Lgl1, a mesenchymal modulator of early lung branching morphogenesis, is a secreted glycoprotein imported by late gestation lung epithelial cells

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Secreted glycoproteins serve a variety of functions related to cell–cell communication in developmental systems. We cloned *LGL1*, a novel glucocorticoid-inducible gene in foetal lung, and described its temporal and spatial localization in the rat. Disruption of foetal mesenchyme-specific *LGL1* expression using antisense oligodeoxynucleotides, which was associated with a 50 % decrease in lgl1 protein levels, inhibited airway epithelial branching in foetal rat gestational day 13 lung buds in explant culture. These findings suggested that lgl1 functions as a secreted signalling molecule. We now provide evidence supporting a role for lgl1 in mesenchymal–epithelial interactions that govern lung organogenesis. Lgl1 is a secreted glycoprotein with a conserved N-terminal secretory signal peptide. Using dual immunofluorescence, intracellular lgl1 was found to co-localize with markers of the Golgi apparatus and endoplasmic reticulum, consistent with its association with secretory vesicles. Using pulse–chase studies, we show that lgl1 is a stable protein with a half-life of 11.5 h. Furthermore, at gestational days 20 and 21 (term = 22), foetal distal lung epithelial cells import lgl1 protein. Taken together, our findings support distinct roles for lgl1 as a mediator of glucocorticoid-induced mesenchymal–epithelial interactions in early and late foetal lung organogenesis.

Key words: cysteine-rich secretory protein (CRISP), glucocorticoid-responsive gene, glycoprotein, lung development, lgl1, mesenchymal–epithelial interactions, secreted factor.

INTRODUCTION

Organogenesis is dependent upon complex and precise mesenchymal–epithelial interactions and signalling between cells and ECM (extracellular matrix) molecules [1–5]. Mammalian lungs originate *in utero* as paired epithelial outgrowths from the ventral foregut into the surrounding mesenchyme. Therein, epithelial branching morphogenesis involves cell proliferation, migration and differentiation, giving rise to functional lungs capable of gas exchange at birth [3]. Although the mechanisms by which mesenchyme instructs the development of the surrounding epithelium are incompletely elucidated, these events are mediated in part by a variety of hormones and soluble/diffusible factors [3,6–8]. The temporal expression of several key mediators of lung morphogenesis also suggests that these molecules may be critical for both early branching morphogenesis and later lung development, including alveogenesis [9,10].

Secreted glycoproteins serve a variety of functions related to cell–cell communication in developmental systems [11]. Tissue-specific regulation of these proteins affects their molecular properties, including localization, half-life and biological activity [11]. Glucocorticoids stimulate and accelerate both lung branching morphogenesis [12] and epithelial cell cytodifferentiation [13,14] in the developing foetal lung. These effects are mediated, in part, by factors released from foetal mesenchyme adjacent to the developing epithelium [15–17]. We previously reported the cloning of *LGL1* (late gestation lung 1) [11,18], a glucocorticoid-inducible gene in foetal rat lung. The *LGL1* gene product (lgl1) belongs to a growing family of CRISPs (cysteine-rich secretory proteins). These proteins are characterized by hydrophobic amino acid clusters at the N-terminus and a cysteine-rich region at the C-terminal half (from which their protein family name is derived) [19]. CRISPs display considerable homology across mammalian species. The functions of mammalian CRISPs remain largely unknown. Recent studies have shown that various members of the CRISP family may be associated with cell-adhesion functions during the development of reproductive organs, and/or as effector molecules in the TGF (transforming growth factor)-signalling pathway during *Caenorhabditis elegans* development [19–22].

We described the spatial and temporal pattern of expression of LGL1 mRNA/lgl1 protein in embryonic rat lung, as assessed by *in situ* hybridization and immunohistochemistry [11]. Neither LGL1 mRNA nor lgl1 protein was detectable in early embryonic lung epithelium [11,18]. We showed that antisense oligodeoxynucleotide disruption of LGL1 mRNA in foetal rat lung explants, which was associated with a 50 % decrease in lgl1 protein levels, inhibited normal lung branching morphogenesis, resulting in lung explants with a decreased number of dilated distal lung buds [11]. In the present study, we investigated the biochemical properties of the lgl1 protein. We used transient transfection and Western blot analysis to demonstrate that lgl1 is secreted, and identified its N-terminal secretory signal peptide. We used dual immunofluorescence to identify its subcellular localization to the Golgi and ER (endoplasmic reticulum) membranes. Using pulse–chase experiments, we showed that lgl1 is a stable protein with a relatively long half-life. Furthermore, we demonstrated

Abbreviations used: CHO cell, Chinese-hamster ovary cell; β-COP, β-coatamer; CRISP, cysteine-rich secretory protein; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FBS, foetal bovine serum; HBSS, Hank's balanced salt solution; HEK-293T cell, human embryonic kidney 293T cell; LGL1, late gestation lung 1 (*LGL1* denotes the late gestation lung 1 gene; lgl1 denotes the late gestation lung 1 gene product); MEM, minimal essential medium; PNGase F, peptide N-glycosidase F.

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uptake by distal airway epithelial cells of lgl1 protein at gestational days 20 and 21, corresponding to the time point when LGL1 mRNA is maximal in foetal lung adjacent fibroblasts. Our findings, taken together with our earlier studies, support the hypothesis that lgl1 functions as a mediator of distinct mesenchymal–epithelial signalling interactions in early compared with late foetal lung organogenesis.

EXPERIMENTAL

Materials

Drugs and chemicals were obtained from the following sources: culture media [DMEM (Dulbecco's modified Eagle's medium), MEM (minimal essential medium), penicillin, streptomycin, agarose, TRIzol®, ethidium bromide, random hexanucleotide primers, Platinum *Taq* polymerase (high fidelity) and restriction endonucleases] were from Gibco BRL Life Technologies, Burlington, ON, Canada; PCR primers were from the Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada; and sequenase, deoxynucleotides, RNA Guard RNase inhibitor, Amplify™ Fluorographic reagent and [^{35}S]Cys/Met Promix were from Amersham Biosciences.

Methods

Construction of lgl1–FLAG and ΔN-lgl1–FLAG plasmids

Full-length LGL1 tagged with the FLAG epitope was prepared by reverse transcriptase-PCR, using d20 adjacent fibroblast cDNA as a template. To introduce an in-frame C-terminal FLAG into the LGL1 open reading frame, PCR amplification was performed using the forward primer 5′-CCGGAATTCATGAGCTGTCTT-CTGAACAATATG-3′ containing an *Eco*RI cut site before the ATG start codon and the reverse primer 5′-CCGGAATTCCTAC-TTGTCATCGTCGTCCTTGTAGTCCTGCCTGACGGGGAA-GATCCG-3′ containing an *Eco*RI cut site along with the FLAG sequence, and *LGL1* stop codon. The PCR fragments were amplified by high-fidelity Platinum *Taq* polymerase and were blunt-end cloned into pcDNA 3.0 vector (Invitrogen). This plasmid, referred to as lgl1–FLAG, was used as a template to generate the ΔN-lgl1–FLAG construct. lgl1–FLAG was digested with *Mlu*I and *Eco*RI to generate a 1277 bp fragment. This fragment was subsequently blunt-end cloned into pcDNA 3.0 vector. *Mlu*I digestion removed nt 1–220 of the LGL1 open reading frame. These nucleotides correspond to the putative signal peptide sequence of lgl1. The integrity of each plasmid was confirmed by sequence analysis.

Cell culture: foetal lung epithelial cells and adjacent fibroblasts

Isolation and primary culture of foetal rat distal lung epithelial cells or adjacent fibroblasts were prepared and maintained as previously described [11]. The term 'adjacent fibroblasts' refers to lung fibroblast cells, isolated in this manner, that are thought to originate in close proximity to the epithelial cells. Wistar rats of known gestational age (day 0 = mating; day 22 = term) were obtained from Charles River (St. Constant, Québec, Canada), and were killed with diethyl ether. The foetuses were aseptically removed from the uterus, foetal lungs were dissected out in cold HBSS (Hank's balanced salt solution) without calcium or magnesium, and were cleared of major airways and vessels. The lungs were rinsed twice in HBSS, minced and suspended in HBSS. The lung tissue was then digested in 0.125 % (w/v) trypsin and 0.4 mg of DNase at 37 °C for 20 min. After trypsin activity was neutralized with MEM containing 10 % (v/v) FBS (foetal bovine serum), the cells were filtered through a sterile mesh filter. The cell mixture was then centrifuged at 120 ***g*** for 3 min at 4 °C. The supernatant, containing peripheral fibroblasts, was discarded. The pellet fraction, containing a mixture of epithelial cells and adjacent fibroblast cells, was resuspended in 1 % (w/v) collagenase and 0.6 mg of DNase in MEM, and was incubated at 37 °C for 15 min. Cells were then centrifuged as above, resuspended in MEM containing 10 % (v/v) FBS and plated in T75 tissue culture flasks for 45 min at 37 °C to allow for differential adherence of the adjacent fibroblasts. Supernatants obtained from this adherence were pelleted as above, and the resulting cell pellet, containing epithelial cells, was resuspended in MEM containing 10 % (v/v) FBS and plated in T75 flasks overnight. Viability and purity of cultures were comparable with previously published data [18].

Cell transfections

HEK-293T (human epithelial kidney-293T) cells were maintained in DMEM containing 10 % (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified 5 % CO_2 in air. Cells were transfected at 50 % confluence using the calcium phosphate precipitation method with a total of 20 μg of DNA per 10 cm diameter dish and 4 μg of DNA per coverslip [23]. Equal amounts of DNA were used per construct. CHO (Chinese-hamster ovary) cells were grown in F12 nutrient mixture containing 10 % (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin and were transfected at 90 % confluence using LIPOFECTAMINE™ 2000 (Invitrogen) with a total of 14.4 μg of DNA per 10 cm diameter dish and 2.4 μg of DNA per coverslip. Cells cultured for 24 or 48 h were then used for immunofluoresence and/or Western blotting experiments.

Electrophoresis and Western blot immunoanalysis

At 48 h after transfection, HEK-293T cells grown in 10 cm diameter dishes were washed twice with cold PBS. Cells were scraped in 300 μl of lysis buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Triton X-100 and 1 % Complete™ protease inhibitor cocktail (Roche Diagnostics)] and incubated on ice for 15 min. After lysis, the supernatant was collected by centrifugation at 14 000 ***g*** at 4 °C for 20 min. Conditioned medium from the cells was centrifuged at 14 000 ***g*** at 4 °C for 5 min and the supernatant was filtered through a 0.22 μm syringe to remove cellular debris. The cleared medium with added protease inhibitors was then frozen in liquid nitrogen and was freeze-dried at − 50 °C overnight.

The total protein concentration of cell lysate or medium samples was determined according to Bradford [23a]. Proteins (50 μg) diluted with sample buffer was loaded in each well on a SDS/10 % (w/v) polyacrylamide gel and transferred on to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Transfer efficiency was determined by Ponceau staining. Membranes were blocked by incubation with 5 % (w/v) non-fat dried milk in PBS-Tx [PBS/0.05 % (v/v) Triton X-100] at room temperature (25 °C) for 1 h to prevent non-specific binding. The membrane was incubated with mouse anti-FLAG M2 antibody (1:10 000 dilution; Sigma) or rabbit anti-lgl1 antibody (1:500 dilution) at room temperature for 1 h, washed four times with PBS-Tx and then incubated with HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG (1:25 000) or goat anti-rabbit IgG (1:10 000) in PBS-Tx containing 3 % (w/v) non-fat dried milk. After 3 PBS-Tx washes, blots were developed with an

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ECL® (enhanced chemiluminescence) detection kit (Amersham Biosciences).

Treatment of lgl1 protein with glycosidases

HEK-293T cells were grown overnight in 10 cm diameter dishes and were transfected with the lgl1–FLAG construct as described above. After 48 h, cells were lysed in lysis buffer and lysates were cleared by centrifugation at 14 000 ***g*** for 20 min at 4 °C as described above. Protein concentration was determined according to Bradford [23a]. Lysate (20 μg) was boiled in sample buffer for 10 min. Samples were then treated or left untreated with 1000 units of either EndoH or PNGaseF (peptide N-glycosidase F) (New England BioLabs) at 37 °C for 1 h. Proteins were separated by SDS/PAGE and were then transferred on to a nitrocellulose membrane for Western blot immunoanalysis using the anti-FLAG antibody M2 (Sigma) as described above.

Localization of lgl1 in transfected cells via immunofluorescence staining

At 48 h post-transfection, HEK-293T cells and/or CHO cells grown on glass coverslips were fixed with 3.7 % (w/v) paraformaldehyde in PBS for 30 min (first 5 min on ice, thereafter at room temperature) and excess aldehyde groups were neutralized with 0.1 M glycine in PBS for 10 min. After permeabilization with 0.5 % (v/v) Triton X-100 in PBS for 5 min, cells were blocked with 5 % (v/v) normal donkey serum (Vector Laboratories, Burlington, CA, U.S.A.) in PBS for 1 h at room temperature, and then incubated for 1 h with the appropriate combination of mouse anti-FLAG M2 (1:1000 dilution), rabbit anti-lgl1 (1:100 dilution), rabbit anti-β-COP (coatamer protein; 1:500 dilution; Affinity Bioreagents, Golden, CO, U.S.A.), rabbit anti-calnexin (1:200 dilution; StressGen Biotechnologies Corp., Victoria, BC, Canada), or mouse anti-calnexin (1:100 dilution; Affinity Bioreagents) antibodies. After extensive washing in PBS, cells were incubated with the appropriate combination of Cy3-conjugated donkey anti-mouse or anti-rabbit (1:200 dilution; Jackson Immunoresearch Laboratories, Mississauga, ON, Canada) and FITC-conjugated donkey anti-mouse or anti-rabbit (1:100 dilution; Jackson Immunoresearch Laboratories) secondary antibodies. Cells were mounted with Dako mount (Dako, Carpinteria, CA, U.S.A.) before acquisition of confocal images.

Confocal image acquisition

Confocal images were acquired using a LSM 510 confocal system mounted on a Zeiss Axiovert 100 microscope. All images were acquired using 488 nm argon laser excitation and fluorescein emission filters. A 40×1.3 oil immersion objective was used to view the cells and the pinhole was set for 1–1.3 units.

Sequence analysis

The lgl1 signal peptide and putative cleavage site were predicted based on analysis of the sequence of the *LGL1* open reading frame, using Signal P computer software (http://www.cbs.dtu.dk/services/SignalP/index.html).

Pulse–chase experiments

To normalize the variation in transfection efficiency, HEK-293T cells transfected with the lgl1–FLAG plasmid were pooled after 24 h, reseeded in 10 cm diameter dishes and were cultured for an additional 24 h at 37 °C in humidified 5 % CO_2 air. Cells were then washed three times for 10 min with warm Cys/Met-deficient DMEM (Gibco/Life Technologies Inc.) and then incubated in the same medium supplemented with 10 % (v/v) FBS and [^{35}S]Cys/Met Promix. A 90 min labelling period (pulse) was followed by washing the cells three times with chasing medium [DMEM containing 10 % (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 20 mM unlabelled methionine and cysteine]. Cells were then chased for the indicated times, washed with PBS and lysed in buffer containing protease inhibitor cocktail. Samples were then centrifuged at 14 000 ***g*** for 15 min at 4 °C to remove insoluble materials and protein concentrations of the lysates were determined using the Bradford protein assay [23a]. Equal amounts of protein were incubated with anti-FLAG antibody M2–agarose beads at 4 °C for 1 h to immunoprecipitate lgl1–FLAG protein. Beads were washed as described above and resolved by reducing SDS/PAGE. Gels were fixed and then treated with Amplify Fluorographic Reagent, dried and exposed to X-ray film. The amount of labelled lgl1–FLAG was quantified using a PhosphorImager (Molecular Dynamics) with ImageQuant software (Molecular Dynamics). Calculation of the LGL1 half-life was based on the blot of log (percentage remaining) against chase time.

Preparation of conditioned medium from HEK-293T cells

HEK-293T cells were seeded at a density of (7–9) × 10^5 cells/10 cm diameter dish 18 h before transfection. Cells were transfected with the lgl1–FLAG construct using the calcium phosphate method as described above. After 48 h, conditioned medium from these cells (containing secreted lgl1–FLAG protein) was filtered for cellular debris and mixed 3:2 with fresh DMEM (Life Technologies) containing 10 % (v/v) FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml). After 48 h, medium from cells transfected with the pcDNA 3.0 vector alone, was filtered for cellular debris and mixed 3:2 as above. This medium is referred to as HEK-293T cell plasmid medium.

Epithelial uptake of lgl1-flag protein

Foetal rat distal lung epithelial cells were plated into a T75 flask and incubated in DMEM/10 % (v/v) FBS with antibiotics as above. At 90 % confluence, epithelial cells were serum-starved for 24 h before treatment with HEK-293T cell-conditioned medium (experimental group) or with HEK-293T cell plasmid medium (control group). After 24 h, cells were washed twice with ice-cold PBS and lysed in 500 μl of lysis buffer. Cell lysates were prepared and protein content was determined as described above. Anti-flag antibody M2–agarose beads (40 μl) were added to 1 mg of cell lysates, and incubated for 2 h at 4 °C. The agarose was pelleted by centrifugation at 6000 ***g*** for 10 s at 4 °C, washed twice with 1 ml of lysis buffer, and centrifuged again at 6000 ***g*** for 10 s at 4 °C. The pellets were then resuspended in 50 μl of sample buffer, boiled for 10 min, and subjected to SDS/PAGE and analysed by Western blot using the polyclonal anti-lgl1 antibody as described [11].

Statistical analysis

All data are presented as means ± S.E.M. Statistical significance was determined by two-way analysis of variance (ANOVA). Pair-wise group comparisons were then assessed using Student–Neuman–Keuls test. Significance was defined as $P < 0.05$.

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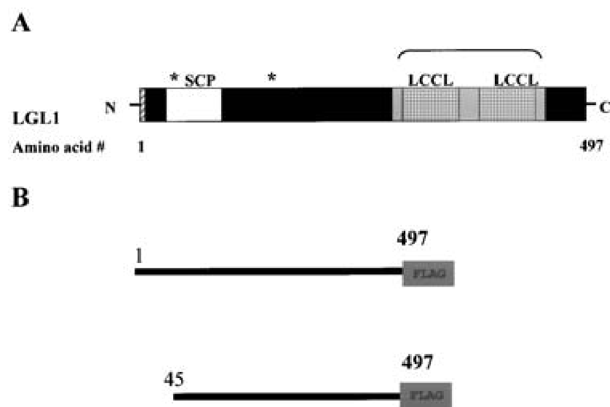

Figure 1 Lgl1 is a CRISP family member

(**A**) Lgl1 belongs to a family of CRISPs, characterized by an N-terminal secreted cysteine protein (SCP) domain (open box) of unknown function, a C-terminal cysteine-rich region (shaded box) and one or more LCCL (*Limulus* factor C, cochlear protein Coch-5b2 and Lgl1) domain(s) (hatched box). Using the Signal P program, a 22-amino-acid signal peptide region (diagonal lines) was identified at the N-terminal region of the lgl1 sequence, along with two putative N-glycosylation sites (asterisk). (**B**) Schematic representation of LGL1 plasmids used: wild-type lgl1–FLAG, and ΔN-lgl1–FLAG mutant.

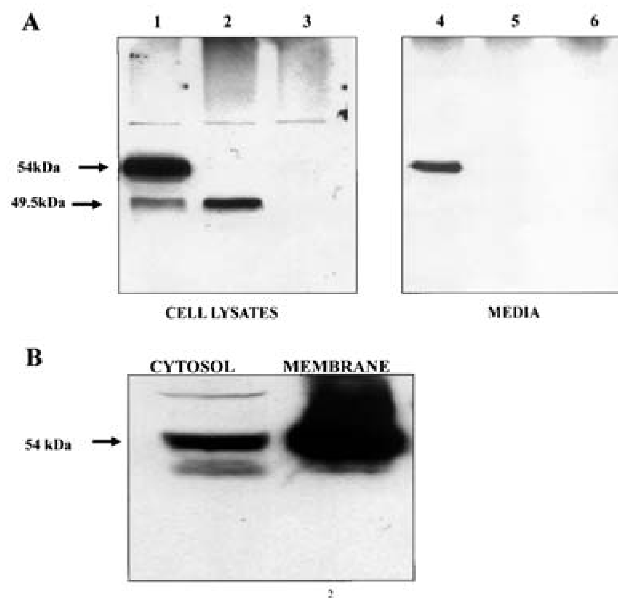

Figure 2 FLAG-tagged lgl1 protein is cytosolic and membrane-associated in HEK-293T cells

(**A**) Full-length lgl1 (lgl1–FLAG) (lanes 1 and 4), ΔN-lgl1–FLAG, lacking the N-terminal 134-amino-acid region of lgl1 (lanes 2 and 5) or pcDNA 3.0 vector alone (lanes 3 and 6) were expressed in HEK-293T cells. After ultrasonic disruption of cells, the cell lysates or the media were analysed by Western blot. The cell lysates (lanes 1–3) and filtered concentrated media (lanes 4–6) were analysed for the presence of the FLAG-tagged proteins with the anti-FLAG antibody M2. (**B**) Lgl1–FLAG protein was a greater proportion of total proteins from the membrane, rather than cytosolic, fraction of transfected HEK-293T cells. Equal amounts of proteins were loaded for Western blot analysis.

RESULTS

Secretion of lgl1 is directed by an N-terminal signal peptide

LGL1 encodes a 497-amino-acid polypeptide with a predicted molecular mass of 52 kDa and significant homology with members of the CRISP family of proteins. CRISPs contain hydrophobic amino acid clusters at the N-terminus and a cysteine-rich region at the C-terminal half, from which their family name is derived (Figure 1A). Analysis using the Signal P software predicted that lgl1 has a signal peptide and identified two potential consensus sites for N-linked glycosylation.

In order to determine whether human cells secrete lgl1, we transfected HEK-293T cells with C-terminally FLAG-tagged expression vectors encoding either full-length, lgl1–FLAG, or N-terminal truncated ΔN-lgl1–FLAG (amino acids 45–497) (Figure 1B). ΔN-lgl1–FLAG was truncated downstream of the predicted signal peptide and extended from nucleotides 220 to 1570. After 48 h in culture, cell lysates and medium from transfected cells were analysed on a Western blot using anti-FLAG antibody M2. The pcDNA 3.0 vector, used as a negative control, showed no protein band as expected (Figure 2A, lanes 3 and 6). Consistent with a requirement for the signal peptide for cellular secretion of lgl1, the lgl1–FLAG protein was detected in the cell lysate (Figure 2A, lane 1) and also in the medium (Figure 2A, lane 4), whereas ΔN-lgl1–FLAG was detected in the cell lysate (Figure 2A, lane 2), but was not present in the medium (Figure 2A, lane 5).

Intracellular lgl1 is cytoplasmic and localizes to Golgi and ER structures

In order to determine the subcellular localization of lgl1 in living cells, we generated a recombinant fusion of the LGL1 cDNA with EGFP (enhanced green fluorescent protein). EGFP–lgl1 was transfected into day 20 foetal lung adjacent fibroblasts (adjacent to the epithelium). Following 48 h in culture, the EGFP–lgl1 fusion protein was detected in the cytoplasm by confocal microscopy (Figure 3A). No EGFP–lgl1 was detected in the nucleus (Figures 3A and 3C). In addition, cytoplasmic staining appeared punctate, consistent with localization of lgl1 in secretory vesicles.

To confirm these findings, we also localized lgl1 by immunofluorescence analysis following transient expression of lgl1–FLAG in HEK-293T cells. After 48 h, expression of the lgl1 protein was assessed by fixation of cells in paraformaldehyde and labelling with the monoclonal anti-FLAG antibody M2. Again, lgl1–FLAG staining was cytoplasmic and punctate (Figure 4A), consistent with association of lgl1 with the Golgi apparatus and ER.

We next used double immunofluorescence staining to further define localization of the punctate lgl1 staining seen within the cells. A polyclonal antibody against calnexin, a well-established marker for the ER, or a polyclonal antibody against β-COP, a coatamer protein responsible for regulating transport between the Golgi and ER were used in conjunction with the monoclonal anti-FLAG antibody M2. A close co-localization for lgl1-FLAG and calnexin or β-COP could be seen in human fibroblast cell lines, HEK-293T and CHO cells (Figures 4A and 4B).

Intracellular maturation of lgl1 protein involves N-linked glycosylation

We identified two putative N-glycosylation sites in the primary sequence of lgl1, using the Signal P software. In order to determine whether or not lgl1 is glycosylated at these sites, intracellular and secreted lgl1 were digested with EndoH and PNGaseF glycosidases. EndoH resistance indicates a mature, complex glycosylated protein, whereas PNGaseF susceptibility indicates an N-linked core glycosylated protein, at the earliest stages of protein transport through the Golgi. Transient expression of

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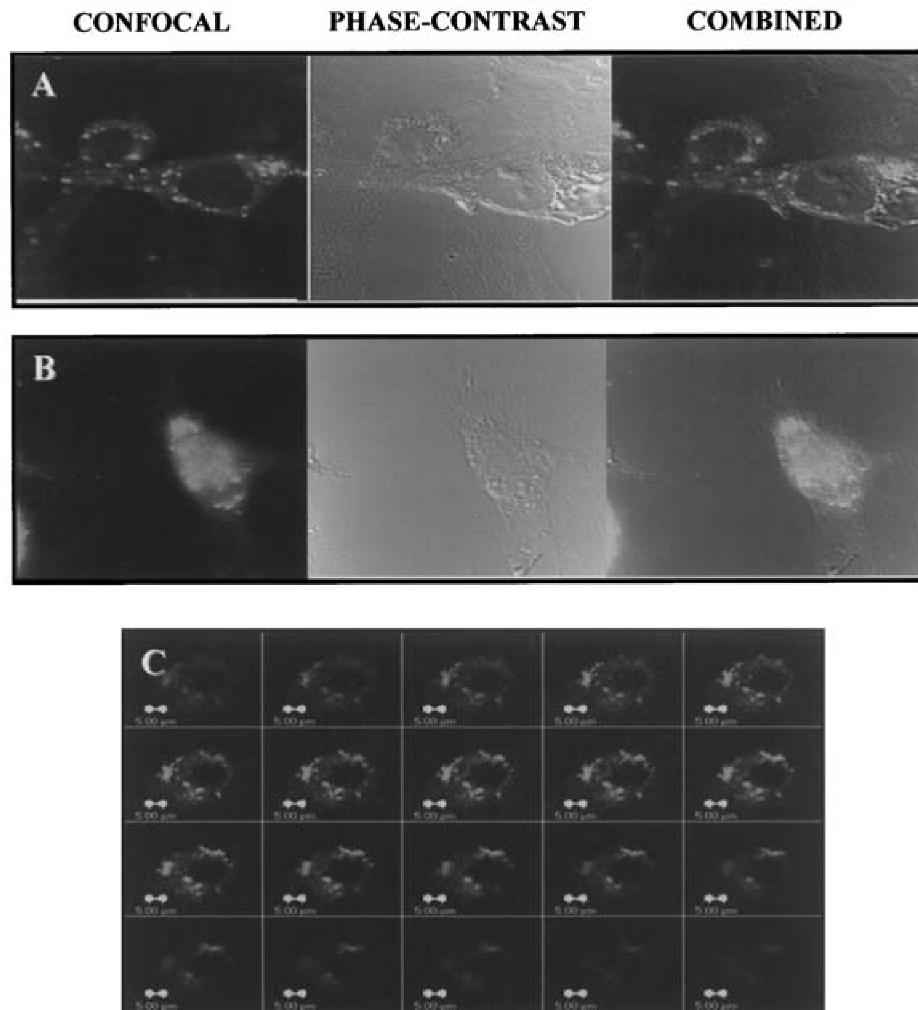

Figure 3 Lgl1 protein is cytosolic in foetal rat lung fibroblasts

Adjacent fibroblasts from day 20 foetal rat lungs were transfected with plasmid pLGL1-EGFP-N3 or pEGFP-N3 alone and fixed with 4 % (w/v) paraformaldehyde after 48 h overexpression. (**A**) Expressed lgl1 protein is localized to the cytoplasm. (**B**) EGFP alone is found throughout the cell, suggesting the cytoplasmic specificity is of lgl1 origin. (**C**) No lgl1 was detectable in the nucleus upon serial sections through the cell.

lgl1–FLAG in HEK-293T cells followed by immunoprecipitation and Western blot analysis identified a major 54 kDa band and a minor 49.5 kDa band. When the intracellular pool of lgl1 was treated with EndoH and/or PNGaseF, the major 54 kDa band disappeared and only the 49.5 kDa band was visible on a Western blot, suggesting the 54 kDa band is derived from the 49.5 kDa precursor by N-linked glycosylation (Figure 5). Interestingly, no EndoH-resistant pool of lgl1 was detected in conditioned medium from these transfected cells, suggesting that secreted lgl1 is core glycosylated, but does not undergo complex glycosylation in the Golgi (results not shown).

Lgl1 is a stable protein

We previously reported that disruption of LGL1 mRNA by antisense oligodeoxynucleotides resulted in only a 50 % decrease in lgl1 protein content in cultured foetal rat lung explants [11]. To test the possibility that this may reflect slow turnover of a stable extracellular protein, we performed pulse–chase experiments on lgl1–FLAG transiently transfected HEK-293T cells. Transfected lgl1 was pulsed with [^{35}S]Cys/Met for 90 min and chased for 0–24 h (Figure 6). Lgl1 expressed in HEK-293T cells has a half-life of 11.5 h.

Lgl1 is taken up from medium by late gestation foetal distal lung epithelial cells in primary culture

No LGL1 mRNA or lgl1 protein was detected in lung epithelium in the early embryonic and late gestation lung [11,18]. Furthermore, we have shown that LGL1 mRNA expression was maximal in adjacent fibroblasts during the saccular stage of lung development, corresponding to foetal day 20 to 22 in the rat [18]. In order to determine whether or not secreted lgl1 is taken up by distal lung epithelial cells in late gestation, we exposed foetal rat distal lung airway epithelial cells to conditioned medium from lgl1–FLAG transfected mesenchymal HEK-293T cells. We showed uptake of lgl1–FLAG protein by foetal lung gestational days 20 and 21 epithelial cells (Figure 7A). No lgl1 protein was detected in negative control epithelial cells exposed to conditioned medium from HEK-293T cells transfected with plasmid vector only (no

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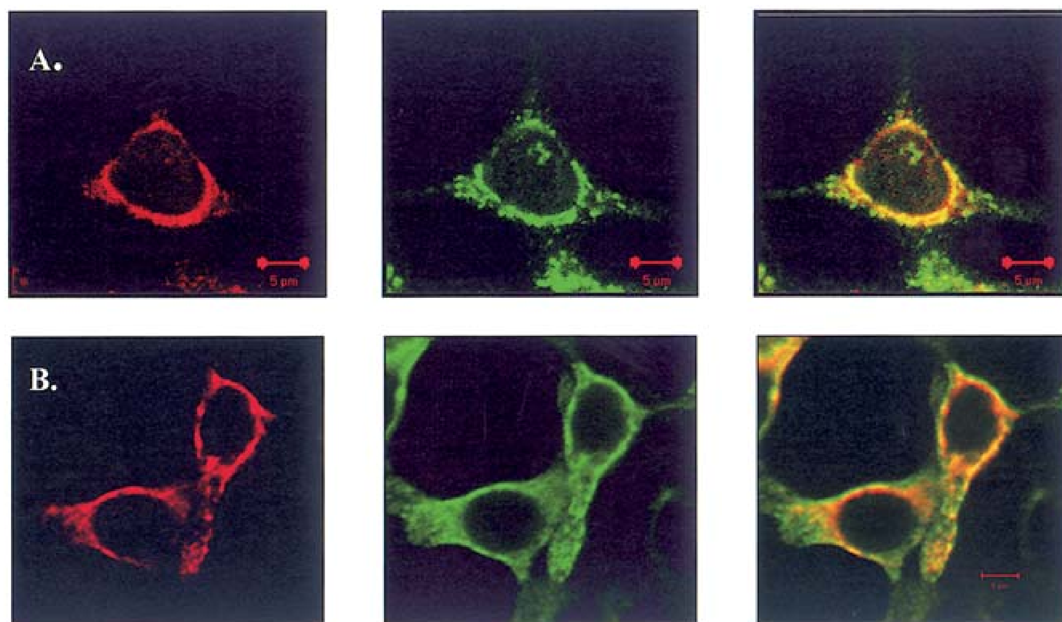

Figure 4 Lgl1 protein co-localizes with the ER and Golgi apparatus

HEK-293T and/or CHO cells were transiently transfected with plasmid lgl1–FLAG and were fixed with 3.7 % (w/v) paraformaldehyde after 48 h overexpression. The localization of lgl1–FLAG was examined by indirect immunofluorescence, and images were collected by confocal microscopy. The images in the first column display the staining for lgl1 expression (red). The images in the second column are the subcellular markers in the same field (green). The images in the third column are the merged images for lgl1 expression and subcellular marker co-localization (yellow). (**A**) Lgl1 co-localizes with η-coat protein, a Golgi marker and (**B**) calnexin, an ER marker. Untransfected cells showed no staining and transfected cells without primary antibody also demonstrated no signal (results not shown).

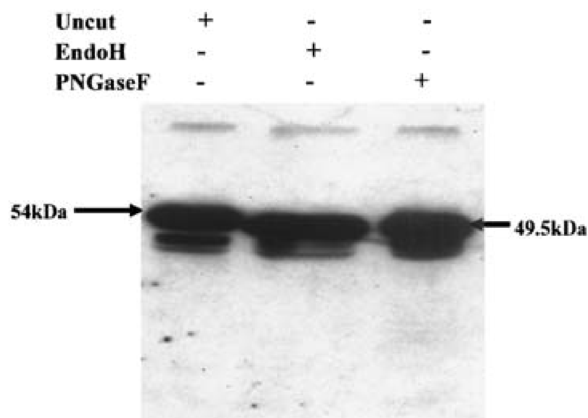

Figure 5 Lgl1 is a glycosylated protein

Equal concentrations of lgl1–FLAG-transfected HEK-293T cell lysates and/or concentrated media were treated with either EndoH or PNGaseF for 1 h at 37 °C. The protein samples were boiled in SDS sample buffer and were separated by SDS/PAGE. Expression of lgl1 was detected using monoclonal anti-FLAG antibody M2.

lgl1–FLAG construct). Neither day 20 nor day 21 epithelial cells expressed detectable LGL1 mRNA by Northern blot analysis (Figure 7B).

DISCUSSION

Consistent with the postulated role of lgl1 as a mesenchymal modulator of lung epithelial morphogenesis, we provide the first empirical evidence that lgl1 is a secreted protein. Expression plasmids encoding lgl1 were labelled with the FLAG epitope and transfected into HEK-293T cells. The resulting fusion protein was detected in the filtered, cell-free conditioned medium of the transfected HEK-293T cells after 48 h of incubation. Furthermore, immunofluorescent staining for the lgl1–FLAG fusion protein showed localization in a punctate cytoplasmic pattern, as would be expected if lgl1 were to be stored in secretory vesicles. Glycosylation occurs in the ER–Golgi complex, organelles dedicated to the production of cell-surface and secreted materials. We used dual immunofluorescence microscopy to clearly demonstrate co-localization of lgl1 protein with either calnexin (a resident ER protein) or β-COP (a Golgi protein), suggesting that lgl1 protein is processed through the Golgi apparatus and ER before secretion.

Given that most secreted proteins undergo post-translational glycosylation in the Golgi and ER, and given that the amino acid sequence of lgl1 has two putative N-glycosylation sites, we next examined whether or not lgl1 protein is in fact glycosylated. Cell lysates from HEK-293T cells transfected with the expression plasmid encoding lgl1–FLAG were digested with PNGaseF and EndoH, two common endoglycosidases. The higher-molecular-mass form of lgl1 (approx. 54 kDa) shifted to a 49.5 kDa form, indicating that native lgl1 is indeed glycosylated. Similarly, glycosidase treatment of HEK-293T conditioned media caused an analogous shift, but no EndoH-resistant pool was detected, indicating that fully processed lgl1 is not complex glycosylated. Taken together, these findings indicate that lgl1 is a secreted glycoprotein.

Our previous observation that disruption of LGL1 by antisense oligodeoxynucleotides in foetal lung explants resulted in only a

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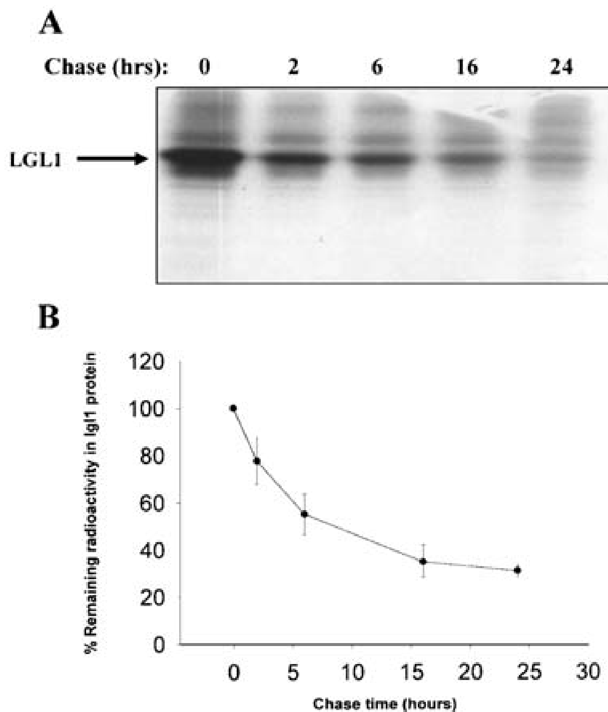

Figure 6 Lgl1 protein has a half-life of about 11.5 h

(**A**) HEK-293T cells were transfected with lgl1–FLAG and after 48 h, cells were metabolically labelled with [^{35}S]Cys/Met and chased with unlabelled Cys/Met for the indicated times. Cell lysates were immunoprecipitated with anti-FLAG antibody M2–agarose beads, which were subsequently washed, resolved by SDS/PAGE, dried and exposed to X-ray film. A representative experiment of three is shown. (**B**) Quantification of pulse–chase experiments. The amount of [^{35}S]lgl1 was determined by phosphorImage analysis and expressed as a percentage of the initial incorporation at zero time (results are means ± S.E.M.; $n = 3$). Calculation of the lgl1 half-life was based on the plot of log (percentage remaining radioactivity) against chase time.

50 % decrease of lgl1 protein levels [11] led us to ask whether lgl1 protein might have a relatively long half-life. To address this question, we performed pulse–chase experiments using HEK-293T cells transiently transfected with lgl1–FLAG. The half-life of lgl1 protein is 11.5 h. This finding could account for the modest decrease in lgl1 protein levels in response to antisense treatment. Moreover, enhanced lgl1 protein stability suggests the function of the protein is both of high biological significance and consistently required throughout a relatively prolonged period of lung development.

Mesenchyme–epithelial interactions provide specific signals critical to epithelial branching and cyto-differentiation [6,7, 24–27]. In classical tissue recombination experiments [6–8,26], grafted distal lung mesenchyme induces denuded tracheal epithelium to branch and reprogrammes tracheal epithelium to express an alveolar type II cell phenotype [7,8]. Conversely, grafting tracheal mesenchyme to denuded distal epithelium prevents branching and induces the expression of ciliated and mucus-producing cells [26]. *In vitro*, trans-filter grafting experiments have provided evidence that distal lung mesenchyme induction of epithelial branching and cytodifferentiation are mediated by a combination of soluble factors, matrix proteins and cell-adhesion molecules [1,3,5,24,27–29]. More recently, the critical role of secreted signalling molecules in regulating the cell–cell and cell–matrix interactions that underlie the branching process has received increased attention [3,28,30–32]. Torday et al. [33] reported the secretion by fibroblasts of leptin, the presence of leptin receptors on the epithelial cell surface, and the augmentation of epithelial surfactant phospholipid and surfactant protein-B synthesis in response to specific ligand binding to these receptors. Although many reports provide convincing evidence for mesenchymal–epithelial interactions, few demonstrations are available of foetal lung mesenchymal synthesis and secretion of a protein that is in turn taken up by epithelial cells that do not express the corresponding mRNA. Our present findings suggest that lgl1 can now be added to the list of mediators of mesenchymal–epithelial interactions in the regulation of lung development. Moreover, our finding of incorporation of lgl1 into the epithelial cell at a developmental stage during which LGL1 mRNA is not detectable, suggests lgl1 protein may act directly from within the target cell itself.

Many key mediators of lung morphogenesis are expressed throughout antenatal lung development, and some persist to adulthood, suggesting that they may also be critical for late lung development and alveogenesis. A given molecule may serve different roles during early compared with late lung development. Examples include the FGF (fibroblast growth factor) pathway, which is involved in both early lung morphogenesis [28] and in later alveogenesis [10], and PDGF-A (platelet-derived growth factor-A), which is implicated in both early lung morphogenesis and alveolar formation [9]. To this list, we propose to add *LGL1*, which we have previously shown is expressed from the earliest stages of lung development [18] and implicated in branching morphogenesis [11]. Maximal foetal *LGL1* expression occurs in late gestation (foetal days 20 and 21 in the rat), when branching of the conducting airways is complete and active alveolarization is beginning. At this time, mesenchymal lgl1 protein is restricted to actin-positive cells (adjacent to airway epithelium) [11] that regulate formation of new alveolar units. Our present evidence for lgl1 protein uptake by late gestation lung epithelial cells provides further support for a distinct role for lgl1 at the time of its maximal expression in late gestation lung.

From its deduced amino acid sequence, we previously identified lgl1 as a member of the CRISP family [18], so-named because of their unique domain structures. They all have a cysteine-rich region at the C-terminal, consisting of six conserved cysteine residues that form a characteristic pattern of disulphide bonds, a SCP (secreted cysteine protein) domain and a signal peptide domain thought to be critical to the secretory process [19,20]. Extracellular glycoproteins are a common means of adhesion and immune recognition between multicellular organisms and parasitic or symbiotic organisms. In this context, it is of interest that a number of CRISP family members are pathogenesis-related proteins [20].

In summary, we provide evidence that lgl1 is a secreted glycoprotein with an 11.5 h half-life. Intracellularly, it maturates to become a core N-linked glycosylated protein. Lgl1 passes through the cytoplasmic compartment of the cell via the Golgi apparatus and ER on its way to being secreted in a process dependent upon a 22-amino-acid N-terminal signal peptide. Foetal rat distal lung epithelial cells of late gestation do not express detectable LGL1 mRNA, but do take up lgl1 protein from culture medium. Our findings are consistent with a role for lgl1 in mesenchymal–epithelial interactions in late gestation, distinct from its role in early airway branching morphogenesis. The molecular mechanisms by which lgl1 protein acts once it has been secreted from the cell remain unclear at present. A precise molecular analysis of the function of lgl1 will require identification of upstream and/or downstream factors interacting with the lgl1 gene product. This will not only provide important clues about the role of lgl1 protein in mesenchymal–epithelial

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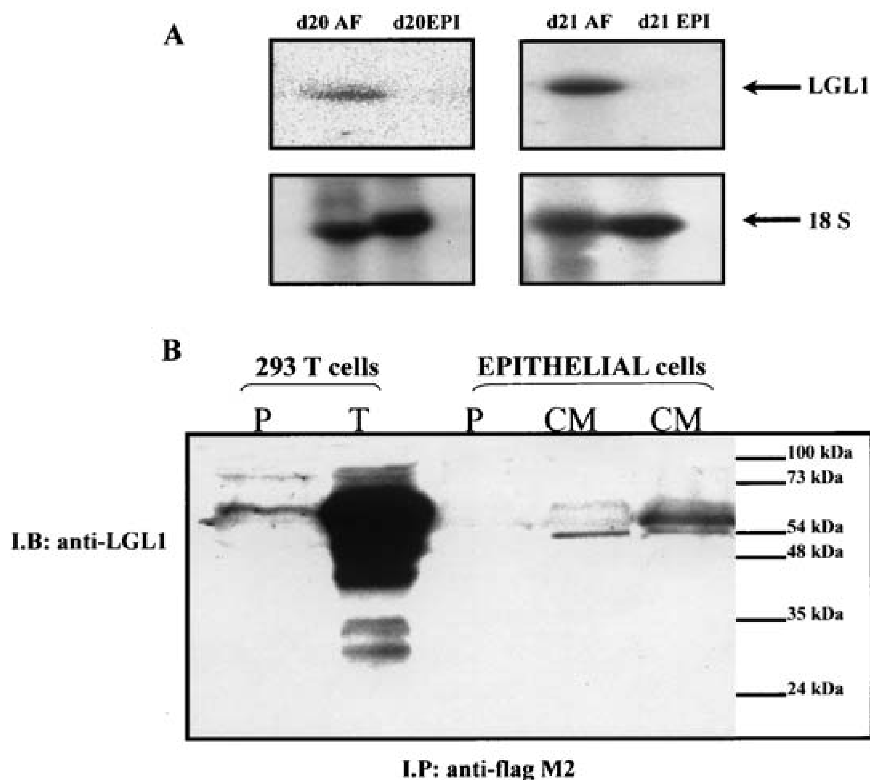

Figure 7 Foetal day 20 and 21 distal lung epithelial cells take up lgl1 protein secreted from mesenchymal cells

(**A**) Foetal rat lung adjacent fibroblasts (AF) but not epithelial cells (EPI) of gestational days 20 or 21 express LGL1 mRNA detectable by Northern blot. Levels of 18 S rRNA were used to control for RNA loading and transfer. (**B**) Immunoblots (I.B.) were performed using anti-lgl1 antibody. Lgl1–FLAG protein is present in d20 and d21 epithelial cells cultured for 24 h in conditioned media (CM) from HEK-293T cells transiently transfected with lgl1–FLAG (T), but not in control cells exposed to medium from HEK-293T cells transiently transfected with the plasmid vector pcDNA3.0 alone (P). Lgl1–FLAG protein is present in HEK–293T cells transfected with lgl1–FLAG (T), but not in cells transfected with the plasmid vector alone (P). See the Experimental section for details.

signalling during foetal lung development, but will also contribute to a more comprehensive understanding of the mechanisms regulating lung development.

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