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A novel inflammation-induced ubiquitin E3 ligase in alveolar type II cells

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Abstract

LINCR was identified as a glucocorticoid-attenuated response gene induced in the lung during endotoxemia. The LINCR protein has structural similarities to *Drosophila* Neuralized, which regulates the developmentally important Notch signaling pathway. Endotoxemia-induced LINCR expression in vivo was localized by in situ hybridization to alveolar epithelial type II cells, and shown to be induced by LPS and inflammatory cytokines in the T7 alveolar epithelial type II cell line. RING domain-dependent ubiquitin E3 ligase activity of LINCR was demonstrated using full-length FLAG-LINCR or a deletion mutant lacking the RING domain expressed in 293T cells, and using a GST-LINCR RING fusion protein expressed in *Escherichia coli*. LINCR preferentially interacted with the ubiquitin-conjugating enzyme UbcH6 and preferentially generated polyubiquitin chains linked via non-canonical lysine residues. We conclude that LINCR is a novel inflammation-induced ubiquitin E3 ligase expressed in alveolar epithelial type II cells, and discuss its potential role in the lung response to inflammation.

Keywords: Alveolar epithelial cells; Bronchopulmonary dysplasia; Inflammation; Lipopolysaccharide; Mouse models; Notch pathway; Ubiquitination

Inflammation is thought to be major contributor to lung injury in bronchopulmonary dysplasia (BPD) in neonates [1,2], as well as in the adult respiratory distress syndrome, chronic obstructive pulmonary disease, asthma, cystic fibrosis, and other diseases in older children and adults. A prominent feature of BPD in the extremely premature infant is an arrest in the normal process of alveolization. However, the mechanisms by which inflammatory pathways may affect normal lung development in these infants, or lung repair and remodeling in response to injury in older individuals, are far from completely understood [2]. The gaps in our knowledge are emphasized by the observation in functional genomic screens of large numbers of inflammation-regulated genes whose roles in lung inflammation are unknown.

In a previous report, we described a functional genomic screening project designed to identify genes whose induction in the lung during endotoxemia is attenuated by glucocorticoids [3,4]. A major part of the anti-inflammatory actions of glucocorticoids is attributable to their ability to attenuate the induction of genes encoding a variety of mediators important in inflammatory and immune responses, including cyclooxygenase-2, the inducible form of nitric oxide synthase, and numerous inflammatory cytokines and chemokines. We refer to genes with these regulatory characteristics as glucocorticoid-attenuated response genes or GARGs. By targeting the screening to the glucocorticoid-attenuated subset of

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LPS-induced genes, we hoped to focus on genes potentially important in glucocorticoid-sensitive lung inflammatory conditions such as BPD. The screening identified 36 GARG cDNAs whose induction in the lung during endotoxemia and attenuation by dexamethasone was confirmed by Northern blotting. Although genes encoding known inflammatory mediators such as cytokines, chemokines, and adhesion molecules were identified, the majority were genes whose roles in the lung response to inflammation have not been investigated. One of these GARG cDNAs encodes a novel protein designated lung-inducible Neuralized-related C3HC4 RING domain protein (LINCR, pronounced "linker"; GenBank Accession No. AF321278).

Two sets of observations led us to pursue further studies of LINCR. First, its expression characteristics strongly suggest a role for LINCR in the lung response to inflammation [3]. LINCR message is strongly and rapidly induced in the lung during endotoxemia, and endotoxemia-induced LINCR expression is attenuated about 50% by pretreatment with dexamethasone. The basal expression of LINCR in adrenalectomized mice is 3-fold higher than in normal or sham-operated mice, indicating that basal lung expression of LINCR is sensitive to the levels of glucocorticoids normally circulating in unstressed mice. In addition, LINCR has a relatively restricted expression pattern, with strongest expression in the lung. LINCR message is also induced in the kidney, but is minimally or not induced in liver, spleen, skeletal muscle, and brain. In contrast, typical inflammatory chemokines and cytokines are strongly induced in a wide variety of cell types and tissues during endotoxemia [5,6].

Second, LINCR is closely related to Neuralized, a modulator of the developmentally important Notch signaling pathway. *Drosophila* Neuralized and its homologs in other species are large proteins (555–754 amino acid residues) that contain two copies of a distinctive 153–156 residue region known as the Neur Repeat (NR) domain plus a C-terminal RING domain [7–9]. LINCR is similar to Neuralized in that it contains an NR domain and a C-terminal RING domain similar to the RING domain of Neuralized [3]. It differs from Neuralized by being much smaller (254 amino acid residues) and having only a single NR domain. Recent studies in *Drosophila* and *Xenopus* have demonstrated that Neuralized is a ubiquitin E3 ligase that modulates Notch signaling by ubiquitinating the Notch ligand Delta [8,10,11]. The Notch pathway is a complex, evolutionarily conserved, and highly regulated signaling system that influences cell differentiation, proliferation, and apoptosis at all stages of development [11,12]. Like *Drosophila* and *Xenopus*, mice and humans have Neuralized homologs containing two NR domains and a C-terminal ring [7,9,13,14]. LINCR, the product of a distinct gene, may have evolved from an ancestral Neuralized gene by deletion of the second NR domain [3].

Ubiquitin (Ub) is a 76 amino acid residue, 8.6 kDa protein, ubiquitously expressed and highly conserved in eukaryotes. The post-translational modification of proteins by covalent conjugation with ubiquitin is a highly regulated cellular process that is essential for numerous cellular functions including cell-cycle progression, cell proliferation and differentiation, signal transduction, transcriptional regulation, receptor down-regulation, apoptosis, and endocytosis [15,16]. Ubiquitination begins with the formation of a thiol-ester linkage between the C-terminal glycine of Ub and the active site cysteine of a ubiqitin-activating enzyme (Uba) or enzyme 1 (E1). This is followed by transfer of Ub to a ubiquitin-conjugating enzyme (Ubc or E2), again via a thiol-ester linkage. Finally, a ubiquitin protein ligase (E3) catalyzes the transfer of Ub from the E2 enzyme to the ε-amino group of a lysine residue on the protein substrate. A polyUb chain may then be elaborated on the protein through the sequential ligation of additional Ub monomers. The E3 ligase determines the timing and substrate specificity of these reactions, and is therefore the key regulatory determinant of ubiquitination, playing a role analogous to that of a kinase in phosphorylation. Proteins tagged with a polyubiquitin chain linked via the canonical lysine 48 of ubiquitin are targeted for rapid degradation by the proteasome. In contrast, polyUb chains linked via other Ub lysine residues, or attachment of a single Ub (mono-ubiquitination), can serve as a signal that regulates the protein activity by a variety of other mechanisms [17,18].

The goals of the present study were to determine the lung cell type(s) in which LINCR is induced during endotoxemia, to establish a system for studying the regulation of LINCR in cell culture, and to test the hypothesis that LINCR, like Neuralized, has ubiquitin E3 ligase activity.

Methods

Materials. Sterile, tissue-culture certified LPS prepared by phenol extraction and gel filtration from *Escherichia coli* serotype O111:B4 was obtained from Sigma (St. Louis, MO). Preservative and pyrogen-free saline (Abbot Laboratories, North Chicago, IL) were used for dilution of LPS and for control injections. Dexamethasone was from Elkins-Sinn (Cherry Hill, NJ). Cell-culture media were obtained from Media Tech (Herndon, Virginia), and fetal calf serum was from Omega Scientific (Tarzana, CA). Cycloheximide, insulin, hydrocortisone, amphotericin B, and tri-iodothyronine were from Sigma. Endothelial cell growth supplement and epidermal growth factor were from BD Biosciences (San Diego, CA). Endothelin-1 was from Bachem (Torrance, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). T7 and SP6 RNA polymerases, proteinase K, and RNase A were from Ambion (Austin, Texas). Rabbit antiserum to pro-surfactant protein C was a gift from Dr. Jeffrey A. Whitsett. Avidin/biotin blocking solution, normal goat serum, biotinylated goat anti-rabbit antibody, streptavidin peroxidase conjugate, and liquid DAB substrate kit were purchased from Zymed Laboratory (S. San Francisco, CA).

Ubiquitin, ubiquitin mutants, and E1 and E2 enzymes were purchased from Boston Biochemical (Cambridge, MA).

Animals. Swiss Webster mice, purchased from Charles River Laboratories (Cambridge, MA), were housed under specific pathogen-free conditions in the UCLA Center for the Health Sciences vivarium. Experiments were conducted in accordance with a protocol approved by the UCLA Animal Research Committee. Lung tissue for in situ hybridization and immunohistochemistry was collected from 4-week-old female mice. Mice were euthanized via halothane inhalation 1 h after the injection of 50 μg LPS in 200 μl saline, or saline alone, via the tail vein. The chest was opened, and the lungs were inflated via a tracheal cannula with 4% paraformaldehyde in DEPC-treated PBS, pH 7.4. After ligation of the trachea, the inflated lungs were removed en bloc, immersed in the fixative at 4 °C for 4 h, and then in DEPC-treated PBS containing 15% sucrose at 4 °C for 20 h. The lungs were then embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Torrance, CA) and frozen at −80 °C. Cryosections (10 μm) were collected on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburg, PA) and stored at −80 °C.

In situ hybridization and immunohistochemistry. The full-length murine LINCR cDNA contains repetitive sequences in the distal portion of the 3′ untranslated region. To construct a plasmid suitable for synthesis of LINCR-specific riboprobes, LINCR clone 024.15 [3] was digested with *Eco*RI and *Pst*I. The 1.6-kb fragment, containing an *Eco*RI–*Sal*I–*Not*I 5′ adapter plus nucleotides 4-1642 of GenBank Accession No. AF321278, was cloned into the *Eco*RI and *Pst*I sites of pGEM4 to generate pGEM4-LINCR. For the synthesis of antisense probe, this plasmid was digested with *Eco*RI and purified by phenol–chloroform extraction. Digoxigenin-labeled antisense probes were synthesized by in vitro transcription using T7 RNA polymerase and digoxigenin-11-uridine-5′-triphosphate (Roche Diagnostics GmbH, Mannheim, Germany). For the sense probe, pGEM4-LINCR was linearized with *Hin*dIII, and in vitro transcription was performed using SP6 RNA polymerase. To reduce the probe fragment size to an average size of about 200 nt, each transcription product was combined with an equal volume of 120 mM sodium carbonate and 80 mM sodium bicarbonate, and incubated for 39 min at 60 °C. The hydrolyzed probes were precipitated by the addition of 5 M ammonium acetate and ethanol, resuspended in formamide, and stored at −80 °C.

In preparation for in situ hybridization, frozen sections were postfixed with 4% paraformaldehyde for 15 min, rinsed in PBS, digested with 5 μg/ml proteinase K at room temperature for 15 min, and then incubated with 4% paraformaldehyde for 5 min. After rinsing with PBS, the sections were acetylated by incubation with 0.1 M triethanolamine containing 0.25% (v/v) acetic anhydride (Sigma, St. Louis, MO) for 10 min.

The riboprobes were diluted to a final concentration of 400 ng/ml in hybridization buffer, heated at 65 °C for 5 min, and incubated with the tissue sections for 16 h at 50 °C. Sense controls were included with each hybridization and were handled identically to the corresponding antisense samples. The hybridization buffer was 50% formamide with 1× Denhardt's solution, 10% dextran sulfate, 3× salt-sodium citrate (SSC; 1× SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.4), 10 mM EDTA, 500 μg/ml yeast transfer RNA, and 500 μg/ml heat-denatured salmon sperm DNA. After hybridization, the sections were washed first with 5× SSC for 5 min at 53 °C and then with 2× SSC/50% formamide for 30 min at 53 °C. To degrade unbound riboprobe, the sections were incubated with 20 μg/ml RNase A at 37 °C for 30 min and then washed twice in 1× SSC/50% formamide for 30 min at 53 °C.

Localization of hybridized probe was determined using the Nucleic Acid Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, slides were incubated with 1% blocking reagent for 30 min at room temperature and then with a 1:4500 dilution of alkaline phosphatase-labeled anti-digoxigenin Fab fragment overnight at 4 °C. After washing, the slides were incubated with a substrate solution containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate for 3–7 h and mounted using Permount (Fisher Scientific, Fair Lawn, NJ).

For immunohistochemical analysis, sections were postfixed with 4% paraformaldehyde for 10 min, rinsed in PBS, and then exposed to 3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase. The sections were incubated with avidin/biotin blocking solution for 10 min and then with 10% normal goat serum for 1 h at room temperature. The blocked sections were incubated overnight at 4 °C with a 1:1000 dilution of the pro-SPC antiserum in PBS with 2% Triton X-100. After washing, the sections were incubated for 30 min at room temperature with a 1:1000 dilution of biotinylated goat anti-rabbit antibody and then with streptavidin peroxidase conjugate for 10 min. The presence of peroxidase was revealed using the liquid DAB substrate kit.

LINCR antibodies. To generate anti-LINCR antibodies, peptides HLDDQRSTARRRSTFHD(C) and (C)EEVAVVSSLKAEEGS matching LINCR residues 30–46 and 240–254, respectively, were synthesized and conjugated to keyhole limpet hemocyanin. The parenthesized cysteines, not present in the LINCR sequence, were added to facilitate conjugation. Rabbits were immunized with a mixture of the two conjugates. Peptide synthesis and antibody production were performed by Alpha Diagnostic International, San Antonio, TX.

Cell culture. T7 cells were propagated and grown to confluence in 10% CO_2/air at 33 °C in permissive medium [19], consisting of DMEM with 2% FCS, penicillin 100 U/ml, streptomycin 1%, 2 mM L-glutamine, amphotericin B 250 μg/ml, IFN-γ 100 U/ml, transferrin 5 μg/ml, epidermal growth factor 25 ng/ml, insulin 10 μg/ml, endothelial cell growth supplement 7.5 μg/ml, endothelin-1 40 nmol/ml, tri-iodothyronine 20 ng/ml, and hydrocortisone 0.36 μg/ml. The cells were then split and incubated in non-permissive medium (lacking IFN-γ) at 39 °C in 10% CO_2/air for 48 h before treatment with LPS or cytokines. After specified intervals, the cells were harvested for RNA extraction.

293T cells were grown in DMEM high glucose containing 10% fetal bovine serum, glutamine, pyruvate, penicillin, and streptomycin. Cells were plated 1 day before transfection at 40–60% confluence in 10 cm plates and transfected with 20 μg DNA per plate for 4 h using the calcium phosphate precipitate method.

Ribonuclease protection assay. RNA was extracted from cell lysates using the acid phenol method and resuspended in formamide. Message expression of LINCR was evaluated by ribonuclease protection assay (RPA), as described [20]. The template for LINCR probe synthesis was constructed using primers GGTGAATTCtggacgatcagcggagc and CGAGCAAGCTTcacccagaagcagaccacat to amplify the 290 nt LINCR cDNA segment corresponding to nucleotides 152–441 of GenBank AF321278. The portions of the primers that match the LINCR sequence are given in lowercase. The LINCR segment was then cloned into the *Eco*RI and *Hin*dIII sites (underlined in the primer sequences) of pGEM-4Z (Promega, Madison, WI). The plasmid used to generate antisense probe for ribosomal protein S2 mRNA (GenBank Accession No. AF283559), used as a control for variations in loading, was described previously [5]. Band intensities were quantitated using a Typhoon 9410 Imager (Amersham Biosciences).

Recombinant protein expression. To construct a plasmid expressing the complete LINCR coding region (254 amino acid residues) with an amino-terminal FLAG epitope tag, the LINCR insert was amplified by PCR using Pfu polymerase (Stratagene) and primers ATCTGCAGgctgatgggttctctcctcagc (FL-F1) and CAGAATTCctgcccaagaagtc atc (FL-R1). The product was digested with *Pst*I and *Eco*RI, and cloned into pCMV-Tag2A (Stratagene) to generate pFLAG-LINCR. Plasmid pFLAG-LINCR-ΔR, expressing a deletion mutant of LINCR lacking the C-terminal RING domain, was constructed similarly, using primers FL-F1 and AAGAATTCcaggcgttggctttg. The protein encoded by pFLAG-LINCR-ΔR has an amino-terminal FLAG tag followed by amino acid residues 1–178 of LINCR. A plasmid expressing the FLAG sequence followed by the C-terminal RING domain, pFLAG-RING, was constructed as above, using primers GGCTGCAGGtggat tcgtagtggtgag and FL-R1. The encoded protein contains an

amino-terminal FLAG tag followed by amino acid residues 179–254 of LINCR. All cloned inserts were verified by sequencing.

Transfected 293T cells were harvested by washing once with PBS and lysed in cell lysis buffer (50 mM Tris–Cl, pH 8, 150 mM NaCl, protease inhibitor cocktail, and 1% Triton X-100). Immunoprecipitation was performed using anti-FLAG monoclonal antibody (M2) conjugated to agarose beads (Sigma). Anti-FLAG beads were prepared according to the manufacturer's instructions. Cellular extracts (200 μg protein) were mixed with 20 μl anti-FLAG M2 beads in 1 ml of cell lysis buffer and rotated for 2 h at 4 °C. The beads were washed three times with 1 ml lysis buffer and then twice with 1 ml of 1× PBS.

To construct a plasmid expressing a fusion of GST with the LINCR RING domain, the LINCR segment encoding amino acid residues 191–254 was amplified using primers CAGAATTCCCgtcatat caggagaggagtgtg and ATAAGTACGCGGCCGCgctgggaatgttgactg aat. The product was digested with *Eco*RI and *Not*I, and cloned into the *Eco*RI and *Not*I sites of pGEX-6P2. To express GST-RING and control GST protein, *E. coli* strain BL21 was transformed with the pGEX6P2-LINCR-RING or pGEX-6P2 plasmids. The bacteria were grown at 37 °C to a density of 1.5–2.0 at 595 nm and then induced at 25 °C for 3 h. Cell lysis and protein isolation using glutathione–agarose beads (Pharmacia) were performed as described by the manufacturer.

Ubiquitination reactions. Reaction mixtures containing 50 mM Tris–Cl, pH 7.5, 2.5 mM $MgCl_2$, 1 mM ATP, 0.1 mM DTT, 20 mM creatine phosphate, 15 U/ml creatine phosphokinase, 39 nM rabbit E1, 6 μM UbcH6 or another E2 enzyme, and 1 mg/ml ubiquitin were incubated at room temperature for specified times in the presence of either 840 mg/ml GST-RING, unmodified GST, or with aliquots of immunoprecipitated FLAG-LINCR or FLAG-LINCR-ΔR. The reaction products were analyzed by SDS–PAGE and immunoblotting with anti-ubiquitin and anti-LINCR, anti-GST, or anti-FLAG antibodies with chemiluminescent detection.

Results

LINCR is induced in alveolar epithelial type II cells during endotoxemia

LINCR message expression in the intact lung is induced approximately 20-fold within 1 h after i.v. injection of LPS, decreasing to about 50% of the peak level after 8 h [3]. To investigate the cellular source of the LPS-induced increase in message expression, lung sections from mice euthanized 1 h after i.v. injection of LPS or saline were hybridized with digoxigenin-labeled antisense and sense RNA probes, and developed using an alkaline phosphatase-labeled anti-digoxigenin antibody as described in Methods. Under the conditions employed, no signal was detected with the sense (control) probe in lung sections from mice injected with either saline or LPS (not shown). LINCR message was not detected in lung sections from saline-treated animals, Fig. 1A, but was strongly expressed in alveolar areas of the LPS-treated mice, Figs. 1B and C. The localization pattern is consistent with expression in AE2 cells, as shown by immunohistochemical staining for pro-surfactant protein C, Fig. 1D. LINCR message was not detected in airway epithelial cells (Fig. 1B and data not shown). Although it remains possible that LINCR could also be expressed at lower levels by other cell types, these experiments indicate that AE2 cells are the main site of LPS-induced LINCR expression.

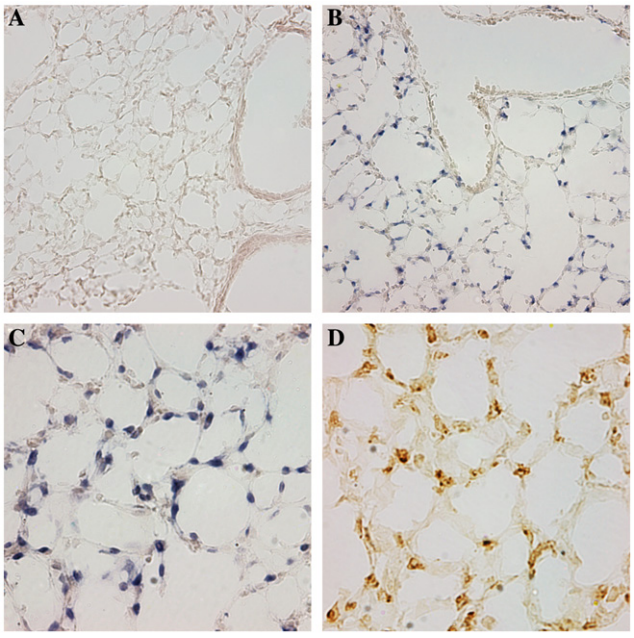

Fig. 1. Localization of induced LINCR message expression. The expression of LINCR and pro-surfactant protein C was compared in frozen sections from lungs collected 1 h after intravenous injection of saline (A) or 50 μg LPS (B–D). LINCR mRNA expression was evaluated by in situ hybridization with digoxigenin-labeled antisense probes for LINCR (A–C), as described in Methods. Under the conditions used, no labeling was detected using a sense probe with lung sections from either saline- or LPS-treated animals (not shown). Pro-surfactant protein C was detected by immunohistochemical staining (D). No LINCR mRNA is detected in the lung from a saline-treated control mouse (A). LINCR mRNA is highly induced in alveolar areas of the lung after LPS injection, but not in the airway epithelium (B). The pattern of LINCR mRNA expression in lung sections from LPS-treated mice is consistent with localization in alveolar epithelial type II cells (C), as shown by comparison with the sections labeled with pro-surfactant protein C antibody, an AE2-cell marker (D).

LINCR is induced by LPS and inflammatory cytokines and attenuated by dexamethasone in the T7 cell line

The T7 cell line is a clonal AE2 cell line derived from transgenic H-2K(b)-tsA58 mice, which express a temperature-sensitive mutant of the simian virus 40 large tumor antigen (SV40T) under the control of the γ-IFN-inducible mouse major histocompatibility complex H-2Kb promoter [19]. T7 cells can be passaged as an immortalized cell line in culture and then induced to differentiate by culturing the cells at 39 °C in the presence of IFN-γ. The differentiated T7 cells have a polarized epithelial cell morphology, tight junctions, apical microvilli, and cytoplasmic lamellar bodies, and they synthesize and secrete phosphatidylcholine and surfactant proteins A, B, and C [19]. To investigate LINCR expression by T7 cells, RNA was harvested at intervals after the addition of

LPS to the culture medium of differentiated T7 cells and analyzed by RNase protection assay as described in Methods. LINCR message was detectable at low levels in untreated T7 cells, and was rapidly but transiently induced following the addition of 500 ng/ml LPS to the culture medium, Fig. 2A (left panel), with peak expression at 1–2 h.

In vivo, LPS rapidly induces the secretion of endogenous inflammatory cytokines including IL-1β, TNF-α, and IFN-γ. To further evaluate the regulation of LINCR in T7 cells, LINCR message expression was measured in T7 cells 2 h after the addition of LPS and these cytokines, singly and in combination. LINCR message was induced by TNF-α but not by IL-1β or IFN-γ alone, Fig. 2B. The combination of LPS and TNF-α or IL-1β and TNF-α induced LINCR message to about twice the level of LPS or TNF-α alone. Although IFN-γ alone did not induce LINCR, IFN-γ co-stimulation together with LPS, IL-1β, or TNF-α, singly or in combination, dramatically enhanced the induction of LINCR by these factors, Fig. 2B. The highest levels of LINCR expression were produced by the combination of TNF-α and IFN-γ together with LPS or IL-1β.

To determine if the kinetics of message induction, as well as the magnitude of the response, was affected by the cytokine treatments, we compared the time courses of LINCR expression in T7 cells following stimulation with LPS and following stimulation with the combination of LPS, TNF-α, and IFN-γ. In both cases, LINCR message peaked at 1–2 h, Fig 2A. However, LINCR expression in response to combined stimulation was sustained much longer (remaining elevated at 6 h at 48% of peak expression) than in response to LPS alone (falling at 6 h to 13% of peak expression), Fig. 2A. The time course seen following combined stimulation is very similar to that produced in vivo during endotoxemia [3].

Dexamethasone attenuated by 40–50% the induction of LINCR by LPS or by the combination of TNF-α and IFN-γ (Fig. 2C and data not shown), a response similar to the ≈50% attenuation observed for lung LINCR expression during endotoxemia [3]. Cycloheximide did not block the induction of LINCR by LPS or by combinations of IFN-γ, LPS, and TNF-α, Fig. 2D and data not shown, indicating that LINCR induction by these stimuli is not dependent on new protein synthesis.

The LINCR RING domain has ubiquitin E3 ligase activity

For many E3 ligases, the isolated RING domain as well as the full-length protein is able to autoubiquitinate itself in vitro, i.e., it catalyzes the attachment of Ub to the RING protein itself and then catalyzes the progressive attachment of additional Ub monomers to elaborate a high-molecular-weight polyubiquitin chain

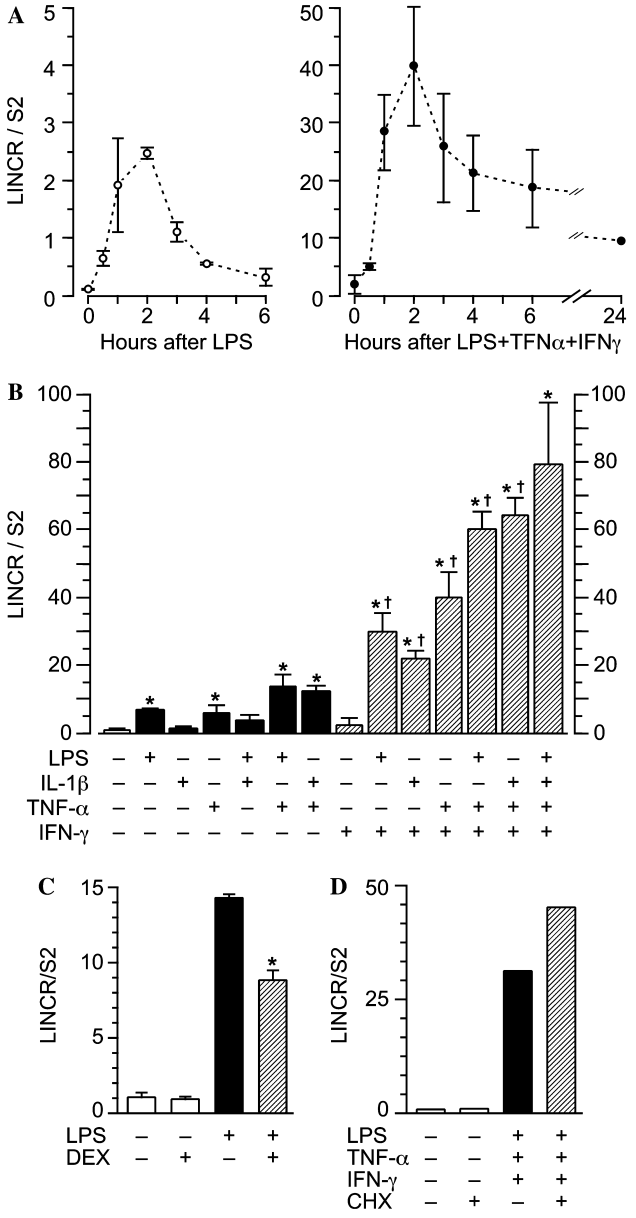

Fig. 2. Regulation of LINCR mRNA expression in the T7 alveolar epithelial type II cell line. T7 cells were cultured as described in Methods. LINCR message expression was evaluated by RNase protection assay at the indicated times following the addition of 500 ng/ml LPS, 10 ng/ml IL-1β, 20 ng/ml TNF-α, and/or 20 ng/ml IFN-γ to the culture medium and quantitated relative to the control S2 message as described in Methods. (A) Time course of LINCR mRNA after LPS (left) or after combined stimulation with LPS, TNF-α, and IFN-γ (right). (B) LINCR message expression 2 h after stimulation with the indicated combinations of LPS, IL-1β, TNF-α, and IFN-γ. $^{*}p < 0.05$ vs unstimulated cells, and $^{\dagger}p < 0.05$ vs. no IFN-γ by *t* test. (C) Induction of LINCR by LPS is attenuated by dexamethasone (DEX, 500 ng/ml), $^{*}p < 0.05$ vs. cells stimulated with LPS, by *t* test. (D) LINCR mRNA induction in T7 cells is not blocked by cycloheximide (CHX, 10 μg/ml). In parts (A)–(C), data are means ± SEM of two independent experiments.

[16,21]. As a first step in testing the ubiquitin ligase activity of LINCR, we constructed a plasmid encoding a fusion of glutathione *S*-transferase (GST) with the

LINCR RING domain. The fusion protein was expressed in *E. coli* and isolated by binding and elution from glutathione–agarose as described in Methods. The GST-RING protein was incubated with ubiquitin, rabbit ubiquitin-activating enzyme (Uba, E1), ATP, and a panel of recombinant E2 enzymes for 90 min. The reaction products were separated by PAGE, and analyzed by immunoblotting with an anti-ubiquitin antibody to detect ubiquitinated products, and with anti-LINCR or anti-GST antibodies to verify equal loading of the gel. A smear of high-molecular-weight polyubiquitinated products was produced in the presence of UbcH6, but not with the other E2s tested, Fig. 3A. The polyubiquitin chains contain large numbers of ubiquitin epitopes, which greatly increases the ability of the anti-ubiquitin antibody to detect small amounts of polyubiquitinated protein, compared to its sensitivity for mono- or oligo-ubiquitinated proteins. For the same reason, the anti-ubiquitin antibody is much more sensitive for detection of the polyubiquitinated protein than antibodies to LINCR or GST. The LINCR blot shows the main band of unmodified GST-LINCR-RING in all lanes, verifying equal loading, plus a band corresponding to mono-ubiquitinated GST-LINCR-RING in the reaction containing UbcH6. Polyubiquitinated protein was not detected in control reactions containing GST in place of GST-RING, nor in control GST-RING reactions from which either the E1, E2 (UbcH6), or ubiquitin was omitted, Fig. 3B. These experiments confirm that the reaction is dependent on the presence of all three enzymes (E1, E2, and the LINCR RING), and that the LINCR RING domain has UbcH6-dependent ubiquitin E3 ligase activity.

The full-length LINCR has RING domain-dependent ubiquitin E3 ligase activity and preferentially utilizes UbcH6

To evaluate the ubiquitin ligase activity of the LINCR RING domain in its native context, we transfected plasmids expressing N-terminal FLAG-tagged LINCR (FLAG-LINCR) or a LINCR mutant lacking the RING domain (FLAG-LINCR-ΔR) into 293T cells. After 24 h, the cell lysates were immunoprecipitated with FLAG antibody. The recovered FLAG-LINCR proteins were tested for in vitro autoubiquitination activity in the presence of rabbit Uba, a panel of E2 enzymes, and ubiquitin, as described above. Polyubiquitinated products were efficiently produced by full-length LINCR in the presence of UbcH6. Polyubiquitinated products were also seen, at much lower levels, in the presence of UbcH5a. However, polyubiquitinated protein was not detected in control reactions containing FLAG-LINCR-ΔR in place of FLAG-LINCR, nor in control reactions with FLAG-LINCR from which either Uba, UbcH6, or ubiquitin was omitted, Fig. 4B. These experiments confirm that the intact LINCR molecule has RING domain-dependent ubiquitin E3 ligase activity, and that LINCR preferentially utilizes the ubiquitin-conjugating enzyme UbcH6. Although the selectivity for UbcH6 is intrinsic to the RING domain, the ability of UbcH5a to support ubiquitination by FLAG-LINCR but not by GST-RING

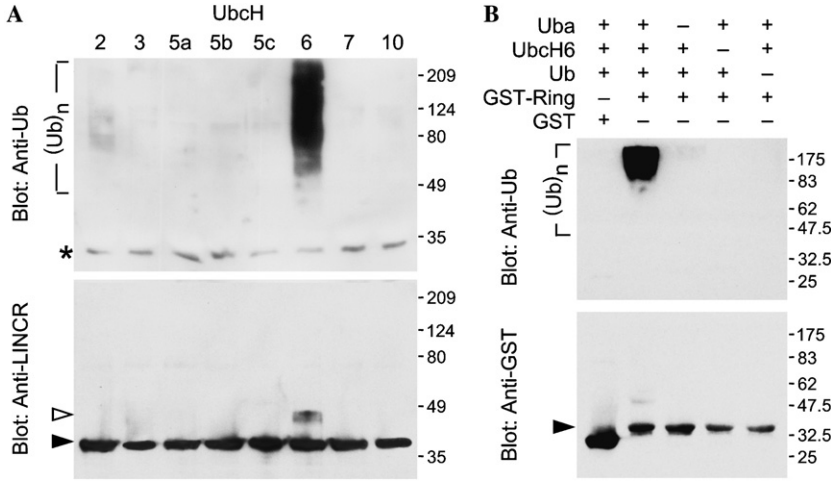

Fig. 3. Ubiquitination activity and ubiquitin-conjugating enzyme usage of the LINCR RING domain. In vitro ubiquitination reactions were performed using recombinant GST-RING or control GST protein. The formation of polyubiquitin chains [$(Ub)_n$] was assessed by immunoblotting with an anti-Ub antibody (upper panels). The membranes were then reblotted with anti-LINCR or anti-GST antibodies to verify equal loading (lower panels). (A) Utilization of ubiquitin-conjugating enzymes (Ubc) by the LINCR RING domain. GST-RING was incubated with ubiquitin, ubiquitin-activating enzyme, ATP, and the specified Ubc for 90 min at 37 °C. Equal samples were analyzed by SDS–PAGE and immunoblotting. The unmodified GST-RING protein band is indicated by the filled arrowhead. The open arrowhead indicates mono-ubiquitinated LING-RING. An irrelevant cross-reacting band is indicated by an asterisk. (B) The ubiquitin ligase activity of the GST-RING fusion protein is mediated by the LINCR RING domain. Ubiquitination reactions containing either GST-RING or GST were incubated with UbcH6 and other reaction components as indicated, and analyzed by immunoblotting. The filled arrowhead indicates the GST-RING band.

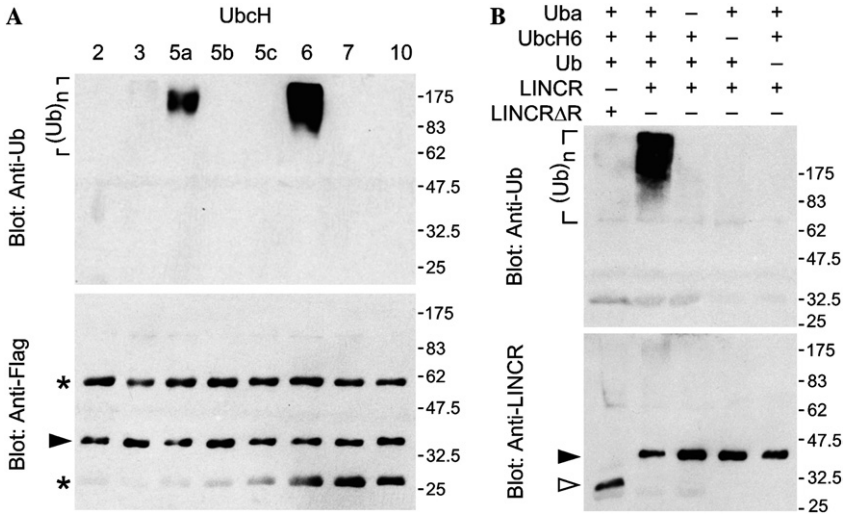

Fig. 4. Ubiquitination activity and ubiquitin-conjugating enzyme usage of full-length LINCR. In vitro ubiquitination reactions were performed using full-length FLAG-LINCR or the RING-deletion mutant FLAG-LINCR-ΔR. The reaction products were evaluated by SDS–PAGE and immunoblotting with anti-ubiquitin (upper panels) and then with anti-FLAG or anti-LINCR antibodies (lower panels). (A) Utilization of ubiquitin-conjugating enzymes by LINCR. The FLAG-LINCR band is indicated by the filled arrowhead. The asterisks indicate IgG bands. (B) The ubiquitination activity of LINCR requires the presence of the C-terminal region containing the RING domain. Ubiquitination reactions contained either FLAG-LINCR-ΔR or FLAG-LINCR, and other reaction components as indicated. The FLAG-LINCR and FLAG-LINCR-ΔR bands are indicated by the filled and open arrowheads, respectively.

indicates that portions of LINCR other than the RING domain can influence the interaction of the LINCR RING domain with E2 enzymes.

LINCR preferentially catalyzes the formation of non-canonical polyubiquitin chains

Attachment of a polyubiquitin chain linked via lysine 48 (K48) of ubiquitin targets a protein for degradation by the proteosome [15]. To determine if LINCR generates K48-linked polyubiquitin chains, we analyzed GST-RING-catalyzed chain formation in reactions with a ubiquitin mutant in which all lysines except K48 were mutated to arginine (K48-only), with a ubiquitin mutant in which K48 was mutated to arginine (K48R) but all other lysines remained intact, and with wild-type ubiquitin. Polyubiquitinated products were detected at 15 min in reactions with Ub and K48R, but not in reactions with K48-only, Fig. 5A. At 30 min, reactions with Ub and K48R produced a band of high-molecular-mass products visible by Coomassie staining (asterisk, Fig. 5A), but this band was not produced in the reaction with K48-only. After 90 min of incubation, high-molecular-weight polyUb chains were detected in reactions with all three ubiquitin species (not shown). These experiments suggest that while the LINCR RING can catalyze ubiquitin chain formation via K48, chain formation via one or more lysine residues other than K48 is favored.

To identify the ubiquitin lysine residues that can be utilized for chain formation by LINCR, GST-RING was incubated for 15, 30, 45, 60, and 90 min with a panel of ubiquitin mutants (K*n*-only) in which all lysine residues except lysine *n* were mutated to arginine. Reactions containing wild-type ubiquitin or a ubiquitin mutant lacking all lysines (no-K Ub) were included as additional controls. The no-K Ub retains the potential for attachment via its C-terminal glycine to lysine residues on other proteins, but lacks the conjugation sites necessary to allow the elaboration of a polyubiquitin chain. Robust polyubiquitin chain formation was observed at all time points in reactions with wild-type ubiquitin and with K11-only ubiquitin, Fig. 5B and data not shown. With incubations of 45 min or longer, high-molecular-weight polyubiquitin chains were also observed in reactions containing K48-only and with K63-only ubiquitin.

To determine the ubiquitin–lysine utilization of full-length LINCR, in vitro experiments similar to those described above were performed using FLAG-LINCR isolated by immunoprecipitation from transfected 293T cells. FLAG-LINCR robustly catalyzed polyubiquitin chain formation with the K48R ubiquitin mutant, but not with K48-only ubiquitin, Fig. 6A. Incubation of FLAG-LINCR in reactions with the K-only ubiquitin mutants for intervals up to 90 min demonstrated that FLAG-LINCR preferentially formed polyubiquitin chains linked by K27 or K63, Fig. 6B and data not shown. Taken together, the experiments in Figs. 5 and 6 indicate that LINCR preferentially catalyzes the formation of polyubiquitin chains linked by lysines other than the canonical lysine 48 involved in the proteosomal degradation pathway, and that this property is intrinsic to the LINCR RING domain. However, the differences in preferential lysine utilization in reactions catalyzed by full-length LINCR and by GST-LINCR suggest that

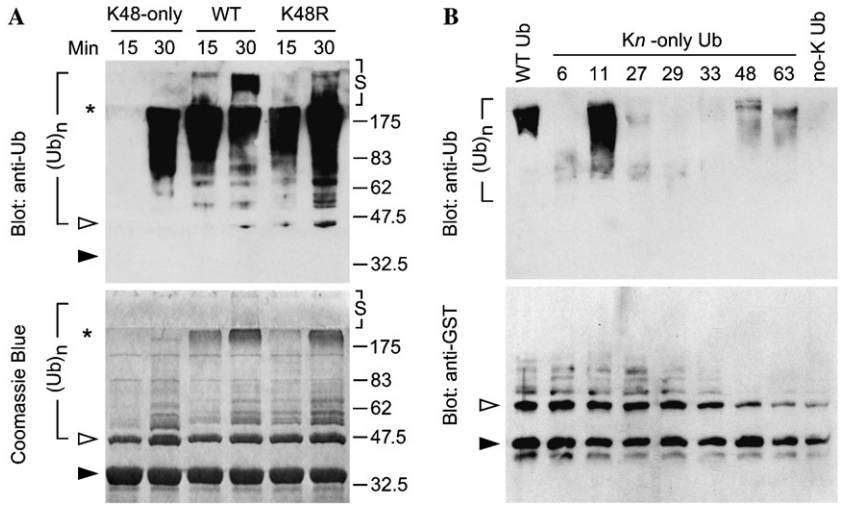

Fig. 5. Lysine usage in polyubiquitin chains catalyzed by GST-RING. (A) GST-RING, rabbit Uba, ATP, and UbcH6 were incubated at 37 °C for 15 or 30 min with ubiquitin K48-only, wild-type ubiquitin (WT), and ubiquitin-K48R. Equal aliquots of the reaction products were subjected to SDS–PAGE, and stained with Coomassie blue (lower panel). Proteins on a duplicate gel were transferred to a nitrocellulose membrane and immunoblotted with anti-ubiquitin antibody (upper panel). The asterisk indicates the band of high-molecular-mass polyubiquitinated protein, just below the stacking gel (S). (B) WT ubiquitin, a ubiquitin mutant in which all but one lysine (K*n*-only Ub, as specified) were mutated to arginine, or a ubiquitin mutant with all lysines replaced by arginine (no-K Ub) were incubated with GST-RING, rabbit Uba, ATP, and UbcH6 at 37 °C for 45 min, and analyzed by SDS–PAGE and immunoblotting with anti-Ub and anti-GST antibodies. In both (A) and (B), the open and filled arrowheads indicate the positions of mono-ubiquitinated and unmodified GST-RING, respectively.

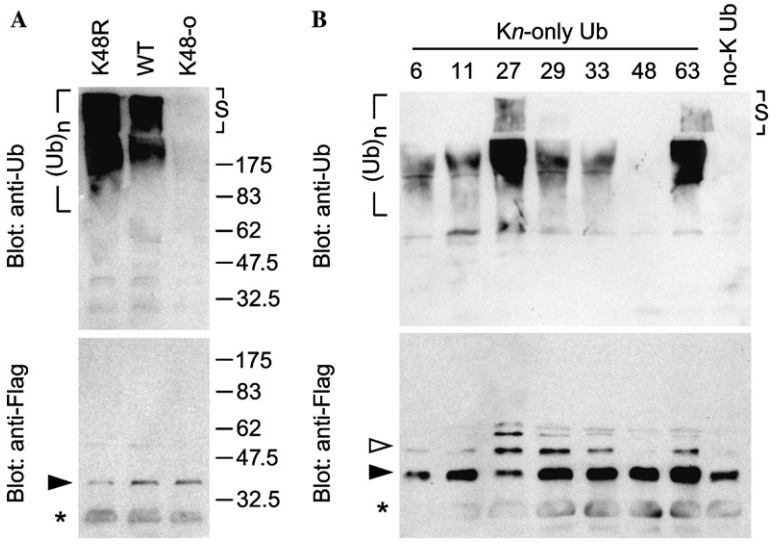

Fig. 6. Lysine usage in polyubiquitin chains catalyzed by FLAG-LINCR. (A) FLAG-LINCR, rabbit Uba, ATP, and UbcH6 were incubated at 37 °C for 45 min with ubiquitin K48-only, wild-type ubiquitin (WT), and ubiquitin-K48R. (B) Ubiquitin mutants with a single lysine (K*n*-only Ub) or no lysines (no-K Ub) were incubated with FLAG-LINCR, rabbit Uba, ATP, and UbcH6 for 60 min at 37 °C. In both (A) and (B), the reaction products were analyzed by SDS–PAGE and immunoblotting with anti-Ub and anti-Flag antibodies. The open and filled arrowheads indicate the positions of mono-ubiquitinated and unmodified FLAG-LINCR, respectively. The asterisk indicates the IgG light chain band.

other domains of LINCR do influence the efficiency with which specific polyubiquitin chain linkages are formed.

Discussion

LINCR is a novel glucocorticoid-attenuated response gene induced in the lung during endotoxemia. At the organ level, endotoxemia-induced LINCR expression is relatively restricted and is most highly expressed in the lung [3]. In this study, endotoxemia-induced LINCR message expression was localized by in situ hybridization to AE2 cells (Fig. 1). Although the possibility cannot be ruled out that LINCR is expressed at lower levels in other lung cell types, our results indicate that AE2 cell expression is likely responsible for the vast majority of

the increase in LINCR message expression seen during endotoxemia. The cells expressing LINCR message are identified as AE2 cells by their typical shape, location at the corners of alveoli, and the similarity of the patterns of cell labeling by the LINCR in situ hybridization probe and by antibodies to pro-surfactant protein C. Antibodies suitable for detecting the LINCR protein by immunohistochemical labeling are not presently available (not shown). However, AE2-cell expression of LINCR is substantiated by the demonstration of LINCR induction in differentiated T7 cells in cell culture. The T7 cell line, which contains a temperature-sensitive mutant of the SV40 T antigen under the control of the $H2K^b$ promoter, can be passaged as an immortalized cell line in culture and then induced to fully differentiate into AE2 cells [19]. However, we were unable to detect induction of LINCR message in alveolar macrophages or RAW 264.7 cells stimulated in vitro with LPS (with or without IFN-γ), or in Swiss 3T3 fibroblasts (data not shown). The lack of expression of LINCR in macrophages is consistent with the absence of LINCR expression in the spleen either in control animals or during endotoxemia [3].

The response to endotoxemia in vivo involves both direct effects of LPS and effects mediated by local or systemic release of endogenous inflammatory mediators including IL-1β, TNF-α, and IFN-γ. We therefore investigated the response of T7 cells to these mediators both singly and in combination. LINCR message is expressed at a low level in unstimulated T7 cells (Fig. 2), just as seen in the in vivo lung [3], and induced in T7 cells treated with LPS or TNF-α alone. Neither IL-1β nor IFN-γ alone produced a statistically significant increase in LINCR expression, but nevertheless, stimulation with IL-1β in combination with either TNF-α or IFN-γ induced LINCR to levels 2- to 4-fold greater than the level induced by TNF-α alone. Strikingly, combinations of IFN-γ with LPS, IL-1β, and TNF-α produced synergistic increases in LINCR induction, up to about 50-fold above the levels in unstimulated control cells. Similar synergistic responses to the combination of IFN-γ with LPS or inflammatory cytokines have been observed for many inflammation-inducible genes, in a variety of cell types [6,22]. Induction by LPS or by combined stimulation with IFN-γ and TNF-α (with or without LPS) was not blocked by cycloheximide, indicating that new protein synthesis is not required for induction of LINCR. The synergistic induction of LINCR by LPS, IL-1β, TNF-α, and IFN-γ in T7 cells, and the similarity of the time courses of the in vivo and in vitro responses, are consistent with the in situ hybridization data indicating that AE2 cells are the main cellular site of LINCR induction during endotoxemia.

The observation of LINCR induction in T7 cells by LPS and inflammatory cytokines is consistent with previous studies showing that lung epithelial cells, including AE2 cells, respond to inflammatory stimuli and participate in lung innate immune responses [23,24]. Skerrett et al. [25] recently showed that mice expressing a dominant negative IκB-α transgene under the control of the SP-C promoter, which selectively inhibited activation of NF-κB in distal airway and alveolar epithelial cells, had impaired inflammatory responses to inhaled LPS, thus confirming a physiological role for respiratory epithelial cells in lung inflammation in vivo. The robust induction of LINCR during endotoxemia in vivo and in response to LPS and inflammatory cytokines in vitro, plus the attenuation of LINCR induction by glucocorticoids both in vivo and in vitro (Figs. 1 and 2, and [3]), strongly suggests that LINCR plays some role in the lung response to inflammation. However, the nature of that role cannot be deduced from these expression characteristics. Although many genes with GARG expression characteristics have known pro-inflammatory functions, others, such as suppressor of cytokine signaling-3 (SOCS-3), have clear anti-inflammatory functions [3,4]. In addition, other GARGs, such as members of the guanylate binding protein (GBP) family [20], do not appear to function primarily in either pro-or anti-inflammatory pathways, but instead have homeostatic functions, including control of proliferative responses [26].

A key feature of LINCR's protein structure is the C-terminal RING domain. RING domains have recently been recognized as the defining feature of a large class of ubiquitin E3 ligases [21]. The ability of RING finger proteins to ubiquitinate themselves in vitro (''autoubiquitination'') in a RING- and E2-dependent manner makes it possible to evaluate E3 ligase activity even when the physiological substrate is unknown. The observation of autoubiquitination by the *E. coli*-expressed GST-RING fusion protein (but not by control GST protein) and by the full-length FLAG-LINCR but not FLAG-LINCR-ΔR mutant lacking the RING domain confirms that this activity is intrinsic to the RING domain of LINCR (Figs. 3 and 4). GST-RING had robust ubiquitination activity in the presence of UbcH6, but no activity was seen in the presence of the seven other E2 enzymes tested. However, ubiquitination by FLAG-LINCR also occurred in the presence of UbcH5a, though much less efficiently than with UbcH6 (Fig. 4 and data not shown). The fact that both GST-RING and FLAG-LINCR preferentially interact with UbcH6 suggests that the interaction between LINCR and this E2 is intrinsic to the RING domain, and that other domains of LINCR are unimportant in determining this specificity. The ability of UbcH5a to support autoubiquitination by intact LINCR but not GST-RING could either be due to steric interference by the GST portion of the fusion protein, or, alternatively, to stabilization of an otherwise unfavorable RING-UbcH5a interaction via interaction of UbcH5a with more proximal portions of LINCR.

In reactions catalyzed by either GST-RING or the full-length FLAG-LINCR protein, polyubiquitin chain formation using a ubiquitin mutant containing only lysine 48 was less efficient than with wild-type ubiquitin or with the K48R ubiquitin mutant, in which lysine 48 is replaced by arginine (Figs. 5A and 6A). Reactions performed using a panel of ubiquitin mutants containing single lysine residues showed preferential formation of lysine 11-linked polyubiquitin chains by GST-RING, and preferential formation of K27 and K63-linked chains by full-length FLAG-LINCR (Figs. 5B and 6B). These studies show that the E3 ligase activity of the LINCR RING domain kinetically favors the generation of polyUb chains linked by non-canonical ubiquitin lysine residues. This suggests that LINCR ubiquitination most likely mediates a function other than tagging its substrate(s) for proteosomal degradation. It remains to be determined whether K11, K27, or K63-linked polyUb chains are in fact generated by LINCR under physiological conditions, or whether LINCR mainly acts to mono-ubiquitinate its substrate. K63-linked chains confer non-proteolytic signals involved in DNA repair, vesicle trafficking, and activation of protein kinases [18], but specific functions have not yet been described for K11 or K27-linked chains.

Multiple components of the Notch signaling pathway have been shown to be expressed in the developing and/or mature lung, including all four mammalian Notch receptors; the Notch ligands Delta-1, Delta-4, Jagged-1, and Jagged-2; the Notch signaling modulators radical fringe and lunatic fringe; and the Notch-regulated bHLH transcription factors Hes-1 and Mash-1 [27–30]. Overexpression of a constitutively active form of Notch-3 in respiratory epithelium inhibits terminal epithelial differentiation and causes perinatal death [31], while mice lacking the Notch-regulated bHLH transcription factor Mash1 (mammalian achaete-scute homolog-1) fail to develop pulmonary neuroendocrine cells [28]. In Foxf1 haploinsufficient mice, a reduction in Notch-2 signaling is associated with abnormalities of lung microvascular development [32]. Antisense inhibition of embryonic lung explants has demonstrated non-redundant roles for several components of the Notch signaling pathway [33]. These studies suggest important roles for Notch signaling in the lung, though a detailed understanding is lacking.

Our interest in LINCR was stimulated by the observation that this inflammation-inducible gene has striking structural similarity to Neuralized, an evolutionarily conserved modifier of Notch signaling that has been shown in *Drosophila* and *Xenopus* to be a ubiquitin ligase that modulates Notch signaling by ubiquitinating the Notch ligand Delta [8,10,11]. The present study strengthens the association between LINCR and Neuralized by showing that LINCR also has ubiquitin ligase activity. A key question that remains to be answered is whether LINCR, like Neuralized, acts as a modulator of Notch signaling. Our attempts to demonstrate a direct interaction between LINCR and Notch ligands by co-immunoprecipitation have been unsuccessful so far (data not shown). For *Drosophila* and *Xenopus* Neuralized, the binding and ubiquitination of Delta requires both NR domains of Neuralized, as well as the RING domain [8,10]. As LINCR has only a single NR domain, it is possible that the binding of LINCR to its physiological substrate may require the participation of additional proteins as part of a complex.

In summary, we have shown that LINCR is cooperatively induced by LPS and inflammatory cytokines in AE2 cells in vivo and in vitro, and that LINCR has RING domain-dependent autoubiquitination activity that defines it as an E3 ligase. LINCR specifically interacts with the E2 enzyme ubcH6 and preferentially generates polyubiquitin chains linked via ubiquitin lysine residues other than K48. We do not yet know whether LINCR functions in vivo as a single-chain E3 or as part of a complex, and the physiological target or targets of LINCR remain to be identified. In particular, it remains to be determined whether LINCR, like Neuralized, functions as a modulator of Notch signaling. However, we have recently shown that overexpression of LINCR in fetal lung epithelium produces marked lung abnormalities incompatible with postnatal survival (T.T. Nguyen and J.B. Smith, unpublished), implying that LINCR does interact with one or more pathways essential in normal lung development. Studies to characterize the altered lung phenotype in these mice and to identify the affected pathways are currently in progress. Because repair and modeling of the lung in response to injury may utilize many of the same pathways that participate in lung development [34,35], LINCR's induction in AE2 cells during inflammation and its interaction with pathways involved in lung development also make it an attractive candidate for future investigation in lung injury and BPD models.

Acknowledgments

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