

Induction of uteroglobin-related protein 2 (*Ugrp2*) expression by EGF and TGF α

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Abstract Uteroglobin-related protein 2 (UGRP2) is thought to play a role in inflammation and/or epithelial cell differentiation in the lung. Induction of *Ugrp2* mRNA expression by epidermal growth factor (EGF) and transforming growth factor α was examined using mouse transformed lung Clara cell-derived mtCC cells. The EGF-induced increase of *Ugrp2* occurred at the transcriptional level that required the EGF receptor and the activation of the ERK-MAPK and phosphoinositide-3 kinase pathways.

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1. Introduction

Uteroglobin-related protein (UGRP) 2, also called high in normal-1 or secretoglobin 3A1 (SCGB3A1), was originally identified as a putative growth inhibitory cytokine gene that is inactivated by methylation in the majority of human breast carcinomas [1]. Independently, UGRP2 was found as a homologous gene to UGRP1 that is a downstream target gene for a homeodomain transcription factor, T/EBP/NKX2.1 [2,3]. In humans, UGRP2 is highly expressed in the trachea, lung, salivary gland, prostate, esophagus, duodenum and mammary gland [1]. In the latter, the expression is specifically found in the luminal mammary epithelial cells of small ducts and lobules [1]. In the adult mouse, UGRP2 is primarily expressed in the trachea and lung, and weakly expressed in the heart, stomach and small intestine [4]. Consistent with the importance of UGRP2 in human breast carcinomas, UGRP2 is expressed in the mouse mammary gland, but its expression is weak compared to that in trachea and lung [4].

Based on amino acid sequences, UGRP1 and UGRP2 belong to a gene superfamily of the Uterogloglobin/Clara cell secretory proteins (UG/CCSP), officially termed secretoglobins (SCGB), thus referring to UGRP1 and UGRP2 as SCGB3A2 and SCGB3A1, respectively [2–4]. UGRP1 is thought to play a

role in lung inflammation [2,5,6]. Further, UG/CCSP, a prototypical protein of the SCGB gene superfamily, to which UGRP1 and UGRP2 are distantly related, exhibits several anti-inflammatory and anti-chemotactic activities [7]. The mouse *Ugrp2* gene is localized at chromosome 11B1 [3], a homologous region to 5q31-q35 in human [8], in which many genes encoding inflammatory cytokines such as interleukin-3, -4, -5, -13 and colony-stimulating factor 2 are located. These facts together with the sites of UGRP2 expression suggest that UGRP2 may play a role in the regulation of inflammation and/or differentiation of epithelial cells [1,4]. However, little is known about the physiological function of UGRP2.

Both epidermal growth factor (EGF) and transforming growth factor α (TGF α) are potent mitogens for most epithelial tissues. Binding of EGF and TGF α to the receptor EGFR promotes dimerization of EGFR, which stimulates intrinsic tyrosine kinase activity, resulting in autophosphorylation of the receptor [9]. The tyrosine autophosphorylated EGFRs exhibit increased binding affinities to src homology 2 regions of substrate molecules. Substrate binding to the receptors is followed by phosphorylation of tyrosine residues on the substrate molecules, which in turn results in the activation of a variety of downstream signaling cascades [10,11]. Activation of the adaptor molecule, Grb2 occurs through its direct binding to the activated EGFR [12]. The Grb2/Sos-1 pathway is well characterized and is responsible for activation of the ERK-MAPK pathway, which ultimately leads to the regulation of gene transcription [13]. Phosphoinositide-3 kinase (PI3K) is also a substrate for activated EGFR [14,15]. Several PI3K isoforms are commonly found that play roles in a number of signal transduction pathways such as activation of PKB/Akt [16].

The aim of the present study was to investigate the mechanisms governing regulation of *Ugrp2* expression by EGF and TGF α . We demonstrate that both EGF and TGF α stimulate the expression of *Ugrp2* through a mechanism dependent on the ERK-MAPK and PI3K pathways.

2. Material and methods

2.1. Materials

Recombinant murine EGF, TGF α and HGF were purchased from PeprTech Inc. (Rocky Hill, NJ). Murine basic FGF (bFGF), actinomycin D (ActD), SB203580, SP600125 and PD098059 were obtained from SIGMA (St. Louis, MO), and LY294002 and AG1478 were from Calbiochem (San Diego, CA). Antibodies against murine ERK1 and ERK2 (ERK1/2), and phospho-ERK (Y204) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Akt and phospho-Akt (Ser473) from Cell Signaling Technology (Beverly, MA).

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Abbreviations: UGRP2, Uteroglobin-related protein 2; mtCC, mouse transformed lung Clara cell-derived cell line

2.2. Cell culture

Mouse transformed Clara cells (mtCC) were maintained in Dulbecco's modified Eagle's medium (Biosource, Camarillo, CA) supplemented with 10% fetal bovine serum (GEMINI Bio-Products, Woodland, CA) and Antibiotic–Antimycotic containing 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 250 ng/ml amphotericin B (Invitrogen Life technology, Carlsbad, CA).

2.3. RT-PCR

To prevent genomic DNA contamination, total RNAs were treated with RNase-free DNase I (Ambion, Austin, TX). For cDNA synthesis, total RNAs were first incubated at 70 °C for 10 min, and chilled on ice. The cDNA synthesis reactions were carried out in a final volume of 20 µl containing RNA, 4 µl of 5× first strand synthesis buffer, 1 µl of mixture of four deoxynucleotide triphosphates (10 mM each), 2 µl of 0.1 M dithiothreitol, and 1 µl of 500 ng/µl N6 random hexamer. After incubation at 37 °C for 2 min, 200 U of Superscript II reverse transcriptase (Invitrogen Life technologies) was added, and the incubation was continued at 37 °C for 60 min, which was then subjected to PCR using Advantage 2 Taq DNA polymerase (BD Biosciences, San Jose, CA) 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 30 cycles for mouse EGFR. Oligonucleotide primers used for RT-PCR were as follows: mouse 18S, 5'-CGGCTACCA-CATCCAAGGAA-3' and 5'-ATTGGAGCTGGAATTACCGC-3'; mouse EGFR, 5'-GGCGTTGGAAAAGAAAGT-3' and 5'-GATGGGGTTGTTGCTGAATCG-3'.

2.4. Northern blot analysis

Five µg of total RNAs extracted from mtCC cells was electrophoresed on a 1% agarose gel containing 0.22 M formaldehyde and blotted onto GeneScreen Plus nylon membranes (Perkin–Elmer Life Sciences, Boston, MA). Filters were serially hybridized with mouse *Ugrp2* and *18S* cDNA probes. Hybridization was performed in Perfect Hybridization solution (SIGMA) overnight at 68 °C. 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 phosphor-imager screen (Molecular Dynamics, Sunnyvale, CA).

2.5. Western blot analysis

mtCC cells were washed with phosphate-buffered saline 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 NaF and 1 mM sodium orthovanadate) with Protease Inhibitor Cocktail Tablets (Complete Mini; Roche Applied Science, Indianapolis, IN). The protein lysates mixed with SDS sample loading buffer containing β-mercaptoethanol were electrophoresed on a 10% SDS–polyacrylamide gel containing 0.8% bis(*N,N'*-methylene-bis-acrylamide), followed by electrotransfer to an Immobilon-P membrane (Millipore). Membranes were blocked with Tris-buffered saline (TBS) containing Tween 20 (TBS-Tween; 20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) with 5% skimmed milk, and were incubated with first antibody in TBS-Tween. Membranes were washed with TBS-Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for enhanced chemiluminescence detection using Western Lighting Chemiluminescence Reagent Plus (Perkin–Elmer Life Sciences). Image visualization was carried out by exposure to Scientific Imaging Film (Kodak, Rochester, NY).

3. Results

3.1. EGF and TGFα induce *Ugrp2* mRNA expression

To study the effect of several mitogens on the levels of *Ugrp1* and *Ugrp2* mRNA expression, mtCC cells were used. These cells, derived from necrotic tumor tissues of lungs obtained from transgenic mice expressing the simian virus 40 large T antigen gene under the control of *Ug/Ccsp* promoter [17,18], constitutively expresses high and modest levels of *Ugrp1* and *Ugrp2* mRNAs, respectively. When treated with 50 ng/ml of EGF, HGF, TGFα, and bFGF for 12 h, both EGF and TGFα

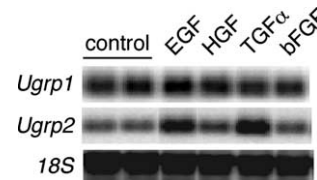


Fig. 1. Induction of *Ugrp1* and *Ugrp2* mRNAs by EGF and TGFα. mtCC cells were treated with 50 ng/ml of selected growth factors (EGF, HGF, TGFα and bFGF) for 12 h. Total cellular RNAs were extracted and mRNA for *Ugrp1*, *Ugrp2* and *18S* were examined by Northern blot analysis.

robustly induced *Ugrp2* mRNA levels, while *Ugrp1* expression stayed at similar levels regardless of addition of mitogens except for a slightly enhanced expression after EGF treatment (Fig. 1). These results suggest that the effect of EGF and TGFα is *Ugrp2*-specific, and thus only *Ugrp2* regulation by these mitogens was studied further.

3.2. Induction of *Ugrp2* mRNA by EGF occurs in a time- and dose-dependent manner

When mtCC cells were treated with different concentrations of EGF and TGFα for 24 h, the slight increase of *Ugrp2* mRNA levels was seen at 6.25 ng/ml for both mitogens (Fig. 2A). EGF induction appeared to plateau with 25 ng/ml while induction by TGFα continued with concentrations of up to 50 ng/ml. A time dependent effect of EGF and TGFα on the induction of *Ugrp2* mRNA was further examined using a fixed concentration of 50 ng/ml EGF and TGFα. In both cases, significant increase of *Ugrp2* mRNA was detected at 6 h after the addition of the mitogen, and levels continued to

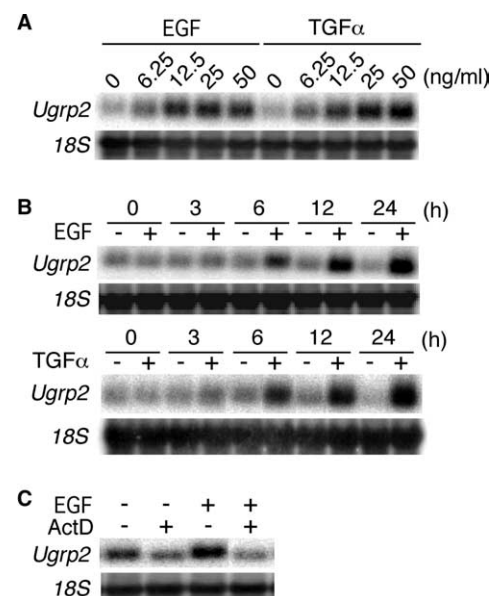


Fig. 2. Dose- and time-dependent increase of *Ugrp2* mRNA by EGF occurs at the transcriptional level. (A) Dose effects of EGF and TGFα. mtCC cells were treated with 6.25, 12.5, 25 and 50 ng/ml EGF or TGFα for 24 h. (B) Time course analysis of EGF and TGFα effects. mtCC cells were treated with 50 ng/ml EGF or TGFα for 3, 6, 12 or 24 h. (C) EGF induces *Ugrp2* mRNA at the transcriptional level. mtCC cells were pre-treated with 5 µg/ml ActD for 1 h, followed by treatment with 25 ng/ml EGF in the presence of ActD for 6 h. Expression of *Ugrp2* and *18S* was detected by Northern blot analysis.

increase up to 24 h (Fig. 2B). These results suggest that time-dependent increase of *Ugrp2* mRNA expression occurs with similar kinetics for both EGF and TGF α . To examine whether the increase of *Ugrp2* mRNA expression by treatment with EGF is due to transcriptional control, mtCC cells were pre-treated with the RNA synthesis inhibitor, ActD, followed by treatment with EGF. ActD abrogated the EGF-induced increase of *Ugrp2* mRNA expression (Fig. 2C), suggesting that the increase of *Ugrp2* mRNA expression by EGF is mediated at the transcriptional level.

3.3. Induction of *Ugrp2* mRNA by EGF is through EGFR

Both EGF and TGF α start a signaling cascade through their binding to a shared EGFR, a cell surface transmembrane protein with tyrosine kinase activity [19,20]. In order to study the mechanism of EGF-induced increase of *Ugrp2* expression, the presence of EGFR was first examined using RT-PCR analysis (Fig. 3A). The results clearly demonstrate that EGFR is present in mtCC cells. When cells were treated with AG1478, a selective inhibitor of EGFR tyrosine kinase activity [21] before the addition of EGF, the increase of *Ugrp2* mRNA by EGF was completely inhibited (Fig. 3B). These results suggest that the induction of *Ugrp2* mRNA by EGF occurs through EGFR activation.

3.4. ERK-MAPK and PI3K pathways are responsible for the induction of *Ugrp2* mRNA by EGF

As typical of growth factors and their receptors, ligand binding to EGFR initiates activation of ERK-MAPK and PI3K pathways. To understand whether ERK-MAPK and PI3K pathways are activated in mtCC cells upon EGF stimulation, phosphorylation of ERK1/2 and Akt was examined for each pathway, respectively. Treatment with the MEK inhibitor, PD098059 and the PI3K inhibitor, LY294002 totally abolished EGF-induced ERK1/2 and Akt phosphorylation (Fig. 4A and B). Interestingly, small amount of activated ERK1/2 was constitutively observed without any treatment, which was also abolished by MEK inhibitor, PD098059 (Fig. 4A). Further, both EGF-induced and constitutive expression of *Ugrp2* were partially and almost completely inhibited by PD098059 and LY294002, respectively (Fig.

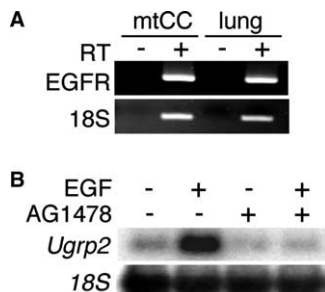


Fig. 3. Induction of *Ugrp2* mRNA by EGF requires activation of EGFR. (A) RT-PCR analysis for the presence of EGFR in mtCC cells. The size of PCR product is 397 bp for EGFR and 193 bp for 18S. RT(-) is shown as a negative control. RNAs extracted from fetal lung were used as a positive control. (B) AG1478, which is a selective inhibitor of EGFR phosphorylation inhibits the induction of *Ugrp2* by EGF. mtCC cells were pre-treated with 1 μ M AG1478 for 1 h followed by treatment with 50 ng/ml EGF in the presence of AG1478 for 6 h. Expression of *Ugrp2* and 18S was detected by Northern blot analysis.

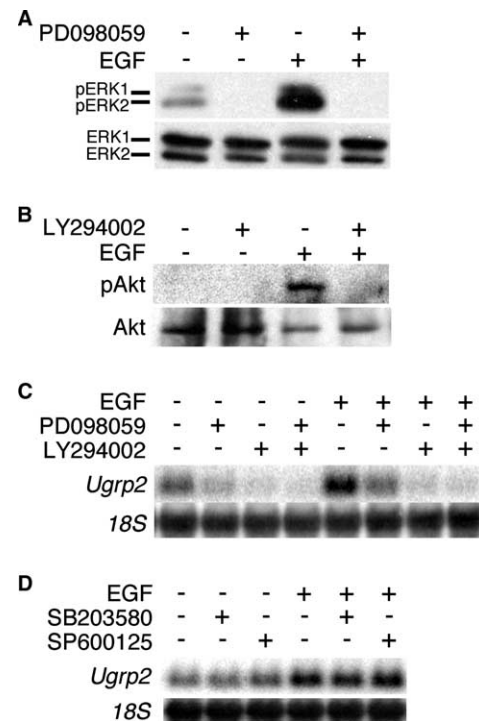


Fig. 4. Induction of *Ugrp2* mRNA by EGF requires activation of both ERK-MAPK and PI3K pathways. (A) ERK1/2 activation in mtCC cells by EGF stimulation. mtCC cells were treated with 25 ng/ml EGF for 5 min. Activation of ERK1/2 was measured by the presence of phosphorylated ERK1/2 (Y204) bands. The total ERK was detected by using anti-ERK antibody. (B) Akt activation in mtCC cells by EGF stimulation. mtCC cells were treated with 25 ng/ml EGF for 30 min. Activation of Akt was measured by the presence of phosphorylated Akt (Ser473) band. The total Akt was detected by using anti-Akt antibody. (C) Both ERK-MAPK and PI3K pathway inhibitors inhibit *Ugrp2* mRNA induction by EGF. mtCC cells were pre-treated with 10 μ M PD098059 (MEK inhibitor) and/or 10 μ M LY294002 (PI3K inhibitor) for 1 h, followed by treatment of 25 ng/ml EGF in the presence of either or both inhibitors for 6 h. Expression of *Ugrp2* and 18S was detected by Northern blot analysis. (D) Neither p38 kinase nor JNK pathway inhibitors inhibit *Ugrp2* mRNA induction by EGF. mtCC cells were pre-treated with 10 μ M SB203580 or 10 μ M SP600125 for 1 h, followed by treatment of 25 ng/ml EGF in the presence of either inhibitors for 6 h. Expression of *Ugrp2* and 18S was detected by Northern blot analysis.

4C). The level of *Ugrp2* expression did not seem to differ by the addition of both inhibitors together as compared to LY294002 alone, suggesting that the latter pathway may be dominant. In order to determine whether other classical MAPKs such as p38 kinase and JNK affect on the induction of *Ugrp2* mRNA, cells were treated with the p38 kinase inhibitor, SB203580 and the JNK inhibitor, SP600125. Neither inhibitors affected EGF-induced induction of *Ugrp2* expression (Fig. 4D). These results suggest that the induction of *Ugrp2* mRNA by EGF occurs through the ERK-MAPK and PI3K pathways, the latter being dominant.

4. Discussion

The present study demonstrates that *Ugrp2* mRNA can be regulated by EGF and TGF α through EGFR activation.

EGF activates both ERK-MAPK and PI3K pathways, leading to the upregulation of *Ugrp2* gene expression in mtCC cells. Both the ERK-MAPK inhibitor, PD098059 and the PI3K inhibitor, LY294002 alone or in combination, partially or completely blocked EGF-induced expression of *Ugrp2*, suggesting that *Ugrp2* transcriptional activation may require the cooperation of two or more transcription factors activated by distinct signal transduction pathways.

The MAPK family of serine/threonine kinases is well known to be composed of ERKs, JNKs and p38 kinases [22]. Activation of EGFR also induces JNK activation in a Rac-dependent manner [23], and p38 activation in a Src- and/or Fyn-dependent manner [24]. The induction of *Ugrp2* mRNA by EGFR activation does not seem to be through the p38 kinase and JNK pathways because no effect was observed on the *Ugrp2* mRNA levels by treatment of cells with their specific inhibitors. Further, these inhibitors did not affect constitutive levels of *Ugrp2* expression while MEK and PI3K inhibitors suppressed constitutive *Ugrp2* expression. In particular, the PI3K inhibitor, LY294002 almost completely suppressed constitutive expression of *Ugrp2*. This complete suppression was also observed for EGF-induced increase of *Ugrp2* expression. This suggests that PI3K pathway is dominant over ERK-MAPK pathway for constitutive as well as EGF-induced expression of *Ugrp2*. Constitutive *Ugrp2* expression may be partly accounted for by low levels of endogenous ERK1/2 activation observed in control cells, which was abolished by MEK inhibitor. On the other hand, Akt phosphorylation was not observed in control cells without EGF stimulation. There remains the possibility that a minute amount of phosphorylated Akt is also present in cells that cannot be detected by western blotting due to antibody sensitivity.

Although it remains to be determined whether other pathways are involved in the regulation of *Ugrp2* mRNA expression, the ERK-MAPK and PI3K pathways are currently responsible for *Ugrp2* gene regulation (Fig. 5). There is a molecular basis for cross talk between ERK-MAPK and PI3K pathways at the level of Raf and Akt, and ERK-MAPK pathway is activated by Akt through c-Raf activation [25]. Whether this cross talk plays a role in the activation of *Ugrp2* gene and whether this is related to the apparent dominance of the PI3K pathway over ERK-MAPK pathway need to be determined.

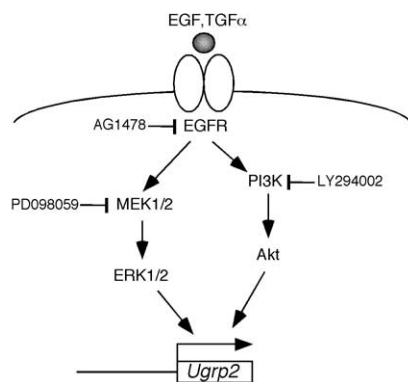


Fig. 5. Signaling pathways leading to the *Ugrp2* upregulation. EGF/TGF α activates ERK-MAPK and PI3K pathways leading to *Ugrp2* activation. Inhibitors at particular steps are shown.

Based on the pattern of *Ugrp2* mRNA expression in terminally differentiated airway epithelial cells of adult and developing mouse embryos, an association between UGRP2 and the terminally differentiated epithelial phenotype was suggested [4]. Thus, the induction of *Ugrp2* mRNA was correlated with upregulation of *Muc2*, a mucinous phenotype marker gene and downregulation of the squamous cell marker gene, transglutaminase I during retinoic acid-induced mucinous differentiation of primary normal human bronchial epithelial cells [4]. Despite these observations, the precise physiological and functional role of UGRP2 in the airways is not fully understood. Whether the regulation of UGRP2 expression by EGF/TGF α is involved in the development of terminally differentiated airway epithelial phenotypes awaits further studies.

In summary, the expression of *Ugrp2* is enhanced by both EGF and TGF α through the mechanism dependent on the ERK-MAPK and PI3K pathways.

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