RLP, a novel Ras-like protein, is an immediate-early transforming growth factor-β (TGF-β) target gene that negatively regulates transcriptional activity induced by TGF-β

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We have described previously the use of microarray technology to identify novel target genes of TGF-β (transforming growth factor-β) signalling in mouse embryo fibroblasts deficient in Smad2 or Smad3 [Yang, Piek, Zavadil, Liang, Xie, Heyer, Pavlidis, Kucherlapati, Roberts and Böttinger (2003) Proc. Natl. Acad. Sci. U.S.A. **100**, 10269–10274]. Among the TGF-β target genes identified, a novel gene with sequence homology to members of the Ras superfamily was identified, which we have designated as RLP (Ras-like protein). RLP is a Smad3-dependent immediate-early TGF-β target gene, its expression being induced within 45 min. Bone morphogenetic proteins also induce expression of RLP, whereas epidermal growth factor and phorbol ester PMA suppress TGF-β-induced expression of RLP. Northern-blot analysis revealed that RLP was strongly expressed in heart, brain and kidney, and below the detection level in spleen and skeletal muscles. At the protein level, RLP is approx. 30 % homologous with members of the Ras superfamily, particularly in domains characteristic for small GTPases. However, compared with prototypic Ras, RLP contains a modified P-loop, lacks the consensus G2 loop and the C-terminal prenylation site and harbours amino acid substitutions at positions that render prototypic Ras oncogenic. However, RLP does not have transforming activity, does not affect phosphorylation of mitogen-activated protein kinase and is unable to bind GTP or GDP. RLP was found to associate with certain subtypes of the TGF-β receptor family, raising the possibility that RLP plays a role in TGF-β signal transduction. Although RLP did not interact with Smads and did not affect TGF-β receptor-induced Smad2 phosphorylation, it inhibited TGF-β-induced transcriptional reporter activation, suggesting that it is a novel negative regulator of TGF-β signalling.

Key words: GTPase, Ras, sorting nexin, transcriptional regulation, transforming growth factor-β.

INTRODUCTION

The Ras superfamily of small guanosine triphosphatases (GTPases/G-proteins) comprises over 100 members that are structurally related and that act as molecular switches, shuttling between an inactive GDP-bound state and an active GTP-bound state to regulate a broad range of biological processes in response to external stimuli, in a temporally and spatially tight controlled manner. The classical Ras proteins are represented by three *ras* genes, N-Ras, H-Ras and K-Ras, the latter two initially identified as the Harvey and Kirsten strains of rat sarcoma viruses. The Ras superfamily comprises the Ras, Rho/Rac/Cdc42, Rab, Ran and Sar1/Arf subfamilies, as well as the recently identified RGK subfamily, which is named after the GTPases Rad, Gem/Kir (RGK) [1,2].

Small G-proteins of the Ras superfamily are characterized by the presence of five conserved loops (G1–G5), which are part of the catalytic domain and important for binding of GDP or GTP, GTP-induced conformational changes and GTP hydrolysis. The G1 motif is also referred to as the 'P-loop', since it harbours the lysine residue that forms bonds with the phosphates of GTP or GDP (reviewed in [3,4]). GTPases are activated by upstream activators, the guanine nucleotide exchange factors, which catalyse dissociation of GDP and promote binding of GTP. This enables the activated G-proteins to interact with and propagate signalling to specific downstream effectors that initiate distinct intracellular signalling cascades. To terminate the action of active G-proteins, hydrolysis of GTP to GDP and free phosphate is necessary. Small G-proteins of the Ras superfamily are endowed with weak intrinsic GTPase activity. GTP hydrolysis is promoted by GAPs (GTPase-activating proteins), which render small G-proteins in an inactive, GDP-bound configuration (reviewed in [2–4]). Mutations that render Ras proteins in a constitutively active, GTPase-impaired configuration, are observed at high frequency in many different types of human cancer (reviewed in [5,6]).

Activation of Ras, followed by activation of downstream effectors (e.g. RalGDS, phosphoinositide 3-kinase and Raf) that initiate distinct signal-transduction pathways, generally occurs after activation of receptor tyrosine kinases and cytokine receptors in association with G-proteins. In addition, activation of the Ras/MAPK pathway (where MAPK stands for mitogen-activated protein kinase) can occur downstream of serine/threonine kinase

Abbreviations used: BMP, bone morphogenetic protein; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular-signal-regulated kinase; FAST-1, forkhead activin signal transducer-1; FBS, fetal bovine serum; GAP, GTPase-activating protein; GST, glutathione S-transferase; HA, haemagglutinin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryo fibroblast; MOI, multiplicity of infection; PDGFRβ, platelet-derived growth factor receptor β; RLP, Ras-like protein; SNX, sorting nexin; TGF-β, transforming growth factor-β; TβR, TGF-β receptor; UTR, untranslated region; WT, wild-type.

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receptors for members of the TGF-β (transforming growth factor-β) superfamily (reviewed in [7,8]). TGF-β is the prototypic member of a large family of cytokines involved in the regulation of many physiological processes, including differentiation, apoptosis and inhibition of cellular proliferation. Besides the TGF-βs, activins and BMPs (bone morphogenetic proteins) constitute this multipotent growth factor family (reviewed in [9]). TGF-β signals through a heteromeric complex of type I and II serine/threonine kinase receptors, whereby the type I receptor specifies downstream signalling by activation of receptor-activated (R-) Smad proteins. Whereas signalling by TGF-β or activin leads to activation of Smad2 and Smad3, BMPs trigger activation of Smad1, Smad5 and Smad8. Phosphorylation of the R-Smads occurs at the C-terminal SSXS motif and relieves the proteins from an autoinhibitory configuration, allowing the R-Smads to interact with the common partner Smad4. These complexes translocate to the nucleus where, in co-operation with other transcription factors, they regulate the expression of target genes (reviewed in [7]).

Although Smads have been recognized as the most predominant signal transducers downstream of the activated TβR (TGF-β receptor) complex, it is also known that the Ras/MAPK pathway can play a role in the translation of TβR activation to target gene regulation, thereby allowing Smad-independent signalling (reviewed in [7,10]). The MAPK pathway, activated by the TβR complex or by other stimuli, can cross-talk to the Smad pathway and thereby regulates the activity of the Smads. Smads harbour consensus sites for phosphorylation by ERK (extracellular-signal-regulated kinase) [-PX(S/T)P-] and JNK (c-Jun N-terminal kinase) (-XXSP-) in their linker region. Phosphorylation at these sites can alter Smad activity, and both inhibition of Smad activity by impaired nuclear translocation [11], as well as enhanced Smad activity [10,12], have been reported. At the nuclear level, Smads can cross-talk with transcription factors activated by the Ras/MAPK pathway, including ATF-2 and AP-1, to regulate expression of TGF-β target genes [13,14]. Moreover, Ras and TGF-β co-operate in the malignant transformation of, e.g. keratinocytes, hepatocytes and breast epithelial cells, promoting their transdifferentiation into fibroblastoid spindle-shaped cells, which are endowed with increased metastatic potentials [15,16].

To identify novel target genes of TGF-β signalling and to analyse the importance of Smad2, Smad3 or MAPK signalling in regulation of these target genes, a broad-scale microarray analysis was performed using MEFs (mouse embryo fibroblasts) [17]. These studies led to the identification of RLP (Ras-like protein), an immediate-early target gene of TGF-β signalling, which shares approx. 30 % homology to members of the Ras superfamily. Compared with prototypic Ras, RLP contains a modified P-loop, lacks the consensus G2 loop and harbours amino acid 'substitutions' at positions that are typically targeted to render prototypic Ras oncogenic. Surprisingly, RLP is not capable of binding GTP or GDP and, accordingly, does not have transforming activity in NIH-3T3 cells or affect activation of MAPK. RLP can interact with type I and II serine/threonine kinase receptors for members of the TGF-β superfamily. Although RLP does not affect TGF-β-induced C-terminal Smad phosphorylation, it suppresses TGF-β-induced activation of the transcriptional reporters ARE-Luc, $(SBE)_4$-Lux and $(CAGA)_{12}$-Luc and might, therefore, be a novel negative regulator of TGF-β signalling.

MATERIALS AND METHODS

Cell culture

Spontaneously immortalized and primary low-passage MEFs derived from embryos deficient in Smad2 (S2KO) or Smad3 (S3KO) and their corresponding WT (wild-type) littermates (S2WT and S3WT) were generated as described previously [17]. Primary mouse dermal fibroblasts established from Smad3-knockout and WT littermates were derived as described by Flanders et al. [18]. These cells, as well as HEK-293 (human embryonic kidney 293 cells), Cos7, C2C12, NMuMG and phoenix Eco cells (A.T.C.C.), were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 50 μg/ml streptomycin. NIH-3T3 cells were cultured in DMEM containing 10 % (v/v) newborn calf serum, 100 units/ml penicillin and 50 μg/ml streptomycin. Primary WT and S3KO murine glomerular mesangial cells were kindly provided by Dr H. W. Schnaper (Northwestern University, Chicago, IL, U.S.A.) and maintained in DMEM/Ham's F12 medium, supplemented with 20 % heat-inactivated FBS, glutamine, penicillin–streptomycin, sodium pyruvate, Hepes buffer and 8 μg/ml insulin. The cells were grown in 5 % CO_2 atmosphere at 37 °C.

Cloning of full-length RLP

RLP (EST clone AA119067) was identified in a microarray screen using 9K mouse cDNA arrays produced by the Albert Einstein College of Medicine cDNA Microarray Facility [17]. Sequence analysis revealed that clone AA119067 contained the 3′-UTR (3′-untranslated region) and only part of the upstream coding sequence. Database searching indicated that the cDNA sequence of AA119067 corresponded to the sequence deposited for EST clone AI608035. Sequence analysis of AI608035 showed that this clone contained the complete coding sequence of RLP. The 5′-end of the mRNA was determined using the 5′-Race System (Invitrogen) according to the manufacturer's method, using primer 5′-TGGAGCTGGCTAATGAGTTC-3′ for first-strand cDNA synthesis. Primer 5′-CTTAAGAATTCACGAGGACGACGGCATC-TGC-3′, containing an *Eco*RI cloning site, was used for subsequent PCR amplification of tailed cDNA. The obtained fragments were restriction-digested and cloned in pBluescript, and were sequence-analysed to identify the most 5′-ATG start codon. PCR primer 5′-ATAGGATCCATGGCTAGCATGACTGGTG-GACAGCAAATGGGTCGCCTCATCCAGAACATGTGTA-3′, containing a *Bam*HI cloning site and T7-epitope sequence, and primer 5′-CGCGAATTCTCAGACCGACGTGACAGTCCTCA-CT-3′, containing an *Eco*RI restriction site and stop codon, were designed to 5′-tag the full-length RLP cDNA with the T7 epitope and to clone the PCR product into *Bam*HI and *Eco*RI sites of pcDNA3. The T7-RLP pcDNA3 construct was sequence-verified.

Multiple-sequence alignment

Sequence alignment of RLP with different Ras superfamily members was achieved using the ClustalW 1.82 multiple-sequence alignment program.

RNA isolation and Northern-blot analysis

The kinetics of induction of RLP expression in primary low-passage MEFs, spontaneously immortalized MEFs and primary dermal fibroblasts was determined by treatment of subconfluent cultures with 8 ng/ml TGF-β1 (a gift from R & D Systems, Minneapolis, MN, U.S.A.), 100 ng/ml BMP-2 (Genetics Institute, Cambridge, MA, U.S.A.) or 100 ng/ml BMP-7 (a gift from Creative Biomolecules, Hopkinton, MA, U.S.A.) for indicated time periods. Reconstitution of Smad3 expression in primary low-passage S3KO MEFs was achieved by adenoviral infection of the cells using an MOI (multiplicity of infection) of 10 or 40 as described previously [19].

Primary dermal fibroblasts were treated for 1 h with 8 ng/ml TGF-β1, 100 ng/ml BMP-2, 100 ng/ml BMP-7, 25 ng/ml activin A (R & D Systems), 50 ng/ml PMA (Sigma), 10 ng/ml EGF (epidermal growth factor; R & D Systems), 10 ng/ml acidic fibroblast growth factor (Sigma), 10 ng/ml muTNFα (where TNF stands for tumour necrosis factor) and 10 ng/ml muIFNγ (where IFN stands for interferon). To check the effect of PMA, EGF, acidic fibroblast growth factor, muTNFα or muIFNγ on TGF-β-induced RLP expression, these factors were added 30 min before treatment of the cells with TGF-β (in the presence of the respective factors) for 1 h.

Total RNA was harvested using an RNeasy kit (Qiagen) according to the manufacturer's instructions. For each sample, 15 μg of total RNA was loaded on 1.2 % agarose/formaldehyde gels and transferred on to Nytran Super Charge membranes (Schleicher and Schuell, Dassel, Germany). Membranes were hybridized in Church buffer [20] with an 800 bp *Eco*RI–*Not*I 3′-UTR fragment derived from EST clone AA119067, which was ^{32}P-labelled using the RadPrime DNA Synthesis random-primed DNA labeling kit (Life Technologies). Tissue distribution of RLP was examined by hybridization of a mouse multiple tissue Northern blot (ClonTech) with an 1100 bp *Eco*RI–*Eco*RI probe fragment of EST clone AI608035, containing the complete coding sequence and flanking parts of 5′- and 3′-UTR. The blots were hybridized overnight at 65 °C and then washed three times for 30 min each at 65 °C. Washed blots were exposed to Kodak X-OMAT AR films for several days at − 80 °C.

GST (glutathione S-transferase)-fusion protein production

GST-fusion protein expression construct for RLP was generated using forward primer 5′-CCCGATATCATGCGCCTCATCCAGAACATGTGTACCATC-3′, containing an *Eco*RV restriction site and lacking the T7-epitope sequence, and reverse primer 5′-TTTGCGGCCGCTCAGACCGACGTGACAGTCCTCACT-3′, containing a *Not*I restriction site and stop codon, to amplify RLP cDNA insert by PCR from the above-described T7-tagged RLP pcDNA3 construct. Restriction-digested PCR fragments were cloned into pGMEX (AMRAD Biotech, Richmond, Victoria, Australia) and sequence verified.

Recombinant RLP was expressed as a GST-fusion protein in BL21DE3 bacteria after isopropyl β-D-thiogalactoside addition and purified on glutathione–Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions. GST–RLP was eluted from the beads in 10 mM gluthatione and 50 mM Tris/HCl (pH 8). GST–RhoA in pGMEX vector and pGMEX empty vector were taken along as controls. Protein concentrations were determined by Bio-Rad Protein Assay and checked by Coomassie Blue staining after SDS/PAGE.

[^{3}H]GDP-binding assay

Binding of [^{3}H]GDP to GST, GST–RLP or GST–RhoA was determined based on the method described by Self and Hall [21]. Briefly, 4 μg of GST-fusion protein was incubated in 320 μl of loading buffer (50 mM Tris, 50 mM NaCl, 5 mM dithiothreitol, 5 mM $MgCl_2$ and 10 mM EDTA) to which 2 μCi of [^{3}H]GDP was added. A 40 μl aliquot ($t = 0$) was immediately transferred into 1 ml of ice-cold termination buffer (50 mM Tris/HCl, pH 7.6, 50 mM NaCl and 5 mM $MgCl_2$). The remaining sample was incubated at 30 °C and 40 μl aliquots were transferred into ice-cold termination buffer after 5, 10, 20, 40 and 60 min of incubation. All aliquots were filtered through prewetted 25 mm nitrocellulose filters (Schleicher and Schuell), washed three times with ice-cold termination buffer, and the amount of radioactivity remaining bound to the proteins on the filters was determined by liquid-scintillation counting.

[^{35}S]GTP-binding assay

Binding of [^{35}S]GTP to GST, GST–RLP or GST–RhoA was determined based on the method described by Zheng et al. [22]. Briefly, 3 μg of GST-fusion protein was incubated in 90 μl of loading buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.2 mM dithiothreitol and 100 μM p[NH]ppA (adenosine 5′-[β,γ-imido]triphosphate)] containing 3 μCi of [^{35}S]GTP. Samples were incubated for 5 min at room temperature (22 °C) after which $MgCl_2$ was added to a final concentration of 5 mM ($t = 0$) and the incubation was continued at 30 °C. Equal aliquots were removed at $t = 0$, 10, 20, 30, 45 and 60 min and the reaction was terminated in 1 ml of ice-cold termination buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl and 10 mM $MgCl_2$). Samples were analysed by the rapid-filtration assay as described above.

Retroviral infections

Retroviral expression construct encoding T7-tagged RLP was generated by PCR amplification using T7-RLP pcDNA3 construct as template in combination with forward primer 5′-TCTCTCGAGACCATGGCTAGCATGACTGGTGGACAGCAAATGGGT-3′, containing an *Xho*I restriction site and T7 sequence, and reverse primer 5′-TCTATCGATTCAGACCGACGTGACAGTCCTCACT-3′, containing a *Cla*I restriction site and stop codon. PCR fragments were restriction-digested and cloned into the *Xho*I and *Cla*I sites of the retroviral LPCX vector (provided by Dr R. Derynck, University of California, San Francisco, CA, U.S.A.), in which the original neomycin cassette was replaced by a puromycin cassette. Retroviral constructs for pBabe and pBabe/RasV12 were provided by Dr D. Peeper (Netherlands Cancer Institute, Amsterdam, The Netherlands). Ecotropic retrovirus was produced in phoenix Eco cells as described by Pear et al. [23].

The day before retroviral infection, NIH-3T3 cells were seeded at a density of 6000 cells/cm^2. Cells were infected with indicated retroviruses for 8 h in the presence of 8 μg/ml polybrene, after which medium was replaced by DMEM/10 % newborn calf serum. Cells were photographed 3 days post-infection.

Transient transfections, immunoprecipitations and Western blotting

The day before transfection, Cos7 cells were plated in 100 mm dishes at a density of 1.5×10^4 cells/cm^2. Cells were transfected with indicated plasmids using Fugene6 (Roche Molecular Biochemicals) or LIPOFECTAMINE™ (Invitrogen) as transfection reagents according to the manufacturer's instructions. After 24 h, cells were washed with PBS and replenished with DMEM/0.2 % FBS, and 14 h later cells were lysed in 0.5 ml of lysis buffer [150 mM NaCl, 25 mM Hepes, pH 7.5, 1 % Triton X-100, 5 mM EDTA and 10 % (v/v) glycerol] including 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM PMSF. Cells were incubated on ice for 20 min, collected in microcentrifuge tubes and centrifuged at 13 000 rev./min for 15 min. Supernatant was collected and 10 % was saved for direct Western-blot analysis. The remaining sample was immunoprecipitated for 4–14 h at 4 °C with 0.4–1 μg of an epitope-specific antibody. Immunocomplexes were collected on Protein G–Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. Lysates and immunoprecipitates were separated by SDS/PAGE and transferred on to Hybond-C Extra membranes (Amersham Biosciences). Proteins were detected

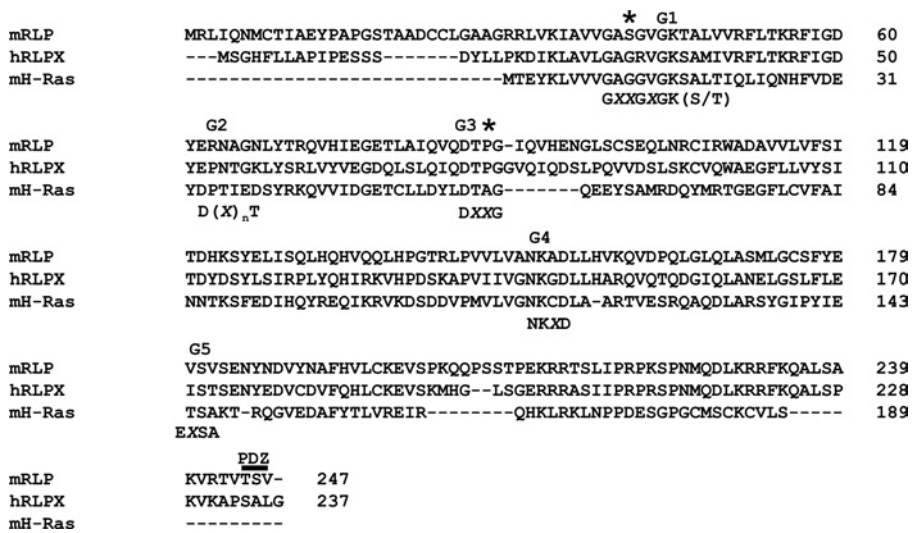

Figure 1 Comparison of the predicted amino acid sequences of mRLP, 'hRLPX' (a novel uncharacterized hypothetical protein) and mH-Ras

The alignment was performed with the ClustalW 1.82 program [49]. Hyphens represent gaps introduced for optimal alignment and numbers are residue numbers. Consensus sequences for GTP-binding regions (G1–G5) are indicated [3]. The positions of Ser-41 and Pro-88 in RLP are indicated with an asterisk (*). The C-terminal PDZ motif in mRLP is indicated by 'PDZ' and underlined. m, murine; h, human. Sequences were derived from GenBank® accession numbers: mRLP, AAH08101; hRLPX, XP_172905; mH-Ras, S57718.

with epitope-specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Amersham Biosciences). Antibodies used include anti-T7 mouse monoclonal (Novagen), anti-H-Ras mouse monoclonal F235 (Santa Cruz Biotechnology), anti-HA (haemagglutinin) rabbit polyclonal Y-11 (Santa Cruz Biotechnology), anti-HA mouse monoclonal F-7 (Santa Cruz Biotechnology), anti-FLAG mouse monoclonal M2 (Sigma), anti-Myc rabbit polyclonal A-14 (Santa Cruz Biotechnology), anti-P-ERK rabbit polyclonal (Cell Signaling Technology), anti-PDGFRβ (platelet-derived growth factor receptor β) goat polyclonal M20 (Santa Cruz Biotechnology), anti-EGFR (EGF receptor) rabbit polyclonal serum 282.7 directed against the EGFR (L. H. Defize, Hubrecht Laboratory, Utrecht, The Netherlands), anti-actin mouse monoclonal (Chemicon, Temecula, CA, U.S.A.), anti-Smad2 rabbit polyclonal and anti-P-Smad2 rabbit polyclonal [24].

Transcriptional reporter assays

NIH-3T3, HepG2 and NMuMG cells were seeded at a density of 20000 cells/cm^2 in 24-well plates. The next day, cells were transiently transfected with the $(SBE)_4$-Lux reporter harbouring four repeats of the CAGACA sequence originally identified as Smad-binding element in the JunB promoter [25], the $(CAGA)_{12}$-Luc reporter, which encodes 12 repeats of the Smad-binding element AGCCAGACA originally identified in the PAI-1 promoter [26], or the ARE-Luc reporter containing part of the Mix.2 gene promoter together with FAST-1 (forkhead activin signal transducer-1) [27] in the absence or presence of T7-RLP pcDNA3 or myc-SNX1 pcDNA3 (where SNX stands for sorting nexin) using LIPOFECTAMINE™ Plus (Invitrogen) or Fugene-6 (Roche Molecular Biochemicals) as transfection reagents. Empty vector pcDNA3 was used to adjust for equal amounts of cDNA to be transfected. pSV-β-galactosidase was co-transfected to normalize transfection efficiency. Cells were washed with PBS 30 h after transfection, put on DMEM/0.2 % FBS and treated with or without 5 ng/ml TGF-β3 for 16 h. Luciferase activity was quantified with a Victor luminometer (Wallac, Turku, Finland) using luciferase assay reagent (Promega).

RESULTS

RLP, a novel TGF-β target gene, is homologous with members of the Ras superfamily

To identify novel target genes of TGF-β signalling and to reveal the relative importance of Smad2, Smad3 and MAPK in the regulation of TGF-β target gene expression, a microarray analysis was set up using MEFs deficient in expression of Smad2 or Smad3 [17]. Out of 360 genes identified as being significantly regulated by TGF-β, EST clone AA119067 represented one of the most strongly regulated genes and an unknown and novel target gene of TGF-β signalling (see Figure 2A for more information and refer to [17] for online support information). Sequence analysis revealed that the EST clone contained only a partial cDNA insert including the 3′-UTR. Database searching, in combination with sequence analysis, identified EST clone AI608035 to encode the full-length cDNA sequence of 744 bp, of which the 5′-ATG start site was revealed by 5′-Race-PCR (results not shown). BLAST analysis of the full-length cDNA sequence in the NCBI nucleotide and protein databases showed that the gene represented by EST clones AA119067 and AI608035 is most homologous with members of the Ras superfamily of GTPases and is, therefore, referred to as RLP (the current NCBI clone ID number for murine RLP is AAH08101). The human RLP gene is composed of four exons (GenBank® accession number AC023154; *Homo sapiens* chromosome 4 clone RP11-752D24) and is located at chromosome 4q12 (GenBank® accession number NM_023940).

RLP cDNA contains an open reading frame of 247 amino acids and encodes a protein with predicted molecular mass of 27.4 kDa, which is conserved from human (94 %) to *Xenopus* (72 %). RLP protein is approx. 30 % identical with both classical representatives of the Ras superfamily of GTPases, such as H-Ras, as well as more atypical members such as Rem and RERG (Figure 1 and results not shown). Interestingly, the highest homology was observed with another uncharacterized protein with homology to members of the Ras superfamily, to which RLP is 52 % (*Mus musculus*, based on cDNA clone AK004371), 54 % (*Rattus norvegicus* clone XP_221886.2) and 53 % (*H. sapiens* clone XP_172905) identical (referred to as hRLPX in Figure 1). Thus

RLP is a novel member of the Ras superfamily, which does not seem to classify to any of the known Ras subfamilies.

The highest homology of RLP with members of the Ras superfamily is recognized within the G domains (Figure 1), regions in GTPases that are of critical importance for GTP–GDP nucleotide exchange, GTP-induced conformational changes and GTP hydrolysis [3]. Although RLP shares significant sequence homology and organization with prototypic H-Ras, it also contains a number of unique features. First, some of the G regions in RLP are distinct from the consensus motifs in Ras GTPases. Particularly, the G2 region, which has the general consensus motif D-X_n-T (in which X can be any amino acid), is defined by 'DPT' in most Ras GTPases. In RLP, the aspartate residue is located at a position two amino acids upstream relative to the position of aspartate in H-Ras, whereas a threonine residue is located six amino acids downstream, relative to the position of threonine in H-Ras (see Figure 1). The threonine residue in the G2 region is critically important for binding of Mg^{2+}, which is essential for GTP hydrolysis [3,4]. Secondly, RLP harbours a serine and a proline residue at positions 41 and 88 respectively, corresponding to Gly-12 and Ala-59 in H-Ras. Substitution of these amino acids in H-Ras renders it in a constitutive active configuration [5]. Finally, RLP lacks the C-terminal CAAX site for prenylation. Prenylation targets H-Ras to the plasma membrane where it can exert its function, whereas non-prenylated mutants of H-Ras are completely inactive. Instead, five myristoylation sites can be identified in RLP and it contains several basic residues in its C-terminal tail, which together might determine post-translational modifications and proper targeting to cellular destinations. In addition, RLP harbours a putative PDZ recognition motif (TSV) at its very C-terminus, which might be important for the interaction with certain PDZ proteins. Together, these results indicate that RLP is a unique member within the Ras superfamily.

RLP is an immediate-early Smad3-dependent target gene of TGF-β signalling

In addition to revealing novel target genes of TGF-β signalling, the microarray screen was also designed to analyse the relative role of Smad2 and Smad3 in TGF-β-mediated regulation of gene expression, using spontaneously immortalized MEFs derived from Smad2 or Smad3 knockout mouse embryos [17]. An overview of the microarray data obtained for RLP in the different MEF genotypes at 1, 4 and 10 h after stimulation with TGF-β is shown in Figure 2(A), indicating that after 1 h TGF-β induces the expression of RLP 14-fold in S2WT MEFs, 8.2-fold in S2KO MEFs, 2.7-fold in S3WT MEFs, but only 1.2-fold in MEFs lacking expression of Smad3 (S3KO). These results suggest that expression of Smad3 is critical for significant induction of RLP by TGF-β. Similar results were obtained by real-time PCR (results not shown). These findings were further corroborated in primary low-passage MEFs as well as spontaneously immortalized MEFs using Northern-blot analysis (Figures 2B and 2C). Induction of RLP expression in the WT MEFs was seen after 1 h of TGF-β treatment and reached maximum levels after 2 h, after which RLP expression levels declined. The induction of RLP expression by TGF-β was reduced in cells lacking expression of Smad2 and the induction was hampered in cells lacking expression of Smad3 (Figures 2B and 2C). Reconstitution of Smad3 expression in primary low-passage S3KO cells by adenoviral transduction with Smad3 led to the recovery of TGF-β-induced RLP expression (Figure 2C), further supporting the observation that RLP is a Smad3-dependent TGF-β target gene.

Interestingly, pretreatment of the primary low-passage MEFs with the MAPK inhibitor U0126 potentiated the induction of RLP expression by TGF-β, suggesting that MAPK negatively regulates RLP expression. In line with these observations, treatment of primary dermal fibroblasts with phorbol ester PMA or EGF, potent activators of MAPK in dermal fibroblasts [28], strongly reduced TGF-β-induced expression of RLP (Figure 2D).

Whereas maximum induction of RLP expression is observed after 2 h of TGF-β treatment (Figure 2B), Northern-blot analysis in primary dermal fibroblasts shows that induction of expression is detectable as early as 45 min after addition of TGF-β (Figure 2E). Addition of CHX (cycloheximide), an inhibitor of *de novo* protein synthesis, did not block TGF-β-induced expression and in fact resulted in a stronger increase in RLP expression by TGF-β (Figure 2E), possibly due to stabilization of the RLP mRNA. Thus RLP is an immediate-early target gene of TGF-β signalling. Interestingly, BMP2 and, to a lesser extent, BMP7 were also capable of inducing expression of RLP in primary dermal fibroblasts (Figure 2E). In conclusion, RLP is a novel Smad3-dependent immediate-early target gene of TGF-β signalling and its expression can also be up-regulated by BMP2 and BMP7.

RLP is mainly expressed in brain and kidney, and in cells of mesenchymal origin

The potency of TGF-β to induce expression of RLP in primary dermal fibroblasts and MEFs was further examined in a broader panel of cell lines of epithelial and mesenchymal origin (Figure 3A). Expression of RLP could not be induced in typical epithelial cell lines such as HaCat and NMuMG, despite their responsiveness to TGF-β [19,29]. Moreover, TGF-β showed no induction of RLP expression in the MDA468 and SW480 cell lines, most probably because they lack expression of Smad4. Among the epithelial cell lines tested, induction of RLP was observed only in rat prostate epithelial NRP-154 cells. Interestingly, basal levels of RLP mRNA were relatively high in WT as well as S3KO primary glomerular mesangial cells and expression of RLP was up-regulated in the WT cells after TGF-β treatment. Thus expression of RLP seems to be most pronounced in cells of mesenchymal origin such as MEFs, dermal fibroblasts and glomerular mesangial cells.

Tissue-distribution analysis of RLP mRNA using a mouse multiple tissue Northern-blot revealed high expression of RLP in kidney, in line with high basal expression of RLP in primary glomerular mesangial cells. Besides the kidney, RLP was particularly highly expressed in heart and brain, but was undetectable in spleen and skeletal muscles (Figure 3B). In conclusion, these expression analysis studies indicate a selective gene expression pattern for RLP in mouse tissues, whereas RLP appears to be mainly expressed in cell lines of mesenchymal origin.

RLP does not affect phosphorylation of MAPK and fails to bind [^{35}S]GTP or [^{3}H]GDP

Recently, a novel Ras superfamily member, ERas [30], was identified, which was shown to have transforming activity in NIH-3T3 cells, similar to H-Ras. To test whether RLP has transforming activity, retroviral RLP was produced and NIH-3T3 cells were infected. Whereas infection with H-RasV12 retrovirus resulted in a transformed cellular phenotype, characterized by an elongated cell morphology and loss of contact inhibition (Figure 4A), infection of the NIH-3T3 cells with RLP retrovirus did not affect cellular morphology. RLP was not capable of affecting transformation of NIH-3T3 cells induced by H-RasV12 (Figure 4A). Similarly, overexpression of H-RasV12 in NIH-3T3 cells by transient transfection resulted in phosphorylation of co-expressed MAPK as expected. In contrast, RLP itself was not capable of

A

Genotype	S3WT				S3KO				S2WT				S2KO			
hr TGF-β1	0	1	4	10	0	1	4	10	0	1	4	10	0	1	4	10
Fold Induction	1	2.73	0.81	1.5	1	1.22	1.1	1.14	1	14.2	1.8	2.91	1	8.15	4.49	2.15

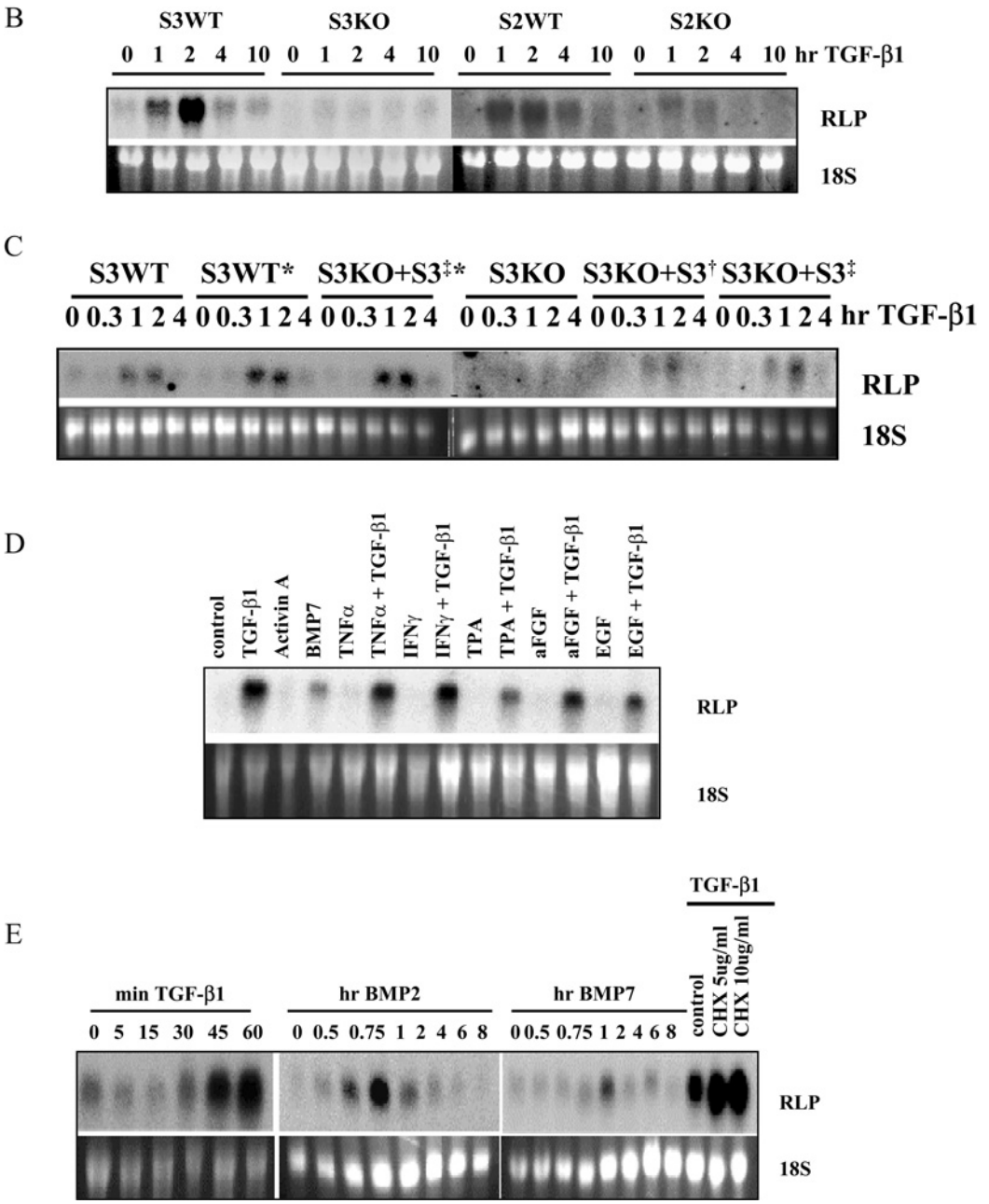

Figure 2 RLP is a Smad3-dependent TGF-β target gene and its expression is negatively regulated by MAPK

(**A**) RLP expression by TGF-β in MEFs derived from S3WT, S3KO, S2WT and S2KO embryos, investigated by microarray analysis as reported previously [17]. Spontaneously immortalized MEFs were treated with 8 ng/ml TGF-β1 for indicated time periods and expression levels were normalized as described in [17] and are expressed as 'Fold induction' relative to zero time for each genotype. (**B**) Northern-blot analysis of TGF-β-induced expression of RLP in spontaneously immortalized S3WT, S3KO, S2WT and S2KO MEFs. MEFs were treated with 8 ng/ml TGF-β1 for indicated time periods after which RNA was extracted and expression of RLP mRNA was analysed by hybridization of the Northern-blot with an 800 bp *Eco*RI–*Not*I 3′-UTR fragment derived from EST clone AA119067. (**C**) Northern-blot analysis of TGF-β-induced expression of RLP in primary low-passage S3WT, S3KO, S2WT and S2KO MEFs, and in S3KO MEFs in which expression of Smad3 was reconstituted after infection of the cells with Smad3 adenovirus (†MOI, 10; ‡MOI, 40) [19]. The role of MAPK in TGF-β-induced RLP expression was analysed by treatment of the cells with MAPK inhibitor U0126 (10 μM), which was added 20 min before addition of TGF-β (*). Cell lysates were harvested and subjected to Northern-blot analysis as described above. (**D**) Regulation of RLP expression in primary dermal fibroblasts by TGF-β and other growth factors, cytokines or the phorbol ester PMA. Fibroblasts were pretreated with indicated stimuli for 30 min, followed by treatment with 8 ng/ml TGF-β1 for 1 h. Northern-blot analysis was performed as described above. (**E**) Regulation of RLP expression by TGF-β, BMPs and CHX in primary dermal fibroblasts. Fibroblasts were treated with 8 ng/ml TGF-β1, 100 ng/ml BMP2 or 100 ng/ml BMP7 for indicated time periods. CHX was added 30 min before TGF-β1 treatment, and after 1 h, cells were harvested for RNA extractions and subsequent Northern-blot analysis.

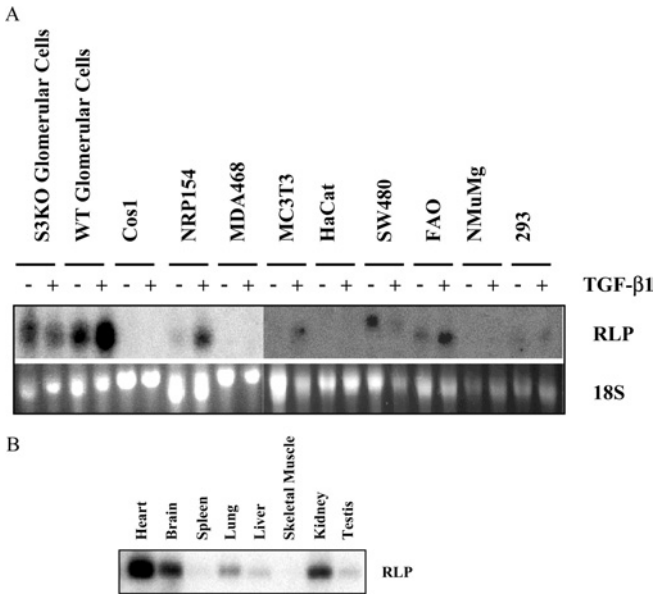

Figure 3 Northern-blot analysis of RLP expression in different cell lines and tissues

(**A**) Analysis of TGF-β-induced RLP expression in cell lines of epithelial and mesenchymal origin. The various cell lines were grown to subconfluency and treated with or without 8 ng/ml TGF-β1 for 1 h. RNA was extracted and expression of RLP mRNA was analysed by hybridization of the Northern-blot with an 800 bp *Eco*RI–*Not*I 3′-UTR fragment derived from EST clone AA119067. (**B**) Analysis of the tissue distribution of RLP. A mouse multiple tissue Northern (ClonTech) was hybridized with an 1100 bp *Eco*RI–*Eco*RI RLP probe fragment of EST clone AI608035, containing the complete coding sequence and flanking parts of the 5′- and 3′-UTR.

inducing phosphorylation of MAPK and had no effect on phosphorylation of MAPK induced by H-RasV12 (Figure 4B). These results further indicate that RLP does not belong to the classical Ras subfamily of Ras GTPases.

GTPases of the Ras superfamily are characterized by their ability to bind GTP which, partly through intrinsic GTPase activity and further catalysis by GAPs, can be hydrolysed to GDP. To test whether RLP is endowed with GTPase activity, recombinant GST protein, GST–RLP and GST–Rho were used to perform [^{35}S]GTP- and [^{3}H]GDP-binding assays [21,22]. As shown in Figure 4(C), GST–RLP was expressed as a fusion protein with expected molecular mass of 51 kDa. GST and GST–Rho, included as negative and positive controls respectively, expressed as proteins with expected molecular mass of 24 and 45 kDa. Analysis of [^{3}H]GDP binding to equal amounts of recombinant GST, GST–RLP or GST–Rho protein (using the method described by Zheng et al. [22]) showed that GST–Rho had very clear affinity for [^{3}H]GDP, reaching peak levels within 20 min (Figure 4D). In contrast, GST–RLP did not show appreciable binding of [^{3}H]GDP, similar to GST only. The experiment was also performed as described by Self and Hall [21] with similar outcome. Analogous to what was observed for binding of the GST-fusion proteins with [^{3}H]GDP, GST–RLP also failed to interact with [^{35}S]GTP, despite the fact that under similar assay conditions GST–Rho efficiently interacted with [^{35}S]GTP reaching peak levels at 20 min after addition of MgCl (Figure 4E).

Possible binding of GDP and/or GTP to RLP was further addressed by performing an *in vitro* P_i labelling of HepG2 cells overexpressing RLP after transient transfection. In these experiments, AU5-tagged H-Ras V12 was included as positive control, clearly showing binding of [^{32}P]GTP and to a lesser extent [^{32}P]GDP, as expected. In contrast, no binding of [^{32}P]GTP or [^{32}P]GDP to RLP could be detected (results not shown). Western-blot analysis suggested that the expression level of H-RasV12 was higher than that of the RLP, but even after prolonged exposure no binding of [^{32}P]GTP or [^{32}P]GDP to RLP could be detected (results not shown). Together, these results indicate that RLP, unlike all other known members of the Ras superfamily of GTPases, lacks appreciable affinity for guanine nucleotides and is, therefore, a unique member of the Ras superfamily.

RLP interacts with different type I and II serine/threonine kinase receptors

As described above, RLP is an immediate-early target gene of TGF-β signalling, being induced within 45 min of addition of TGF-β1. This raises the possibility that RLP might have an impact on the TGF-β signal-transduction pathway. Therefore we first analysed whether RLP was able to interact physically with Smad proteins. However, no co-immunoprecipitation of RLP with Smad2, Smad3, Smad4, Smad6 or Smad7 could be detected after their concomitant overexpression in Cos7 cells (results not shown). Moreover, co-expression of constitutive active TGF-β type I receptor could not promote an interaction between RLP and Smad2, Smad3 or Smad4 (results not shown).

We next tested whether RLP could physically interact with the type I or II TβRs after their co-expression in Cos7 cells. As shown in Figure 5(A), TβRI, TβRII, as well as kinase inactive or constitutive active forms of the receptors co-immunoprecipitated with RLP. No immunoprecipitation of TβRI was observed when RLP was not co-expressed (Figure 5A, lane 13), indicating the specificity of the interaction between RLP and the receptors. Furthermore, after co-expression of RLP with both TβRI and TβRII, both receptor types were pulled down after immunoprecipitation of RLP (Figure 5A, lanes 6–12, numbering from the left), whereas addition of TGF-β did not affect this interaction (Figure 5A, lane 7). Interestingly, RLP was also capable of interacting with the kinase-dead or constitutively active forms of TβRI as well as with the kinase-dead form of TβRII, suggesting that RLP does not discriminate between the different functional conformations of the receptors.

To test whether the interaction of RLP with TβRI and TβRII was unique for these two serine/threonine kinase receptors, we also investigated the interaction of RLP with type I and II serine/threonine kinase receptors for other members of the TGF-β superfamily (Figures 5B and 5C). As shown in Figure 5(B), RLP could also interact with ALK2, ALK3 and ALK6, type I receptors for BMPs. The interaction of RLP with type II receptors was more restricted and, in addition to TβRII, only ActRIIB (a type II receptor for BMPs) could interact with RLP. Tyrosine kinase receptors for EGF or PDGF were unable to interact physically with RLP (Figure 5D). These results indicate that RLP interacts selectively with a subset of serine/threonine kinase receptors for members of the TGF-β superfamily.

RLP interacts with SNX1

In an attempt to reveal the possible role of RLP, putative interaction partners of RLP were analysed, including members of the SNX family of vesicular-trafficking proteins. Some members of the Ras superfamily, particularly Rab and Sar1/Arf family members, are involved in vesicular trafficking of cargo proteins to proper intracellular destinations (reviewed in [2]). Moreover, SNXs, like RLP, have recently been described to interact with receptors for ligands of the TGF-β superfamily [31]. Co-expression of RLP with SNX1 or SNX2 in Cos7 cells, followed by immunoprecipitation of cell lysates with an antibody to

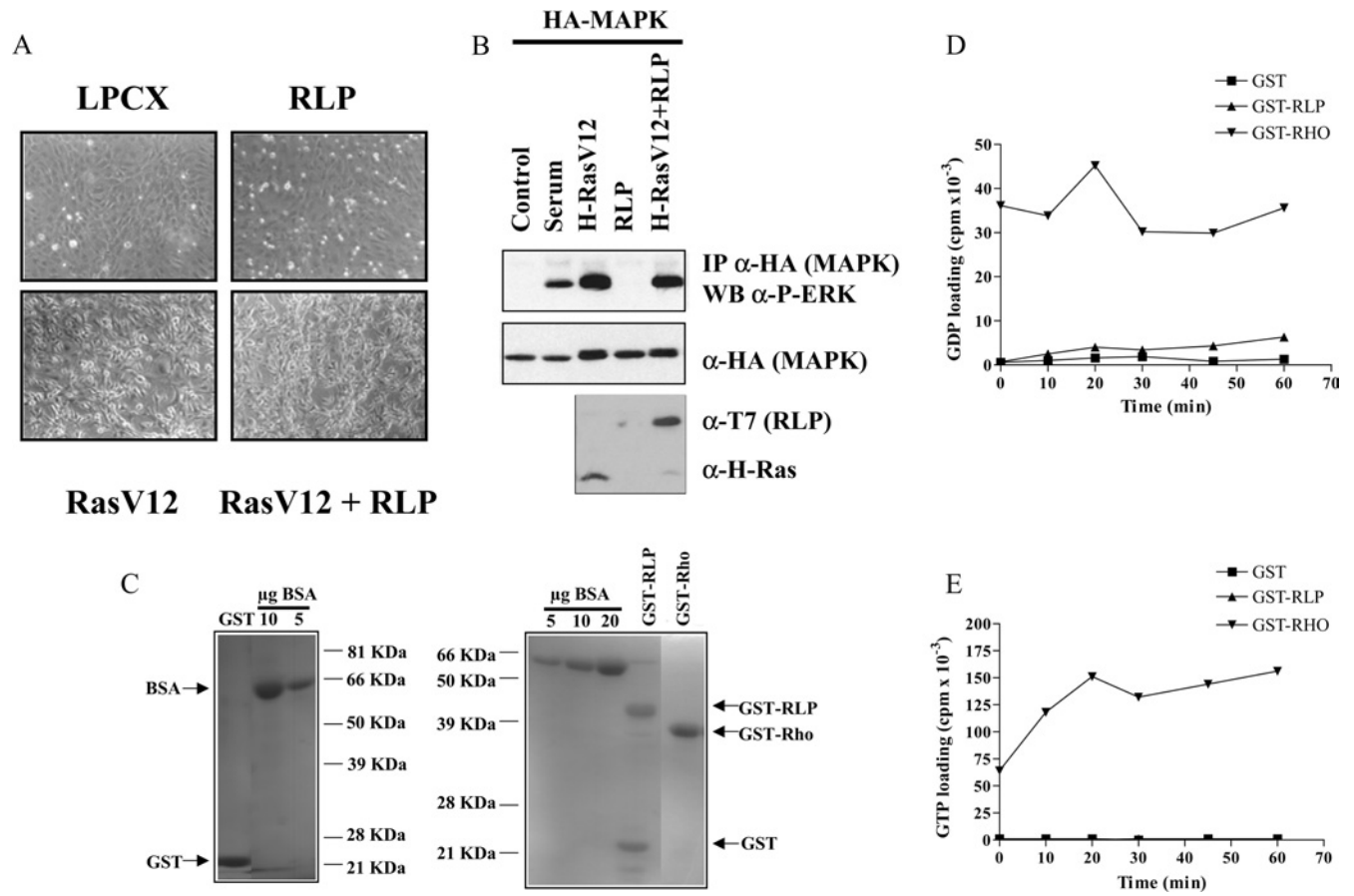

Figure 4 RLP is not a classical GTPase

(**A**) Analysis of transformation of NIH-3T3 cells after retroviral infection with H-RasV12, RLP or both. NIH-3T3 cells were infected with the respective retroviruses for 8 h after which the medium was replaced. Photographs were taken after 3 days. (**B**) Western-blot analysis of MAPK phosphorylation regulated by H-Ras or RLP. Cos7 cells were transfected with HA-tagged MAPK in the absence or presence of T7-tagged RLP and AU5-tagged H-Ras. As a negative control, Cos7 cells transfected with HA–MAPK were left untreated, and as a positive control Cos7 cells transfected with HA–MAPK were treated with 20 % FBS for 10 min. Cell lysates were immunoprecipitated with anti-HA antibody and blotted with anti-P-ERK antibody to detect phosphorylated MAPK. To check the expression levels of the various overexpressed proteins, 10 % of the initial cell lysates was used for direct Western-blot analysis (middle and bottom panels). (**C**) Coomassie Blue-stained SDS/PAGE of recombinant GST (24 kDa), GST–Rho (51 kDa) and GST–RLP (57 kDa) proteins. Recombinant proteins were dissolved in glutathione buffer and thereby ran at lower molecular masses than expected. Owing to partial proteolysis between GST and RLP of the GST–RLP-fusion protein, GST could also be detected in the GST–RLP sample. (**D**) Analysis of [^{3}H]GDP binding to GST, GST–Rho and GST–RLP. Each recombinant protein (4 μg) was incubated with 2 μCi of [^{3}H]GDP and equal fractions of the reaction mixtures were removed at the indicated time points. Binding of [^{3}H]GDP to the recombinant proteins was assessed by the rapid filtration method as described in the Materials and methods section. (**E**) Analysis of [^{35}S]GTP binding to GST, GST–Rho and GST–RLP. Each recombinant protein (3 μg) was incubated with 3 μCi of [^{35}S]GTP and equal fractions of the reaction mixtures were removed at the indicated time points. Binding of [^{35}S]GTP to the recombinant proteins was assessed by the rapid filtration method as described in the Materials and methods section.

pull-down RLP indicated that only SNX1 specifically co-immunoprecipitated with RLP (Figure 6A, results not shown). No specific interaction could be observed with SNX2 (Figure 6A) or SNX4 and SNX6 (results not shown).

Deletion analysis of SNX1 indicated that SNX1Δ214, lacking the coiled-coil domains, was still capable of interacting with RLP, whereas SNX1Δ249, lacking the coiled-coil domains and the linker region, could no longer interact with RLP (Figures 6B and 6C; results not shown). These results indicate that SNX1 can be an interaction partner of RLP, and the linker region connecting the PX domain and the coiled-coil domains in SNX1 contributes to the interaction with RLP.

RLP negatively regulates TGF-β-induced luciferase reporter activation

In view of the fact that RLP could interact with type I and II receptors for TGF-β, we investigated whether RLP could influence transcriptional regulation mediated by the TGF-β signal-transduction pathway. Therefore the effect of RLP on TGF-β-inducible luciferase reporters, including the ARE-luc, the $(SBE)_4$-Lux and the $(CAGA)_{12}$-luc constructs, was analysed in the NMuMG, HepG2 and NIH-3T3 cell lines. Whereas the $(SBE)_4$-Lux and the $(CAGA)_{12}$-Luc reporter constructs are activated by binding of Smad3–Smad4 complexes, the ARE-Luc reporter is positively regulated by Smad2–Smad4 complexed with FAST-1.

As shown in Figure 7, TGF-β efficiently induced luciferase expression from the ARE-Luc, $(SBE)_4$-Lux and the $(CAGA)_{12}$-Luc reporter constructs in the tested cell lines. Interestingly, co-expression of RLP negatively regulated luciferase activation induced by TGF-β (Figures 7A–7E).

In view of the fact that SNX6 has been reported to regulate negatively TGF-β-mediated transcriptional activity [31], we also tested the effect of SNX1 on activation of the $(SBE)_4$-Lux and ARE-Luc reporters. As shown in Figures 7(C)–7(E), SNX1 also repressed luciferase reporter activation by TGF-β, albeit that the inhibition of the $(SBE)_4$-Lux reporter by SNX1 was marginal. Interestingly, co-expression of RLP and SNX1 further

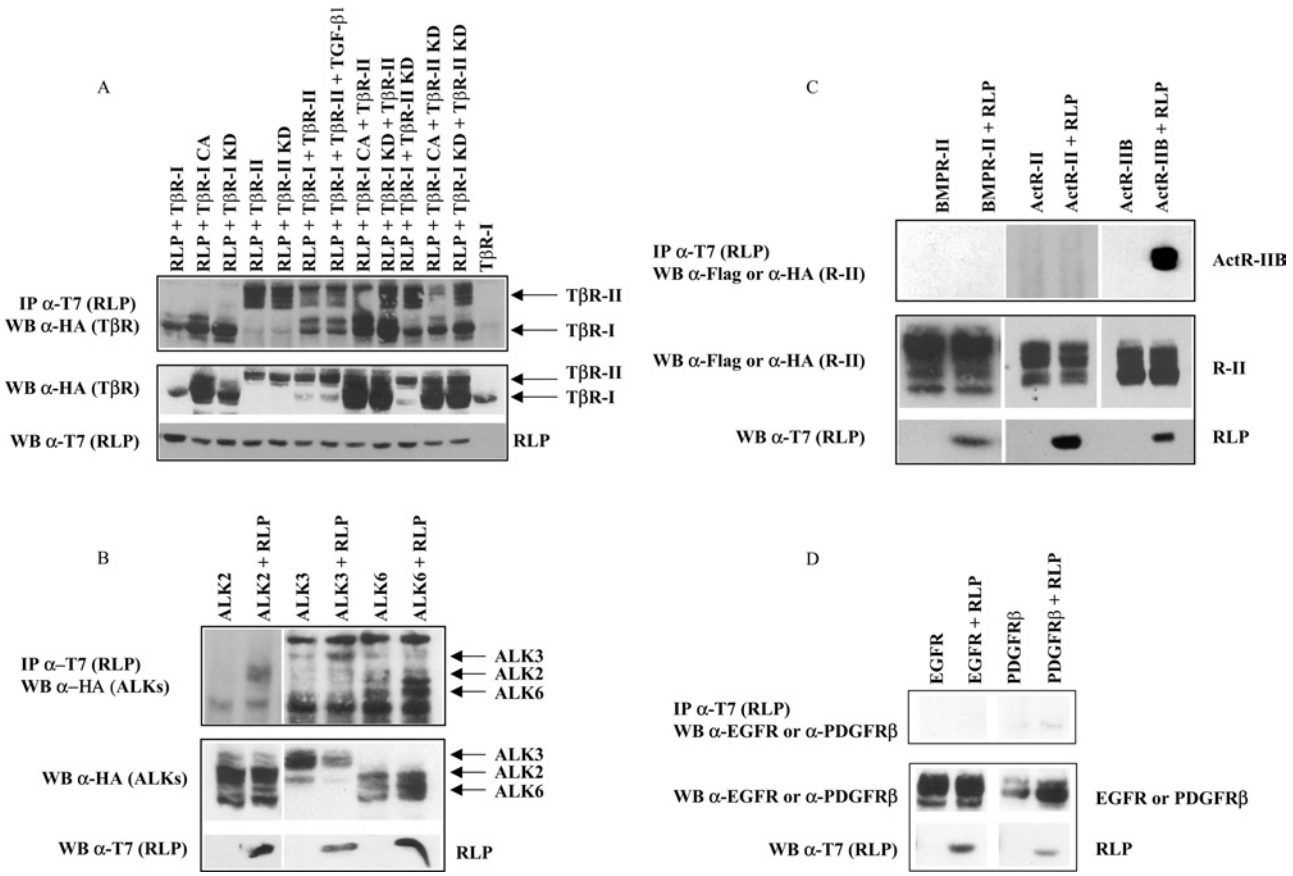

Figure 5 RLP interacts with type I and II serine/threonine kinase receptors

(**A**) Cos7 cells were transfected with T7-tagged RLP in the absence or presence of WT, CA (constitutively active) or KD (kinase-dead) variants of the HA-tagged TGF-β type I and II receptors. One plate of cells overexpressing RLP, TβRI and TβRII was treated with 5 ng/ml TGF-β3 for 30 min. Cell lysates were immunoprecipitated with an anti-T7 antibody and blotted with an anti-HA antibody to demonstrate the interaction of RLP with the type I and II TβRs. (**B**) Cos7 cells were transfected with T7-tagged RLP and HA-tagged ALK2, ALK3 or ALK6, type I serine/threonine kinase receptors for BMPs. Cell lysates were immunoprecipitated with the anti-T7 antibody and blotted with the anti-HA antibody to demonstrate the interaction of RLP with the different ALKs. (**C**) Cos7 cells were transfected with T7-tagged RLP and HA-tagged ActRII, HA-tagged BMPRII or FLAG-tagged ActRIIB, type II serine/threonine kinase receptors for activins and BMPs. Cell lysates were immunoprecipitated with the anti-T7 antibody and blotted with the anti-HA or anti-FLAG antibody to investigate the interaction of RLP with the different type II receptors. (**D**) Cos7 cells were transfected with T7-tagged RLP and the EGFR or the PDGFRβ, both tyrosine kinase receptors. Cell lysates were immunoprecipitated with the anti-T7 antibody and blotted with an EGFR antibody or a PDGFRβ antibody to investigate the possible interaction of these tyrosine kinase receptors with RLP. For all experiments, 10 % of the initial cell lysates was used for direct Western-blot analysis to check the expression levels of the various overexpressed proteins (middle and bottom panels in each Figure).

repressed TGF-β-induced reporter activation (Figures 7C–7E). These results indicate that RLP does inhibit TGF-β signal transduction as revealed at the level of reporter gene activation.

DISCUSSION

In the present study, we report the initial characterization of a novel Ras-like protein, designated RLP, which was identified as a novel TGF-β target gene in MEFs using microarray analysis [17] (Figure 2A). We show that RLP is an immediate-early target gene of TGF-β and BMP. The RLP protein has a molecular mass of 27 kDa and is encoded by 247 amino acids. To our knowledge, RLP is the first protein to be identified with sequence homology to Ras GTPases, which lacks detectable affinity for GTP or GDP, thereby constituting a unique member of the Ras superfamily.

Induction of expression of RLP by TGF-β is dependent on Smad3 and the expression levels of RLP peak after 1–2 h of TGF-β treatment. Moreover, in MEFs, expression of RLP is increased by addition of an MAPK inhibitor (Figure 2C), whereas in dermal fibroblasts RLP expression is inhibited by PMA or EGF (Figure 2D), suggesting that activation of MAPK negatively regulates TGF-β-induced expression of RLP. Whereas other members of the Ras superfamily, including RERG [32], Rheb [33] and members of the RGK subfamily of GTPases [34–38], have been shown to be transcriptionally regulated, RLP is the first Ras-like GTPase known to be transcriptionally regulated by members of the TGF-β superfamily, particularly in cell lines of mesenchymal origin.

Expression analysis of RLP in mouse tissues reveals that RLP has a restricted expression pattern, being highly expressed in brain, kidney and heart, but below detection in spleen and skeletal muscles. In this respect, RLP again shares similarities with the RGK subfamily of Ras-like GTPases, which are characterized by a tissue-specific expression pattern [1,35–37]. It will be interesting to reveal the biological significance of the selective expression pattern of RLP.

RLP shares significant sequence homology and organization with prototypic H-Ras. The guanine nucleotide consensus sequences GX_4GK(T/S) (G1 motif), the D*XX*G (G3 motif) and the NK*X*D (G4 motif) are well conserved in RLP (Figure 1), although RLP does contain amino acid substitutions at positions in these motifs which render prototypic Ras oncogenic. Despite these substitutions, RLP does not have transforming activity and does not cause phosphorylation of MAPK. As has been reported

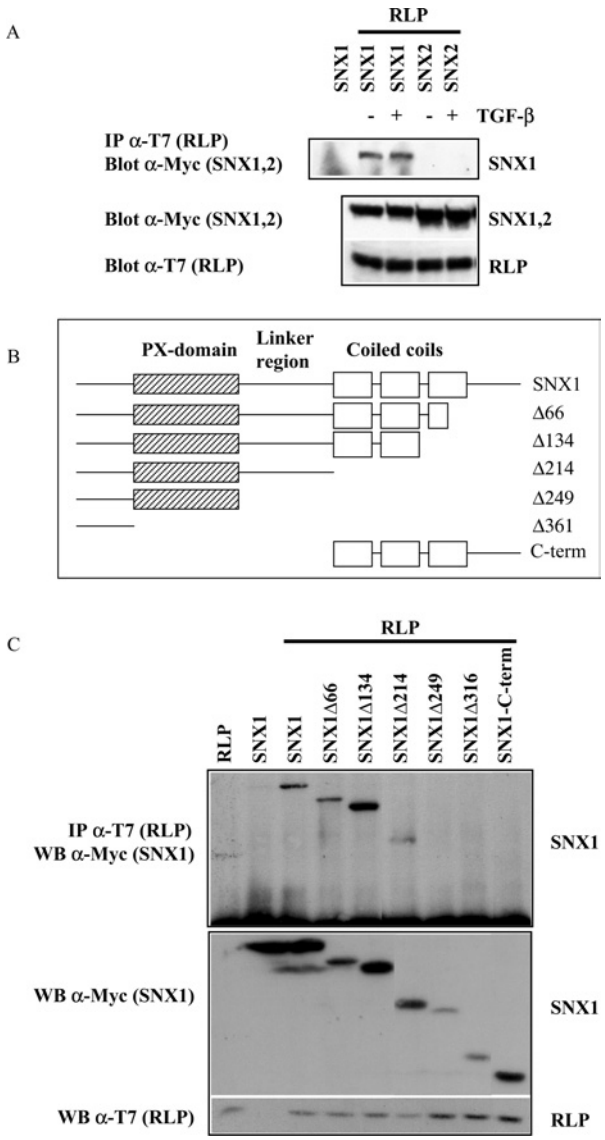

Figure 6 RLP interacts with SNX1

(**A**) Cos7 cells were transfected with T7-tagged RLP and Myc-tagged SNX1 or Myc-tagged SNX2 in the presence or absence of 5 ng/ml TGF-β1. Cell lysates were immunoprecipitated for RLP using the T7 antibody. Subsequent Western-blot analysis of the immunoprecipitated samples was performed using a Myc antibody to investigate the possible association of SNX1 and SNX2 with RLP. To determine expression levels of the overexpressed proteins, 10 % of the initial cell lysates was used for direct Western-blot analysis (middle and bottom panels). (**B**) Overview of the deletion constructs used to determine the region in SNX1 that is responsible for the interaction of RLP with SNX1. The PX domain (hatched boxes) and coiled-coil regions (open boxes) are connected by the linker region. (**C**) Immunoprecipitation and Western-blot analysis of RLP interacting with different fragments of SNX1. The respective proteins were overexpressed in Cos7 cells, lysates were immunoprecipitated with the T7 antibody recognizing the T7 tag coupled with RLP, and Western-blot analysis was performed using a Myc antibody to detect Myc-tagged SNX1 fragments. IP, immunoprecipitated.

for the Ras GTPase Rap and for certain members of the RGK subfamily, deviations from the consensus amino acids can be tolerated without loss of GTPase activity [1,39]. To our surprise, recombinant GST–RLP failed to bind [^{35}S]GTP or [^{3}H]GDP in the presence of 5 mM $MgCl_2$, conditions under which GST–Rho strongly bound the guanine nucleotides. In addition, overexpressed RLP, in contrast with H-Ras V12, also failed to show binding of [^{32}P]GDP or [^{32}P]GTP after radioactive P_i labelling of the transfected cells. This apparent lack of affinity for guanine nucleotides makes RLP a very unique Ras-like protein. In view of the fact that no ^{32}P-labelled nucleotides were detected on the TLP plate after analysis of P_i-labelled cells overexpressing RLP, it seems unlikely that RLP would bind other nucleotides, such as ATP or UTP, instead of GTP.

Unlike the G1, G3 and G4 motifs in RLP, the G2 and G5 domains are not conserved when compared with prototypic Ras. The G5 motif of RLP is defined by EVSVS, whereas the consensus sequence for Ras GTPases is E[A/C/S/T]SA(K/L) [3]. The G5 region has only one direct contact with GTP through Ala-146 in prototypic Ras. The absence of this alanine residue in the region corresponding to the G5 motif in RLP could possibly contribute to the fact that RLP lacks affinity for GTP. More importantly, RLP contains a unique G2 domain, encoded by 'ERN', which diverges greatly from the consensus motif 'DPT' in typical Ras GTPases [3]. The G2 motif, which is the effector domain, has a critical role in defining interactions with effector proteins, thereby controlling downstream signal propagation. In addition, the threonine residue in this region is critically important for binding of Mg^{2+}, which is essential for GTP hydrolysis [3,4]. Although divergence from the consensus G2 motif in members of the RGK subfamily does not impair their affinity for guanine nucleotides or their GTP hydrolysis [1], the absence of this threonine residue in the G2 motif of RLP could possibly render RLP unable to bind Mg^{2+}, resulting in lack of affinity for guanine nucleotides.

Whereas the general structure of RLP resembles that of prototypic Ras, the flanking N- and C-terminal sequences in RLP are extended relative to those in Ras and account for the larger molecular mass of RLP. Interestingly, members of the RGK subfamily of RLPs are also characterized by the presence of extended, partially conserved N- and C-terminal tails, but RLP does not share homology with these domains. Thus although RLP shares several features with members of the RGK subfamily, it differs sufficiently to represent a novel unique subfamily of Ras GTPases. At the protein level, RLP is most homologous with the uncharacterized 'RLPX', which shares amino acid substitutions (Gly-13 and Ala-59 in prototypic Ras are substituted by an arginine and a proline in RLPX) and altered G2 (EPN) and G5 (EISTS) domains similar to RLP. Thus RLP and RLPX possibly constitute a novel, unique subfamily within the Ras superfamily of GTPases. On the basis of the similarities between RLP and RLPX, it will be interesting to analyse whether RLPX also lacks affinity for guanine nucleotides, possibly reflecting a unique feature of members of the RLP subfamily.

Interestingly, RLP lacks the C-terminal CAAX site for prenylation and, instead, it contains a putative PDZ recognition domain (TSV), which suggests that RLP might interact with PDZ proteins. Remarkably, the C-terminal 37 amino acids in RLP are highly conserved with those in RLPX, with the exception of the last five amino acids resulting in the absence of a PDZ-recognition motif in RLPX. RLP contains five myristoylation sites and the C-terminus harbours several basic residues, which together might determine post-translational modifications and proper targeting to cellular destinations. Preliminary results regarding the subcellular localization of RLP suggest that RLP localizes both to the cell membrane, as well as to the actin cytoskeleton (results not shown).

RLP was identified as a novel target gene of TGF-β signalling and we show in the present study that expression of RLP is not only induced by TGF-β but also by BMPs. Moreover, RLP interacts with specific type I and II serine/threonine kinase receptors for TGF-β and BMPs. For various cell types, it has been reported that activation of the TβR complex or the BMP–receptor complex can result in downstream signal propagation through activation of the Ras signal-transduction pathway and/or distinct MAPK pathways, including ERK1/ERK2, JNK and p38 MAPK [13,40–42].

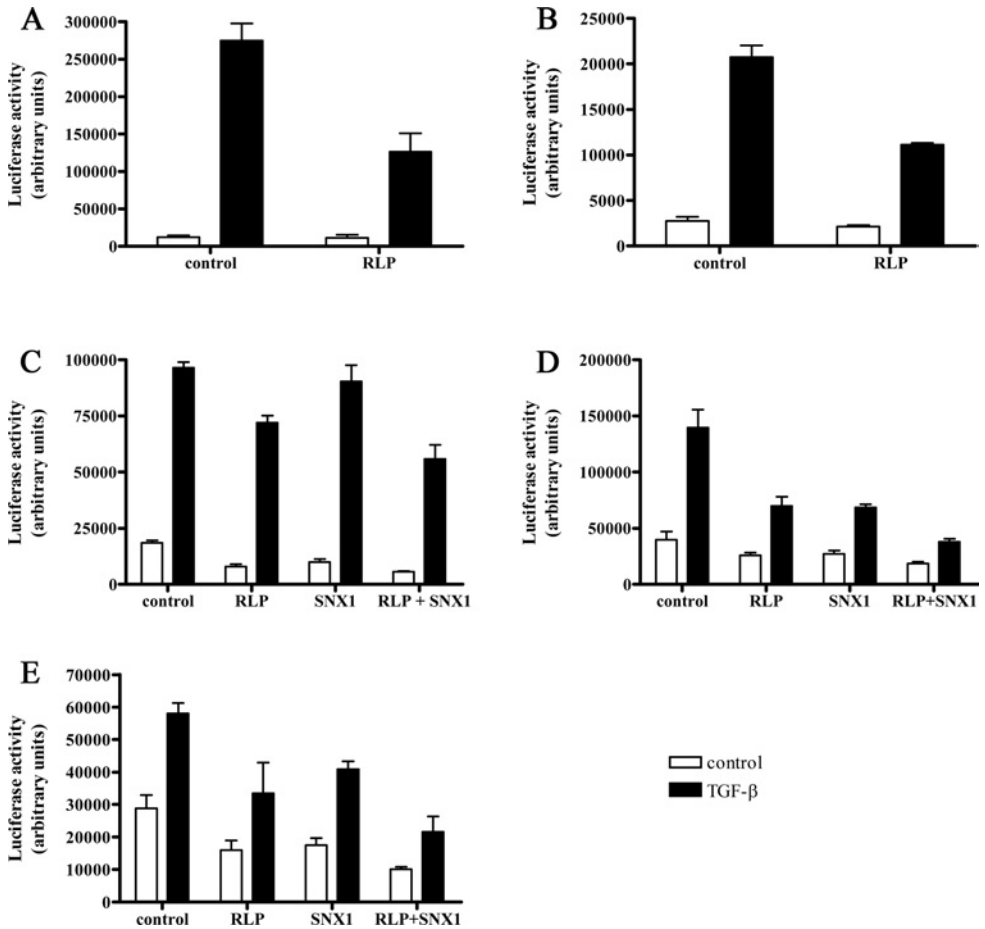

Figure 7 RLP and SNX1 negatively regulate TGF-β-induced luciferase reporter activation

(**A**) Effect of RLP on TGF-β-induced $(CAGA)_{12}$-Luc reporter activation in NIH-3T3 cells. Cells were transfected with $(CAGA)_{12}$-Luc reporter in the absence or presence of RLP. The cells were serum-starved for 8 h after which half of the wells were treated with 5 ng/ml TGF-β3 for 16 h. (**B**) Effect of RLP on TGF-β-induced ARE-Luc reporter activation in HepG2 cells. Cells were transfected with ARE-Luc reporter together with FAST-1 in the absence or presence of RLP. Cells were treated as described above. (**C**) Effect of RLP and SNX1 on TGF-β-induced $(SBE)_4$-Lux reporter activation in NIH-3T3 cells. Cells were transfected with $(SBE)_4$-Lux reporter in the absence or presence of RLP or SNX1. Cells were treated as described above. (**D**) Effect of RLP and SNX1 on TGF-β-induced ARE-Luc reporter activation in NIH-3T3 cells. Cells were transfected with ARE-Luc reporter together with FAST-1 in the absence or presence of RLP or SNX1. Cells were treated as described above. (**E**) Effect of RLP and SNX1 on TGF-β-induced ARE-luc reporter activation in NMuMG cells. Cells were transfected with ARE-luc reporter together with FAST-1 in the absence or presence of RLP or SNX1. Cells were treated as described above. For each assay, β-galactosidase values were used to normalize for transfection efficiency and the results are presented as relative luciferase values. Error bars indicate S.D.

However, information is incomplete concerning identification of upstream links of these pathways to the serine/threonine receptor complex [43,44]. It is therefore intriguing that RLP is actually capable of interacting, directly or indirectly through a docking protein, with the TβR complex. RLP itself does not activate the ERK1/ERK2 signal-transduction pathway (Figure 4B) and in view of its lack of affinity for guanine nucleotides, RLP probably does not link up with any of the MAPK signalling pathways. Whether the activity of RLP can be regulated by the TβR complex, and in that case, how its functional activity is controlled is subject for further studies.

RLP inhibits TGF-β-induced transcriptional activation of Smad-dependent luciferase reporters, such as (SBE)4-Lux, (CAGA)12-Luc and ARE-Luc. This suggests that RLP interferes, directly or indirectly, with activation, heteromerization and/or nuclear translocation of the R-Smads. Whereas RLP interacts with type I and II receptors, it did not interact with Smads or affect their C-terminal phosphorylation. In addition, we did not find indications that overexpression of RLP would interfere with heteromeric complex formation among Smad2, Smad3 and Smad4. Furthermore, we did not find indications that TβR or Smad expression levels were affected by overexpression of RLP (results not shown).

In view of the fact that RLP does interact with the TβR complex, either directly or indirectly through a docking protein, it seems probable that the interference of RLP with TGF-β signalling would occur at this level. A possible mechanism whereby RLP could ultimately interfere with TGF-β-induced transcriptional regulation is by modulating phosphorylation of the ERK or JNK phosphorylation sites located in the linker region of R-Smads. Phosphorylation at these sites has been shown to alter the activity of the Smads [10–12]. Although it does not seem probable that RLP itself might directly affect this phosphorylation, it is possible that RLP interferes with docking of signal mediators upstream of ERK or JNK to the TβR complex. Another possibility is that by interaction of RLP with the TβR complex, RLP might interfere with docking of proteins that could introduce other, possibly unknown, modifications to the Smads, which are of critical importance for their functioning.

Alternatively, the mechanism whereby RLP interferes with TGF-β signal transduction could relate to the observation that RLP can interact with SNX1. SNXs are hypothesized to function

in the intracellular trafficking of plasma membrane receptors. They consist of a Phox homology (PX) domain, which interacts with phosphoinositides in membranes, and a variable number of coiled-coil domains, which are involved in protein–protein interactions. Recently, SNX6, but also SNX1 and SNX2, were shown to interact with serine/threonine kinase receptors for ligands of the TGF-β superfamily [31]. SNX1 and SNX2 share 70% amino acid homology in their PX domain and C-terminal regions, but despite their high homology they can exert distinct functions [45–48]. The fact that RLP interacted only with SNX1 and not with SNX2 shows the high specificity of the interaction. The domain in SNX1, which is responsible for the interaction with RLP, is localized to the linker region that connects the PX domain and the coiled-coil domains. Although RLP and SNX1 can interact with each other, both proteins are also capable of interacting with TβRI, which suggests that RLP and SNX1 could possibly interact simultaneously with the TβR complex.

The interaction of RLP with SNX1 suggests that RLP, like SNXs, could possibly affect endosomal targeting of components of the TGF-β signal-transduction pathway. Although overexpression of SNX1 or RLP did not affect expression levels of overexpressed Smad2 and TβRI (results not shown), this does not exclude the possibility that reduced transcriptional reporter activity might result from enhanced proteosomal degradation of critical components involved in the TGF-β signal-transduction pathway. However, it should be noted that RLP did not show co-localization with markers for early endosomes (EEA1 and the FYVE domain of Hrs) or late endosomes (mannose-6-phosphate). However, these studies need further investigation before conclusions can be drawn on the mechanism(s) by which RLP, a novel TGF-β regulated Ras-like protein, interferes with Smad-dependent transcriptional reporter activity.

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