Interleukin-10Induces Uteroglobin-related Protein (UGRP) 1 Gene Expression in Lung Epithelial Cells through Homeodomain Transcription Factor T/EBP/NKX2.1*[]

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UGRP1 is a downstream target gene for homeodomain transcription factor T/EBP/NKX2.1, which is predominantly expressed in lung epithelial cells, and may play an anti-inflammatory role in lung inflammation. To understand the role of UGRP1 in inflammation, its expression was investigated in relation to cytokine signaling. In vivo experiments using mouse embryonic lung organ culture and intranasal administration of interleukin (IL) 10 revealed that constitutive expression of Ugrp1 mRNA is enhanced by IL-10. Increase of protein levels was also demonstrated by immunohistochemistry using embryonic lungs. This IL-10 induction of Ugrp1 gene expression occurs at the transcriptional level when examined using mouse embryonic lung primary cultures. In human lung NCI-H441 cells that in contrast to mouse lung cells, do not exhibit constitutive expression of the gene, expression of the UGRP1 gene was induced in a rapid and stable fashion. Two T/EBP, but not STAT3, binding sites located in the human UGRP1 gene promoter are responsible for IL-10 induction of the UGRP1 gene as judged by transfection, gel shift, and chromatin immunoprecipitation analyses. The IL-10 receptor chains, IL-10R1 and IL-10R2, are expressed in H441 cells, however, STAT3 was only weakly activated upon IL-10 treatment. In contrast, STAT3 was strongly activated when the cells were treated with other cytokines such as IL-22 and interferon-β but UGRP1 expression was not increased. Together these results demonstrate that IL-10 induces UGRP1 gene expression in lung epithelial cells through a T/EBP/NKX2.1-dependent pathway. The results further suggest that UGRP1 might be a target for IL-10 anti-inflammatory activities in the lung.

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Uteroglobin-related protein 1 (UGRP1) was originally identified as a downstream target gene for homeodomain transcription factor T/EBP (thyroid-specific enhancer-binding protein), also called thyroid transcription factor-1 (TTF-1) or NKX2.1 (1). T/EBP regulates the expression of thyroid- and lung-specific genes including thyroglobulin (2), thyroid peroxidase (3, 4), thyrotropin receptor (5), and Na/I symporter (6) in the thyroid, and surfactant proteins A (7), B (8), and C (9), and Clara cell secretory protein (10) in the lung. T/EBP is expressed in brain, thyroid, and lung epithelium during embryogenesis and is essential for the genesis of these organs (11). In adult lung, the expression of T/EBP is confined to both the conducting airways and type II alveolar epithelial cells (12).

UGRP1, officially named Secretoglobin gene family 3A2 (13), is a novel gene encoding a homodimeric secretory protein of ~10 kDa that is highly expressed in epithelial cells of the trachea, bronchus, and bronchioles (1). Based on our previous results, there is considerable evidence to suggest that UGRP1 may function in the regulation of the local immune response in the lung. First, the human UGRP1 gene is located on chromosome 5q31-q32, a region where one of the asthma susceptibility genes was assigned and genes coding for several Th2-type cytokines such as interleukin (IL)-4, IL-5, and IL-13 are located (13). Second, the UGRP1 amino acid sequence exhibits similarity to the UG/Clara cell secretory protein, which is known to function as an anti-inflammatory agent via inhibition of phospholipase A2 (1). Third, a polymorphism (G/A) was identified in the human UGRP1 gene promoter that is associated with an increased risk of asthma in a Japanese population of adult asthmatic patients (14). Lastly, the mRNA level of Ugrp1 is decreased in inflamed mouse lungs, and returns to basal levels following dexamethasone treatment (1). IL-10 is a pleiotropic cytokine that was shown to mediate anti-inflammatory, immunosuppressive, and tissue protective functions. The known IL-10 signaling is through binding to specific receptors IL-10R1 and IL-10R2, which leads to activation of the JAK-STAT (Janus kinase-signal transducers and activators of transcription) signal transduction pathways (15). An anti-inflammatory role for IL-10 was demonstrated by lung cells and bronchoalveolar lavage fluid obtained from IL-
4 °C overnight and embedded in paraffin. Sections (4 μm) with 50 ng/ml IL-10 for 2 days were fixed with 4% paraformaldehyde at 37 °C overnight. The membranes were washed twice with 2× SSC containing 0.5% SDS at 65 °C for 20 min, followed by exposure to a Storm PhosphorImage screen and the signals were visualized with ImageQuant software (Amersham Biosciences).

**PCR-based Nuclear Run-on Experiments—** Mouse embryonic lung primary culture was used as a source for PCR nuclear run-on experiments. Briefly, E16.5 mouse embryonic lungs were incubated in Dulbecco’s modified Eagle’s/F-12 media containing 10% fetal bovine serum, 20 units/ml Dispase I (Roche Applied Science), and 10,000 units/ml collagenase (Sigma) at 37 °C for 30 min with shaking. Dispersed cells were washed three times in Dulbecco’s Modified Eagle’s/F-12 media containing 10% fetal bovine serum by centrifugation at 3,000 rpm at room temperature. Individual cells were plated out at the density of 1 × 10^6 cells per well in a 6-well dish and cultured in a CO2 incubator at 37 °C overnight. Cells were then treated with 100 ng/ml IL-10 or mock-treated with PBS for 2 h before harvest in PBS. Harvested cells were lysed by pipetting 10–15 times in 5 volumes of nucleus isolation buffer containing 10 mM Tris, 50 mM NaCl, 3 mM MgCl2, 0.5% NP-40, and 1 mM PMSF (Sigma), and nuclei were collected by centrifugation at 14,000 × g for 5 min at 4 °C. Nuclei and the supernatant were separately subjected to RNA isolation using TRIzol to obtain nuclear and cytosolic total RNAs.

Quantitative RT-PCR analysis was performed with ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) to determine nuclear level Ugrp1 pre- and/or post-processed transcript in the nuclei and/or cytosol. Total RNAs isolated from either nuclei or cytosol were treated with DNase I (Ambion, Austin, TX) to eliminate contaminating genomic DNAs. Reverse transcription of isolated RNAs was carried out by using random hexamers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). To detect the Ugrp1 transcript, two sets of primers were used (Fig. 2D): F1 (5′-AGTTCCATCCGAGTTGAAGACAGG-TATGC-3′) and R1 (5′-TTTGCTCACAGACAGGC- TGTGTCATTTGCGGGGGC-3′) are specific to the sequences in intron 1 and amplify only pre-spliced transcripts, whereas F2 (5′-GGTTATTCTGCACTGCTCCTTCTC-3′) and R2 (5′-TACCGAGTGGAAGACGCTCAG-3′) primer sequences are derived from the junctions of exon 1 and 2 and 2 and 3, respectively, and amplify only post-spliced transcripts. Reactions were performed using SYBR Green master mixture (Applied Biosystems, Foster City, CA) and the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s (pre-processed specific primers) or 62 °C for 30 s (post-processed specific primers) for 30 s, and 72 °C for 30 s. Data were analyzed by the standard curve method, and normalized for 18 S rRNA, which was measured by using the following primer pair: 5′-GGCTCTACACTCCAAGGGAA-3′ (forward) and 5′-ATGAGCAGCGAGCGCCACAG-3′ (reverse).

**RT-PCR Analyses—** The RT reactions were performed in a final volume of 20 μl containing DNase I-pretreated RNA (2.5 μg), 4 μl of 5× first strand synthesis buffer (Invitrogen, Carlsbad, CA), 1 μl of a mixture of four dNTPs (2 μM each), 2 μl of 0.1× dithiothreitol, and 50 ng of oligo(dT) primers. After incubation at 42 °C for 2 min, 200 units of Superscript II reverse transcriptase (Invitrogen) was added, and the incubation was continued for 50 min. PCR was carried out using 0.5 μl of the reaction mixture, Advantage 2 polymerase (BD Biosciences, Palo Alto, CA), and the following oligonucleotide primers: human IL-10 R1 (forward, 5′-GCTCTAGGTAGTTGAATAGATCTG-3′; reverse, 5′-TA-TGTGCTATTTGGCAGGGGC-3′), human IL-10 R2 (forward, 5′-TGGATTGACACACATTGGACCC-3′; reverse, 5′-TTTGTCACAGCAAGCGGTT-3′; forward, 5′-GGACACAGTGACTGAC-CAGGTTG-3′; reverse, 5′-TTCCTCCCTTCCTCCACCAGG-3′), and human IL-12 R1 (forward, 5′-AATGTGGAAGAGTGTCGGCGGAC-3′; reverse, 5′-TGGCGAGATGACGAAATGAGC-3′).

Luciferase Reporter Plasmid Construction and Transfection—Sequence-specific primers were used to design a reporter construct in pGL3 plasmid (Promega, Madison, WI) containing various lengths of regulatory elements of human UGRP1 gene promoter as follows: -31 bp UGRP1, -5′-GGCTGTCATGAGATCTTGCCTTGCCTTCAGG-3′; -76 hUGRP1, 5′-GGGTGCTACGGGGTGTACTAGGACTAG-3′; -100 hUGRP1, 5′-GGGTGCTACGGGGTGTACTAGGACTAG-3′; -178 hUGRP1, 5′-GGGTGCTACGGGGTGTACTAGGACTAG-3′; -222 hUGRP1, 5′-GGGTGCTACGGGGTGTACTAGGACTAG-3′; -5′AGATCGGTTTGGGATTCG-3′.

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TTTC-3’, (common reverse primer). The construct – 209 hUGRP1 was as previously described (1).

The following oligonucleotides were used to make pGL3-M1, M2, M1’ and –76M5 mutant plasmids (mutated nucleotides boldfaced with underline): M1, 5’-GGGCTCTAGCAGGAGGTTTTATAGGCGGAGGAGAGGAAATACTG-3’, M2, 5’-GGGCTCTAGCAGGAGGTTTTATAGGCGGAGGAGAGGAAATACTG-3’, M3, 5’-GGGCTCTAGCAGGAGGTTTTATAGGCGGAGGAGAGGAAATACTG-3’, M4, 5’-AGATCTGGTCCCTGGAATATTTTTC-3’, and M5, 5’-AGATCTGGTCCCTGGAATATTTTTC-3’. (common reverse primer). The PCR fragments were digested with NheI (5’ side) and XhoI, and inserted into the NheI and XhoI sites of the pGL3 plasmid. For mutants M1’, M2’, M3’, and M2*2’, the reverse primer 5’-CTGAACTTGTCCCTACACCTGAGCTACCCACC-C-3’ was used in combination with the above forward primers, and the resultant PCR fragment was digested with NheI and EcoRI (located downstream of the 3rd T/EFP binding site), and swapped with the NheI-EcoRI fragment of the –209 hUGRP1 construct.

A series of UGRP1 luciferase reporter constructs and plasmid pRL-TK as a normalization control for transfection efficiency, and pCMV4-T/EFP (22) or pCMV4 were co-transfected using Opti-MEM media and Lipofectamine (Invitrogen) into H441 cells that were preincubated without serum for 24 h. IL-10 (25 ng/ml) was added 12 h after transfection, and 48 h later, cells were lysed, and luciferase activities were assayed using the luciferase assay system (Promega).

Nuclear extracts of NCI-H441 cells were prepared using NE-PER (Pierce, Rockford, IL) according to the manufacturer’s instructions. The following oligonucleotide sequences were used as probes or competitors: GRR (5’-AGTATTGGCCACAGAAA-3’) (23), PRL (5’-AGGATTCGTTGGG-3’); Nkx2.1-dependent IL-10 Induction of UGRP1 Gene Expression

**RESULTS**

IL-10 Induces UGRP1 Gene Expression—UGRP1 mRNA is constitutively expressed in adult as well as embryonic mouse lungs (Fig. 1) (1). The mRNA levels were found to be augmented by IL-10 when embryonic day (E) 15.5 lungs were organ-cultured in the presence of IL-10 and mice were intranasally administered IL-10. Thus, the organ-cultured embryonic lungs exhibited an increase in Ugrp1 mRNA levels in a statistically significant time-dependent manner at 48 h after the initiation of IL-10 treatment (Fig. 1A), and intranasal IL-10 administration demonstrated a dose-dependent increase of Ugrp1 mRNA levels after 24 h, with 600 ng being statistically significant (Fig. 1B). When embryonic lungs were subjected to immunohistochemistry, the increase in UGRP1 protein expression, which correlated with increased Ugrp1 mRNA expression, was observed in mouse lungs. In the nuclei, the levels of pre-spliced and post-spliced Ugrp1 mRNA were not significantly different between IL-10-treated and control mice (Fig. 1C).

Northern blotting after an additional 6-h incubation period. Northern blotting after an additional 6-h incubation period. Anti-IL-10R1 Antibody Treatment—

**Phospho-STAT3 Western Blots—** The levels of tyrosine-phosphorylated STAT3 were measured by Western blotting as previously described (28). Briefly, H441 cells were treated with various cytokines (50 ng/ml each) for 30 min at 37 °C. At the end of this incubation period, the cells were lysed, and STAT3 protein was immunoprecipitated using a rabbit anti-STAT3 antibody (Santa Cruz Biotechnology). The levels of activated STAT3 were measured using a mouse monoclonal anti-phospho-STAT3 (Tyr-705) antibody (Cell Signaling Technology, Beverly, MA).

Anti-IL-10R1 Antibody Treatment—H441 cells were pretreated for 30 min at 37 °C with or without a monoclonal antibody (3F9) (5 ng/ml) that was obtained in purified form from BD Pharmingen (San Diego, CA) and is known to specifically block the binding of IL-10 to the IL-10 receptor (29). IL-10 (50 ng/ml) was then added, and total RNA was prepared for Northern blotting after an additional 6-h incubation period.

**Analysis of UGRP1 Gene Promoter—** To determine the transcriptional regulatory elements in the UGRP1 promoter region, we used the luciferase assay to measure transcriptional activity of various reporter constructs. The UGRP1 promoter region was divided into three fragments (Fig. 2A). The first fragment, from –803 to –209 bp, was found to be essential for the induction of Ugrp1 mRNA expression by IL-10 in H441 cells. The second fragment, from –209 to –3 bp, was also found to be essential for the induction of Ugrp1 mRNA expression by IL-10 in H441 cells. The third fragment, from –3 to +500 bp, was found to be necessary for the induction of Ugrp1 mRNA expression by IL-10 in H441 cells. The results of these experiments demonstrated that IL-10 enhances Ugrp1 gene expression in mouse lungs. To completely determine the IL-10 induced increase in UGRP1 mRNA expression, various lung cell lines were analyzed. Human pulmonary adenocarcinoma NCI-H441 cells, which do not demonstrate any constitutive expression of UGRP1, were found to express UGRP1 mRNA albeit at low levels, upon treatment with IL-10 at 25 and 50 ng/ml (Fig. 2A). The UGRP1 mRNA expression was first observed as early as 2 h after the initiation of treatment, was drastically increased by 4 h, and continued at elevated levels up to 24 h. Interestingly during this period, T/EFP mRNA levels did not significantly change (Fig. 2B). Next, H441 cells were pretreated with either the RNA synthesis inhibitor, actinomycin D, or the protein synthesis inhibitor, cycloheximide (Fig. 2C). The IL-10 induction of UGRP1 mRNA expression was not observed in the presence of actinomycin D, whereas cycloheximide did not have any effect on IL-10-induced UGRP1 mRNA levels, demonstrating that induction of UGRP1 gene expression by IL-10 in H441 cells requires transcription but not a newly synthesized protein(s). Also, of interest was that T/EFP mRNA expression was completely abolished by actinomycin D treatment.

PCR-based nuclear run-on experiments (30) were performed to confirm that the IL-10-induced increase in Ugrp1 mRNA occurred at the transcriptional level. RNA was isolated from nuclei and cytoplasm of primary mouse embryonic lung cells treated with and without IL-10 for 2 h. Specific primers were designed that selectively recognize either pre-processed or post-processed transcripts (Fig. 2D, illustration in upper panel). In the nuclei, the levels of pre-spliced and post-spliced transcripts were, respectively, 13- and 1.6-fold higher with IL-10 treatment than without, whereas in the cytosol, a 3-fold increase of Ugrp1 mRNA levels was obtained as compared with no treatment (Fig. 2D, lower panels). Taken together, the results indicate that IL-10 modulation of UGRP1 gene expression takes place at the transcriptional level.

**Analysis of UGRP1 Gene Promoter—** To determine the transecriptional control of UGRP1 gene expression, a series of reporter constructs were generated using the luciferase assay system. The constructs were then transfected into a mouse embryonic lung cell line, and luciferase activities were assayed after 24 h. The results indicated that IL-10-enhanced UGRP1 gene expression was mediated by the T/EFP binding site. The T/EFP binding site was found to be essential for the induction of Ugrp1 mRNA expression by IL-10 in H441 cells.
scriptional control site(s) for IL-10 induction, the human UGRPI gene promoter activity was examined by transient transfection analysis with reporter plasmids containing various lengths of the human UGRPI gene promoter in the presence or absence of IL-10 in H441 cells that have an endogenous low level expression of T/EBP. Treatment with IL-10 increased reporter gene activity driven by constructs containing sequences between 179 and 209 bp of DNA upstream of the transcription start site of the UGRPI gene (Fig. 3A), suggesting a potential transcriptional control site for IL-10 signal transduction in this region.

In an attempt to identify possible transcription factors that bind to DNA located between −179 and −209 bp that may be responsible for IL-10 induction of UGRPI gene expression, electrophoretic mobility shift analysis (EMSA) was performed using nuclear extracts prepared from H441 cells treated with IL-10 and oligonucleotides containing sequence between −179 and −209 bp of the UGRPI gene promoter, or the binding sites for GRR (STAT1, -3, and -5 binding site), SIE (STAT-1 and -3 binding sites), PRL (STAT5 binding site), C/EBP, Cdx/A, or T/EBP as probes or competitors. The 209-179 probe formed a strong specific complex with nuclear extracts prepared from cells treated with IL-10. Interestingly, the mobility of the complex was identical to that obtained with the T/EBP consensus sequence as a probe (Fig. 4A). The specific protein-DNA complex was only efficiently competed away by unlabeled oligonucleotide (209-179) or T/EBP-specific oligonucleotide, and not by other oligo probes (Fig. 4B). A competition assay was also performed using HTE-1, an enhancer binding element, which IL-10-activated IL-10E1 binds to and induces expression of the tissue inhibitor of matrix metalloproteinase 1 (Fig. 4B, right panel) (27). HTE-1 did not compete with T/EBP for binding to the 209-179 probe, suggesting that IL-10 induction of UGRPI gene expression is...
Mouse embryonic lung primary cultures were treated with 100 ng/ml IL-10 for 2 h. Total RNAs were separately shown in the GAPDH dehydrogenase. All Northern blots were hybridized serially with UGRP1, T/EBP, and GAPDH probe as a loading control. IL-10 treatment only is shown as a positive control. The representative results from two independent experiments are shown.

The 3rd T/EBP binding site is capable of activating gene transcription when T/EBP is present, whereas IL-10 did not enhance promoter activity. Introduction of a mutation into the 3rd T/EBP binding site (−76M3) abolished the T/EBP-stimulated promoter activity as expected. The activity of the −209 construct increased to similar levels by IL-10 treatment or by co-transfection of T/EBP. Both IL-10 and T/EBP overexpression together further increased the promoter activity. Interestingly, promoter activity of the construct −209M3, which has a mutation at the 3rd (proximal) T/EBP binding site, completely abolished the IL-10 effects, suggesting involvement of the 3rd T/EBP binding site in IL-10 activation of the UGRP1 gene.

Co-transfection analysis was further carried out using various mutant constructs: M1 (1st T/EBP binding site mutated), M2 (2nd site mutated), M1*2 (both 1st and 2nd sites mutated), M1*3 (both 1st and 3rd sites mutated), M2*3 (both 2nd and 3rd sites mutated), and M1*2*3 (all three sites mutated). Among three mutants (M1, M2, and M1*2) that have one or two of the distal (−179 to −209 bp) T/EBP binding sites mutated, only mutant M1 exhibited IL-10 induction of the promoter activity in the absence of the co-transfected T/EBP. The M1 construct in the
presence of T/EBP, or the M2 and M1*2 constructs with or without T/EBP did not exhibit any increase upon IL-10 treatment. These results suggest that the 2nd T/EBP binding site is essential for IL-10 induction of UGRP1 gene expression, whereas the 1st site is dispensable, but required for maximal IL-10 induction of UGRP1 gene expression. When the proximal 3rd T/EBP binding site was mutated in addition to the distal sites (M1*3, M2*3, and M1*2*3), IL-10 induction was not observed with any of the constructs, further suggesting a critical role for the 3rd binding site for IL-10 induction of the gene.

Co-transfection of the T/EBP expression plasmid increased promoter activities albeit to different degrees in all mutants having at least one intact T/EBP binding site. The M1*2*3 that has all T/EBP binding sites mutated has almost completely lost both T/EBP and IL-10 induction of promoter activity. These data indicate that both the 2nd and 3rd T/EBP binding sites are essential for IL-10 induction of UGRP1 gene expression, however, all three sites are required for maximal IL-10 induction of promoter activity.

When human and mouse UGRP1 gene promoter sequences are compared, the areas containing the 2nd and 3rd T/EBP binding sites in human UGRP1 gene promoter aligned very well with T/EBP binding regions II and IV in the mouse Ugrp1 gene promoter, previously identified by DNase I footprinting analysis (1) (Fig. 5C). This suggests that IL-10 induction of mouse Ugrp1 gene expression is likely to occur through a similar, if not the same, T/EBP-mediated mechanism.

The requirement of the 2nd and 3rd T/EBP binding sites for IL-10 signal transduction was further confirmed by EMSA using probes 209-179, 80-51, and oligonucleotides having mutations at the 1st (Mut 1), 2nd (Mut 2), both 1st and 2nd (Mut 1*2), and 3rd (Mut 3) T/EBP binding sites as probes and/or competitors (Fig. 5B). As expected, only the Mut 1 oligonucleotide competed for the formation of a specific DNA-protein complex (Fig. 5D).

To determine whether IL-10-induced binding of T/EBP to the UGRP1 gene promoter actually takes place in situ, a ChIP assay was performed. Three sets of primers were used to specifically amplify either distal (1st and 2nd together) or proximal (3rd) T/EBP binding sites or exon 3 of the UGRP1 gene as a nonspecific control (Fig. 6A). Both distal and proximal T/EBP binding sites were specifically amplified only in the IL-10 treated group after precipitating with anti-T/EBP antibody (Fig. 6, B and C). These results are consistent with co-transfection and EMSA, indicating that T/EBP binds in situ upon IL-10 stimulation to the UGRP1 gene promoter at the distal and proximal binding sites.

IL-10 Induces UGRP1 Gene Expression through the IL-10 Receptor, but Does Not Require JAK-STAT Signaling—To understand the mechanism of IL-10 induction of UGRP1 gene expression, RT-PCR was performed to first examine whether the IL-10 receptor chains, IL-10R1 and IL-10R2, are expressed by H441 cells. RT-PCR yielded products of 291 bp for IL-10R1 and 300 bp for IL-10R2 from both H441 cells and human

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**Fig. 3. Determination of IL-10 responsive region.** A, UGRP1 gene promoter deletion analysis in the presence (closed box) or absence (open box) of IL-10 treatment. Constructs are illustrated on the left with possible transcription factor binding sites depicted in B. The relative luciferase activity of cells transiently transfected with the indicated constructs is shown based on the activity obtained with the basic pGL3 vector that was arbitrarily set as 1. Values are the means of three independent experiments ± S.E., each done in triplicate. B, human UGRP1 gene promoter sequence. Relative positions of the 5’ end of each construct are shown by arrowheads. The IL-10 responsive region (−179-209 bp) contains binding sites for transcription factors C/EBP (underlined), Cdx/A (overlined), and T/EBP (boxes 1 and 2). The proximal T/EBP binding site (box 3) is also shown, and the TATA box and translation initiation codon ATG are indicated in boldface. The polymorphic nucleotide previously reported (14) is marked with an asterisk (*).
monocytes (positive control), demonstrating that H441 cells express both receptors (Fig. 7A). The presence of IL-10R1 and IL-10R2 subunits was also confirmed in mouse embryonic and adult lungs by RT-PCR (data not shown).

A family of IL-10-related cytokines, including IL-10, IL-22, IL-26, and IL-28, share a common receptor subunit, IL-10R2 but have distinct receptor 1 subunits (28, 32, 33). The presence of IL-22R1 and IL-28R1 was demonstrated by RT-PCR in H441 cells but not in human monocytes. Low level expression was observed in liver and NCI-A549 cells that were used as positive controls (Fig. 7B). IL-22R1 and IL-28R1 are known to be expressed by epithelial cell lines such as H441 and A549, but are not expressed on hematopoietic cells such as monocytes.

To determine whether IL-10 and other IL-10 family members activate the JAK-STAT pathway in H441 cells, cells were treated with IL-10, IL-19, IL-20, IL-22, or IFN-β/H9252, and the levels of phosphorylated STAT3, the major STAT protein activated by all five cytokines, were examined. It is known that IL-10 stimulation does not result in serine phosphorylation of STAT3 (34). Furthermore, it is interesting to note that H441 cells have constitutive serine phosphorylation of STAT3.2 When the tyrosine phosphorylation of STAT3 was examined, it was predominantly observed in IL-22- and IFN-β/H9252-treated cells, whereas only low levels of activation were detected in IL-10-treated cells (Fig. 7C), indicating that the STAT3 signal transduction pathway is active in H441 cells. Of particular interest is the fact that the induction of UGRP1 mRNA expression was observed only in IL-10-treated cells despite the low level of STAT3 activation compared with IL-22 or IFN-β-treated cells (Fig. 7D). These results indicate that IL-10 induction of UGRP1 gene expression is STAT3-independent.

Finally, to confirm that the induction of UGRP1 gene expression by IL-10 is mediated by signaling through IL-10 receptors, the effect of anti-IL-10R1 antibody on IL-10 induction of UGRP1 mRNA expression was examined (Fig. 7E). This antibody has been shown to specifically block the binding of IL-10 to the IL-10 receptor (29). Northern blotting analysis clearly demonstrated that IL-10 induces UGRP1 mRNA expression by signaling through the IL-10 receptor.

**DISCUSSION**

We report here a novel IL-10 signal transduction pathway for UGRP1 gene expression in lung epithelial cells that is mediated through the IL-10 receptor and the homeodomain transcription factor T/EBP. UGRP1 gene expression was enhanced by IL-10 in *in vivo* and *ex vivo* lungs that have constitutive expression of Ugrp1, whereas the expression was induced by IL-10 in H441 cells where no constitutive expression was found. It is not surprising that we found a difference in expression patterns between *in vivo*/*ex vivo* tissues and a tumor cell line, the latter of which may not express factor(s) that are necessary for constitutive expression of UGRP1. We further found that the IL-10 modulation of UGRP1 gene expression is transcriptional, and T/EBP binding sites, present in the promoter region of the UGRP1 gene, are essential for induction of this gene by IL-10. Our results demonstrate for the first time that T/EBP plays a role in a cytokine signaling pathway in addition to the established roles for this transcription factor in regulating thyroid (2–6) and lung-specific gene expression (7–28).

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**Fig. 4. EMSA.** A, various oligonucleotides were used as probes, including a sequence between −179 and −209 bp of the UGRPI gene promoter (probe 209-179), GRR, SIE, PRL, T/EBP, Cdx/A, and C/EBP. H441 cells were incubated with or without 25 ng/ml IL-10 for 12 h before harvest for nuclear extract (NE) preparation. B, EMSA competition or antibody supershift (Ab) assay using the labeled 209-179 oligonucleotide as a probe and 50-fold excess of various unlabeled specific oligonucleotides as competitors or antibodies as indicated. A supershifted band obtained with antibody against T/EBP is shown by an asterisk. Right panel, competition assays using the HTE-1 oligonucleotide as a competitor. C, EMSA time course. H441 cells nuclear extracts were prepared at various time points after the addition of 25 ng/ml IL-10. All EMSA experiments were repeated at least 3–4 times.

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A. Srisodsai, R. Kurotani, Y. Chiba, F. Sheikh, H. A. Young, R. P. Donnelly, and S. Kimura, unpublished observation.
and in the genesis of the thyroid, lung, and ventral forebrain during embryogenesis (11).

Our results further demonstrate the constitutive expression of IL-10R1 by H441 cells. The IL-10 receptor complex is composed of two receptor chains, IL-10R1 and IL-10R2 (15, 35). The IL-10R1 subunit is primarily expressed by hematopoietic cells such as B cells, T cells, NK cells, monocytes, and macrophages, whereas IL-10R2 is constitutively expressed by most cells and tissues (15, 35). The presence of IL-10R1 in H441 cells and mouse lungs is an addition to a short list of non-hematopoietic cells that express IL-10R1 that includes fibroblasts, epidermal cells, and keratinocytes (35). The constitutive IL-10R1 expression has been described in placental cytotrophoblasts (36) and colonic epithelium (37). The level of IL-10R1 expression in H441 cells may be low because IL-10 treatment yielded weak STAT3 activation as compared with IL-22 or IFN-β. Under low IL-10R1 expression, no tyrosine phosphorylation of STAT3 and STAT1, nor induction of the IL-10 target gene suppressor of cytokine signaling (SOCS) 3 were observed in human neutrophils (38). In our case, the expression of UGRP1 mRNA was highly induced by IL-10 despite low levels of IL-10R1 and STAT3 activation. It is interesting to note that SOCS3 was constitutively expressed in H441 cells.2 We attempted small interfering RNA experiments to suppress STAT3 activity to further demonstrate that STAT3 is not involved in the novel IL-10 signaling pathway, however, the experiments did not work. Further work is required before any conclusions can be reached regarding this point. Furthermore, although our EMSA results did not suggest the involvement of STAT1 or STAT5 in the current novel IL-10 signaling pathway, this possibility cannot be excluded and further experiments are required to address this question.

Currently, the known IL-10 signal transduction pathway involves IL-10R, the receptor-associated Janus tyrosine kinases, JAK1 and TYK2, and the latent transcription factor STAT3 (15, 35). STAT3-independent IL-10 signaling pathways

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**Fig. 5. Analysis of mutant T/EBP binding sites.** A, co-transfection analysis of constructs −76 and −209, and their mutants in the presence or absence of T/EBP and/or IL-10. Mutant constructs are illustrated on the left. The relative luciferase activity of cells transiently transfected with the indicated constructs is shown based on the activity obtained with the basic pGL3 vector that was arbitrarily set as 1. Values are the means of three independent experiments ± S.E., each done in triplicate. B, sequences of mutants used in transfection analysis and EMSA. The putative T/EBP consensus binding site and mutated sequences are shown in boldface. C, alignment of mouse and human UGRP1 gene promoter sequences. T/EBP binding regions or sites for mouse Ugrp1 (II, III, and IV) and human UGRP1 (1, 2, and 3) gene promoters are overlined and underlined, respectively. D, EMSA competition assay using probe 209-179 or 80-51 and 50-fold excesses of unlabeled wild-type or specific mutated oligonucleotides. A T/EBP antibody supershifted band is shown by an asterisk.
have been described for the macrophage de-activation responses to IL-10 in the J774 mouse macrophage cell line (39) and the IL-10 induction of tissue inhibitor of matrix metalloproteinase 1 expression in human prostate cells (27). The mechanism for IL-10 de-activation of macrophage has not yet been fully defined, although phosphorylation of certain cytoplasmic tyrosine residues is required for this process to occur. In the case of tissue inhibitor of matrix metalloproteinase 1, phosphorylated tyrosine residues further phosphorylate the IL-10E1 protein (27), which triggers rapid translocation of IL-10E1 to the nucleus where it binds to a specific enhancer element, termed HTE-1, located in intron 1, upstream of the 5’-ATG translation start site of the tissue inhibitor of matrix metalloproteinase 1 gene. Because the HTE-1 oligonucleotide did not compete for binding to the IL-10 responsive region of the UGRP1 gene in the EMSA binding reaction, it is unlikely that IL-10E1 is involved in the IL-10 signal transduction pathway of UGRP1 induction in H441 cells. On the other hand, T/EBP clearly bound to this region although the T/EBP-specific antibody produced a very faint supershifted band. The finding that only a small component was supershifted upon antibody addition was previously observed when recombinant T/EBP was used in EMSA with mouse Ugrp1 gene promoter binding sequences as probes (1). One of these T/EBP binding sites corresponds very well to the 2nd T/EBP binding site described in the current study. We have no explanation for the weak supershift in this study, but it might be because of conformational differences in the binding of the protein to this specific site, thus reducing the ability of the antibody to recognize the protein or possibly the interaction of other proteins with T/EBP, thus masking the epitope.

The novel IL-10 signaling in H441 cells occurs very rapidly and is stable because the specific DNA-T/EBP complex is formed as early as 2 min, and remains elevated even after 24 h of IL-10 treatment. Whereas the mechanism responsible for IL-10 signaling observed in our study is unknown, T/EBP could be phosphorylated by kinases such as JAK1 and TYK2, and then translocated to the nucleus where it binds to its specific binding sites. In fact, several studies on phosphorylation of T/EBP and its relation to DNA binding and transcriptional activity have been reported. The results vary from phosphorylation by protein kinase C without any changes in binding affinity (40), to phosphorylation by protein kinase A and increase of DNA binding activity and transcription (41, 42). Mammalian sterile 20-like 2 kinase (43) and extracellular signal-regulated kinase (44) were also shown to phosphorylate T/EBP. In all cases, phosphorylation occurs at serine residues.

To determine whether T/EBP is phosphorylated upon IL-10 treatment, we performed the following experiments: 1) addition of the general kinase inhibitor staurosporin (1 μM) in culture media to examine its effect on IL-10 induced UGRP1 mRNA levels; 2) in vivo phosphorylation assays; and 3) Western blotting to demonstrate the effect of IL-10 on the phosphorylation status of T/EBP (Supplemental Materials Fig. 1A). These re-
Results, although limited, did not indicate an involvement of T/EBP phosphorylation in the novel IL-10 signaling pathway. Furthermore, T/EBP is a nuclear protein and is located in the nucleus regardless of IL-10 treatment (Supplemental Materials Fig. 1, B and C), suggesting that T/EBP may not be a protein that translocates to the nucleus upon IL-10 treatment. Alternatively, there is a possibility that an unknown protein may be phosphorylated upon IL-10 signaling, which then translocates to the nucleus and phosphorylates or binds directly to T/EBP or T/EBP interacting protein(s) such as co-activators, resulting in enhancement of binding affinity of T/EBP to its binding site and a subsequent increase in target gene transcriptional activity. To understand the mechanism of IL-10 induction of UGRP1 gene expression, additional experiments must be conducted as to determine: 1) whether phosphorylation of T/EBP is indeed involved, and if so, what are the type and site of phosphorylation and kinases involved; 2) whether other factors might be interacting with T/EBP to transduce IL-10 signals; and 3) what roles JAKs might play in this novel signaling pathway.

Lastly, our findings may provide a rationale to further exploring the possibility that the anti-inflammatory activity of IL-10 is mediated through UGRP1 expression in lung tissues. Our current results clearly demonstrate that UGRP1 expression is induced in lung epithelial cells by IL-10, a cytokine known to have potent anti-inflammatory and immunoregulatory activities. We previously hypothesized that UGRP1 may be involved in regulating lung inflammation (1, 14). In support of our hypothesis, intranasally administered IL-10 reduced production of Th-2 cytokines such as IL-4 and IL-5, and eosinophilia in bronchoalveolar lavage fluid of ragweed-induced allergic mice (45). Similarly, IL-10 abrogated ovalbumin-induced airway inflammation and tumor necrosis factor-α production (46). Interestingly, IL-10 induction of UGRP1 gene expression is independent of the polymorphism in the UGRP1 gene promoter that was demonstrated to be associated with an increased risk of asthma (14). Recently, macrophage scavenger receptor with collagenous structure (MARCO) expressed by alveolar macrophages in the lung was identified as a receptor for UGRP1 (47). Lipopolysaccharide, a previously identified MARCO ligand, competes with UGRP1 for binding to MARCO and bacteria, thus suggesting that the UGRP1-MARCO ligand-receptor pair is likely involved in inflammation and pathogen clearance in the lung. Whether UGRP1 is a mediator of anti-inflammatory activity of IL-10 awaits further experiments.

In conclusion, a novel IL-10 induction of UGRP1 gene expression was found in lung epithelial cells that is mediated by a homeodomain transcription factor T/EBP. This signaling pathway might play a role in the anti-inflammatory activities of IL-10 in the lung.
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