Glucocorticoid-attenuated response genes induced in the lung during endotoxemia

JEFFREY B. SMITH,1 TAM T. NGUYEN,1 HEATHER J. HUGHES,1 HARVEY R. HERSCHMAN,2 DANIEL P. WIDNEY,1 KIM C. BUI,1 AND LEONOR E. ROVAI1

1Department of Pediatrics, UCLA School of Medicine and Mattel Children’s Hospital at UCLA; and 2Departments of Biological Chemistry and Molecular Pharmacology, and Molecular Biology Institute, University of California, Los Angeles, California 90095

Received 31 December 2001; accepted in final form 11 April 2002

Inflammation is also thought to have important roles in the pathophysiology of bronchopulmonary dysplasia (BPD) in neonates (14, 29, 60) and of acute respiratory distress syndrome (ARDS) (11, 68, 70). Demonstrable short-term improvements in respiratory function, sometimes dramatic, resulted in widespread use of postnatal glucocorticoids for attempted prevention or treatment of BPD during the past decade. Improvement in long-term outcome has not been demonstrated, however. Because of the potential for serious adverse effects, including neurodevelopmental impairment, the routine use of corticosteroids for prevention or treatment of BPD in preterm infants is no longer recommended (3, 4, 6, 20). The results of using glucocorticoids for prevention or treatment of acute ARDS have also been disappointing (11, 68, 70), although a large multicenter trial is currently evaluating the use of glucocorticoids in the late, fibrosing phase of ARDS. The limited effectiveness of glucocorticoids in BPD and ARDS may be due, in part, to adverse effects of glucocorticoids on lung growth and repair that negate the potential benefits of reduced lung inflammation. If specific sets of inflammatory mediators important in the pathophysiology of these diseases could be identified, it might be possible to develop selective anti-inflammatory treatments that are safer and more effective.

One approach to finding new targets for anti-inflammatory treatments of lung diseases may be to identify lung-expressed genes whose activity is modulated by glucocorticoids. Although other mechanisms also contribute, a major part of the anti-inflammatory actions of glucocorticoids is attributed to their ability to attenuate the induction of genes encoding a variety of mediators important in inflammatory and immune responses (1, 5). Glucocorticoids inhibit the induction of the inducible form of prostaglandin H synthetase (cyclooxygenase 2), the inducible form of nitric oxide syn-
glucocorticoid attenuated GARGs remained to be identified. We subsequently verified that endotoxemia-induced LIX expression was strongly glucocorticoid attenuated in the lungs of adrenalectomized (ADX) mice (51). In contrast, the related chemokines KC and macrophage inflammatory protein (MIP)-2 were LPS induced but not glucocorticoid attenuated in the lungs of the ADX or normal mice, despite the ability of glucocorticoids to attenuate the induction of KC and MIP-2 induction in cell culture studies. These results emphasized the importance of in vivo studies for determining which genes are subject to attenuation by glucocorticoids in specific models of lung inflammation.

The goal of the present study was to identify GARGs induced in the lung during endotoxemia. We hoped to identify novel genes, as well as genes not previously implicated in lung inflammation, as candidates for further study in other models. We also hoped to gain a better understanding of characteristics of the GARG class as a whole. An advantage of the endotoxemia model for this study is that intravenous injection of LPS triggers the prompt release of endogenous mediators including TNF-α, IL-1β, and interferon (IFN)-γ, so downstream genes responsive to these mediators, as well as those directly responsive to LPS, are induced. Although the timing and pattern of gene expression will differ in specific disease models, many of the genes induced during endotoxemia are expected to participate in a wide variety of acute and chronic inflammatory lung diseases. ADX mice were used for the screening to eliminate the effects of endogenous glucocorticoids and maximize potential message attenuation in response to an exogenous steroid. The expression characteristics of candidate GARG cDNAs were evaluated in normal as well as ADX mice.

Anticipating that GARG messages would constitute only a small fraction of the total mRNA population in the lung, we used a two-stage screening strategy. First, a subtracted library enriched in endotoxemia-induced genes was constructed with lung RNA from ADX mice. The library was then screened by differential hybridization to select candidate GARG clones. Northern analysis confirmed glucocorticoid attenuation of endotoxemia-induced lung expression for 36 GARG cDNAs. The majority were genes of unknown function not previously implicated in the pulmonary response to inflammation. Four were new murine sequences, including the murine ortholog of the human IFN-inducible T-cell α-chemoattractant (I-TAC) chemokine (CXCL11), as we reported previously (71), and a new member of the guanylate binding protein (GBP) family, murine GBP-5. We report here the complete cDNA sequences of a new member of the 2′−5′-oligoadenylate synthetase-like family (murine OASL-2), and a novel lung-inducible Neutralized-related C3HC4 RING domain protein (LINCR) and its predicted human homolog. The results of this study indicate that the network of glucocorticoid-attenuated genes participating in the lung response to endotoxemia is much more extensive and diverse than previously thought.

METHODS

Animals. ADX, sham-operated, and nonoperated male Swiss-Webster mice purchased from Charles River Laboratories (Cambridge, MA) were studied at 8–12 wk of age and at least 2 wk after operation. Mice were housed under specific pathogen-free conditions in the UCLA Health Sciences Center vivarium for at least 1 wk before experimental manipulation. ADX mice were supplied with normal saline instead of water to drink. All procedures were performed in accordance with protocols approved by the UCLA animal care and use committee.

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 (Abbott Laboratories, North Chicago, IL) was used for dilution of LPS and for control injections. Dexamethasone sodium phosphate (Dex, 4 mg/ml of dexamethasone phosphate equivalent) for injection was prepared from ADX mice killed 1, 2, or 4 h after tail vein injection of LPS (4 μg/ml of sterile saline) or saline alone was injected via the tail vein. Two 400-μg doses of Dex in 100 μl of saline or saline alone were injected subcutaneously 18–20 h before and 5 min before LPS. Lungs and other organs were harvested for RNA preparation as described (51).

Construction of subtracted library. A cDNA population enriched in endotoxemia-induced messages was generated using the suppression subtraction hybridization (SSH) technique (17, 18). Lung RNA for the “tester” population was prepared from ADX mice killed 1, 2, or 4 h after tail vein injection of LPS (4–5 mice per group). Lung RNA for the “driver” population was prepared from ADX mice treated with two doses of Dex as above, and the mice were killed 1, 2, and 4 h after the second dose (4 mice per group). Poly-A-selected RNAs were prepared from the pooled tester and pooled driver RNAs using the PolyATtract I kit (Promega, Madison, WI). After first- and second-strand synthesis, the tester and driver cDNAs were digested with *Rsa*I, and aliquots of the tester cDNA were separately ligated to the SSH adapters I and II from the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA). Sequential 7- and 16-h hybridizations of the driver with the adapted tester cDNAs and PCR amplification of the subtracted cDNAs were performed as described in the PCR-Select manual.
The PCR-amplified subtracted cDNAs had an XmaI site and an EagI site at opposite ends of the cDNA insert within adapter-derived sequences. The subtracted cDNAs were sequentially digested with XmaI and EagI and then ligated to EcoRI-SalI-XmaI and XhoI-NcoI-EagI adapters we designed. The EcoRI-SalI-XmaI adapter was prepared by annealing nonphosphorylated AATTCTCCAGCGTGTCGACT with 5′-phosphorylated CGGAGTCGACACCGTGGAG. Nonphosphorylated TCGAGTGCTGTGTAGGTATCCAT and 5′-phosphorylated GGCTATGGGACATCTACACACGACC were then phosphorylated. The primary library, containing 4 × 10⁶ phage (95% recombinants), was amplified once. The cDNA insert sizes of 18 randomly selected phages ranged from 460 to 1,230 nt (mean 690).

Analysis and analysis. Diluted aliquots from the subtracted library were plated at a density of ~950 plaques per 150-mm petri dish. One additional dish was plated with ~200 plaques. Each plate was also inoculated at specific locations with plaque-pure control phages containing inserts of LIX, monocyte chemoattractant protein (MCP)-1, and ribosomal protein S2 (rpS2) cDNA. Poly-A-selected RNA from 10 ADX mice killed 2 h after LPS injection. RNA for the “LPS-probe” was prepared from pooled lung RNA from ADX mice treated with Dex. The criteria for selection of those whose nearest neighbor was ≥3 mm from the nearest neighbor on the low-density phage map was described (59).

For PCR, candidate phages were replated at low density. We amplified inserts from well-separated progeny plaques using vector primers TAATAGCTCTAGATAAGG and ATTAACCTCCTAAAGGGA or using primer pairs targeted to the EcoRI-SalI-XmaI and XhoI-NcoI-EagI adapter sequences. Primers jDW (GTCGACTTCGGGCGAGGT) and jDX (AGGTATCCATGGCCCGAGGT) terminate at the junction between the subtracted cDNAs and the SSH adapters I and II, so Rsal digestion of jDW/jDX-amplified inserts removed all adapter-derived sequences from the ends of the cDNAs. To amplify inserts lacking the Rsal junction, such as those derived from cDNAs cut by EagI or XmaI during the cloning process, we used the primers jE5 (GAATTCTCACGGGTGTCGACT) and jE6 (TGGTACGGTGGTAGGTATCCAT). Digestion of jE5/jE6-amplified inserts with SalI and NcoI left 8–16 nt of adapter-derived sequences on the ends of the cDNAs. An annealing temperature of 60°C was used for all three primer pairs. The ends of vector-primer-amplified cDNA inserts were sequenced with adapter primers jDW or jDX and with jDX or jE6.

Analysis of candidate phages was performed in stages. First, inserts were amplified by PCR from 42 plaques (group 1) that were ≥3 mm from the nearest neighbor on the low-density phage filter. To identify redundant phages within group 1, we hybridized replicate filter arrays of the phase inserts with selected insert probes. Group 1 replicates among the remaining candidates were identified by hybridizing a replicate filter from the library screening with pooled group 1 probes (after removal of adapter sequences). Among the 83 remaining candidates, the 32 group 2 plaques were those whose nearest neighbor was ≥1 mm distant and for which the major plaque component could be identified unambiguously by PCR. The 35 candidates remaining after elimination of group 2 replicates were plaque purified by differential hybridization (group 3). A nonsubtracted cDNA library in λ Zap II, prepared from lung RNA of LPS-treated ADX mice as described (71), was screened by hybridization with the candidate LINCR insert. Potential full-length phages were converted to plasmid form and sequenced. IMAGE Consortium (37) clones for OASL-2 and human LINCR were obtained from Incyte Genomics (Palo Alto, CA).

Northern analysis and ribonuclease protection assay. Northern analysis of total cellular RNA, 10 μg/lane, was performed as described (51, 58). Autoradiographic signal intensities were quantitated by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA). Corrections for lane-to-lane variations in loading were made using the signal intensity of the same filter reprobed with rpS2. We verified that LPS and Dex did not affect lung expression of rpS2 message under the conditions used in this study (51, 71). Signal intensities for IFN-γ and IL-6 were determined by ribonuclease protection assay (MCK-1 probe set; BD Pharmacien, San Diego, CA) and corrected for loading variations using the rpL32 signal.

Sequence analysis. Sequence analysis and contig assembly were performed with MacVector and AssemblyLign (Accelrys, Madison, WI). We performed multiple alignment and phylogenetic analysis using Clustal W (64) and drew tree diagrams from the Clustal W output using TreeView (43). The human LINCR cDNA was predicted from genomic sequences using the GENSCAN (12) and Fgenes (http://genomic.sanger.ac.uk/) programs. We determined the positions of ubiquitin-like domains in the OASL proteins by searching the National Center for Biotechnology Information conserved domain database (2).

RESULTS

Identification of candidate GARG cDNAs. To identify candidate GARG cDNAs, we constructed a subtracted library enriched in endotoxemia-induced genes and then screened the library by differential hybridization. The subtraction was performed using the SSH technique (17, 18), as described in METHODS. The tester and driver were Rsal-digested cDNA populations prepared from lung RNA of LPS-treated and of Dex-treated ADX mice, respectively. The differential hybridization screening was performed by hybridizing replicate filter lifts of the subtracted library phage with 1) an LPS cDNA probe synthesized from lung RNA of ADX mice killed 2 h after intravenous injection of LPS, 2) an LPS-Dex probe from lung RNA of ADX mice pretreated with Dex and killed 2 h after intravenous injection of LPS, and 3) a control cDNA probe from lung RNA of ADX mice treated with Dex. The criteria for selection of GARG candidate phage plaques were 1) increased signal density on the LPS filter compared with the control filter (induction during endotoxemia) and 2) decreased signal on the LPS-Dex filter compared with the LPS filter (glucocorticoid attenuation). More than 90% of the library clones screened satisfied the first criterion, verifying that the library was highly enriched in endotoxemia-induced genes. From a screening of 6,600 plaques, we identified 599 candidate GARG phage satisfying both criteria (9% of the phage screened).

The candidate phage were analyzed in three stages (see METHODS). The 42 group 1 clones yielded cDNAs for...
12 different genes, 10 of which were subsequently confirmed as GARGs by Northern analysis (below). The group 2 and group 3 clones yielded 19 and 24 additional genes, respectively, of which 13 in each group were confirmed as GARGs. In accordance with the expectation that the randomly picked group 1 clones were likely to be those most abundant in the subtracted library, all had been cloned previously. There were two novel cDNAs (GBP-5 and LINCR) in group 2 and two (I-TAC and OASL-2) in group 3. Because of the method used to eliminate replicate phage, the abundances of clones for the cDNAs in groups 1 and 2 were not determined. However, 11 of the 13 confirmed group 3 GARGs were represented by a single clone. The large proportion of single isolates among the group 3 clones indicates that the screening was not exhaustive.

Candidate gene expression in normal and ADX mice. To confirm the identification of GARG cDNAs and eliminate false positives among the 55 final candidates, we evaluated their expression characteristics by Northern analysis. First, induction during endotoxemia and attenuation by Dex of all candidates were evaluated in normal (nonoperated) mice. Our criteria for a GARG message were 1) an endotoxemia-induced increase in lung message expression of twofold or more and 2) attenuation by Dex of 25% or more of the endotoxemia-induced increase. Thirty-five of the 55 candidate cDNAs met both of these criteria in normal mice (Table 1).

Next, we evaluated the effect of adrenalectomy on the expression characteristics of the remaining candidates. We hoped that for some of these candidates, the absence of endogenous glucocorticoids in ADX mice would allow detection of attenuation by Dex, as we had previously observed for LIX (51). A selection of candidates already confirmed as GARGs in normal mice was also studied in this second, independent experiment comparing normal, ADX, and sham-ADX mice. First, we evaluated the expression of LIX and the novel cDNA LINCR using Northern blots containing individual RNA samples from all mice in each treatment group (Fig. 1A). Although LIX expression in sham-operated and normal mice was similar, the endotoxemia-induced expression of LIX was 10.3-fold greater in ADX mice than the level expressed during endotoxemia in normal mice. Dex attenuated the LPS-induced expression in ADX mice to a level comparable with that of LPS-treated normal mice (with or without Dex), confirming the results of our previous study (51). In contrast to LIX, endotoxemia-induced LINCR message was not increased in ADX compared with normal and sham-operated mice and was attenuated by Dex to a similar extent in all three groups of mice. The remaining candidates and a number of the GARG cDNAs already identified were evaluated by replicate Northern blots of pooled RNA samples (Table 1, Fig. 1B).

All of the candidates tested that met the criteria for GARGs in normal mice in the first experiment were confirmed as GARGs in both normal and ADX mice in this second, independent experiment. Only one additional candidate, P-selectin, was identified as glucocorticoid-attenuated in ADX mice but not normal mice, bringing the total of confirmed GARGs isolated in the screening to 36. However, the evaluation revealed marked differences in the effects of adrenalectomy on LPS-induced expression among GARGs. Adrenalectomy produced marked enhancement of LPS-induced expression of LIX (10-fold) and IL-6 (eightfold) and twofold or greater enhancement of LPS-induced expression of intracellular adhesion molecule (ICAM)-1, P-selectin, IL-1β, MCP-1, MIP-1α, and IFN-inducible GTPase (IIGP). The ADX-enhanced LPS-induced expression of these genes was reduced by Dex to levels similar to those in LPS-Dex-treated normal and sham-operated mice (Fig. 1), which indicates that the enhancement of LPS-induced expression in ADX mice was due to loss of the endogenous glucocorticoid response and not to other effects of adrenalectomy.

Characteristics of the GARG cDNAs. The 36 GARG cDNAs identified in this study include diverse categories of genes (Table 1). Although all were induced by LPS and attenuated by Dex, they exhibited wide quantitative differences in responses to LPS and Dex in normal mice and qualitative differences in the effect of adrenalectomy. IFN-γ, IL-6, LIX, and monokine induced by IFN-γ (MIG), previously identified as GARGs and included in Table 1 for comparison, were not among the 36 cDNAs identified in the screen. This provides a further indication that the screening was not exhaustive. Four of the cDNAs cloned in the GARG screening were new murine sequences: I-TAC, GBP-5, OASL-2, and LINCR. Our results for murine I-TAC screening were new murine sequences: I-TAC, GBP-5, OASL-2, and LINCR. Our results for murine I-TAC were reported previously (71). The sequence features and expression characteristics of murine GBP-5, a new member of the GBP family of large GTPases (46, 72), will be described separately (T. T. Nguyen and J. B. Smith, unpublished data). The complete cDNA sequences of OASL-2 and LINCR are described below.

Our screening focused on genes expressed in the lung but was not designed to select lung-specific genes. Message expression of 15 GARGs was evaluated in brain, lung, heart, liver, spleen, kidney, small intestine, and skeletal muscle. Except for OASL-2 and LINCR (discussed below), expression levels were low or undetectable in all organs of control mice. All 15 GARGs evaluated were substantially induced in at least two other organs in addition to the lung, and most were detectably induced in all organs examined (not shown). We conclude that most GARGs expressed in the lung during endotoxemia are not lung specific.

Identification of a new OASL gene, murine OASL-2. One of the group 3 clones had an incomplete open reading frame with similarity to the human 2′-5′-OASL and murine M1204/OASL-p54 proteins (26, 49, 65). We refer to M1204 and its presumed alternative splice form (GenBank AK010034) as murine OASL-1. To clarify the relationship of our partial cDNA to these genes, we searched the GenBank expressed sequence tag database and identified what proved to be a full-length OASL-2 cDNA (IMAGE clone 3661036). The complete sequence was assigned GenBank accession number AF426289. The OASL-2 cDNA is 2,070 nt,
including a 15-nt poly-A tail after the consensus polyadenylation signal AATAAA at nt 2,031–2,036. The cDNA contains a 1,533-nt open reading frame encoding the 511-amino acid (AA) residue OASL-2 protein, which has a molecular mass of 59 kDa and a predicted isoelectric point of 7.53. The initiating ATG at nt 27–30 is the first ATG in the sequence and occurs in the context GCCatgG, which matches the strong consensus

| Table 1. Identities and expression characteristics of confirmed GARG messages |
|---------------------------------|------------------|-----------------|------------------|
|                                  | Normal           | Adrenalectomized | Adrenalectomized |
|                                  | Induction Factor | Dex Attenuation % | ADX-LPS Enhancement | Dex Attenuation % |
| Adhesion and matrix proteins     |                  |                  |                  |
| ICAM-1 (X52264)                  | 5.9              | 27               | 2.0              | 29               |
| P-selectin (M87861)              | >100             | 16               | 2.3              | 52               |
| Thrombospondin-1 (M87276)       | 7.5              | 60               |                  |                  |
| VCAM-1 (X67783)                  | 6.8              | 61               |                  |                  |
| Cytokines/Chemokines             |                  |                  |                  |
| IFN-γ (K00983)†                  | 37               | 94               | 1.5              | 93               |
| IP-10 (M86829)                   | >100             | 93               | 1.1              | 74               |
| IL-1β (M15131)                   | 9.3              | 29               | 2.1              | 71               |
| IL-6 (J03783)†                   | 14               | 69               | 7.9              | 92               |
| I-TAC (AF179872)                 | >100             | 94               | 0.60             | 87               |
| JEMCP-1 (J04467)                 | >100             | 62               | 3.5              | 73               |
| LIX (U72267)†                    | 5.6              | 11               | 10.3             | 95               |
| MIG (M84815)†                    | >100             | 93               | 0.47             | 84               |
| MIP-1α (J04491)                  | >100             | 61               | 2.3              | 73               |
| IFN-induced GTP-binding proteins|                  |                  |                  |
| GBP family                       |                  |                  |                  |
| GBP-2 (AJ007970)                 | 26               | 69               | 1.1              | 34               |
| GBP-3 (U44731)                   | 30               | 74               | 0.77             | 36               |
| GBP-5 (AF422243)†               | 83               | 73               | 1.2              | 27               |
| IRG-47 family                    |                  |                  |                  |
| IRG-47 (M63630)                  | 21               | 65               |                  |                  |
| GTP2/Tgtp (U15636)               | 13               | 59               |                  |                  |
| GTP1 (AJ007972)                  | 6.7              | 56               |                  |                  |
| IIGP (AJ007971)                  | 63               | 81               | 2.1              | 60               |
| IGTP (U53219)                    | 18               | 40               | 0.85             | 26               |
| MX family                        |                  |                  |                  |
| MX2 (J03368)                     | >100             | 76               |                  |                  |
| Signal transduction              |                  |                  |                  |
| CD40 (M83312)                    | 21               | 75               | 1.3              | 66               |
| IRF-1 (M21065)                   | 17               | 73               | 0.92             | 80               |
| MAD (X53106)                     | 13               | 48               |                  |                  |
| RhoC (X06388)                    | 4.5              | 37               | 1.2              | 27               |
| SOCS-3 (U88328)                  | 11               | 65               | 0.91             | 54               |
| IFN-induced TPR domain family    |                  |                  |                  |
| GARG-16/IFI56 (U43084)           | 91               | 79               | 1.6              | 30               |
| GARG-39 (U43085)                 | 29               | 85               |                  |                  |
| GARG-49/IRG2 (U43086)            | 27               | 56               |                  |                  |
| Other IFN or viral induced       |                  |                  |                  |
| IRG-1 (L32821)                   | >100             | 78               |                  |                  |
| ISG-15 (X56602)                  | 100              | 61               |                  |                  |
| Cig5/Best5/Vig1 (L3989981)       | 53               | 79               | 1.6              | 59               |
| Various                          |                  |                  |                  |
| BRP39 (X90305)                   | 3.3              | 46               |                  |                  |
| Histone 3.3 rep. var. (X13605)   | 2.8              | 82               |                  |                  |
| LINCR (AF321278)*               | 23               | 64               | 1.1              | 82               |
| MacMARCS/SIF2 (X61399)          | 14               | 31               | 1.5              | 36               |
| Ninjurin (U91513)                | 3.7              | 72               |                  |                  |
| OASL-2 (AF426289)*              | >100             | 70               | 1.2              | 40               |
| Thykin (L32973)                  | 19               | 71               |                  |                  |

The table summarizes the results of 2 independent experiments characterizing message expression of the 36 genes identified in the screening plus four additional glucocorticoid-attenuated response gene (GARG) messages studied for comparison. Lung message expression was evaluated 4 hours after intravenous injection of lipopolysaccharide (LPS) or saline with or without dexamethasone (Dex) pretreatment as described in METHODS, using pooled RNA samples (4 mice/group). GenBank accession numbers are in parentheses after the gene names. * New cDNAs cloned in this screening project. Induction factor is the ratio of LPS-induced message level to the level in saline-treated normal (nonoperated) mice. Dex attenuation is the difference between expression levels in LPS-treated and LPS-Dex-treated mice, divided by the difference between expression levels in LPS-treated and saline-treated mice. ADX-LPS enhancement is the ratio of LPS-induced expression in ADX mice to LPS-induced expression in normal mice. Blanks indicate absent data for genes not studied in ADX mice. ADX, adrenalectomized; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; IFN, interferon; IP, IFN-α-inducible protein; IL, interleukin; I-TAC, IFN-inducible T-cell α-chemoattractant; MCP, monocyte chemoattractant protein; LIX, LPS-induced C-X-C chemokine; MIP, macrophage inflammatory protein; GBP, guanylate binding protein; LINCR, lung inducible Neuralized-related C3HC4 RING protein; OASL, oligoadenylate synthetase like; Cig, cytomegalovirus-induced gene; IGTP, IFN-γ-inducible GTPase; IIGP, IFN-inducible inductive protein; SOCS, suppressor of cytokine signaling. ** New cDNAs cloned in this screening project. Induction factor is the ratio of LPS-induced message level to the level in saline-treated nonoperated mice. Dex attenuation is the difference between expression levels in LPS-treated and saline-treated mice. ADX-LPS enhancement is the ratio of LPS-induced expression in ADX mice to LPS-induced expression in normal mice. Blanks indicate absent data for genes not studied in ADX mice. ADX, adrenalectomized; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; IFN, interferon; IP, IFN-γ-inducible protein; IL, interleukin; I-TAC, IFN-inducible T-cell α-chemoattractant; MCP, monocyte chemoattractant protein; LIX, LPS-induced C-X-C chemokine; MIP, macrophage inflammatory protein; GBP, guanylate binding protein; LINCR, lung inducible Neuralized-related C3HC4 RING protein; OASL, oligoadenylate synthetase like; Cig, cytomegalovirus-induced gene; IGTP, IFN-γ-inducible GTPase; IIGP, IFN-inducible inductive protein; SOCS, suppressor of cytokine signaling.
context (A/G)NNatG for translation initiation (32). Murine OASL-2 message was induced >100-fold in lungs of LPS-treated mice and attenuated by Dex (Table 1, Fig. 1). The ~2.5-kb message was expressed in the small intestine of control mice and strongly induced during endotoxemia in the heart, liver, spleen, small intestine, and kidneys (not shown), as well as in the lung (Fig. 1).

The murine OASL-2 and human OASL proteins are highly similar throughout their lengths (Fig. 2), containing 71% identical and 83% similar AA residues. In contrast, murine OASL-2 and OASL-1 share only 45% identical and 18% similar residues. Like the other OASL family members (Fig. 3A), murine OASL-2 contains a ~340-residue amino terminal domain similar to human 2'-5'-oligoadenylate synthetase 1 (OAS-1). Human OAS-1 consists largely of a single OAS domain (Fig. 3A), whereas OAS-2 has two and OAS-3 has three tandem OAS repeats (not shown). The OASL domains of the OASL family members are 37–43% identical to human OAS-1. In contrast, the murine OAS-1 and human OAS-1 proteins are 67% identical.

The COOH-terminal regions of the murine and human OASL proteins consist of two tandem ubiquitin-like domains not present in the OAS proteins in these species (Fig. 3A). The protein we refer to as chicken OASL, encoded by what was originally described as the chicken OAS-A gene, has a similar pair of ubiquitin-like domains in its COOH-terminal region (62, 73). The exon-intron structures of this chicken gene and human OASL are identical (62). A phylogenetic analysis of the OASL and OAS-1 proteins in several species, plus the individual OAS domains of human OAS-2 and OAS-3, confirms the close relationship of murine OASL-2 to human OASL (Fig. 3B). The murine and human OASL proteins cluster in a distinct subgroup adjacent to chicken OASL. The preservation of high similarities among the human, murine, and chicken OASL genes suggests that their COOH-terminal ubiquitin-like domains have an important, conserved function.

Identification of a novel Neuralized-related RING domain protein, LINCR. The LINCR cDNA was represented by a single group 2 candidate clone containing a 2,078-nt insert with an incomplete open reading frame at the 5'-end. Northern blotting using the insert as a probe identified a single 2.8-kb band in lung RNA from LPS-treated mice. Basic local alignment search tool (BLAST) searching revealed no significant matches to known sequences. Four full-length LINCR clones were obtained by screening a nonsubtracted library prepared from the same RNA used for the tester population of the subtracted library. Two clones were completely sequenced on both strands and were identical except for a silent polymorphism at nt 105 of the 5'-oligoadenylate synthetase 1 (OAS-1). Human OAS-1 consists largely of a single OAS domain (Fig. 3A). The protein we refer to as chicken OASL, encoded by what was originally described as the chicken OAS-A gene, has a similar pair of ubiquitin-like domains in its COOH-terminal region (62, 73). The exon-intron structures of this chicken gene and human OASL are identical (62). A phylogenetic analysis of the OASL and OAS-1 proteins in several species, plus the individual OAS domains of human OAS-2 and OAS-3, confirms the close relationship of murine OASL-2 to human OASL (Fig. 3B). The murine and human OASL proteins cluster in a distinct subgroup adjacent to chicken OASL. The preservation of high similarities among the human, murine, and chicken OASL genes suggests that their COOH-terminal ubiquitin-like domains have an important, conserved function.

Identification of a novel Neuralized-related RING domain protein, LINCR. The LINCR cDNA was represented by a single group 2 candidate clone containing a 2,078-nt insert with an incomplete open reading frame at the 5'-end. Northern blotting using the insert as a probe identified a single 2.8-kb band in lung RNA from LPS-treated mice. Basic local alignment search tool (BLAST) searching revealed no significant matches to known sequences. Four full-length LINCR clones were obtained by screening a nonsubtracted library prepared from the same RNA used for the tester population of the subtracted library. Two clones were completely sequenced on both strands and were identical except for a silent polymorphism at nt 105 of the 5'-oligoadenylate synthetase 1 (OAS-1). Human OAS-1 consists largely of a single OAS domain (Fig. 3A). The protein we refer to as chicken OASL, encoded by what was originally described as the chicken OAS-A gene, has a similar pair of ubiquitin-like domains in its COOH-terminal region (62, 73). The exon-intron structures of this chicken gene and human OASL are identical (62). A phylogenetic analysis of the OASL and OAS-1 proteins in several species, plus the individual OAS domains of human OAS-2 and OAS-3, confirms the close relationship of murine OASL-2 to human OASL (Fig. 3B). The murine and human OASL proteins cluster in a distinct subgroup adjacent to chicken OASL. The preservation of high similarities among the human, murine, and chicken OASL genes suggests that their COOH-terminal ubiquitin-like domains have an important, conserved function.

Identification of a novel Neuralized-related RING domain protein, LINCR. The LINCR cDNA was represented by a single group 2 candidate clone containing a 2,078-nt insert with an incomplete open reading frame at the 5'-end. Northern blotting using the insert as a probe identified a single 2.8-kb band in lung RNA from LPS-treated mice. Basic local alignment search tool (BLAST) searching revealed no significant matches to known sequences. Four full-length LINCR clones were obtained by screening a nonsubtracted library prepared from the same RNA used for the tester population of the subtracted library. Two clones were completely sequenced on both strands and were identical except for a silent polymorphism at nt 105 of the 5'-oligoadenylate synthetase 1 (OAS-1). Human OAS-1 consists largely of a single OAS domain (Fig. 3A). The protein we refer to as chicken OASL, encoded by what was originally described as the chicken OAS-A gene, has a similar pair of ubiquitin-like domains in its COOH-terminal region (62, 73). The exon-intron structures of this chicken gene and human OASL are identical (62). A phylogenetic analysis of the OASL and OAS-1 proteins in several species, plus the individual OAS domains of human OAS-2 and OAS-3, confirms the close relationship of murine OASL-2 to human OASL (Fig. 3B). The murine and human OASL proteins cluster in a distinct subgroup adjacent to chicken OASL. The preservation of high similarities among the human, murine, and chicken OASL genes suggests that their COOH-terminal ubiquitin-like domains have an important, conserved function.

Identification of a novel Neuralized-related RING domain protein, LINCR. The LINCR cDNA was represented by a single group 2 candidate clone containing a 2,078-nt insert with an incomplete open reading frame at the 5'-end. Northern blotting using the insert as a probe identified a single 2.8-kb band in lung RNA from LPS-treated mice. Basic local alignment search tool (BLAST) searching revealed no significant matches to known sequences. Four full-length LINCR clones were obtained by screening a nonsubtracted library prepared from the same RNA used for the tester population of the subtracted library. Two clones were completely sequenced on both strands and were identical except for a silent polymorphism at nt 105 of the 5'-oligoadenylate synthetase 1 (OAS-1). Human OAS-1 consists largely of a single OAS domain (Fig. 3A). The protein we refer to as chicken OASL, encoded by what was originally described as the chicken OAS-A gene, has a similar pair of ubiquitin-like domains in its COOH-terminal region (62, 73). The exon-intron structures of this chicken gene and human OASL are identical (62). A phylogenetic analysis of the OASL and OAS-1 proteins in several species, plus the individual OAS domains of human OAS-2 and OAS-3, confirms the close relationship of murine OASL-2 to human OASL (Fig. 3B). The murine and human OASL proteins cluster in a distinct subgroup adjacent to chicken OASL. The preservation of high similarities among the human, murine, and chicken OASL genes suggests that their COOH-terminal ubiquitin-like domains have an important, conserved function.

Identification of a novel Neuralized-related RING domain protein, LINCR. The LINCR cDNA was represented by a single group 2 candidate clone containing a 2,078-nt insert with an incomplete open reading frame at the 5'-end. Northern blotting using the insert as a probe identified a single 2.8-kb band in lung RNA from LPS-treated mice. Basic local alignment search tool (BLAST) searching revealed no significant matches to known sequences. Four full-length LINCR clones were obtained by screening a nonsubtracted library prepared from the same RNA used for the tester population of the subtracted library. Two clones were completely sequenced on both strands and were identical except for a silent polymorphism at nt 105 of the 5'-oligoadenylate synthetase 1 (OAS-1). Human OAS-1 consists largely of a single OAS domain (Fig. 3A). The protein we refer to as chicken OASL, encoded by what was originally described as the chicken OAS-A gene, has a similar pair of ubiquitin-like domains in its COOH-terminal region (62, 73). The exon-intron structures of this chicken gene and human OASL are identical (62). A phylogenetic analysis of the OASL and OAS-1 proteins in several species, plus the individual OAS domains of human OAS-2 and OAS-3, confirms the close relationship of murine OASL-2 to human OASL (Fig. 3B). The murine and human OASL proteins cluster in a distinct subgroup adjacent to chicken OASL. The preservation of high similarities among the human, murine, and chicken OASL genes suggests that their COOH-terminal ubiquitin-like domains have an important, conserved function.

Identification of a novel Neuralized-related RING domain protein, LINCR. The LINCR cDNA was represented by a single group 2 candidate clone containing a 2,078-nt insert with an incomplete open reading frame at the 5'-end. Northern blotting using the insert as a probe identified a single 2.8-kb band in lung RNA from LPS-treated mice. Basic local alignment search tool (BLAST) searching revealed no significant matches to known sequences. Four full-length LINCR clones were obtained by screening a nonsubtracted library prepared from the same RNA used for the tester population of the subtracted library. Two clones were completely sequenced on both strands and were identical except for a silent polymorphism at nt 105 of the 5'-oligoadenylate synthetase 1 (OAS-1). Human OAS-1 consists largely of a single OAS domain (Fig. 3A). The protein we refer to as chicken OASL, encoded by what was originally described as the chicken OAS-A gene, has a similar pair of ubiquitin-like domains in its COOH-terminal region (62, 73). The exon-intron structures of this chicken gene and human OASL are identical (62). A phylogenetic analysis of the OASL and OAS-1 proteins in several species, plus the individual OAS domains of human OAS-2 and OAS-3, confirms the close relationship of murine OASL-2 to human OASL (Fig. 3B). The murine and human OASL proteins cluster in a distinct subgroup adjacent to chicken OASL. The preservation of high similarities among the human, murine, and chicken OASL genes suggests that their COOH-terminal ubiquitin-like domains have an important, conserved function.
scribed below, between LINCR and other conserved proteins. In contrast, translations of other open reading frames in the LINCR cDNA had no detectable homology to other proteins in any species. There is no in-frame upstream stop codon before ATG 61–63, so the possibility of an upstream ATG not included in the LINCR cDNA sequence cannot be ruled out entirely. However, the observed mRNA size of 2.8 kb is consistent with the 2,573-nt length of the cDNA plus a 200-nt poly-A tail.

The predicted murine LINCR protein contains 254 AA residues, with a molecular mass of 28 kDa and isoelectric point of 6.50. No transmembrane regions are predicted. The COOH-terminal region contains a C3HC4-type RING domain (21, 27, 28), with the spacing C-X(2)-C-X(11)-C-X(1)-H-X(3)-C-X(2)-C-X(10)-C-X(2)-C

Fig. 4. LINCR has sequence similarities to Drosophila Neuralized (Neur), human and murine homologs of Neur, and Caenorhabditis elegans F10D7.5 (10, 42, 47, 52). Each of these proteins contains a COOH-terminal RING domain with similar spacing and ~30% identity to the RING domain of murine LINCR. These proteins also contain two copies of a distinctive 153–156 AA region, the Neur repeat (NR) domain, Fig. 5A. In contrast, murine LINCR contains a single NR domain. In Neur and its homologs, the two NR domains are related but not identical. The first (NR1) and second (NR2) domains of murine Neur are only 25% identical to each other but are 97 and 95% identical, respectively, to the NR1 and NR2 domains in human Neur. The phylogenetic analysis shows that the single NR domain of murine or human LINCR occupies a position intermediate between the NR1 and NR2 domains of the other proteins (Fig. 5B). The murine and human LINCR domains are 27–33% identical to both the NR1 and NR2 domains of Drosophila Neur and C. elegans F10D7.5. However, the murine and human LINCR NR domains have greater similarity to the NR1 domains of
the murine and human Neur proteins (39–41% identical) than to their NR2 domains (26–29% identical). This suggests that an ancestral mammalian LINCR gene may have evolved from a duplicated Neur gene by deletion of its second NR domain.

Murine LINCR mRNA was strongly induced in the lung during endotoxemia and attenuated by Dex (Table 1, Fig. 1). These characteristics were essentially unaffected by adrenalectomy or sham operation. However, expression of LINCR in saline-treated ADX mice was threefold higher than in normal or sham-operated mice (Fig. 1A), indicating that basal lung expression of LINCR is sensitive to the levels of glucocorticoids normally circulating in unstressed mice. LINCR mRNA was also induced in heart and kidney and was constitutively expressed in the small intestine (not shown).

LINCR expression in the lung was evaluated 1, 2, 4, and 8 h after LPS injection and in controls (3 mice per group). After a rapid rise to a peak at 1 h, LINCR expression remained high for 4 h then decreased at 8 h to 50% of the initial peak value (not shown).

Identification of a predicted human LINCR homolog. A translated BLAST search of the GenBank draft human genome sequence database using the murine LINCR protein sequence identified a closely related gene on human 2q56 (AF321278), the predicted human LINCR sequence in Fig. 4, D. melanogaster Neur (S35503), human Neur (U87864), murine Neur (m-neu1, Y15160), and C. elegans Neur (F10D7.5 (T16028)).
man chromosome 2p11 (GenBank NT026970.3). Analysis of the genomic sequence identified exons encoding the predicted sequence of human LINCR (Fig. 4). Within the region shown, human LINCR is 67% identical and 9% similar to murine LINCR. The NH2-terminal region of human LINCR is not shown in Fig. 4 because alternative exon predictions for this region could not be distinguished. To date, we have not identified a cDNA clone encoding the complete human LINCR. However, the portion of human LINCR shown in Fig. 4 is encoded by a segment of IMAGE clone 3346442 (GenBank BC012317). Despite our uncertainty about its amino terminal region, human LINCR is likely to be similar to murine LINCR in containing only a single NR domain, because no second NR domain occurs within the 60 kb of contiguous sequence upstream of this gene, or indeed anywhere in the available human chromosome 2 sequence.

**DISCUSSION**

Glucocorticoid hormones act via the glucocorticoid receptor to modulate the expression of a large number of genes involved in inflammatory and immune responses and other processes (1, 39). This study focuses on the subset of genes whose induction in response to inflammatory stimuli is attenuated by glucocorticoids, which we refer to as GARGs to distinguish them from noninduced genes whose expression is reduced by glucocorticoids. Our underlying hypothesis is that GARG expression characteristics define a large subset of inflammation-related genes and that identifying and determining the functions of GARGs participating in specific disease processes will reveal new targets for therapeutic intervention.

The results of this study, the first to attempt the systematic identification of GARGs in an in vivo model, indicate that genes with these regulatory characteristics are numerous and diverse. As expected, some of the 36 cDNAs cloned and verified as GARGs by Northern analysis in this study (Table 1) represent well-known proinflammatory mediators, including IL-1β, the chemokines IP-10, MCP-1, and MIP-1α, and adhesion proteins ICAM-1, P-selectin, IL-1β, MCP-1, MIP-1α, and IIGP (Table 1). ADX-induced enhancement of message expression has been described for IFN-γ, IL-1β, IL-6, and a few other cytokines in the spleen during murine cytomegalovirus infection (53) and for IL-1β and IL-6 in spleens of LPS-treated rats (45, 55). Interestingly, the LPS-induced expression of the IFN-γ-induced chemokines MIG and I-TAC was reduced 40–50% in ADX compared with normal and sham-operated mice (Table 1, Fig. 1), whereas expression of the closely related IFN-γ-induced chemokine IP-10 was unaffected by adrenalectomy. The mechanisms responsible for these gene-specific effects of adrenalectomy have not been studied. Perhaps the genes showing the largest ADX-induced increases in LPS-induced expression are those most sensitive to attenuation by endogenous glucocorticoids at the levels induced during endotoxemia. Alternatively, the LPS-induced expression of specific genes might be affected by loss of adrenal catecholamines and increased noradrenaline secretion or by other compensatory responses to adrenalectomy.

The idea of finding new potential targets for anti-inflammatory treatments by identifying genes with GARG regulatory characteristics is applicable to other inflammatory models and is not limited to the specific techniques used in this study. Microarrays are now more widely available than at the time this study was initiated and would likely be the method of choice for future studies. Nevertheless, microarrays remain ex-
pensive and do not, as yet, provide true genome-wide coverage, so screening of subtracted libraries should continue to be valuable for certain applications. Some of the techniques developed for this study may be useful to other investigators. Although the subtracted cDNAs produced by the SSH procedure can be easily cloned into plasmid vectors, differential screening of λ-phages is more sensitive and reproducible in our experience (59). Unfortunately, the standard SSH adapters do not include sites convenient for cloning into λ. Rather than alter the SSH adapters, which might have unpredictable effects on the efficiency of the suppression PCR (17), we designed adapters that facilitated both the cloning into λ Zap II and, by incorporating convenient restriction and primer sites, the downstream analysis of phage inserts. The approaches we used to identify phage inserts by PCR and to eliminate redundant phage by hybridization avoided repeated differential screenings for plaque purification and greatly reduced sequencing costs.

One of the cDNAs we cloned encoded a new OASL gene (26, 49, 65), which we call murine OASL-2. The functions of the OASL genes are unknown, but the OASs have a well-defined function in a regulated RNA decay pathway involved in the antiviral and growth inhibitory effects of IFNs (30, 48, 56). When activated by double-stranded (ds) RNA, the OAS enzymes catalyze the production of a mixture of 2′–5′-linked oligomers of adenylate (2–5A), whose only known function is to activate the latent ribonuclease RNase L. RNase L activation requires 2–5A oligomers containing three or more adenylates.

The OASL genes contain an NH2-terminal OAS-like domain plus two characteristic COOH-terminal ubiquitin-like domains not present in the OAS genes (Figs. 2 and 3A). Intrinsic ubiquitin-like domains may participate in regulating the activity or stability of some proteins (26), but the role of the ubiquitin-like domains in the OASL proteins has not been determined. The function of the OAS domain in the OASL proteins is uncertain also. Neither the p-59 and p-56 forms of human OASL had any detectable RNase L activity (26, 49). The murine OASL-1 (M1204), cloned from a murine spleen dendritic cell library, was expressed in thymus, bone marrow, and mature dendritic cells, but not in the other leukocytes tested, and was localized by in situ hybridization to dendritic cells in spleen and lung (65). Future studies directly comparing the expression and regulation of murine OASL-1 and OASL-2 may provide useful clues about their functions.

We found that murine OASL-2 message was easily detected in the small intestine but not the spleen in control mice and, during endotoxemia, was strongly induced in the lung, heart, liver, spleen, and kidneys (not shown). In contrast, expression of human OASL message in small intestine was low, with greater expression in spleen, peripheral blood leukocytes, and other tissues (26). In cell lines, human OASL message was induced by IFN-α or IFN-γ (26, 49). Murine OASL-1 (M1204), cloned from a murine spleen dendritic cell library, was expressed in thymus, bone marrow, and mature dendritic cells, but not in the other leukocytes tested, and was localized by in situ hybridization to dendritic cells in spleen and lung (65). Future studies directly comparing the expression and regulation of murine OASL-1 and OASL-2 may provide useful clues about their functions.

The LINCR cDNA encodes a novel protein related to the Drosophila neuralized gene product, which is involved in Notch pathway-dependent cell fate decisions (34, 35, 75). The Neur protein is localized primarily to the plasma membrane (34, 75). It contains two copies of the distinctive NR domain, plus a COOH-terminal C3HC4 RING domain (10, 47). Recent studies have shown that the RING domains of Drosophila and Xenopus Neur have ubiquitin E3 ligase activity and that at least one of the targets of ubiquitination by Neur is the Notch ligand Delta (16, 33, 44, 74). Human and murine homologs of Drosophila neur have also been identified recently. Human Neur was cloned as potential tumor suppressor gene on chromosome 10 at a site frequently deleted in malignant astrocytomas (42). Remarkably, the murine and human Neur proteins are 94% identical (52, 67), suggesting that they serve a critical, highly conserved function.

Murine LINCR and its predicted human homolog on chromosome 2 (Fig. 4) share a COOH-terminal RING domain similar to those in murine, human, and Drosophila Neur, and in C. elegans F10D7.5 but have only a single NR domain (Fig. 5). Murine Neur was expressed in multiple tissues in the embryo but was detected in adults only in brain (52), consistent with functions in development and the nervous system. In contrast, LINCR mRNA was expressed in the adult mouse small intestine, a tissue constantly exposed to inflammatory stimulation by luminal bacteria. LINCR expression was also induced in the lung during endotoxemia and attenuated by Dex (Fig. 1 and data not shown). LINCR’s expression pattern and structural similarity to Neur suggest the hypothesis that it may act as an E3 ligase to target for degradation one or more components of signaling pathways involved in innate immune or cell differentiation responses.

The 36 cDNAs identified in this study probably represent only a fraction of the GARGs involved in the lung response to endotoxemia. We know that the differential screening was not exhaustive, because eleven of the 13 confirmed GARGs from the group 3 candidate pool were single isolates. Furthermore, known GARGs including IFN-γ, IL-6, ILIX, and M1G (Table 1) were not identified in the screening, perhaps because their mes-
sage expression was low at the 2-h time point chosen for the differential screening. Injection of LPS is expected to trigger a cascade of immediate-early, early, and late gene induction, so we would expect to identify different sets of GARGs if the evaluation were performed at other time points. Together, our results suggest that future studies using microarrays or other techniques could identify many additional glucocorticoid-attenuated response genes whose functions and roles in lung inflammation are presently unknown.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-57008 to J. B. Smith.

Present addresses: Heather Hughes, Dept. of Neurobiology and Behavior, Univ. of California, Irvine, CA; Leonor Rovai, Manufacturing Research & Technology, Alpha Therapeutic Corp., Los Angeles, CA.

REFE RENCES

38. Masferrer JL, Seibert K, Zweifel B, and Needleman P. Endogenous glucocorticoid-attenuated response genes whose functions and roles in lung inflammation are presently unknown.


