Published in final edited form as:

Biochem J. ; 421(2): 283–292. doi:10.1042/BJ20090061.

The RhoU/Wrch1 Rho GTPase gene is a common transcriptional target of both the gp130/STAT3 and Wnt-1 pathways

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Abstract

STAT3 (signal transducer and activator of transcription 3) is a transcription factor activated by cytokines, growth factors and oncogenes, whose activity is required for cell survival/proliferation of a wide variety of primary tumours and tumour cell lines. Prominent among its multiple effects on tumour cells is the stimulation of cell migration and metastasis, whose functional mechanisms are however not completely characterized. RhoU/Wrch1 (Wnt-responsive Cdc42 homologue) is an atypical Rho GTPase thought to be constitutively bound to GTP. RhoU was first identified as a Wnt-1-inducible mRNA and subsequently shown to act on the actin cytoskeleton by stimulating filopodia formation and stress fibre dissolution. It was in addition recently shown to localize to focal adhesions and to Src-induced podosomes and enhance cell migration. RhoU overexpression in mammary epithelial cells stimulates quiescent cells to re-enter the cell cycle and morphologically phenocopies Wnt-1-dependent transformation. In the present study we show that Wnt-1-mediated RhoU induction occurs at the transcriptional level. Moreover, we demonstrate that RhoU can also be induced by gp130 cytokines via STAT3, and we identify two functional STAT3-binding sites on the mouse *RhoU* promoter. RhoU induction by Wnt-1 is independent of β -catenin, but does not involve STAT3. Rather, it is mediated by the Wnt/planar cell polarity pathway through the activation of JNK (c-Jun N-terminal kinase). Both the so-called non-canonical Wnt pathway and STAT3 are therefore able to induce RhoU, which in turn may be involved in mediating their effects on cell migration.

Keywords

chromatin immunoprecipitation (ChIP); mouse embryonic fibroblast; promoter analysis; RhoU; signal transducer and activator of transcription 3 (STAT3); Wnt-1

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AUTHOR CONTRIBUTION

Davide Schiavone, Sarah Dewilde and Francesco Vallania performed the experiments. Ferdinando Di Cunto analysed the data. James Turkson provided an essential reagent. Valeria Poli designed the experiment, provided funding and wrote the paper.

INTRODUCTION

STATs (signal transducers and activators of transcription) are transcription factors that play a major role in the signalling from cytokine receptors. STAT transcription factors become activated via tyrosine phosphorylation by receptor-associated JAK (Janus kinase) kinases [1], dimerize, concentrate into the nucleus and bind to STAT-responsive elements on the promoters of cytokine-induced genes. STAT3 was first isolated as the mediator of acute-phase gene induction in the liver in response to IL (interleukin)-6 and subsequently was identified as the main STAT transcription factor activated by all gp130 cytokines [2,3]. STAT3 is also activated by a wide variety of other cytokines and growth factors [e.g. leptin, IL-12, IL-17, IL-10, interferons, G-CSF (granulocyte colony-stimulating factor), EGF (epidermal growth factor) and PDGF (platelet-derived growth factor)] and by a number of oncogenic proteins such as c-Src, c-Abl, v-Sis, v-Fps, v-Ros, HER-2/neu and Met [4]. A *Stat3*-null mutation in the mouse leads to early embryonic lethality, and conditional inactivation has revealed complex roles linked to inflammation, regeneration, proliferation, migration and energy homeostasis [5].

Although STAT activity downstream of cytokines and growth factors is normally tightly controlled in both intensity and duration, STAT3 is found to be constitutively active in the vast majority of human and mouse tumours of both haematologic and solid origin, where its inhibition often leads to growth arrest and apoptotic cell death [6]. STAT3 exerts its pro-oncogenic role both directly, inducing the expression of anti-apoptotic and cell-cycle genes in the tumoral cells, and indirectly, through down-modulation of tumour immune surveillance [7]. STAT3 can also regulate cell movement, contributing to cytoskeleton reorganization and controlling cell adhesion properties, and is thought to play a role in tumour invasion and metastasis by inducing the expression of matrix metalloproteinases and promoting the EMT (epithelial-mesenchymal transition) (recently reviewed in [8]). In the zebrafish embryo, STAT3 activity is required for cell movements during gastrulation, a defect partially rescued by activation of the Wnt/PCP (planar cell polarity) signalling [9].

The evolutionarily conserved Wnt signal transduction pathway plays important roles in development and is strongly implicated in tumorigenesis. As for STAT3, aberrant Wnt signalling occurs in tumours of diverse origin and is likely to represent an important generic step in tumour development, contributing to functions as diverse as resistance to apoptosis, tissue invasion and metastasis, growth-factor-independent growth, evasion from immune response and sustained angiogenesis [10]. Wnt proteins, acting through the frizzled family of seven-pass transmembrane receptors and through recently identified alternative receptors such as Ryk and Ror2 [11], can activate three different pathways: (i) the so-called canonical Wnt signalling pathway, leading to the stabilization and nuclear concentration of β -catenin and to the activation of LEF (lymphoid enhancer factor)/TCF (T-cell factor)-mediated transcription (this is the best characterized pathway mediating the proliferative effects of Wnt ligands); (ii) the Wnt/PCP pathway, involved in controlling cell polarity and movement, particularly during embryogenesis, and mediated by the activation of the JNK (c-Jun N-terminal kinase) pathway; and (iii) the Wnt/ Ca^{2+} pathway, resulting in increased intracellular calcium and activation of PKC (protein kinase C).

Recently, a new Rho family member homologous with Cdc42 named Wrch1 (Wnt-regulated Cdc42 homologue)/RhoU has been isolated as a Wnt-1-responsive mRNA by means of subtraction cloning [12]. RhoU is an atypical Rho GTPase with no detectable GTPase activity and high intrinsic guanine nucleotide exchange, and is thus likely to be constitutively GTP-bound [13,14]. Its regulation occurs both at the level of mRNA expression [12] and through release of the inhibitory effects of the N-terminal domain [14]. RhoU acts on the actin cytoskeleton by stimulating the formation of filopodia, stimulates

quiescent cells to re-enter the cell cycle and phenocopies Wnt-1-dependent morphological transformation of mouse mammary epithelial cells [12]. Recently, RhoU was shown to localize to focal adhesions in HeLa cells and fibroblasts and to podosomes in osteoclasts and Src-expressing cells; its depletion causes an increase in focal adhesion numbers and inhibits cell migration [15,16].

In the present study we report that RhoU can also be induced by gp130 cytokines and that both its basal and cytokine-induced transcription in MEF (mouse embryonic fibroblast) cells requires STAT3, which binds to two STAT3-responsive elements on the mouse *RhoU* promoter. We also show that Wnt-1-mediated RhoU induction is regulated at the transcriptional level but is independent of STAT3, does not involve the canonical β -catenin-dependent pathway and requires JNK activity instead. Finally, we provide evidence for STAT3-mediated RhoU regulation also in human tumour or non-transformed epithelial cell lines.

EXPERIMENTAL

Cell lines and treatments

Stat3^{+/+}, *Stat3*^{-/-}, *Stat3*Rev immortalized MEFs [17], HEK (human embryonic kidney)-293, HEK-293-Wnt1, HeLa, PC3, DU145 and SKBR3 cells were grown in DMEM (Dulbecco's modified Eagle's medium; Gibco-BRL). T47D, SKOV3, MDAMB-231 and MDA-MB-468 were grown in RPMI 1360 (Gibco-BRL). Both media were supplemented with 10 % (v/v) heat-inactivated FCS (foetal calf serum; Gibco-BRL), 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. MCF10A cells were grown in DMEM F12 (Gibco-BRL) supplemented with 5 % (v/v) horse serum (Gibco-BRL), 20 ng/ml EGF, 0.5 μ g/ml hydrocortisone and 10 μ g/ml insulin. All cell lines were maintained at 37°C in a 5 % CO₂ atmosphere. The HEK-293-Wnt1 stable transfectants were generated by stable transfection with a Wnt-1 expression vector. Cells were treated with 20 ng/ml OSM (oncostatin M; R&D Systems) or with recombinant IL-6 (500 ng/ml) plus soluble receptor (250 ng/ml) [18,19] for different lengths of time. MEFs were treated with the following inhibitors: 50 μ M SP600125 (Sigma-Aldrich), 10 μ M Y-27632 (Sigma-Aldrich) and 100 μ M myristoylated PKC peptide inhibitor (Promega) for the times indicated. DU145 and SKBR3 were treated with 50 mM S31-201 (a STAT3 inhibitor) for the times indicated.

RNA extraction, retrotranscription and qRT-PCR (quantitative real-time PCR)

Total RNA was prepared with the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) according to the manufacturer's protocol. Samples were treated with DNase I Amp Grade (Invitrogen) before retrotranscription to eliminate genomic DNA contamination. To produce template cDNA, 125 ng of total RNA was reverse-transcribed using the RT High Capacity kit (Applied Biosystems) according to the manufacturer's protocol. qRT-PCR reactions were performed with the ABI Prism 7300 real-time PCR System (Applied Biosystems) using Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen). The reactions were carried out in a total volume of 25 μ l using a TaqMan assay (Mm00505976_m1; Applied Biosystems) specific for murine RhoU. Human RhoU was detected by using the Universal Probe Library system (Roche) with the following primers and probe: forward primer, 5'-GACTCCAACCTCTGTGACACTGC-3'; reverse primer, 5'-ATGAGGGGCTCACGACACT-3'; and probe #4. Results were analysed with the 2^{- $\Delta\Delta$ Ct} method using the 18S rRNA pre-developed TaqMan assay (Applied Biosystem) as an internal control.

EMSA (electrophoretic mobility-shift assay) competition assays

EMSA probes/competitors consisted of double-stranded DNA oligonucleotides formed by a 9 bp long STAT3-binding site flanked by 3 bp on both sides and by a GATC protruding sequence on the 5' end. Labelling and EMSAs were performed as described previously [2]. Briefly, the HA-SIE (high-affinity sisinducible element) probe (1 pmol) was labelled using [³²P]dCTP and Klenow and purified from free nucleotides on a Sephadex spin column. Nuclear extracts (4 µg) were pre-incubated on ice for 10 min in 80 mM KCl, 2 mM MgCl₂, 2 mM EDTA, 40 mM Hepes, 20 % (v/v) glycerol, 3 µg/reaction poly dI/dC, 5 mM DTT (dithiothreitol) and 1 µg/µl BSA. Competitor (1 µl), corresponding to concentration ratios 10-, 50- or 100-fold higher with respect to that of the labelled probe, was added and left to interact for 10 min on ice followed by incubation with the labelled probe (10000 c.p.m.) for 10 min at room temperature (25 °C). Samples were fractionated on a 5 % (v/v) polyacrylamide gel in 0.25 × TBE buffer (4.65 µg/µl Tris, 30 µg/µl boric acid and 60.5 µg/µl EDTA, pH 8.3) at room temperature. Gels were dried and autoradiographed. The oligonucleotide sequences used are indicated below: site1, upper 5'-GATCCATTTCCAAGACAGC-3' and lower 5'-GATCGCTGTCTTGGAATG-3'; site 2, upper 5'-GATCATATTAAAGGAATTC-3' and lower 5'-GATCGAATTCCTTTAATAT-3'; site 3, upper 5'-GATCGGTTGCTGGGAATTG-3' and lower 5'-GATCCAATTCCCAGCAACC-3'; site 4, upper 5'-GATCCTTTTCAGATAATAC-3' and lower 5'-GATCGTATTATCTGAAAAG-3'; site 5, upper 5'-GATCAGGAGCCGGGAAAGG-3' and lower 5'-GATCCCTTTCCCGGCTCCT-3'; site 6, upper 5'-GATCCGTTTCGAGGCGCCG-3' and lower 5'-GATCCGGCGCCTCGAAACG-3'; HA-SIE, upper 5'-GGGCATTTCCCGTAAATCGT-3' and lower 5'-CCCACGATTTACGGGAAATG-3'; and unrelated, upper 5'-GATCGGTTTCCGGACCAGC-3' and lower 5'-GATCGCTGGTCCGGAAACC-3'.

Western blot analysis

Total protein extracts were obtained in 50 mM Tris/HCl (pH 7.4), 10 % (v/v) glycerol, 1 % (v/v) Nonidet P40, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.4 mM Na₃VO₄, 10 mM NaF, 0.5 mM PMSF and 40 µg/ml protease inhibitor cocktail (Sigma–Aldrich). Proteins were cleared by centrifugation (12000 g for 10 min at 4°C) and the concentration was measured using the Bradford assay (Bio-Rad). Samples were fractionated on SDS/PAGE and transferred on to PVDF membrane (Millipore) for immunoblotting. The following antibodies were used: anti-phospho-STAT3 (Tyr⁷⁰⁵; Cell Signaling Technology), total STAT3 (Santa Cruz Biotechnology), actin (Santa Cruz Biotechnology) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Millipore).

Generation of luciferase reporter plasmids

RhoU promoter fragments were generated by PCR using a genomic BAC vector as a template. The following primers were used: –1765 forward (5'-ACTAGGTACCGAATTCAGGTGGCCCAAGGGC-3'); –1300 forward (5'-GGCCGGTACCGCATCTCTGTACTTCAGGTTGTCC-3'); –770 forward (5'-GGCCGGTACCCGCTAACAAAGGGTGGTATCCTGC-3'); –200 forward (5'-GGCCGGTACCGGTCTCGGATAGAAGAAGGAGCC-3'); and reverse (5'-TATCTCGAGGCCGCGAGACCAGCTGCC-3'). Amplified fragments were digested with KpnI/XhoI, cloned into pBlueScript (Stratagene), sequenced and subcloned into the promoter-less luciferase reporter vector pGL3basic (Promega). Mutant constructs were made by site-directed mutagenesis using the QuikChange® Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. The following primers were used for mutagenesis (mutated bases are underlined): –1300 M2 sense (5'-GGGTATAAGTAATGCTAATATTAAAGCCCTTCGTTTGACAGAATGGTTA-3'); –1300 M2 antisense (5'-

TAACCATTCTGTCAAAACGAAGGGCTTTAATATTAGCATTACTTATACCC-3');
 -1300 M3 sense (5'-
 CGTGAGTCACCATGTGGTTGCTGGCCCTTGAAGTCAGGACC-3'); -1300 M3
 antisense (5'-GGTCCTGAGTTCAAGGGCCAGCAACCACATGGTGACTCACG-3');
 -1300 M5/-770 M5 sense (5'-
 GGAAGCTTGGAAGGAGCCGGCCAGGGGGGTGG-3'); -1300 M5/-770 M5
 antisense (5'-CCACCCCCCTGGGCCGGCTCCTTCCCAAGCTTCC-3'); -1300 MA sense
 (5'-CAACAACATCTGGGACATAAACCAGGCTCAGTATTAACTAGCTCTTGTC-3');
 -1300 MA antisense
 (5'-GACAAGAGCTAGTTAAATACTGAGCCTGGTTTATGTCCCAGATGTTGTTG-3');
 -770 MB sense (5'-
 CCAAAGAGAGGGAGCGCTAAACCAGGGTGGTATCCTGCATC-3'); -770 MB
 antisense (5'-GATGCAGGATACCACCCTGGTTTAGCGCTCCCTCTCTTTGG-3'); -770
 MC sense (5'-
 CAAAGGGTGGTATCCTGCATCCAAATGAGAATACCTGATTTTAAACAG-3'); -770
 MC antisense (5'-
 CTGTTAAAAATCAGGTATTCTCATTGATGCAGGATACCACCCTTTG-3'). All
 mutant constructs were controlled by sequencing.

Transient transfections, co-culture and luciferase assays

MEFs and HeLa cells were seeded in 24-well plates and transfected 24 h later with Lipofectamine™ reagent (Invitrogen). Each promoter construct was mixed at a 10:1 ratio with the pSEAP vector [20] as an internal control for transfection efficiency for a total amount of 0.4 µg of DNA and incubated with 5 µl of Lipofectamine™ according to the manufacturer's protocol. In cotransfection experiments, promoter constructs were mixed at a ratio of 5:5:1 (reporter/expression vector/pSEAP). At 24 h after transfection, the cells were treated with OSM or detached and re-seeded with an equal amount of Wnt1-expressing HEK-293 or wild-type control cells for co-culture experiments. At 48 h after transfection, the cells were washed twice with PBS and lysed in 50 µl of passive lysis buffer (Promega). Luciferase assays were performed using a luciferase assay system (Promega) according to the manufacturer's protocol. SEAP assays were performed using the phospho-light system (Applied Biosystems) according to the manufacturer's protocol. Luciferase and SEAP chemiluminescent activity were quantified using a Mithras LB940 multilabel reader (Berthold Technologies). SEAP values were used to normalize for differences caused by unequal transfection efficiency.

ChIP (chromatin immunoprecipitation) assay

Stat3^{+/+} and *Stat3*^{-/-} MEFs were treated or not with OSM as described above. ChIP assays were performed with the fast ChIP method [21] with some modifications. Briefly, cross-linking was performed with 1 % (v/v) formaldehyde for 10 min at room temperature. The reaction was quenched by the addition of glycine to a final concentration of 125 mM and incubation for 10 min at room temperature. Cells were lysed and sonicated at 4°C for six 10 s rounds followed by a 60 s pause, at an amplitude of 40 % (Ultrasonic Processor VCX-750, Sonics and Materials).

Immunoprecipitations were performed by incubating overnight at 4°C 1 ml of sheared chromatin with anti-STAT3 serum (R&D Systems, 5 µl), anti-acetyl-histone H3 (Upstate Cell Signaling Solutions, 2 µg) or negative control IgG (ChIP-IT Control Kit-Mouse; Active Motif, 2 µg). Primers for PCR amplification were designed using the program Primer3 [22] to obtain an amplicon size of approx. 150 bp. PCR products were fractionated on a 2 % (w/v) agarose gel and stained with ethidium bromide. PCR reactions were performed using 5 µl of immunoprecipitated DNA using the following primers: RhoU site 3, forward 5'-

GGCACCAGATCCCATTACAG-3' and reverse 5'-TTTTGTGCTGCCCTTTCTCT-3'; RhoU site 5, forward 5'-ATCGCAAGGTCAGCTTTCAC-3' and reverse 5'-CTTGCTTGGCCATTACCAT-3'; Socs3 -65, forward 5'-GGAGAGACAGCGGTCGTAAG-3' and reverse 5'-CACAGCCTTTCAGTGCAGAG-3'; and β -globin, forward 5'-CTCCCCCTCACTCTGTTCTG-3' and reverse 5'-AGGAGGAGGGGAAGCTGATA-3'.

RESULTS

RhoU is a STAT3 transcriptional target induced by gp130 cytokines

Gene expression profiling of *Stat3*^{+/+} or *Stat3*^{-/-} MEFs either untreated or stimulated with different cytokines has led us to identify, among others, the atypical Rho GTPase RhoU/Wrch1 as a STAT3-dependent target of gp130 cytokine stimulation [23]. Moreover, RhoU displayed a striking degree of conserved co-expression with STAT3 across hundreds of human and mouse microarray experiments [24], further suggesting a close functional correlation. Quantitative PCR analysis of *RhoU* mRNA levels showed indeed profoundly defective expression in *Stat3*^{-/-} MEFs already under basal conditions (Figure 1a). In addition, whereas both IL-6 and OSM treatment induced *RhoU* mRNA in *Stat3*^{+/+} MEFs, they were completely ineffective in the absence of *Stat3*. As a result, *RhoU* expression levels were 4–5-fold lower in the *Stat3*^{-/-} MEFs following cytokine stimulation. These defects are indeed STAT3-dependent since reintroducing *Stat3* by stable transfection in the *Stat3*^{-/-} MEFs (revertant MEFs) completely rescued both *RhoU* basal expression and cytokine inducibility (Figure 1a). *RhoU* mRNA induction in response to OSM stimulation was already appreciable 30 min after OSM treatment, peaked at 6 h and decreased to basal levels 24 h later (Figure 1b).

The *RhoU* promoter contains two functional STAT3-binding sites fully responsible for OSM inducibility

Since *RhoU* expression is induced by gp130 cytokines/STAT3, we searched the mouse *RhoU* promoter region for potential STAT3 regulatory elements. Bioinformatics analysis [23] identified six potential STAT3-binding sites (sites 1–6) that were assessed for *in vitro* binding capacity by EMSA competition assays (Figure 2a). Liver nuclear extracts from LPS-treated mice were used as a source for activated STAT3, with a labelled HASIE [25] double-stranded oligonucleotide as a probe. STAT3 binding to the HA-SIE probe was then competed with non-labelled double-stranded oligonucleotides based on the sequences of the six predicted STAT3-binding sites. Site 3 showed the greatest *in vitro* binding activity, with full competition at a ratio of 100:1 between non-labelled competitor and labelled probe, and partial competition at the ratios of 50:1 and 10:1. Sites 5 and 2 were also able to compete, albeit with lower efficiency/affinity, whereas sites 1, 4 and 6 did not show any significant competing activity. Self-competition with a unlabelled HA-SIE oligonucleotide or competition with an unrelated sequence were used as positive and negative controls respectively.

In order to analyse *RhoU* promoter activity, mouse *RhoU* promoter deletion fragments were fused to a luciferase reporter and transiently transfected in *Stat3*^{+/+} and *Stat3*^{-/-} MEFs (Figure 2b). Transfected cells were either stimulated with OSM or left untreated for 24 h before measuring luciferase activity. In keeping with the mRNA expression results, transcriptional activity of all promoter fragments was drastically reduced in *Stat3*-deficient MEFs as compared with their wild-type controls already under basal conditions. In addition, the constructs -1765, -1300 and -770 were induced by OSM treatment in the *Stat3*^{+/+} MEFs, confirming that cytokine-dependent *RhoU* mRNA induction occurs transcriptionally. In contrast, OSM-dependent induction of all constructs was completely defective in the

Stat3-deficient cells. Both basal and OSM-stimulated activity of *RhoU* promoter constructs were comparable with those of the mouse *Socs3* promoter [26], a well-recognized STAT3 target. *RhoU* -200, the shortest promoter fragment tested, displayed reduced activity in both *Stat3*^{-/-} and *Stat3*^{+/+} MEFs and was unresponsive to OSM treatment, suggesting that the region(s) required for OSM induction must be located upstream of the -200 position. Interestingly, and only in the presence of *Stat3*, both basal transcription levels and OSM inducibility increased with the progressive shortening of the promoter up to position -770, before dropping at -200. This might suggest the presence of repressor element(s) in the distal portions of the promoter. To assess the functional contribution of the tested STAT3-binding sites (see Figure 2a), sites 2, 3 and 5 were mutagenized in the context of the *RhoU* -1300 construct and the transcriptional activity of the resultant *RhoU* -1300 M2, M3 or M5 constructs tested in wild-type MEFs (Figure 2c). The site 2 mutation did not have any effect on either basal or OSM-induced expression, suggesting that this site is not involved in STAT3-mediated regulation. Indeed, site 2 was the one with the lowest *in vitro* binding activity (Figure 2a). Disruption of sites 3 or 5 significantly decreased transcriptional activation in response to OSM stimulation, and the compound double mutation (-1300 M3 + M5) completely abolished promoter inducibility and decreased its basal activity (Figure 2c). Thus both STAT3-binding sites 3 and 5 co-operate to confer full promoter activation in response to OSM stimulation. On the other hand, site 5 also plays a prominent role to confer OSM inducibility to the *RhoU* -770 promoter construct, since its mutation in this context (-770 M5) almost completely abolished OSM inducibility (Figure 2c). Site 6 did not appear to contribute to this residual induction since neither a site 6 single mutation nor a compound site 5/site 6 double mutation were able to abolish it (-770 M6 and M5+M5 mutants; Figure 2c).

To further analyse the *in vivo* functionality of sites 3 and 5 we assessed STAT3 *in vivo* occupancy by ChIP. ChIP assays were performed with chromatin samples prepared from *Stat3*^{+/+} and *Stat3*^{-/-} MEFs, either untreated or stimulated with OSM for 30 min. With respect to the negative IgG controls, both sites showed weak STAT3 binding already in untreated cells (Figure 2d). Similar to what was observed for the well-characterized SOCS3 -65 STAT3-responsive element [26], STAT3 binding to both site 3 and site 5 was strongly induced by OSM, confirming their *in vivo* functional role in mediating *RhoU* promoter activity. Importantly, no STAT3 binding was ever detected with chromatin from *Stat3*^{-/-} MEFs, thus confirming the specificity of the ChIP assay used in the present study.

RhoU induction by Wnt-1 occurs at the transcriptional level, but does not require STAT3

Since *RhoU* was initially isolated as a Wnt-1-regulated gene, but its transcriptional regulation was never characterized, we decided to investigate the mechanisms involved in *RhoU* up-regulation downstream of Wnt-1 and the potential co-operation with the STAT3 pathway. Thus *RhoU* mRNA expression levels were measured in *Stat3*^{+/+} and *Stat3*^{-/-} MEFs stimulated with OSM and Wnt-1 either alone or in combination (Figure 3a). Wnt-1 stimulation was obtained by co-culturing MEFs with HEK-293 cells stably expressing Wnt-1, whereas control cells were co-cultured with wild-type HEK-293 cells. In keeping with previous observations, *RhoU* mRNA expression was dependent on STAT3 already under basal conditions and failed to be induced by OSM stimulation in *Stat3*^{-/-} cells. In contrast, Wnt-1 stimulation could similarly induce *RhoU* mRNA in both *Stat3*^{+/+} and *Stat3*^{-/-} MEFs. Likewise, Wnt-1 could induce *RhoU* promoter transcription at similar levels in the presence or absence of *Stat3* (Figure 3b). These results suggest that Wnt-1-mediated *RhoU* induction occurs at the transcriptional level and is independent of STAT3. This was further supported by the observation that the *RhoU* -770 M5 construct, which has lost OSM inducibility, is still fully responsive to Wnt-1 stimulation (Figure 4a). Thus both the Wnt-1 and the gp130/STAT3 pathways can activate transcription of the *RhoU* promoter, but they

act through independent mechanisms. Of note, RhoU induction could not be obtained using the supernatants of the Wnt-1-expressing HEK-293 cells (results not shown), suggesting that specific stimulation by membrane-bound Wnt-1 and not secretion of soluble mediators by the Wnt-1-expressing cells is involved.

Similar to what was observed for OSM, Wnt-1-mediated induction progressively increased with promoter shortening, suggesting that the inhibitory sequence(s) already postulated in the case of OSM induction may be also active in the context of Wnt-1 stimulation (Figure 3c). As in the case of OSM responsiveness, the RhoU -200 construct was not anymore able to respond to Wnt-1 stimulation, indicating that the Wnt-1-responsive element(s) must lie in the region between positions -770 and -200. The reporter vector Super8X-TOPFlash and its negative control Super8X-FOPFlash [27] were used in all co-culture experiments to monitor Wnt pathway activation (results not shown).

RhoU promoter induction by Wnt-1 does not involve the canonical β -catenin pathway

The molecular mechanisms leading to *RhoU* gene activation downstream of Wnt-1 are currently unknown. Among the many signalling events activated by Wnt ligands, the canonical Wnt/ β -catenin pathway which leads to the up-regulation of several target genes mediated by the LEF/TCF transcription factor is the best characterized [28,29]. Mouse *RhoU* promoter analysis using the TRANSFAC open-source program [30] identified three consensus sequences for LEF/TCF (sites A, B, C; Figure 2b). However, none of the three sequences appears to play a role in *RhoU* promoter activity, as shown by transient transfection assays of the relative promoter mutants -1300 MA, 770 MB or -770 MC (Figure 4a). Moreover, downstream activation of the Wnt-1/ β -catenin pathway by expression of a stabilized mutant form of β -catenin did not have any effect on *RhoU* promoter transcription, whereas it strongly activated the TOPFlash control construct (Figure 4b). Thus the canonical Wnt/ β -catenin pathway does not appear to be involved in mediating RhoU transcriptional induction.

A JNK-mediated non-canonical Wnt pathway is responsible for RhoU activation

Besides the canonical β -catenin pathway, Wnt ligands are known to activate the Wnt/ Ca^{2+} and the PCP non-canonical pathways (as illustrated in Figure 7). In order to assess their potential involvement in RhoU induction by Wnt-1, we have used available specific chemical inhibitors to interfere with the activities of the main mediators involved.

First, *RhoU* mRNA levels were measured by qRT-PCR in wild-type MEFs treated or not with specific inhibitors for PKC (Wnt/ Ca^{2+} pathway), ROCK (Rho-associated kinase) and JNK (PCP pathway) in the presence or absence of Wnt-1 stimulation (Figure 5a). Wnt-1-mediated *RhoU* mRNA induction was unaffected by PKC and ROCK inhibitors, whereas it was completely abolished by inhibiting JNK activity (Figure 5a). A similar effect was also observed at the level of *RhoU* promoter activation, since Wnt-1-mediated induction of the -770 promoter construct was strongly reduced in the presence of the JNK inhibitor (Figure 5b).

Thus RhoU transcriptional activation by Wnt-1 appears to be mainly mediated by the non-canonical PCP pathway, with a central role played by the MAPK (mitogen-activated protein kinase) JNK. Since the activation of JNK downstream of Wnt is known to be mediated by the small GTPases Cdc42 and Rac (see Figure 7), we performed co-transfection experiments of the *RhoU* promoter with constitutively active Rac1 and Cdc42 mutants (Figure 5c). Both Rac1-Val¹² and Cdc42-Val¹², but not the control RhoA-Val¹⁴, were able to significantly activate the *RhoU* promoter and this induction was abolished by JNK inhibition,

strengthening the idea that Wnt-1 can activate the PCP pathway leading to the induction of the *RhoU* promoter through Rac1 and Cdc42-mediated JNK activation.

STAT3/gp130-dependent regulation of RhoU in human epithelial cell lines

In order to extend our results to more biologically relevant experimental systems, we analysed *RHO* mRNA expression and its correlations with the gp130/STAT3 pathway in human transformed and non-transformed epithelial cell lines. OSM treatment led to *RHO* mRNA up-regulation also in HeLa cells, albeit with different kinetics as compared with MEFs (Supplementary Figure S1a at <http://www.BiochemJ.org/bj/421/bj4210283add.htm>). In the same cells, additionally, both OSM and Wnt-1 stimulation were able to induce *RHO* promoter activity (fragment -770) to a similar extent as observed in MEFs (Supplementary Figure S1b and S1c).

Since RhoU is thought to mediate Wnt-1 oncogenic properties and might therefore play a similar role downstream of STAT3, we decided to correlate STAT3 activation and *RHO* expression levels in a panel of human tumour cell lines well-characterized for STAT3 constitutive activation. *RHO* expression levels (Figure 6c) were always elevated in cell lines with high (DU145, SKOV3, MDA-MB-231 and MDA-MB-468) or moderate (SKBR3) levels of constitutively active STAT3 (Figures 6a and 6b), all of which, with the exception of SKOV3 cells, have been reported to be addicted to STAT3 activity for survival/proliferation [31–33]. Conversely, cell lines with no detectable phosphorylated STAT3 showed variable *RHO* mRNA levels (Figures 6a–6c). In particular, *RHO* mRNA was almost undetectable in the breast cancer cells T47D, whereas its levels were comparable with those of the other cell lines in the prostate cancer PC3 cells. Of note, these are known to display constitutive Wnt-1 expression, which may be responsible for RhoU up-regulation in the absence of STAT3 activity [34].

In order to assess the functional correlation between STAT3 activity and *RHO* expression, DU145 and SKBR3 cells were then treated with the specific STAT3 inhibitor S3I-201 [33]. Both phospho- and total STAT3 levels were drastically reduced in both cell lines after S3I-201 treatment (Figure 6d), correlating with a dramatic decrease of *RHO* mRNA levels as determined by qRT-PCR (Figure 6e; 78% and 93% reduction at 24 h in DU145 and SKBR3 cells respectively).

Finally, IL-6 stimulation triggered STAT3 phosphorylation correlating with a strong up-regulation of *RHO* mRNA expression in MCF10A cells, a non-transformed human mammary cell line displaying almost undetectable levels of STAT3 phosphorylation under basal conditions (Figures 6f and 6g).

DISCUSSION

The atypical Rho GTPase RhoU was first identified as a Wnt-1-responsive gene by subtractive cloning [12]. RhoU overexpression can trigger cellular transformation and phenocopies the effects of Wnt-1 in mammary epithelial cells, suggesting a potential role in Wnt-mediated tumorigenesis [12]. Rather than being regulated by GDP/GTP exchange like most Rho GTPases, RhoU activity can be modulated by acting on its N-terminal domain, which exerts a partial inhibitory effect on the RhoU ability to activate PAK (p21-activated kinase) and to cause cellular transformation [14]. This inhibition can be relieved by interaction of the PxxP motif in the N-terminal domain with SH3-containing protein adaptors such as Grb2 and Nck β , suggesting functional interactions with receptor tyrosine kinase-activated pathways. Moreover, unlike most Rho GTPases that are constitutively expressed, RhoU expression is tightly controlled [35,36]. In addition to being induced by Wnt-1, RhoU mRNA has been recently shown to accumulate in osteoclasts in response to

RANKL (receptor activator for nuclear factor κ B ligand)-induced differentiation [37]. However, the molecular mechanisms for either induction have not been characterized. In the present study, we demonstrate for the first time that RhoU induction by Wnt-1 occurs at the transcriptional level. Moreover, we show that RhoU is also a prominent target for STAT3-mediated transcription downstream of the gp130 cytokines IL-6 and OSM (Figure 7). Intriguingly, although both *in vivo* functional STAT3-binding sites 3 and 5 are required to confer full OSM inducibility to the -1300 RhoU promoter construct, site 5 alone is sufficient in the context of the shorter -770 promoter fragment, which lacks site 3. Both sites might indeed be required both *in vivo* and in the longer promoter construct to overcome the observed inhibitory effect of sequence(s) located between the -1300 and -770 positions. Interestingly, STAT3 was required not only for RhoU cytokine-mediated induction, but also for its basal expression, and both functionally tested STAT3-binding sites, site 3 and site 5, displayed a low level of constitutive binding following ChIP analysis. Constitutive STAT3 binding to a subset of sites was recently shown in NIH 3T3 cells [38], although a correlation with basal gene expression was not tested. Low amounts of Tyr⁷⁰⁵-phosphorylated STAT3 are present in growing MEFs and could be responsible for the constitutive binding observed. Alternatively, non-canonical mechanisms recruiting unphosphorylated STAT3 may be involved. At any rate, the ability of STAT3 to transcriptionally regulate a subset of target genes under basal conditions may have important implications for its physiology.

Both Wnt ligands and STAT3 play surprisingly similar multiple roles in tumorigenesis. In particular, both are involved in stimulating cell movements in tumoral cells as well as during development [8,39]. RhoU is known to act on the actin cytoskeleton, triggering filopodia formation and stress fibre dissolution. In addition, RhoU was recently shown to localize to focal adhesions and podosomes and to regulate their genesis promoting cell migration [15,16]. Intriguingly, STAT3 was also shown to localize to focal adhesions in HeLa and ovary tumour cells, where it interacted with activated focal adhesion kinase and paxillin and promoted cell migration [40], and we could demonstrate that activation of the gp130/STAT3 pathway induces both RhoU mRNA and promoter activity also in HeLa cells. Thus RhoU transcriptional induction might be one of the mechanisms through which STAT3 acts on focal adhesions and ultimately triggers cell migration. Indeed, the same *Stat3*^{-/-} MEFs used in the present study, exhibiting low basal RhoU levels, also display impaired migration in a wound healing assay [41]. In addition, the convergence of both Wnt-1 and STAT3 signalling on RhoU may represent a point of cross-talk between the two pathways, which has been proposed in several systems. STAT3 expression was induced by the Wnt/ β -catenin pathway in oesophageal cancer cells and in embryonic stem cells [42,43]. STAT3 was also proposed to act upstream of the Wnt/PCP pathway during the gastrulation of zebrafish embryos [9] and to promote β -catenin nuclear accumulation in colorectal cancer cells [44]. Moreover, the negative regulator of Wnt signalling Duplin was recently shown to also suppress STAT3 activity [45]. Indeed, both STAT3 and Wnt-1 aberrant activation are often observed in the same tumours, suggesting that the two pathways can co-operate in different ways depending on the cellular context.

However, the convergence of the Wnt-1 and gp130/STAT3 pathways on RhoU induction must involve distinct mechanisms, since STAT3 is not required for Wnt-1-mediated induction of the RhoU promoter. In addition, as previously suggested by Tao et al. [12], RhoU induction by Wnt-1 appears also to occur independently of β -catenin, suggesting the involvement of one of the non-canonical pathways. We could identify this pathway with the Wnt/PCP pathway, since Wnt-1-mediated RhoU induction is abolished by an inhibitor of JNK activity. Interestingly, RhoU itself was reported to be able to activate JNK [12], suggesting the existence of a positive-feedback loop whereby activated RhoU may enhance its own transcription by means of JNK activation. Supporting and extending this observation, we could demonstrate that both constitutively active Rac1 and Cdc42, which lie

upstream of JNK in the Wnt/PCP pathway, can activate RhoU transcription in a JNK-dependent way. Thus, at least in MEFs, Wnt-1 can activate both the canonical β -catenin pathway and the non-canonical PCP pathway at the same time, as shown by the simultaneous activation of RhoU and TOPFlash in our system. It is intriguing that the Wnt/PCP pathway is best characterized as regulating cell movements during embryonic gastrulation and that the main functions of RhoU appear to be linked to focal adhesions and cell migration.

At present, the transcription factors mediating the effects of the PCP pathway are unknown, and our system may provide a relatively straightforward way to identify at least some of them. However, bioinformatic analysis of the RhoU promoter region involved in Wnt-1 responsiveness did not disclose any obvious candidate, and co-transfection experiments with transcription factors potentially activated by JNK such as c-fos, c-jun and CREB (cAMP-response-element-binding protein) did not modify promoter activity (D. Schiavone and V. Poli, unpublished work). More detailed promoter analysis will therefore be required in order to identify the PCP-activated transcription factors. The identification of RhoU as a common transcriptional target of both STAT3 and Wnt-1, together with the known roles of both pathways in stimulating cell migration in normal and tumoral cells, suggests the intriguing idea that RhoU may represent a common mediator of STAT3 and Wnt effects on cell motility under both physiological and pathological conditions, being potentially a common therapeutic target. Interestingly, we have observed a positive correlation between STAT3 phosphorylation and RhoU expression levels in all tested tumour-derived cell lines. Indeed, phosphorylated STAT3 was undetectable in T47D cells, the only ones displaying very low *RHO* mRNA levels. The metastatic prostate cancer cell line PC3 in contrast, also lacking detectable STAT3 activation, displayed high *RHO* mRNA expression. In these cells Wnt-1 expression was reported to be extremely elevated, and could thus play a dominant role in RhoU induction bypassing the need for STAT3 activity [34]. A direct correlation between STAT3 activity and RhoU transcription in tumour cell lines is also supported by our observation that specific STAT3 inhibition triggered a dramatic reduction of *RHO* mRNA in DU145 and SKBR3 cells. Accordingly, acute STAT3 activation by IL-6 treatment could efficiently up-regulate *RHO* mRNA in the non-transformed mammary epithelial cell line MCF10A. Taken together, these results strengthen the hypothesis that RhoU may represent a common transcriptional and functional target of both the Wnt-1 and STAT3 pathways in physiological, as well as pathological, contexts. Further studies addressing the role played by RhoU in mediating specific features of STAT3 and Wnt-mediated cell transformation will be required to address this issue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Dr I. Barbieri for help with the MCF10 cells, Dr C. J. Auernhammer for kindly providing the SOCS3 mouse promoter construct, Professor M. Orlandini for the mutant β -catenin (S33A) and the Wnt-1 constructs, Professor S. Rose-John and Professor C. Toniatti for the gift of recombinant plasmids and Professor G. Merlo, Professor E. Calautti and Professor D. Taverna for critically reading the manuscript prior to submission.

FUNDING

This work was supported by the Italian Ministry of University and Research (MIUR COFIN and FIRB) and by the Italian Cancer Research Association (AIRC).

Abbreviations used

ChIP	chromatin immunoprecipitation
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EGF	epidermal growth factor
EMSA	electrophoretic mobility-shift assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HA-SIE	high-affinity sis-inducible element
HEK	human embryonic kidney
IL	interleukin
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LEF	lymphoid enhancer factor
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
OSM	oncostatin M
PCP	planar cell polarity
PKC	protein kinase C
qRT-PCR	quantitative real-time PCR
ROCK	Rho-associated kinase
STAT	signal transducer and activator of transcription
TCF	T-cell factor
Wrch1	Wnt-responsive Cdc42 homologue

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Figure 1. RhoU mRNA expression is defective in *Stat3*^{-/-} MEFs

Total RNA was extracted from *Stat3*^{+/+}, *Stat3*^{-/-} and revertant (rev) MEFs either untreated or stimulated with IL-6 or OSM for the indicated times. *RhoU* mRNA was measured by qRT-PCR. Expression was normalized to 18S, and results are shown as means $\pm$ S.E.M. of two independent experiments performed in duplicate. Asterisks indicate statistically significant differences between selected groups (* P < 0.05 and ** P < 0.01). A.U., arbitrary units.

Figure 2. Functional STAT3 binding sites in the RhoU promoter

(a) EMSA competition assays. A radioactively labelled HA-SIE probe was incubated with liver nuclear extracts containing activated STAT3 and the binding was competed with increasing amounts of unlabelled oligonucleotides based on the sequence of the six predicted STAT3-binding sites on the mouse *RhoU* promoter, whose sequence and position from the TSS (transcription start site) are indicated. Unlabelled unrelated or HA-SIE oligonucleotides were used as negative or positive competing controls respectively. (b and c) Transient transfection assays. The indicated constructs were co-transfected with a pSEAP expression vector as an internal control. At 24 h later, cells were stimulated or not with OSM and cultured for a further 24 h. Luciferase activity was normalized to SEAP activity as

a control for transfection efficiency. Results shown are means $\pm$ S.E.M. of at least two independent experiments performed in duplicate. **(b)** Transfections were performed with *Stat3*^{+/+} and *Stat3*^{-/-} MEFs. Numbers over the bars indicate the induced fold-change of stimulated over unstimulated cells. The empty pGL3 vector and the previously characterized *Socs3* promoter-luciferase construct [26] were used as controls. **(c)** *Stat3*^{+/+} MEFs were transfected with the indicated wild-type or mutant (M) RhoU promoter constructs. **(d)** ChIP assays were performed with *Stat3*^{+/+} and *Stat3*^{-/-} MEFs, either unstimulated (NT) or treated with OSM for 30 min. Immunoprecipitations were performed with antibodies against acetylated histone H3 (α -Ac. H3), STAT3 (α -STAT3) antisera or control IgG. Non-immunoprecipitated chromatin was used as the total input control (T.I.). Specific primers were used to amplify regions of approx. 150 bp containing the indicated STAT3-binding sites (see the Materials and methods section). Primers amplifying the second intron of the silent β -globin gene (shown) or the non-coding transcript Xist (inactive X specific transcript) (not shown) were used as negative controls. A.U., arbitrary units.

Figure 3. RhoU induction downstream of the Wnt-1 and STAT3 pathways

(a) qRT-PCR analysis. *Stat3*^{+/+} and *Stat3*^{-/-} MEFs were co-cultured with wild-type or Wnt-1-expressing HEK-293 cells for 24 h with or without OSM stimulation. *RhoU* mRNA levels were measured by qRT-PCR and normalized to 18S RNA. Results are shown as means $\pm$ S.E.M. of two independent experiments performed in duplicate. (b) Transient transfection assays. *Stat3*^{+/+} and *Stat3*^{-/-} MEFs were transfected with the RhoU -770 construct and the pSEAP vector. At 24 h after co-transfection, cells were treated as in (a) for a further 24 h. Results shown are means $\pm$ S.E.M. of normalized luciferase activity from at least two independent experiments performed in duplicate. (c) *Stat3*^{+/+} MEFs were transfected with the following vectors: the indicated RhoU promoter deletion fragments and

TOPFlash and FOPFlash reporters. Transfected MEFs were Wnt-1-stimulated as in (a). Results shown are means $\pm$ S.E.M. of normalized luciferase activity from at least two independent experiments performed in duplicate. A.U., arbitrary units.

Figure 4. RhoU induction downstream of Wnt-1 is independent from STAT3 and β -catenin
(a) *Stat3*^{+/+} MEFs were transfected with the RhoU -1300 and -770 vectors, either wild-type or mutated (M) in the indicated LEF/TCF or STAT3-binding sequences. Wnt-1 stimulation and data analysis were performed as described for Figure 3. **(b)** The RhoU -770 construct and TOPFlash and FOPFlash reporters were co-transfected with a stable mutant β -catenin (S33A) or with an empty control vector in Stat3 wild-type MEFs. Luciferase activity was normalized and reported as above. A.U., arbitrary units.

Figure 5. Wnt-1-mediated RhoU activation follows a non-canonical pathway involving JNK
(a) qRT-PCR analysis. Wild-type MEFs were stimulated or not with Wnt-1 as described for Figure 3 and treated with specific inhibitors for PKC (myristoylated peptide inhibitor, 100 μ M), ROCK (Y-27632, 10 μ M) and JNK (SP600125, 50 μ M) for 24 h. RhoU expression levels were measured by qRT-PCR and normalized to 18S RNA. Values are means $\pm$ S.E.M. of two independent experiments performed in duplicate. **(b)** and **(c)** Transient transfection assays. Wild-type MEFs were transfected with the promoter fragment -770 alone **(b)** or in combination with plasmids encoding the constitutively active GTPases RhoA (RhoA V14), Rac1 (Rac1 V12), Cdc42 (Cdc42 V12) or a control empty vector **(c)**. At 24 h later, cells were stimulated or not with Wnt-1 as described for Figure 3 and treated with 50 μ M

SP600125 (**b**) or simply treated or not with 50 μ M SP600125 (**c**) and cultured for a further 24 h. Luciferase activity was quantified and normalized as described for Figure 2. A.U., arbitrary units.

Figure 6. *RHO* mRNA regulation by STAT3 in human tumour cell lines

(a) Total and phospho-STAT3 were measured by Western blot analysis along with GAPDH as a loading control in the indicated tumour cell lines. (b) Quantification of the phospho-STAT3 signal in (a) normalized to GAPDH and reported as means $\pm$ S.E.M. of two replicates. (c) *RHO* mRNA from the same samples analysed in (a) was measured by qRT-PCR and normalized to 18S. Results shown are means $\pm$ S.E.M. of two independent experiments performed in duplicate. (d–g) Total and phospho-STAT3 protein levels (d and f) and *RHO* mRNA (e and g) were measured by Western blot analysis and qRT-PCR respectively, in DU145 and SKBR3 cells treated or not with the STAT3 inhibitor S3I-201 (d and e) or in MCF10A cells treated with IL-6 plus soluble IL-6 receptor. A.U., arbitrary units.

Figure 7. Schematic representation of the best known Wnt signal transduction pathways
 The Wnt signal branches into three main pathways: the canonical β -catenin-dependent, the non-canonical planar cell polarity (PCP) and Wnt- Ca^{2+} . All of these pathways are thought to occur through the Frizzled receptor and Dishevelled. Other receptors have recently been shown to be able to activate additional non-canonical pathways. In particular the Ror2 receptor, depicted here, converges on to JNK in a similar manner to the Wnt/PCP pathway. In the present study we have demonstrated that RhoU is transcriptionally induced both by Wnt-1, through the PCP pathway involving the Rho GTPases Rac1/Cdc42 and the MAPK JNK, and by gp130 cytokines through the JAK/STAT3 pathway (thick lines). APC, adaptor protein complex; CaCN, calcineurin; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; Dsh, Dishevelled; GSK3 β , glycogen synthase kinase 3 β ; LPR 5/6, lipoprotein receptor 5/6; NF-AT, nuclear factor of activated T-cells; PLC, phospholipase C; TK, tyrosine kinase.