. (B) Purified GST-Smad3 constructs and GST alone were separated by SDS–PAGE. Gel was stained with Coomassie Brilliant Blue to visualize proteins. Migration positions of proteins are shown by arrows. Migration positions of molecular mass markers are shown on the side of the gel.

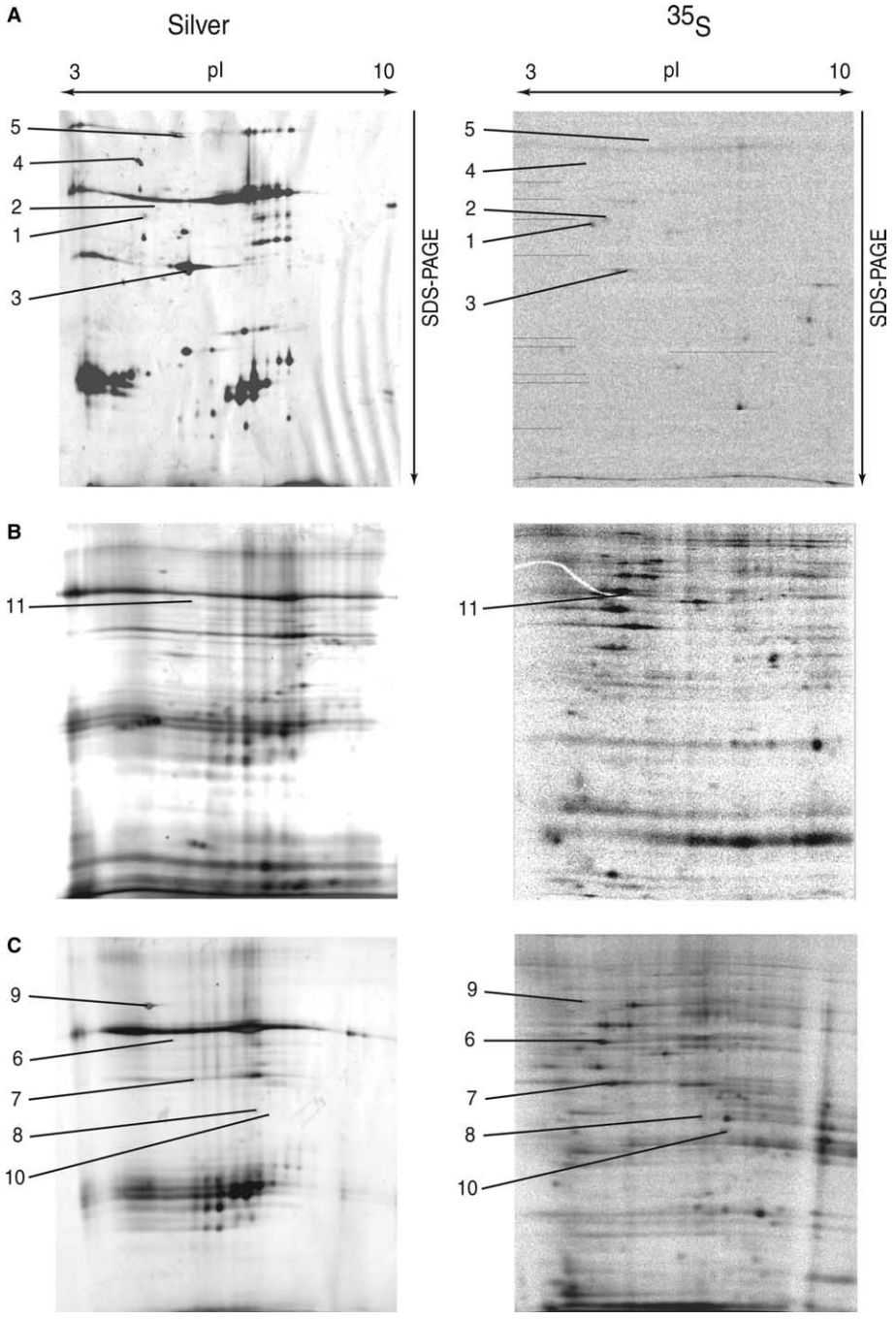

Fig. 2. Two-dimensional gels with proteins co-precipitated with Smad3 constructs. Images of two-dimensional gels stained with silver or after exposure in a phosphorimager are shown. The pH gradient of the separation in the first dimension is shown on the top of gels, and direction of SDS–PAGE is shown on the side of the gels. Migration positions of identified proteins are shown by lines. Annotation of spots is given in Table 1. Due to variations in intensities of silver staining and intensities of ^{35}S labelling, some of the spots may be poorly visible on the images of whole gels; lines indicate migration positions of the spots. Images of 2D gels obtained with GST-Smad3-MH1L (A, B, C), GST-Smad3-LMH2 (D, E, F), and GST only (G) are shown; the left panels show silver stained gel and the right panels show an image of the same gel after exposure in a phosphorimager, e.g., ^{35}S labelled proteins. Mv1Lu cells were ^{35}S labelled for 4 h (A, D) or for 6 h (B, C, E, F, G). SDS–PAGE was performed in 10% (A, B, D, E, G) or in 12% (C, F) polyacrylamide gels. Two gels per each condition were generated, totally 6 gels per one Smad3 construct and 3 gels for GST alone, and protein spots which appeared on both gels for each running condition were considered for further analysis. Representative gels out of 15 generated gels from three experiments are shown.

3. Results

3.1. Selection of proteins specifically interacting with Smad3

The N-terminal MH1 and C-terminal MH2 domains of Smad3 have distinct structural and functional characteristics; thus, we generated two deletion constructs of Smad3. The first construct lacks the MH1 domain (GST-Smad3-LMH2) and the second lacks the MH2 domain (GST-Smad3-MH1L) (Fig. 1). It has to be noted, that the Smad3 constructs were not modified by activated TGFβ receptors, as they were purified

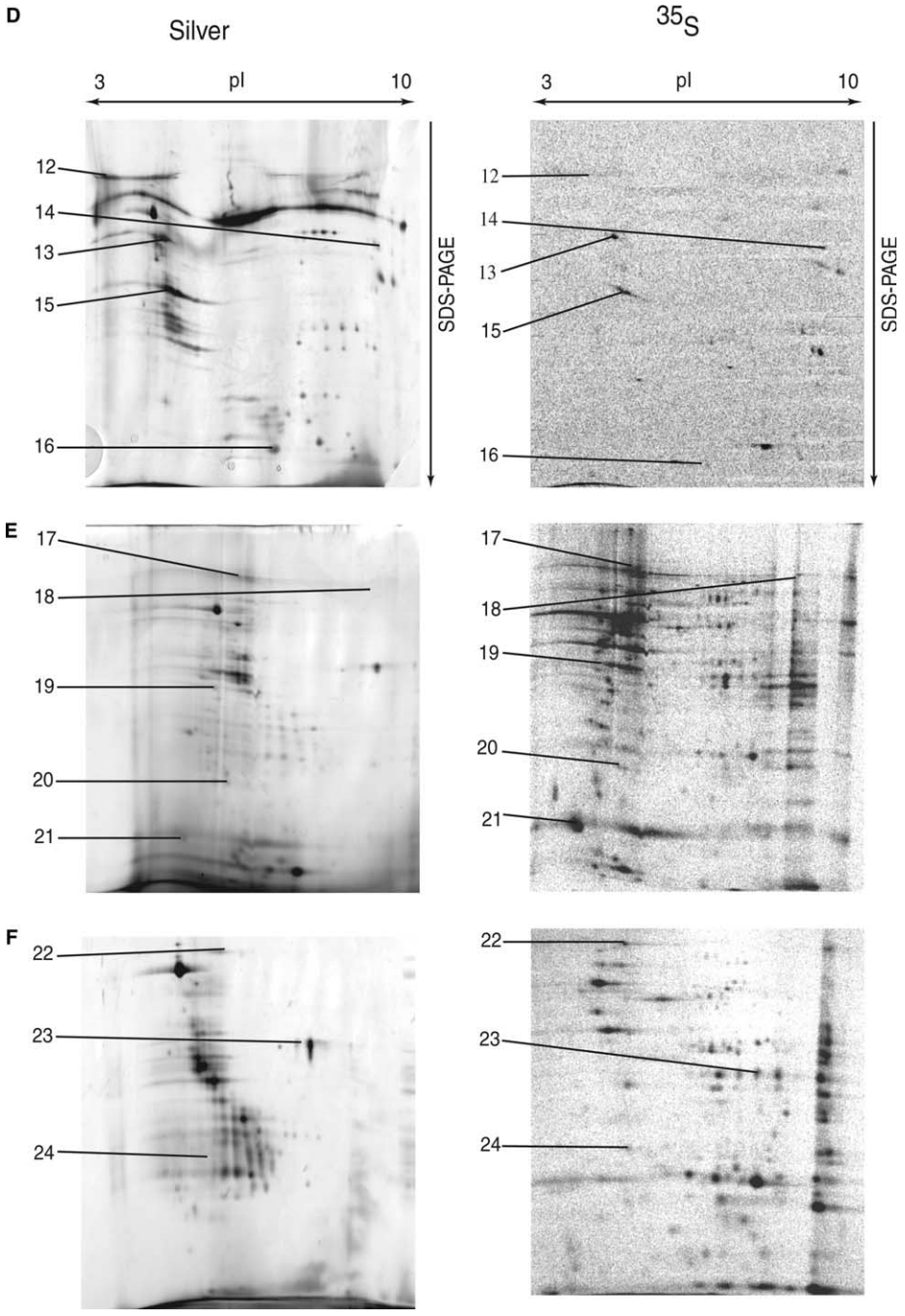

Fig. 2 (*continued*)

from bacteria and were not in contact with TGFβ receptors. As a receptor-dependent phosphorylation is essential for activation of Smad3, we consider that the constructs used in this work represent Smad3 in non-activated state. Thus, interacting proteins would represent pre-activation complexes of Smad3.

We observed a number of proteins that co-precipitated with the generated GST fusion constructs of Smad3, as well as with the GST alone (Fig. 2). We used 2D gels of 10% and 12% polyacrylamide concentration, as they showed to be more efficient in separation of proteins, as compared to 1D SDS–PAGE (data not shown). An analysis of silver stained gels and images of ^{35}S labelled proteins was performed to select specifically interacting proteins. Notably, only ^{35}S labelled proteins, co-precipitating with GST-Smad3 constructs, but not with GST alone, were selected. As a source of interacting proteins, we used Mv1Lu cells, which have been widely used in studies of TGFβ signalling in epithelial cells and have intact TGFβ/Smad-dependent signalling [18]. A number of proteins was found to interact with GST constructs non-specifically, as they were precipitated with GST alone (Fig. 2; data not shown). Totally, we selected 45 protein spots for identification of proteins by peptide mass fingerprinting. Twenty-five pro-

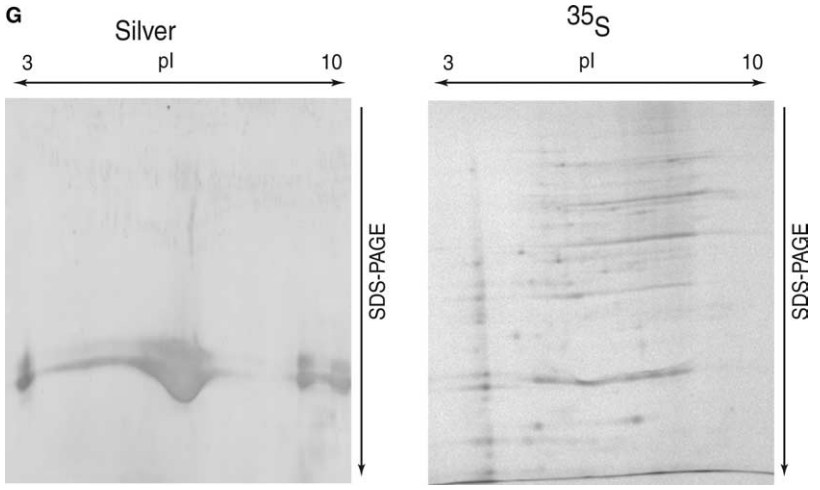

Fig. 2 (*continued*)

teins were identified (Table 1); vimentin interacted with both Smad3 constructs (p06 and p13; Table 1). The remaining 19 proteins were identified ambiguously and will not be discussed. Fourteen proteins formed a complex with the GST-Smad3-LMH2 construct and 12 proteins interacted with the GST-Smad3-MH1L construct.

Table 1
Smad3 interacting proteins Proteins forming complexes with various constructs of GST-Smad3 were identified by 2D-GE and MALDI TOF MS

Number of spot[a]	Construct used[b]	Name of protein	Accession No.[c]	Probability[c]	"*Z*" Value	Sequence coverage (%)	Experimental values		Theoretical values	
							p*I*	M_r	p*I*	M_r
P01	MH1L	AGP7 (APG7)	NP_083111	1.0e + 000	2.43	8	5.0	70.0	6.0	78.6
p02	MH1L	KIAA1354	NP_061335	1.0e + 000	1.94	8	5.3	75.0	5.8	72.0
p03	MH1L	Ornithine decarboxylase, chain A	7ODCA	1.0e + 000	2.41	9	5.7	50.0	5.6	47.8
p04	MH1L	EPB41	AAH39079	1.0e + 000	2.43	9	5.0	85.0	5.6	81.8
p05	MH1L	Oocyte-testis gene 1	NP_739563	1.0e + 000	1.48	10	5.7	100.0	5.4	105.7
p06	MH1L	Vimentin (source: fibroblast)	AAA61281	9.8e – 001	1.01	23	5.0	65.0	4.8	41.7
p07	MH1L	Smad3	NP_005893	1.0e + 000	2.07	17	6.0	50.0	6.7	48.9
p08	MH1L	KIAA1119	BAA86433	9.6e – 001	0.56	7	8.0	50.0	6.9	215.9
p09	MH1L	Tropomyosin 3	NP_689476	1.0e + 000	2.43	25	4.0	30.0	4.7	28.2
p10	MH1L	CT-10 related kinase 3	AAL27153	9.6e – 001	0.63	7	8.0	30.0	5.5	31.5
p11	MH1L	SREBP-2[d]	AAA85718	7.3e – 003	0.05	7	5.0	55	5.5	95.00
p12	LMH2	Lamin A	CAA53945	9.9e – 001	0.76	11	4.0	100.0	6.2	72.9
p13	LMH2	Vimentin	A25074	9.9e – 001	0.80	27	5.0	65.0	5.1	53.7
p14	LMH2	Splicing factor 3A	NP_005868	9.8e – 001	0.71	8	9.0	70.0	5.2	88.9
p15	LMH2	Actin, beta	AAH08633	1.0e + 000	2.06	28	5.5	45.0	5.6	41.3
p16	LMH2	Sex-determining region Y (SRY)	AAL68650	1.0e + 000	2.43	34	9.0	25.0	10.0	24.6
p17	LMH2	Glycogen phosphorylase	NP_005600	1.0e + 000	0.84	18	6.0	100.0	6.6	97.5
p18	LMH2	Junctophilin 3	NP_065630	1.0e + 000	1.84	7	9.0	100.0	9.4	81.6
p19	LMH2	Keratin 10, type I	KRHU0	1.0e + 000	1.96	12	5.0	40.0	5.2	59.7
p20	LMH2	Interleukin 1	NP_055254	1.0e + 000	1.93	13	5.5	25.0	6.1	24.6
p21	LMH2	Pregnancy-associated endometrial α2-globulin	AAA35802	1.0e + 000	2.04	15	4.5	20.0	5.1	18.6
p22	LMH2	Hypothetical protein	XP_297429	9.8e – 001	0.73	19	8.0	50.0	10.7	33.9
p23	LMH2	Keratin 2a	NP_000414	9.6e – 001	0.58	12	8.5	50.0	8.3	66.1
p24	LMH2	Zinc finger, DHHC domain containing 4 protein	NP_082655	9.8e – 001	0.65	11	9.0	45.0	9.6	40.4
p25	LMH2	α1,3-Fucosyltransferase	BAB68651	9.9e – 001	0.70	27	n.a.	60.0	10.2	44.8
p26	MH1L	RBAP2	AAF21796	9.7e – 001	0.63	9	n.a.	110.0	6.4	106.9

[a] Numbers of selected protein spots from silver-stained and ^{35}S labelled gels are presented.
[b] LMH2, GST-Smad3-LMH2; MH1L, GST-Smad3-MH1L.
[c] NCBInr sequence identification numbers. Probability, *Z*-value, coverage and theoretical p*I* and M_r were obtained from the ProFound search. The calculation of experimental p*I* and M_r was based on migration of proteins on a 2D gel. n.a., data are not available, as p25 and p26 were identified after resolving of Smad3 interacting proteins in 1D SDS–PAGE.
[d] SREBP-2 is included in the Table 1, as its interaction with Smad3 was confirmed by co-immunoprecipitation of the proteins expressed in mammalian cells.

The main group consists of 8 proteins which are components of the cytoskeleton, or proteins regulating cytoskeleton (p04, p06, p09, p12, p13, p15, p18, p19 and p23; Table 1). Vimentin was identified as a protein which interacted with both Smad3 constructs (p06 and p13), suggesting that the most probable vimentin-interacting region is in the linker of Smad3. Identification of components of the cytoskeleton is in agreement with the previously reported interaction of Smad3 with the cytoskeleton [19].

Seven of the identified proteins have assigned functions in various signalling processes; four of the proteins are involved in regulation of transcription (p07, Smad3; p11, SREBP-2; p16, SRY and p26, RBAP-2), splicing factor 3 (p14) regulates activation of snRNA, and CT-10 related kinase 3 (CrkIII, p10) is an adaptor protein. AGP7 protein (p01) has been described as a protease. SREBP-2 is included in the list of identified proteins (Table 1), as its complex formation with Smad3 was confirmed by co-immunoprecipitation of the proteins expressed in mammalian cells (Fig. 4). Smad3 protein forms homo-oligomers in cells [20], and identification of endogenous Smad3 as an interacting protein suggests that conditions of the assay allowed preservation of physiologically relevant interactions.

Enzymes regulating metabolism of carbohydrates (p17, glycogen phosphorylase; p25, α1,3-fucosyltransferase) and ornithine (p03, ornithine decarboxylase) were identified. The importance of the Smad3 interaction with metabolic enzymes remains to be elucidated. Five of the interacting proteins do not have assigned functions (p02, KIAA1354; p05, oocyte-testis gene 1; p08, KIAA1119; p22, novel protein; and p24, zinc finger, DHHC-domain containing 4 protein).

Twenty-four of the identified proteins have not been described as Smad3 binding molecules; the 25th protein, Smad3 (p07), is suggested to have an intra-molecular interaction between the MH1 and the MH2 domains. However, 5 of these 24 proteins (25 protein spots, not including Smad3, p07; Table 1) do not have assigned functions in cells and 13 proteins are involved in activities which have been described to involve Smad proteins. The interacting proteins with activities previously not associated with Smad3 are metabolic enzymes (p03, p17 and p25), splicing factor 3A (p14) and CT-10 related kinase 3 (CrkIII, p10). Detection of the latter two proteins suggests that Smad3 may affect activation of snRNA and may interact with the adaptor protein involved in recognition of a phosphotyrosine.

3.2. Confirmation of interaction of SRY, actin β, AGP7 and SREBP-2 proteins with Smad3 by immunoblotting

To confirm observed interactions by an alternative technique, we performed immunodetection of co-precipitated proteins in GST pull-down assays. We selected for a confirmation study AGP7, SRY, and actin β proteins, as specific antibodies were available for these proteins. We also used human HeLa and mink Mv1Lu cells to explore whether both mink and human proteins would form a complex with Smad3. We detected AGP7, SRY, actin β and SREBP-2 proteins co-precipitated with Smad3 constructs (Fig. 3). SRY and actin β interacted with a GST-Smad3LMH2 construct, and AGP7 and SREBP-2 interacted with a GST-Smad3MH1L construct. This is in agreement with the specificity of interactions observed using 2D-GE and mass spectrometry (Table 1). Weak interactions of AGP7 with GST-Smad3LMH2 and SRY with GST-Smad3MH1L suggest that the linker region of Smad3 may contribute to the interactions; use of specific antibodies increases sensitivity of detection and allows detection of even weak interactions. Detection of human SRY, actin β, AGP-7 and SREBP-2 proteins in a complex with Smad3 constructs (Fig. 3) confirmed the findings made with mink proteins (Figs. 2 and 3, Table 1). This also supports the significance of peptide mass fingerprinting of mink proteins (Table 1) using NCBI database pool of mammalian proteins.

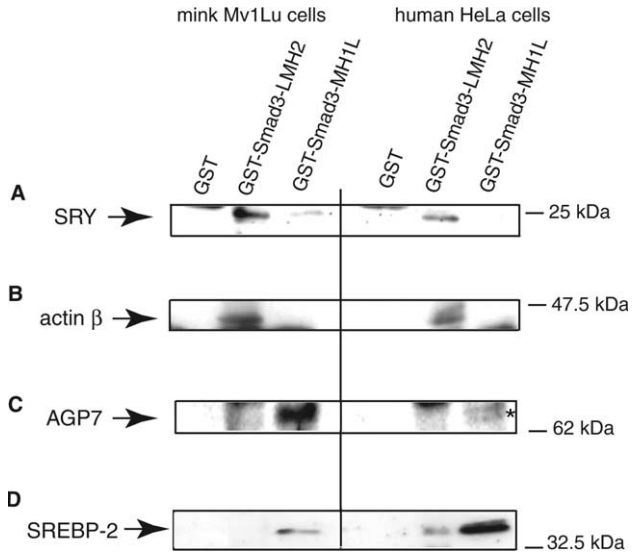

Fig. 3. Confirmation of sex-determining region Y protein, actin β, AGP7 and SREBP-2 interactions with Smad3. GST pull down assays were performed with cell extracts from mink Mv1Lu and human HeLa cells, as indicated. Cells were not treated with TGFβ, to identify proteins which may form pre-activation complexes with Smad3. Interacting proteins were subjected to SDS–PAGE, transferred to membrane, and immunoblotted with antibodies, as indicated. Specific antibodies to SRY (A), actin β (B), AGP7 (C) and SREBP-2 (D) were used to detect co-precipitated proteins, as indicated in panels. Arrows show migration positions of proteins with expected molecular masses of SRY, actin β, AGP7 and SREBP-2 proteins. Migration positions of molecular mass markers are indicated. In panel C, * indicates a protein band of predicted molecular mass, which was recognized by anti-AGP7 antibodies. GST-Smad3-MH1L, GST-Smad3-LMH2, and GST constructs are indicated. Representative experiments out of 2 performed, are shown.

3.3. SREBP-2 forms a complex with Smad3 and inhibits its transcriptional activity

To further confirm SREBP-2 and Smad3 interaction in vivo, we transfected Flag-SREBP-2 and myc-Smad3 in COS7 cells, and performed a co-immunoprecipitation assay. We found that myc-Smad3 and Flag-SREBP-2 formed a complex in non-treated cells, while TGFβ1 treatment decreased complex formation (Fig. 4). Ligand-independent complex formation between Flag-SREBP-2 and myc-Smad3 in COS7 cells is in agreement with the detection of SREBP-2 in the GST pull-down assay (Figs. 2 and 3D; Table 1), as the bait GST-Smad3 construct resembles a non-activated Smad3.

Smad3 regulates transcription of genes by binding to a promoter DNA and by interacting with other proteins [1–4]. To explore whether the interaction with SREBP-2 affects the transcriptional activity of Smad3, we performed assays with luciferase reporters, sensitive to Smad3 (Fig. 5). CAGA(12)-luc

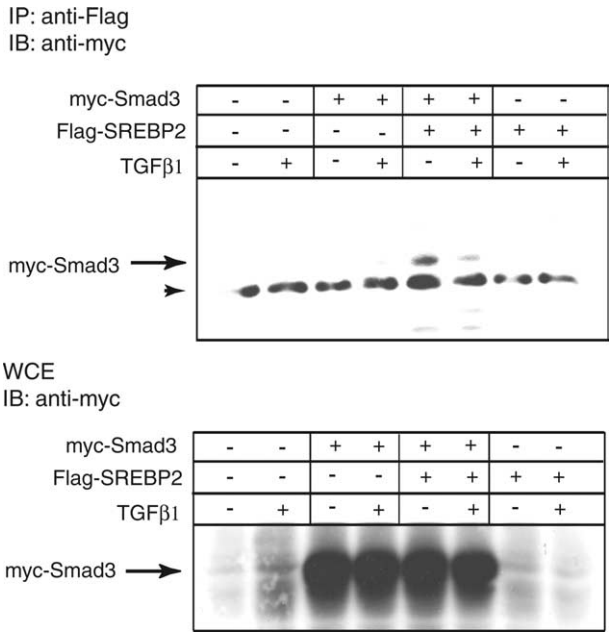

Fig. 4. Flag-SREBP-2 and myc-Smad3 form a complex in cells. Flag-SREBP-2, myc-Smad3 and constitutively active mutant of TβR-I were transfected in COS7 cells. Expression of constitutively active TβR-I and treatment with TGFβ1 (10 ng/ml) were performed to induce TGFβ-dependent signalling, as indicated. myc-Smad3, co-precipitated with Flag-SREBP-2, was detected with anti-myc antibodies (upper panel). Expression of myc-Smad3 was monitored by immunoblotting of a whole cell extract (lower panel). Migration positions of myc-Smad3 are shown by arrows. Arrowhead shows a non-specific band which was detected in all samples. A representative experiment out of 2 performed is shown. WCE, whole cell extract; IP, immunoprecipitation; IB, immunoblotting.

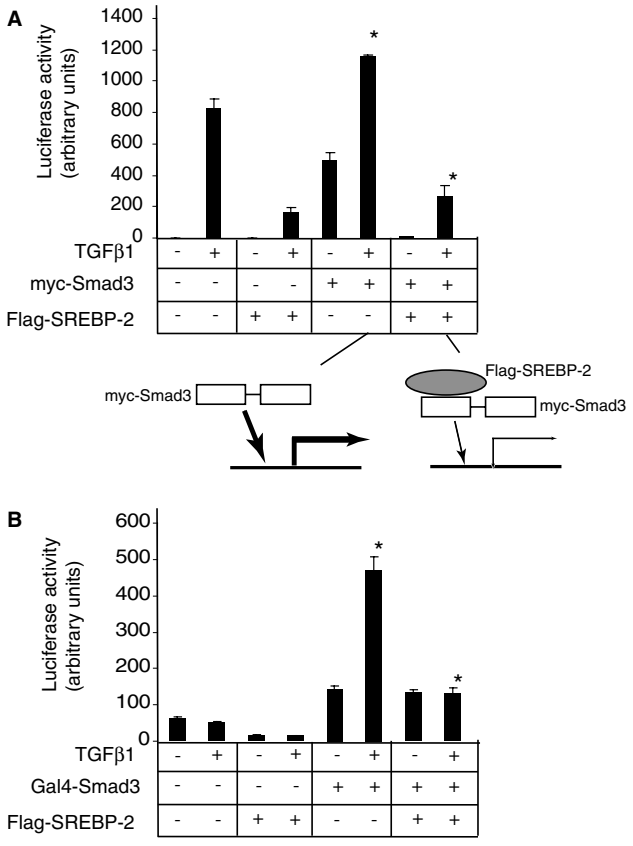

Fig. 5. SREBP-2 inhibits transcriptional activity of Smad3. Assays with CAGA(12)-luc (A) and Gal4(TK)-luc (B) luciferase reporters were performed in HepG2 cells. Cells were transfected with myc-Smad3 and Flag-SREBP-2 constructs, and treated with TGFβ1 (10 ng/ml), as indicated. Luciferase activity was normalized to the expression of β-galactosidase. Schematic presentation of activation of CAGA(12)-luc reporter upon transfection of cells with myc-Smad3 and Flag-SREBP-2, and treatment with TGFβ1 are shown in panel A. *, $P < 0.01$, cells transfected with myc-Smad3 compared to cells transfected with myc-Smad3 and Flag-SREBP-2, and treated with TGFβ1.

reporter contains CAGA elements in the promoter, which bind activated Smad3 directly [21]. Treatment of HepG2 cells with TGFβ1 activated the reporter via endogenous Smad3, as expected. Expression of myc-Smad3 increased activation and co-transfection of SREBP-2 with myc-Smad3 inhibited Smad3-dependent activation of CAGA(12)-luc reporter (Fig. 5A). Similar results were obtained with Mv1Lu cells (data not shown).

We also studied the effect of SREBP-2 on the transcriptional activity of Smad3 in another reporter assay; the Gal4(TK)-luc reporter is activated specifically when Smad3 fused to the Gal4 DNA-binding domain is transfected in cells which are treated with TGFβ1 (Fig. 5B) [22]. We found that in this assay SREBP-2 also inhibited Smad3-dependent transcriptional activity induced by the ligand. Thus, in two independent assays we observed inhibitory effect of SREBP-2 on transcriptional activity of Smad3. Stronger interaction of SREBP-2 with Smad3 in the absence of ligand treatment, as compared to TGFβ1-treated cells (Fig. 4), suggests that SREBP-2 may sequester Smad3 to inhibit its transcriptional activity (Fig. 5).

4. Discussion

Description of protein interaction networks is of importance for the understanding of protein functions. Here, we identified 25 proteins that co-precipitated with Smad3 constructs fused to GST. As GST-Smad3 constructs were not subjected to the receptor-specific phosphorylation, they could be considered as representing a non-activated Smad3. Smad3 in inactive state was earlier found to interact with microtubules and treatment with TGFβ leads to dissociation of Smad3 from microtubules [19]. Our finding that 8 Smad3-interacting proteins are components of the cytoskeleton (Table 1) supports the notion that Smad3 may be bound to the cytoskeleton prior to activation. Five of these proteins were found to bind to the GST-Smad3-LMH2 construct and 3 were bound to the GST-Smad3-MH1L. Confirmation of interactions of Smad3 with SRY, actin β, AGP7 and SREBP-2 proteins obtained from mink and human cells by immunoblotting (Fig. 3) suggests that our proteomics/mass spectrometry-based findings with mink proteins are also relevant for human proteins. Interaction of actin β with GST-Smad3LMH2 construct, and no interaction with GST-Smad3MH1L and GST alone (Fig. 3), suggests that the identified components of the cytoskeleton form a complex with Smad3 specifically.

Smad3 is a transcription factor and, therefore, interactions with signalling proteins would be expected. We identified 7 proteins which have regulatory roles in gene transcription, splicing of snRNA, and protein degradation. Twenty-four

proteins identified in 25 protein spots have not been previously described as Smad3-binding molecules, increasing the number of Smad3-interacting proteins to more than 50 [23]. We do not exclude that some of the interactions with Smad3 may not be direct, but rather reflected formation of multiprotein complexes. Further studies of Smad3 complexes purified directly from cells are required to map changes in protein interaction upon activation of Smad3.

Smad3 interacts with a number of transcriptional regulators, which modulate Smad3-dependent gene expression [1–4,23]. SREBP-2 is a transcription factor, which stimulates expression of a number of enzymes of sterol synthesis [24,25]. An interaction of Smad3 with SREBP-2, observed by us, may be involved in the inhibitory effect of SREBP-2 on the transcriptional activity of Smad3 (Figs. 2, 4 and 5). As TGFβ is a potent inhibitor of steroidogenesis [26], it is tempting to suggest that SREBP-2 may inhibit the anti-steroidogenic action of TGFβ/Smad3 by sequestering Smad3, thus enhancing SREBP-2-specific stimulation of the steroidogenesis. To support this suggestion, further exploration of Smad3 interaction with SREBP-2 is required.

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