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Induction of Uteroglobin-Related Protein 2 (*Ugrp2*) Gene Expression by the Th2 Cytokines IL-4 and IL-13¹

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Abstract

Uteroglobin-related proteins 1 and 2 (UGRP1 and -2) are thought to play important roles in inflammation and immunologic responses in the lung. In this study we demonstrate that IL-4 and IL-13 enhance *Ugrp2* gene expression in the mouse transformed Clara cell line, mtCC, in a time- and dose-dependent manner. Addition of actinomycin D abrogated the IL-4- and IL-13-induced increase of *Ugrp2* expression, demonstrating that this increase occurs at the transcriptional level. When mtCC cells were pretreated with IFN- γ before the addition of IL-4 or IL-13, IL-4- and IL-13-induced *Ugrp2* mRNA increase was markedly decreased. IL-4 and IL-13 induced phosphorylation of STAT6 in mtCC cells, which binds to the proximal STAT-binding element (SBE) in the *Ugrp2* gene promoter, leading to transcriptional activation of this gene. Mutations of the proximal SBE abrogated the binding of activated STAT6 to this site and the IL-4-induced increase in *Ugrp2* gene promoter activity. IFN- γ -activated STAT1 binds to the same SBE in the *Ugrp2* gene promoter to which STAT6 binds and decreases the binding of STAT6 to this site. Furthermore, an IL-4-induced increase in *Ugrp2* expression was not observed in primary cultures of lung cells derived from STAT6-deficient mice. These results indicate that *Ugrp2* expression is enhanced by IL-4 and IL-13 through STAT6 binding to the proximal SBE located in the *Ugrp2* gene promoter.

Uteroglobin-related protein 2 (UGRP2),⁵ also called high in normal-1 (HIN-1) or secretoglobin 3A1, was identified as a candidate tumor suppressor gene that is silenced by methylation in the majority of breast carcinomas (1). Subsequently, it was found as a homologous gene to UGRP1, which is a downstream target gene for T/EBP/NKX2.1 homeodomain transcription factor (2). Mouse UGRP2 shows 33% amino acid sequence identity to mouse UGRP1 (2).

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Based on amino acid sequences, both UGRP1 and UGRP2 belong to a gene superfamily of the uteroglobin/Clara cell secretory proteins (UG/CCSP), officially termed secretoglobins (3-5).

In humans, the highest level of *UGRP2* expression is detected in trachea, followed by salivary gland, lung, and mammary gland (3). In contrast, *Ugrp2* expression in mice is predominantly found in trachea and lung (3,5), with low levels of expression reported in other tissues, such as heart, stomach, small intestine, and mammary gland (5). *Ugrp2* mRNA is detected on embryonic day 17.5 during mouse development in the tracheal epithelium and later also in the submucosal glands of the proximal trachea and bronchi (4,5). *Ugrp2* mRNA expression is up-regulated by retinoic acid, which induces mucinous differentiation in primary normal human bronchial epithelial cells (5). It was suggested that UGRP2 may have a role in the acquisition or maintenance of the terminally differentiated proximal airway epithelial phenotype (5).

The mouse *Ugrp2* gene is localized at region 11A5-B1, which is homologous to human chromosome 5q35 (3). The human chromosome region 5q31-q34 was reported to contain a number of genes associated with inflammatory cytokines, such as IL-3, IL-4, IL-5, IL-13, and CSF-2. Furthermore, UG/CCSP, a prototypical protein of the secretoglobin gene superfamily to which UGRP2 is distantly related, exhibits several anti-inflammatory properties, including inhibition of phospholipase A₂ (6), IFN- γ production (7), and neutrophil inflammation and chemotactic response (8). These facts together with the site of UGRP2 expression suggest that UGRP2 may play a role in the regulation of inflammation and immunologic responses. However, little is known about the physiological function of UGRP2 in the lung.

IL-4, a Th2 cytokine, is a pleiotropic lymphokine produced by Ag-activated T cells, which acts on hemopoietic, endothelial, dendritic, osteoblastic, and fibroblastic cells and plays a particularly important role in allergic sensitization (9). IL-4 has a role in B cell class switching to IgE production (10). Together with IL-3, IL-4 functions as a growth factor for mast cells (11) and is able to up-regulate VCAM-1 expression, leading to preferential migration of eosinophils into tissues (12).

⁵Abbreviations used in this paper:

UGRP2	uteroglobin-related protein 2
βc	cytokine receptor common β -chain
CCSP	Clara cell secretory protein
γc	common γ -chain
HIN-1	high in normal-1
mtCC	mouse transformed Clara cell line
SBE	STAT-binding element
UG	uteroglobin
HNF3β	hepatocyte nuclear factor 3 β

IL-4 activates gene expression by signaling through either type 1 or type 2 IL-4R complexes on the surface of IL-4-responsive target cells. Binding of IL-4 to the IL-4R α -chain induces subsequent heterodimerization with either the common γ -chain (γ_c) (13,14) or the γ -chain-like receptor, IL-13R α 1 (15-17). This dimerization activates one or more members of the receptor-associated JAKs that initiates the phosphorylation cascade, subsequently resulting in the activation of secondary effector molecules, including STAT6 (18-21). Activated STAT6 then homodimerizes and translocates to the nucleus, where it binds with high affinity to STAT-binding elements (SBE) in the promoters of various IL-4-responsive genes (18,22-29). In recent years it was found that IL-13 induces the expression of the same repertoire of genes that are inducible by IL-4 in monocytes (30).

The aim of the present study was to investigate the mechanisms governing the regulation of *Ugrp2* expression by the Th2 cytokines, IL-4 and IL-13. We demonstrate that both IL-4 and IL-13 stimulate the expression of *Ugrp2* through a mechanism dependent on the transcription factor STAT6 that is able to bind to one of the SBEs present in the *Ugrp2* gene promoter and up-regulates the promoter activity. Furthermore, we show that IFN- γ markedly reduces the IL-4- and IL-13-induced increase in *Ugrp2* mRNA expression.

Materials and Methods

Materials

Pregnant BALB/c mice were purchased from Charles River Laboratories, and STAT6-deficient mice were bred onto the BALB/c background by Dr. M. J. Grusby (Harvard Medical School, Boston, MA) (31). We obtained the breeding pairs from Dr. W. E. Paul (National Institute of Arthritis and Infectious Diseases, National Institutes of Health, Bethesda, MD). Recombinant murine IL-4, IL-13, and IFN- γ were purchased from R&D Systems. Actinomycin D was purchased from Sigma-Aldrich. Abs against murine STAT1, STAT6, and phospho-STAT6 were purchased from Santa Cruz Biotechnology. All animal studies were conducted after approval by the institutional animal care and use committee.

Cell culture

Mouse transformed Clara (mtCC) cells (32) were maintained in DMEM (BioSource International) supplemented with 10% FBS (Gemini Bio-Products) and antibiotic-antimycotic containing 100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, and 250 ng/ml amphotericin B (Invitrogen Life Technologies).

Lung primary culture

Mouse embryonic lung primary culture was prepared using the method previously described (33). In brief, embryos were obtained by dissection of pregnant females on embryonic day 17.5. Fetal lungs were dispersed by 10 U/ml collagenase (Sigma-Aldrich) and 1 U/ml dispase (dispase I; Roche) and were cultured in DMEM/Ham's F-12 medium (BioSource International) supplemented with 10% FBS (Gemini Bio-Products).

Isolation and culture of bone marrow-derived macrophages

Monolayers of mouse bone marrow-derived macrophage were generated according to standard procedures. Briefly, bone marrow aspirates from the tibias and femurs of wild-type mice were flushed with sterile DMEM to dislodge the cells. The harvested cells were then cultured in DMEM containing 10% FBS, 2 mM L-glutamine, antibiotics, and 100 ng/ml recombinant human M-CSF. The cultures were incubated at 37°C under a 5% CO₂ atmosphere for 5-7 days.

RT-PCR

To prevent genomic DNA contamination, total RNA was treated with RNase-free DNase I (Ambion). For cDNA synthesis, total RNA was first incubated at 70°C for 10 min, then chilled on ice. The cDNA synthesis reaction was conducted in a final volume of 20 µl containing 1 µg of RNA, 4 µl of 5× first-strand synthesis buffer, 1 µl of a mixture of four deoxynucleotide triphosphates (10 mM each), 2 µl of 0.1 M DTT, and 1 µl of 500 pg/µl N6 random hexamers or oligo(dT)₂₀ (Invitrogen Life Technologies). After incubation at 37°C for 2 min, 200 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies) was added, and incubation was continued at 37°C for 60 min. Cells were then subjected to PCR using Advantage 2 *Taq* DNA polymerase (BD Biosciences) under the following conditions: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, 20 cycles for 18S and GAPDH, and 30 cycles for IL-2R α , IL-4R α , IL-9R α , IL-10R1, IL-13R α 1, and γ c. For IL-5R α , IL-10R2, and common β -chain receptor (β c), annealing temperatures of 50, 58, and 55°C were used instead of 60°C, and the reaction was run for 35, 30, and 35 cycles, respectively.

Oligonucleotide primers used for RT-PCR were as follows: mouse 18S, 5'-CGGCTACCACATCCAAG GAA-3' and 5'-ATTGGAGCTGGAATTACCGC-3'; mouse GAPDH, 5'-ACCA CAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTTGCT GTA-3'; mouse IL-2R α , 5'-AGGTTTCCGAAGACTAAAGG-3' and 5'-CCAC GAAATGATAGATTCTC-3'; mouse IL-4R α , 5'-ATCTGCGTG CTTGCTGGTTCT-3' and 5'-CTGGTATCTGTCTGATTGGACCG-3'; mouse IL-5R α , 5'-CATGCTGGTTTCCCAGGAC-3' and 5'-TTTCCCA CATAAATAGGTTG-3'; mouse IL-9R α , 5'-AGTCTCGGTCCGAGAG CAAG-3' and 5'-GTCTCCTGGGCAGGTACTGT-3'; mouse IL-10R1, 5'-GTCTGAGAGCACCTACTATG-3' and 5'-TTGTAAACTCGGAG ATCCTT-3'; mouse IL-10R2, 5'-GAGACGTGGACCTTGAAGAA-3' and 5'-ACGGAGACTATGAGGATGAT-3'; mouse IL-13R α 1, 5'-CAT CTTCTCCTCAAAAATGGTGCC-3' and 5'-GGATTATGACTGCCAC TGCGAC-3'; mouse γ c, 5'-GTTCTGAGCCTCAGGCAACC-3' and 5'-CAGATTGCTGAGTGTTAGAT-3'; and mouse β c, 5'-GCTACATG GCACTGGTGGCT-3' and 5'-GCTTATAAGCCACCTCAAAC-3'.

Northern blot analysis

Total RNAs from mtCC cells (5 µg) or lung primary culture cells (20 µg) were electrophoresed on 1% agarose gels containing 0.22 M formaldehyde and blotted onto GeneScreen Plus nylon membranes (PerkinElmer). Filters were serially hybridized with mouse *Ugrp2* and *18S* cDNA probes. Hybridization was performed in Perfect Hybridization Solution (Sigma-Aldrich) at 68°C overnight. The membrane was washed twice with 2× SSC containing 0.1% SDS at 68°C for 30 min, once with 2× SSC at 68°C for 30 min, followed by exposure to a Storm PhosphorImager screen (Amersham Biosciences). Signals were visualized and quantitated using ImageQuant software (Amersham Biosciences).

Western blot analysis

The mtCC or lung primary culture cells were washed with PBS and lysed in radio-immuno-protein-assay buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 10 mM sodium fluoride, and 1 mM sodium orthovanadate) with Protease Inhibitor Cocktail Tablets (Complete Mini; Roche). The protein lysates were mixed with SDS sample loading buffer containing 2-ME, electrophoresed on 10% SDS-polyacrylamide gels containing 0.1 or 0.8% bis-(*N,N'*-methylene-bis-acrylamide), and then electrotransferred to an Immobilon-P membrane (Millipore). Membranes were blocked with TBS containing Tween 20 (TBS-Tween; 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) and 5% skin milk, and were incubated with the first Ab in TBS-Tween. Membranes were washed with TBS-Tween, then incubated with HRP-conjugated secondary Ab (Santa Cruz

Biotechnology). Protein bands were detected using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer) and were exposed to scientific imaging film (Eastman Kodak).

Construction of mouse UGRP2-luciferase promoter plasmids

A *Bam*HI fragment containing 3.2 kb of the 5'-flanking sequence of the mouse *Ugrp2* genomic DNA was subcloned into pBluescript II, and PCR was performed with T7 primer (5'-GTAATACGACTCACTATAGGGC-3') and a *Ugrp2* gene-specific primer (5'-TGGTAGAATGAGTCTCAG-3'; +52/+35 based on the transcription start site as +1) (3). The PCR product was subcloned into pCR2.1 (Invitrogen Life Technologies), and an *Spe*I-*Xho*I fragment from this plasmid was inserted into *Nhe*I-*Xho*I site of the luciferase reporter vector pGL3-basic (Promega) to generate pGL3-3185 plasmid. A *Bgl*II fragment containing 420 bp of the 5'-flanking sequence of the mouse *Ugrp2* genomic DNA was subcloned into pBluescript II, and PCR was performed with T3 primer (5'-AATTAACCCTCACTAAA GGG-3') and a *Ugrp2* gene-specific primer (+52/+35). The PCR product was subcloned into pCR2.1, and an *Sma*I-*Xho*I fragment from this plasmid was inserted into the *Sma*I-*Xho*I site of pGL3-basic to generate pGL3-411 plasmid. This construct was further digested with *Kpn*I and *Nhe*I for preparation of deletion plasmids using Exonuclease III (New England Biolabs) and S1 nuclease (Invitrogen Life Technologies). Four deletion constructs thus obtained (pGL3-65, -154, -212, and -264) were sequenced to determine the exact sequences. To generate the pGL3-1158 construct, a -1158/+52 bp fragment was amplified by PCR using the primers -1158 m*Ugrp2* (5'-CGCCCTTCAGAGGAAGAGGCT-3') and GLprimer2 located in the pGL3 basic as a reverse primer (5'-CTTTATGTTTTGGCGTCTTCC-3') and subcloned into pGL3-basic. Mutations were introduced into STAT binding elements (SBEs), -1143/-1154 (5'-CTTCAGAGGAAG-3') and -201/-209 (5'-TTCCTGGAA-3'; putative STAT6 binding sequences are underlined), in the *Ugrp2* gene promoter using PCR with primers 5'-CGC CATGCAGAGTACGAGGCT-3' and 5'-CCGTGGTACCTGCAAGAAA GG-3' (mutations are in italics), respectively. All plasmids were confirmed by nucleotide sequencing.

Luciferase reporter assay

The mtCC cells were seeded in 24-well tissue culture plates, grown to 90% confluence, and transfected with pGL3 reporter plasmids containing various lengths of the mouse *Ugrp2* gene promoter using Lipofectamine 2000 (Invitrogen Life Technologies). As an internal control, plasmid phRL-TK containing the *Renilla* luciferase gene (Promega) was cotransfected. Cells were cultured in 0.5% FBS-containing medium in the presence or the absence of cytokine for 48 h, then lysed with passive lysis buffer (Promega). Luciferase activity was measured according to instructions in the technical manual for the Dual-Luciferase Reporter Assay System (Promega).

EMSA

Nuclear protein extracts were prepared from cytokine-treated cells using a modification of the original method described by Schreiber et al. (34). Double-stranded oligonucleotide (21 nt), prepared based on the DNA sequences present in the mouse *Ugrp2* gene promoter, was used as a probe in gel-shift assays. This oligonucleotide contains a SBE that can bind to STAT6 and STAT1. Binding reactions were performed at room temperature for 30 min by incubating radiolabeled probe DNA (>10⁵ cpm) with 5 µg of nuclear extracts prepared from IL-4-, IL-13-, IFN-γ-, or IL-4/IL-13- and IFN-γ-stimulated cells (30 min at 37°C) in the presence of 2 µg of polydeoxyinosinic-polydeoxycytidylic acid poly(dI:dC) (Amersham Biosciences), 10 mM DTT, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 50% glycerol in a final volume of 20 µl. After incubation, 8 µl of each mixture was electrophoresed on nondenaturing 6% polyacrylamide gels (Novex; Invitrogen Life Technologies) using 0.25× TBE electrophoresis

buffer containing 22 mM Tris-HCl (pH 8.0), 22 mM borate, and 0.5 mM EDTA. Gels were then dried and visualized by autoradiography.

Results

IL-4 and IL-13 enhance *Ugrp2* mRNA expression

The induction of *Ugrp2* mRNA by selected Th1 (IL-2) and Th2 (IL-4, IL-5, IL-9, IL-10, and IL-13) cytokines was examined in mtCC cells that were generated from tumor cells derived from transgenic mice expressing the SV40 large T Ag gene under the control of the *Ug/Ccsp* promoter (32). This cell line constitutively expresses modest levels of *Ugrp2* mRNAs (Fig. 1A). After 12-h incubation in DMEM containing reduced serum (0.5%), cells were treated with 20 ng/ml IL-2, IL-4, IL-5, IL-9, IL-10, and IL-13 for 12 h. Among them, IL-4 and IL-13 were found to enhance the levels of *Ugrp2* expression (Fig. 1A). Because cytokine signaling starts with the binding of cytokines to their specific cell surface receptor complexes (35), the presence of receptors for each cytokine was examined in mtCC cells using RT-PCR (Fig. 1B). The results suggest that no induction of *Ugrp2* gene expression by IL-2, IL-5, and IL-9 in mtCC cells is due to the lack of at least one receptor component in each receptor complex. In contrast, the *Ugrp2* gene was induced by IL-4 and IL-13 in the absence of the γ_c because mtCC cells express IL-13R α 1. This indicates that IL-4 and IL-13 activate *Ugrp2* gene expression in mtCC cells by signaling through type 2 IL-4R complexes (15-17).

Increase in *Ugrp2* expression occurred at the transcriptional level in a time- and dose-dependent manner

Ugrp2 mRNA levels were increased in the presence of as little as 1 ng/ml IL-4 or IL-13 and were additionally increased in a dose-dependent manner, with the level reaching a plateau at ~10 ng/ml (Fig. 2A). The time course for *Ugrp2* mRNA induction by IL-4 and IL-13 was determined with a fixed concentration of 20 ng/ml IL-4 and IL-13 and at various time points between 3 and 24 h. The increase in *Ugrp2* expression was detected 3 h after the addition of IL-4 and IL-13, and the expression level was already close to the maximum (Fig. 2B). To examine whether the increase in *Ugrp2* mRNA expression by treatment with IL-4 and IL-13 occurs at the transcriptional level, mtCC cells were pretreated with the RNA synthesis inhibitor, actinomycin D, followed by treatment with IL-4 and IL-13 (Fig. 2C). Actinomycin D abrogated the IL-4- and IL-13-induced increase in *Ugrp2* mRNA expression, suggesting that the enhancement of *Ugrp2* gene expression by IL-4 and IL-13 in mtCC cells is mediated at the transcriptional level.

Activation of STAT6 by IL-4 and IL-13 in mtCC cells

IL-4 and IL-13 bind to cell surface receptor complexes (15-17), leading to the activation of STAT6 (18-21) that is capable of binding to SBEs in the promoters of various IL-4- and IL-13-responsive genes (18,22,23). To determine whether STAT6 is involved in the IL-4- and IL-13-induced increase in *Ugrp2* gene expression, the nuclear protein extracts prepared from IL-4- and IL-13-stimulated mtCC cells were analyzed by Western blotting using anti-phospho (Y)-STAT6 and anti-STAT6 Abs (Fig. 3). Because mtCC cells express IL-4R α and IL-13R α 1, but not γ_c , these results also demonstrate that STAT6 is activated by IL-4 and IL-13 by signaling through the type 2 IL-4R complexes.

IFN- γ inhibits IL-4- and IL-13-induced increases in *Ugrp2* mRNA

IFN- γ is a major product of activated Th1 cells, whereas IL-4 and IL-13 are primarily produced by Th2 cells. IL-4 and IFN- γ exhibit mutual antagonism in many systems. For example, IFN- γ can markedly inhibit IL-4- and IL-13-stimulated expression of IL-1RI and IL-1RII (36), 15-lipoxygenase (29), and Fc ϵ RIIb (CD23) genes (37). When mtCC cells were treated with IFN-

γ , the constitutive level of *Ugrp2* expression was reduced (Fig. 4). Furthermore, IFN- γ pretreatment before the addition of IL-4 or IL-13 markedly reduced the IL-4- and IL-13-induced increase in *Ugrp2* mRNA levels. These results demonstrate an effect of IFN- γ on the regulation of *Ugrp2* gene expression by IL-4 similar to those seen with other IL-4-regulated genes. It is also notable that costimulation of cells with IL-4 and IL-13 did not further increase the level of *Ugrp2* expression above that achieved with IL-4 or IL-13 treatment alone, suggesting that IL-4 and IL-13 signal through the same receptor complex.

Identification of IL-4-responsive elements in mouse *Ugrp2* gene promoter

A transcription start site of the mouse *Ugrp2* gene was previously determined by 5'RACE analysis (3). Several *Ugrp2* promoter-luciferase reporter plasmids were constructed and analyzed by transient transfection with or without IL-4 using mtCC cells that constitutively express *Ugrp2*. The reporter activity of the *Ugrp2* gene promoter without IL-4 gradually increased up to -412 bp of the promoter sequence. With the construct -1158, the baseline activity decreased to a level similar to that of the construct -212, suggesting the possible presence of an element that represses the promoter activity in between -412 and -1158 bp of the *Ugrp2* gene promoter (Fig. 5A). In the presence of IL-4, the reporter activity of the construct -212 was ~2-fold higher than that with no IL-4, whereas the promoter activity of the -154 construct was similar regardless of the addition of IL-4 (Fig. 5A). All other constructs containing upstream sequences up to -264, -412, -1158, and -3185 bp exhibited ~2-fold higher reporter activities with IL-4 compared without IL-4, suggesting that an element(s) responsible for the IL-4-induced increase in *Ugrp2* gene promoter activity resides between -154 and -212 bp from the transcription start site of the *Ugrp2* gene. Within this region, one putative SBE was found with the consensus sequence of TTC(N₃)GAA (5'-TTCCTGGAA-3') located at position -209 to -201 bp (proximal SBE) relative to the transcription start site of the gene. To confirm that the -209 to -201 bp SBE is responsible for the IL-4-induced increase in *Ugrp2* promoter activity, mutations were introduced to modify two nucleotides (5'-TTCCTGGAA-3' to 5'-TACCTGCAA-3'; putative STAT6 binding sequences are in italics, and mutations are underlined) in this SBE. The construct -212M1 carrying these mutated nucleotides exhibited almost complete suppression of the IL-4-induced increase in promoter activity (Fig. 5B), suggesting that the proximal SBE plays an important role in the induction of *Ugrp2* promoter activity by IL-4. Furthermore, within 1.2 kb of the mouse *Ugrp2* gene promoter, another putative SBE, 5'-CTTCAGAG GAAG-3' (TTC(N₄)GAA), was found at -1154 to -1143 bp (distal SBE) relative to the transcription start site. Mutations were also introduced into this distal SBE by modifying four nucleotides (5'-CTTCAGAGGAAG-3' to 5'-ATG CAGAGTACG-3'). When the construct -1158M2 with these mutated nucleotides was subjected to transfection analysis, IL-4-induced reporter activity similar to that of a construct without the mutation (-1158 construct) was obtained, whereas construct -1158M1, which has the proximal SBE mutated, but with intact distal SBE, exhibited similar promoter activities both with and without IL-4, suggesting that the distal SBE is dispensable for IL-4-induced expression of the *Ugrp2* gene. These results demonstrate that the proximal SBE, -209 to -201 bp relative to the transcription start site, is important for the induction of mouse *Ugrp2* gene promoter activity by IL-4.

Binding of STAT6 to proximal SBE in mouse *Ugrp2* gene promoter

To confirm whether STAT6 binds to the proximal SBE (-209 to -201 bp), EMSA was conducted using a ³²P end-labeled, double-stranded oligonucleotide of -215 to -195 bp of the mouse *Ugrp2* gene promoter (Fig. 6). Nuclear extracts prepared from IL-4-treated macrophages produced a shifted band that was supershifted by the addition of STAT6 Ab, but not STAT1 Ab (Fig. 6, lanes 2, 3, and 6). IL-13 produced the same shifted band corresponding to STAT6; however, it was less potent than IL-4 (Fig. 6, lanes 12 and 15). Interestingly, a DNA-protein complex corresponding to STAT1 was obtained when IFN- γ -treated nuclear extracts were

used, which was supershifted by the addition of Ab for STAT1, but not STAT6 (Fig. 6, lanes 4, 5, 7, and 13). Introduction of mutations into the proximal SBE resulted in complete loss of both STAT6- and STAT1-specific bands (Fig. 6, lanes 9 and 10). These results demonstrate that both IL-4-activated STAT6 and IFN- γ -activated STAT1 can bind to the proximal SBE in the *Ugrp2* gene promoter. When the cells were cotreated with IFN- γ and IL-4 or IL-13, the binding of STAT1 to the *Ugrp2* SBE decreased the binding of STAT6 to the same element due to the apparently higher affinity of STAT1 for this site (Fig. 6, lanes 14 and 16). These results may explain the inhibitory effects of IFN- γ on the IL-4 induced expression of the *Ugrp2* gene.

Requirement of STAT6 in the regulation of *Ugrp2* gene expression by IL-4

To demonstrate the role of STAT6 in the increased expression of *Ugrp2* by IL-4, *Ugrp2* expression levels were compared between wild-type and STAT6-deficient mice using embryonic lung primary culture cells (Fig. 7). First, the lack of STAT6 expression was confirmed in these primary culture cells prepared from STAT6-deficient mice (Fig. 7A). The presence of IL-4R α and IL-13R α 1 and the lack of γ_c expression were confirmed by RT-PCR in wild-type and STAT6-deficient mouse lung primary cells (Fig. 7B). Activation of STAT6 by IL-4 was shown in wild-type primary cells (Fig. 7C). Treatment of wild-type and STAT6-deficient lung primary cells with IL-4 clearly demonstrated that IL-4 enhanced *Ugrp2* mRNA levels in wild-type, but not STAT6-deficient, primary cells (Fig. 7D), indicating that IL-4 enhancement of *Ugrp2* gene expression is dependent on the STAT6 pathway.

Discussion

This study demonstrates that *Ugrp2* expression is enhanced by treatment with IL-4 and IL-13 in mtCC cells that express endogenous *Ugrp2*. Other selected Th2 cytokines, such as IL-5, IL-9, and IL-10, and the Th1 cytokine IL-2 did not induce the expression of *Ugrp2* mRNA in this cell line. The lack of expression of at least one of their specific receptor components appears to be responsible for the lack of induction by IL-2, IL-5, and IL-9, whereas both IL-10R1 and IL-10R2 are present, suggesting that the *Ugrp2* gene promoter does not respond to IL-10 signaling. Although IL-4 signal transduction can take place through a pathway involving IL-4R α and the γ_c , the absence of a γ -chain in mtCC cells and the lack of an additive effect of IL-4 and IL-13 on the induction of *Ugrp2* gene expression suggest that the IL-4 or IL-13 signal goes through the heterodimeric type 2 IL-4R complex, leading to activation of STAT6. A binding site for STAT6 was identified at position -209 to -201 bp relative to the transcription start site of the *Ugrp2* gene by transient transfection assays and EMSA, which is responsible for the IL-4-induced increase in *Ugrp2* gene expression. Furthermore, experiments using STAT6-deficient lung primary culture cells demonstrated that STAT6 is necessary for the IL-4-induced increase in *Ugrp2* gene expression.

RT-PCR analysis revealed the presence of both IL-10R1 and IL-10R2 in mtCC cells. IL-10R1 is known to be primarily expressed by hemopoietic cells, such as B cells, T cells, NK cells, monocytes, and macrophages, whereas IL-10R2 is constitutively expressed by most cells and tissues (38,39). We previously demonstrated the presence of IL-10R1 in the human lung adenocarcinoma-derived cell line NCI-H441 (33). It appears that lung epithelial cells express IL-10R1.

Promoters of IL-4-inducible genes contain unique DNA sequences that constitute specific binding sites for STAT6. These DNA sequences, referred to as SBEs, are characterized by a palindromic sequence, 5'-TTC(N₄)GAA-3'. The symmetrical GAA half-sites with a 4-bp spacer, called an N4 motif, distinguish the STAT6 site from binding sites for other STAT proteins, where the GAA half-sites usually have 3-bp spacers, called an N3 motif (22). However, genes whose promoters contain 3-base spacer SBEs may still be selectively activated

by STAT6. For example, the N3 motif SBE present in the human FcεRIIb (CD23) gene promoter can be bound by multiple STATs, yet only STAT6 appears to transactivate this gene (40). In the case of the *Ugrp2* gene, the proximal SBE, located ~200 bp upstream of the transcription start site, belongs to an N3 motif SBE, binds to the IL-4-activated STAT6, and activates transcription. Interestingly, STAT1 activated by IFN-γ can also bind to this site. Clearly, the presence of a 3- or 4-base spacer does not by itself define the specificity of an SBE.

To confirm STAT6 binding to the proximal SBE of the *Ugrp2* gene promoter, we used nuclear extracts prepared from IL-4-stimulated macrophages instead of mtCC or lung primary culture cells for EMSA, because the latter two nuclear extracts did not produce a clear band for a specific binding complex between STAT6 and an oligonucleotide containing the proximal SBE. The exact reason for this is not known. However, in the case of mtCC cells, STAT1 is constitutively activated in the absence of any stimulation and can bind to SBE in the *Ug/Ccsp* promoter (32). Thus, the binding of STAT6 to the *Ugrp2* gene-proximal SBE may be masked by constitutively activated STAT1. In fact, nuclear extracts prepared from control mtCC cells without IL-4 treatment produced a prominent protein-DNA complex that corresponds to STAT1 (A. Yamada and S. Kimura, unpublished observation). Furthermore, IFN-γ-activated STAT1 appears to bind to the same SBE in the *Ugrp2* gene promoter and reduce STAT6 binding when macrophage nuclear extracts were used.

In contrast to the stimulatory effects of IL-4 and IL-13 on *Ugrp2* expression, IFN-γ was found to repress both basal and IL-4-stimulated *Ugrp2* expression. IFN-γ is a well-known antagonist that inhibits the expression of several IL-4- and IL-13-inducible genes, including IGF-1 (41), FcεRIIb (CD23) (37), IgE (42), 15-lipoxygenase (29), IL-1R1 and IL-1RII (36), and IL-1R antagonist (43). One mechanism to explain the antagonistic effect of IFN-γ is that it induces the expression of suppressor of cytokine signaling-1, which inhibits IL-4-induced activation of JAK1 and STAT6 (44,45). Alternatively, there could exist a competition between STAT1 and STAT6 for binding to the SBE (N3 site). This mechanism occurs in the promoter of the IFN regulatory factor-1 gene (46). This appears to be the case for the inhibitory effect of IFN-γ observed with IL-4- and IL-13-induced *Ugrp2* gene expression. It should be noted, however, that suppression of basal *Ugrp2* expression by IFN-γ can suppress induction of *Ugrp2* gene expression independently of IL-4 or IL-13.

In lungs, the expression of the *Ug/Ccsp* gene, a prototypical member of the secretoglobin gene superfamily to which *Ugrp2* belongs, is regulated by IL-4 and IFN-γ (32,47-49). However, the mode of regulation of *Ug/Ccsp* expression by IL-4 and IFN-γ appears to differ from what we observed for *Ugrp2* in mtCC cells. Thus, transgenic mice overexpressing IL-4 in the airways had decreased expression of *Ug/Ccsp* (47), whereas IFN-γ increased the expression of *Ug/Ccsp* both in vivo, by intratracheal administration of IFN-γ to mice, and in vitro, using mtCC cells (32,49) and the human bronchial epithelial cell line BEAS-2B in the presence of IFN-γ (48). In the latter, no induction of *Ug/Ccsp* expression was observed by IL-4 and IL-13. Two mechanisms for IFN-γ stimulation of *Ug/Ccsp* expression have been described. First, IFN-γ activates STAT1, which directly binds to SBE in the distal promoter of the *Ug/Ccsp* gene, leading to activation of the gene. Secondly, IFN-γ-activated STAT1 stimulates transcription of IFN regulatory factor-1, which, in turn, activates transcription of hepatocyte nuclear factor 3β (HNF3β), an essential transcription factor for *Ug/Ccsp* gene expression (50). These findings indicate that the expressions of *Ug/Ccsp* and *Ugrp2* are differentially regulated by IFN-γ. It is also known that UGRP2 feedback inhibits IFN-γ expression and activity in lung (7). It is possible that UGRP2 mediates a similar feedback inhibitory function to control IL-4/IL-13 activity in lung. Although the physiological consequence of differential regulation by IL-4/IL-13 and IFN-γ between *Ugrp2* and *Ug/Ccsp* genes is not known, a compensatory mechanism may account for this phenomenon. Similar differential regulation was reported between

Ugrp1 and *Ug/Ccsp* genes in the lungs of *Ug/Ccsp*-knockout mice, in which increased *Ugrp1* expression was observed (51).

Transcription analysis of the *Ugrp2* promoter-reporter constructs showed that the construct -154 has ~4-fold higher promoter activity than the -65 construct regardless of IL-4 treatment, and the basal promoter activity gradually increased up to -412 bp. Within the -412 bp of the *Ugrp2* gene promoter, there are putative HNF3 β (-71 to -61 bp) and C/EBP (-74 to -64 bp) binding sites detected by the transcription factor Search program (52). Many lung-specific genes are known to be regulated by HNF3 β and C/EBP (50,53). A basal increase in *Ugrp2* gene promoter activity could be due to binding of these transcription factors to their binding sites. Furthermore, an element that represses *Ugrp2* promoter activity appears to be located in between -412 and -1158 bp. We do not know what protein binds to this element that causes repression of promoter activity. Additional studies are required to define the transcription factors that positively and negatively regulate *Ugrp2* gene expression.

Finally, *Ugrp2*, also called *HIN-1*, was found to be significantly down-regulated due to hypermethylation of the *HIN-1* gene promoter in a majority of human primary breast carcinomas, including in situ and invasive ductal and lobular carcinomas and breast cancer cell lines (1). Reintroduction of *HIN-1* by transfecting expression plasmid to various breast cancer cell lines inhibited the growth of cells (1). Based on these results, it was proposed that *HIN-1* is a candidate tumor suppressor gene (1). In normal adult mice and humans, the expression of *Ugrp2* was found at the highest level in the epithelial cells of trachea and lung (3,5). *Ugrp2* mRNA expression was induced by retinoic acid, which correlates with up-regulation of mucinous cell phenotypes when examined in primary normal human bronchial epithelial cells, suggesting that *Ugrp2* may have a role in the acquisition or maintenance of the terminally differentiated proximal airway epithelial phenotype (5). In this regard, we previously reported that *Ugrp2* expression can also be induced by epidermal growth factor and TGF- α , suggesting a possible involvement of *Ugrp2* in epithelial development (54). Despite these observations, the precise physiological and functional significance of *Ugrp2* expression in the airways is not fully understood. In the present study, *Ugrp2* gene expression is up-regulated by the Th2 cytokines, IL-4 and IL-13, which play a major role in the development of asthma (55,56). Cotreatment with IL-4 and epidermal growth factor together did not increase *Ugrp2* gene expression above that induced by either cytokine alone (A. Yamada and S. Kimura, unpublished observation). It appears therefore that the expression of the *Ugrp2* gene can be positively or negatively regulated by multiple cytokines. Overall, our current findings are consistent with a potential role for this gene in the pathogenesis of inflammatory lung diseases, such as asthma. Additional studies are required to define the pathophysiological role of elevated *Ugrp2* expression in lung disease.

In conclusion, we have demonstrated that both IL-4 and IL-13 stimulate the expression of the *Ugrp2* gene through the binding of STAT6 to an SBE present in the *Ugrp2* gene promoter.

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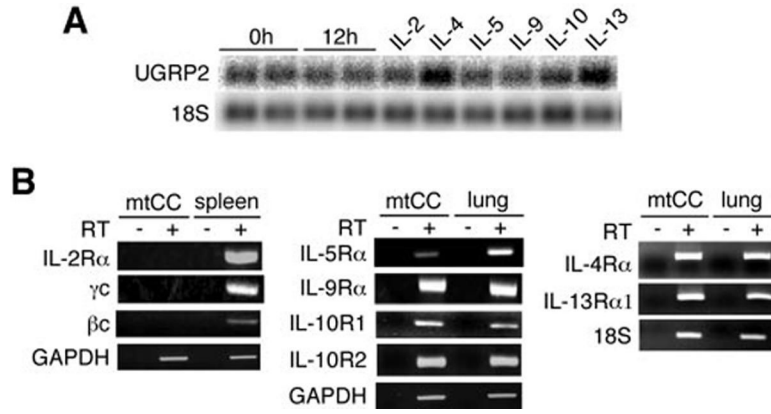


FIGURE 1. Induction of *Ugrp2* mRNA by cytokines and analysis of their receptors. *A*, The mtCC cells were treated with 20 ng/ml selected cytokines (IL-2, IL-4, IL-5, IL-9, IL-10, and IL-13) for 12 h. Total cellular RNAs were extracted, and mRNAs for *Ugrp2* and *18S* were examined by Northern blot analysis. *B*, RT-PCR analysis of IL-2Rα, IL-4Rα, IL-5Rα, IL-9Rα, IL-10R1, IL-10R2, IL-13Rα1, γc, and βc using RNAs prepared from mtCC cells, and adult mouse lung and spleen as controls. The sizes of the PCR products are 260 bp (IL-2Rα), 432 bp (IL-4Rα), 416 bp (IL-5Rα), 317 bp (IL-9Rα), 360 bp (IL-10R1), 272 bp (IL-10R2), 385 bp (IL-13Rα1), 266 bp (γc), 504 bp (βc), 452 bp (GAPDH), and 191 bp (18S).

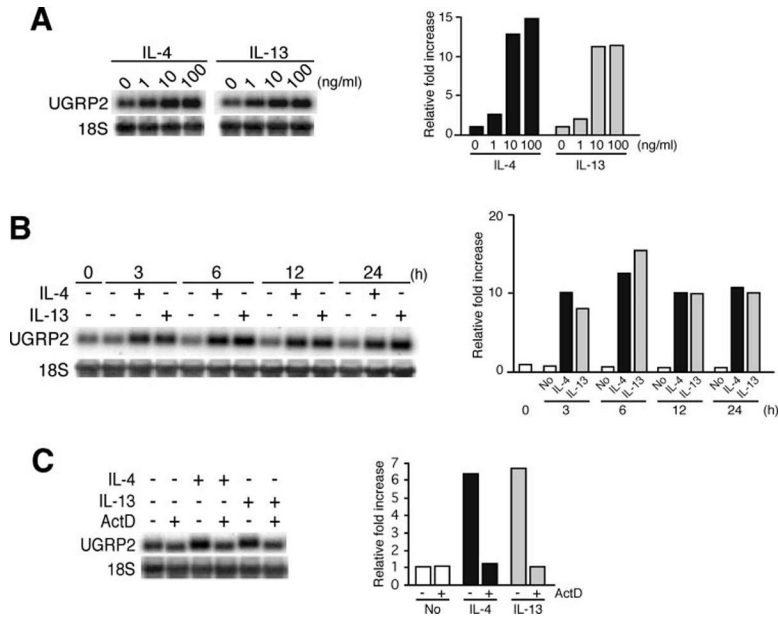


FIGURE 2.

Dose- and time-dependent increase in *Ugrp2* mRNA by IL-4 and IL-13 occurs at the transcriptional level. *A*, Dose effects of IL-4 and IL-13. The mtCC cells were treated with 1, 10, or 100 ng/ml IL-4 or IL-13 for 12 h. *B*, Time-course analysis of the effects of IL-4 and IL-13. The mtCC cells were treated with 20 ng/ml IL-4 or IL-13 for 3, 6, 12, or 24 h. *C*, IL-4 and IL-13 induced *Ugrp2* mRNA at the transcriptional level. The mtCC cells were pretreated with 5 μ g/ml actinomycin D (ActD) for 1 h, followed by treatment with 20 ng/ml IL-4 or IL-13 for 6 h. The expressions of *Ugrp2* and *18S* were detected by Northern blot analysis, and signals were quantitated using ImageQuant software. The relative fold increase in *Ugrp2* mRNA levels after normalizing to those of *18S* is plotted on the *right* in each section. Experiments were repeated twice, and each time a robust increase (*A* and *B*) or a decrease (*C*) in *Ugrp2* mRNA expression was obtained when cells were treated with 10 ng/ml IL-4/IL-13 (*A*), 3 h after initiation of treatment with IL-4/IL-13 (*B*), and with ActD treatment together with IL-4/IL-13 (*C*).

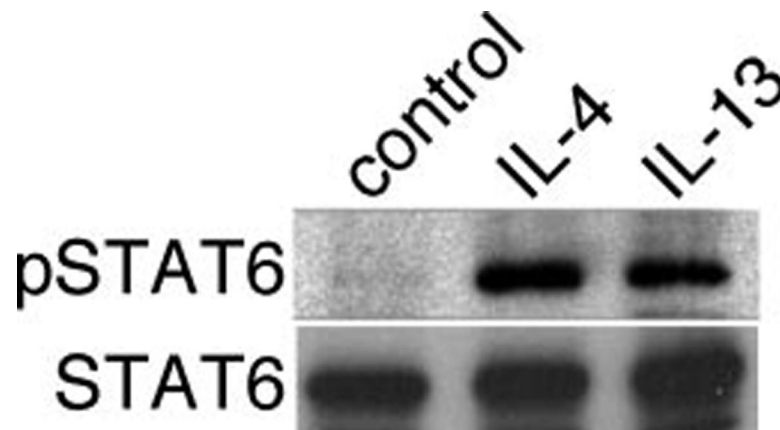


FIGURE 3. STAT6 is activated by IL-4 and IL-13 in mtCC cells. The mtCC cells were treated with 20 ng/ml IL-4 and IL-13 for 30 min. The level of activated STAT6 was measured by Western blotting using a rabbit polyclonal anti-phospho-STAT6 (Tyr641) Ab.

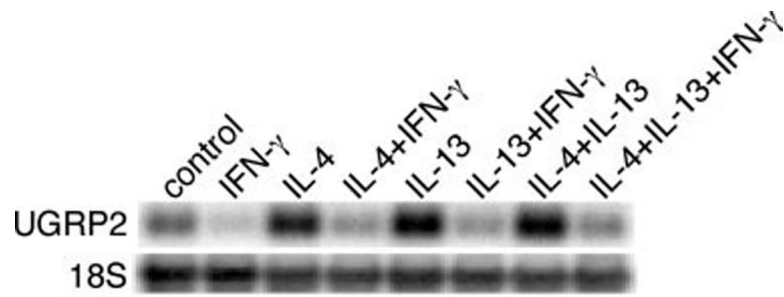


FIGURE 4.

IFN- γ inhibits the increase in *Ugrp2* mRNA caused by IL-4 and IL-13. The mtCC cells were pretreated with 20 ng/ml IFN- γ for 1 h, followed by treatment with 20 ng/ml IL-4 or IL-13 for 6 h. The expressions of *Ugrp2* and *18S* were detected by Northern blot analysis.

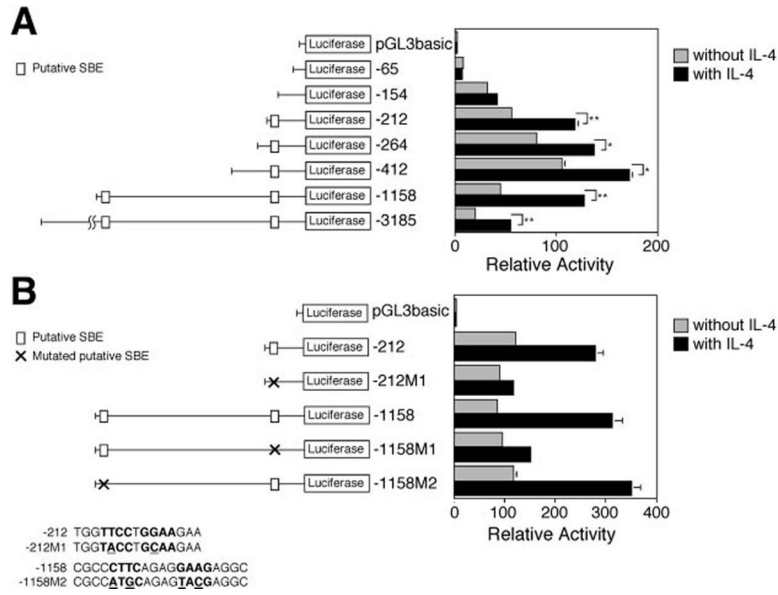
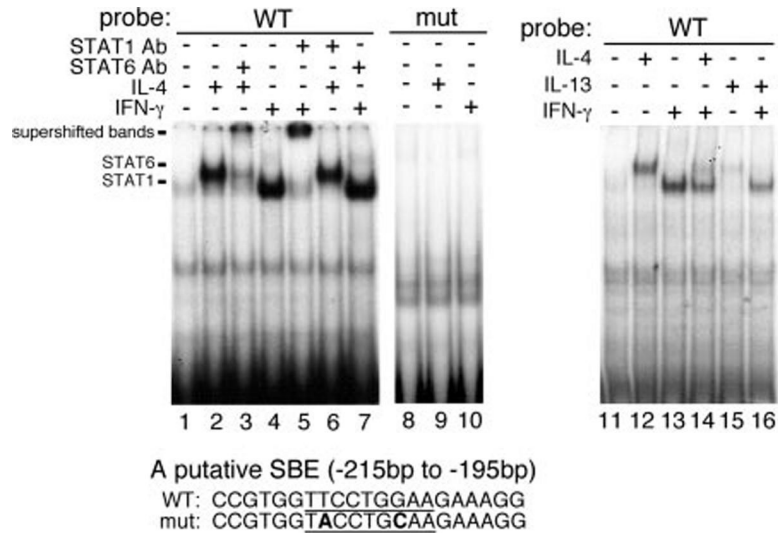
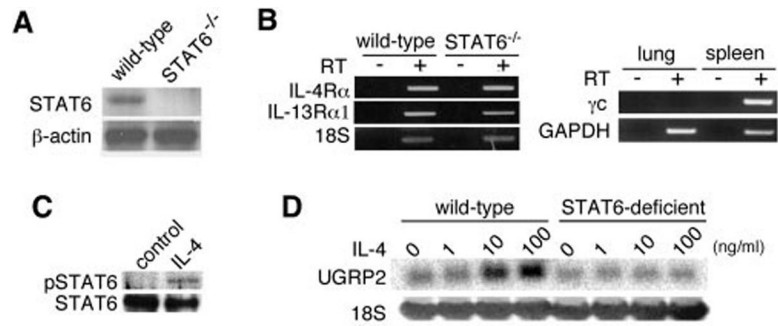


FIGURE 5. Determination of the IL-4-responsive region in the mouse *Ugrp2* gene promoter. *A*, Transfection analysis of a series of luciferase reporter plasmids containing various lengths (-3185, -1158, -412, -246, -212, -154, and -65/+52 bp) of the mouse *Ugrp2* gene promoter with or without IL-4. Putative distal (5'-**TTCAGAGGAA**-3'; -1154 to -1143 bp) and proximal SBEs (5'-**TTCCTGGAA**-3'; -212 to -154 bp) are indicated by the illustration on the *left*. Putative SBEs are characterized by a palindromic sequence shown in bold. Luciferase assays were performed in mtCC cells with (■) or without ([unk]) stimulation with 50 ng/ml IL-4. Results shown are the mean \pm SE from three independent experiments. A significant difference was obtained in the values with and without IL-4 treatment: *, $p < 0.05$; **, $p < 0.01$. *B*, Mutation analysis. The putative SBE sites and mutated sequences (underlined) are shown in bold. The mutation of proximal SBE abrogates the IL-4-induced promoter activities of -212 and -1158 constructs.

**FIGURE 6.**

EMSAs for STAT6 binding site in the *Ugrp2* gene promoter. A putative SBE is underlined. In the mutated SBE oligonucleotide (mut), mutations were introduced by changing two nucleotides (bold). Nuclear extract proteins (5 μ g) from macrophages stimulated with 20 ng/ml IL-4 (lanes 2, 3, 6, 9, and 12), IL-13 (lane 15), IFN- γ (lanes 4, 5, 7, 10, and 13), or IL-4/13 and IFN- γ together (lanes 14 and 16) for 30 min at 37°C were incubated with labeled wild-type (WT; lanes 1-7 and 11-16) and mutant (mut; lanes 8-10) SBE oligonucleotide. For supershift assays, 2 μ g of STAT6 (lanes 3 and 7) or STAT1 (lanes 5 and 6) Ab was added to probe-extract complexes. After incubations, DNA/protein complexes were resolved by 6% PAGE.

**FIGURE 7.**

STAT6 is required for IL-4 up-regulation of *Ugrp2* gene transcription. *A*, The presence of STAT6 in wild-type, but not STAT6-deficient (STAT6^{-/-}), mouse embryonic lung primary culture cells. Expression of STAT6 was detected by Western blotting using a rabbit polyclonal anti-STAT6 Ab. *B*, Expression of IL-4Rα and IL-13Rα1 and absence of γc in lung primary culture cells. RT-PCR was conducted using RNAs prepared from embryonic lung primary culture cells of wild-type and STAT6-deficient (STAT6^{-/-}) mice for IL-4Rα and IL-13Rα1, and from wild-type mice for γc. Spleen was used as a positive control for γc. The sizes of PCR products are 432 bp (IL-4Rα), 385 bp (IL-13Rα1), 191 bp (18S), 266 bp (γc), and 452 bp (GAPDH). *C*, STAT6 is activated by IL-4 in lung primary culture cells. Wild-type lung primary culture cells were treated with 50 ng/ml IL-4 for 30 min. The level of activated STAT6 was measured by Western blotting using a rabbit polyclonal anti-phospho-STAT6 (Tyr641) Ab. *D*, Northern blot analysis of total RNAs (20 μg/lane) prepared from wild-type or STAT6^{-/-} mouse embryo lung primary culture cells treated with IL-4 (0, 1, 10, and 100 ng/ml) for 24 h. Hybridization was serially conducted using a probe for *Ugrp2* and *18S*.