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Signal Peptide of Eosinophil Cationic Protein Upregulates Transforming Growth Factor-Alpha Expression in Human Cells

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Abstract Eosinophil cationic protein (ECP) is a major component of eosinophil granule protein that is used as a clinical bio-marker for asthma and allergic inflammatory diseases. Previously, it has been reported that the signal peptide of human ECP (ECPsp) inhibits the cell growth of *Escherichia coli* (*E. coli*) and *Pichia pastoris* (*P. pastoris*), but not mammalian A431 cells. The inhibitory effect is due to the lack of human signal peptide peptidase (hSPP), a protease located on the endoplasmic reticulum (ER) membrane, in the lower organisms. In this study, we show that the epidermal growth factor receptor (EGFR) is upregulated by the exogenous ECPsp-eGFP as a result of the increased expression of the transforming growth factor-alpha (TGF-α) at both transcriptional and translational levels in A431 and HL-60 clone 15 cell lines. Furthermore, the N-terminus of ECPsp fragment generated by the cleavage of hSPP (ECPspM1-G17) gives rise to over threefold increase of TGF-α protein expression, whereas another ECPsp fragment (ECPspL18-A27) and the hSPP-resistant ECPsp (ECPspG17L) do not show similar effect. Our results indicate that the ECPspM1-G17 plays a crucial role in the upregulation of TGF-α, suggesting that the ECPsp not only directs the secretion of mature ECP, but also involves in the autocrine system. J. Cell. Biochem. 100: 1266–1275, 2007.

Key words: eosinophil cationic protein; signal peptide; signal peptide peptidase; transforming growth factor-alpha

Human eosinophil cationic protein (ECP) is located in the secretory granules of the eosinophil and thus is recognized as an eosinophil granule protein [Olsson and Venge, 1974]. The mature ECP protein is a polypeptide of 133 amino acids or approximately 18 to 22 kDa depending on the level of post-translational modification [Peterson et al., 1988]. ECP is also known as RNase3 belonging to ribonuclease A superfamily with a low ribonucleolytic but a high cytotoxic activity. The ribonucleolytic activity is not essential for the cytotoxicity of ECP [Rosenberg, 1995], suggesting that different mechanisms for the cytotoxic activity exist.

ECP has a signal peptide of 27 amino acids preceding the mature ribonuclease polypeptide [Rosenberg et al., 1989]. The signal peptide of ECP (ECPsp), like signal peptides of other

Abbreviations used: BA, butyric acid; DTE, dithioerythritol; ECP, eosinophil cationic protein; EGFR, epidermal growth factor receptor; eGFP, enhanced green fluorescent protein; hSPP, human signal peptide peptidase; Q-PCR, quantitative real time polymerase chain reaction; RNase, ribonuclease; TGF-α, transforming growth factor-alpha.

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secretory proteins, contains a short, positively charged amino-terminal region, a central hydrophobic region, and a polar carboxy-terminal region. The hydrophobic region of ECPsp contains a helix-breaking glycine residue which serves as a cleavage site of human signal peptide peptidase (hSPP), an intramembrane presenilin-like aspartic protease located in the membrane of animal and plant cells [Weihofen et al., 2002]. SPP is a glycoprotein containing seven to nine putative transmembrane regions with a molecular weight of approximately 40 kDa. In a previous study, we show that ECPsp plays a role in the cytotoxicity to inhibit the growth of bacterial and fungal cells. The ECPsp fusion proteins inhibit the growth of *Escherichia coli* (*E. coli*) and *Pichia pastoris* (*P. pastoris*), but not in mammalian cells, presumably due to lack of SPP in the lower organisms [Wu and Chang, 2004].

It has been postulated that ECPsp might be processed into two fragments ECPspM1-G17 (ECPsp1-17) and ECPspL18-A27 (ECPsp18-27) by hSPP, resulting in the loss of cytotoxicity in mammalian cells. However, the ECPspG17L peptide derived from site-directed mutagenesis could escape the proteolysis by hSPP, which in turn led to growth inhibition of A431 cells. In addition, knockdown of hSPP by small interference RNA (siRNA) also resulted in the inhibitory effect of cell growth [Wu and Chang, 2004]. In this study, investigation on the biological functions of hSPP-processed ECPsp fragments led to discovery of ECPsp1-17-induced expression of transforming growth factor-alpha (TGF-α) in A431 and HL-60 clone 15 cells at both transcription and translation levels. Since A431 cell line has been previously established and demonstrated to be able to process the signal peptide of eosinophil cationic protein (ECPsp) and is readily available [Wu and Chang, 2004], to prove the concept that transforming growth factor alpha (TGF-α) is upregulated as a consequence of intramembrane cleavage of ECPsp, the same cell line is chosen for all comparative studies in this study. In addition, among all leukemia cell lines, HL-60 is a promyelocyte leukemia cell line and upon treatment with butyric acid (BA), it is known as a characteristic to convert to eosinophil-like cells where ECP are secreted from. To monitor the function of ECPsp and evaluate the TGF-α induction event in cells possessing characteristic properties of eosinophil granules, HL-60 cell line is further tested in the absence and presence of BA. The overexpression of TGF-α in turn upregulates the expression of epidermal growth factor receptor (EGFR) in A431 cells. TGF-α is an eosinophil-derived protein involved in airway remodeling and has the ability to stimulate the proliferation of cells [Wong et al., 1990; Booth et al., 2001; Lordan et al., 2002; Kay et al., 2004]. Our results provide the first evidence that the N-terminal fragment of hSPP-processed ECPsp possesses novel cellular function that may contribute to the molecular mechanisms of proliferation of epithelial cells in airway remodeling related to asthma.

MATERIALS AND METHODS

Construction of ECPsp1-17-eGFP, ECPsp18-27-eGFP, and ECPspG17L-eGFP

The ECPsp variants were amplified by PCR using the primer pairs (Table I). The PCR products were digested by *Eco*RI and *Bam*HI and were separately ligated into the pEGFPN1 vector (Clonetech). The sequence was confirmed by using DNA sequencing (ABI).

Cell Culture

Human epidermoid carcinoma cell line A431 and human promyelocytic leukemia cell line HL-60 clone 15 were purchased from American Type Culture Collection (ATCC). A431 was cultured in DMEM (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS,

TABLE I. The Primers for Construction of Recombinant ECPsp Variants

Primer ID	Sequence (5′-3′)
ECPspG17L-F′	ATAT**GAATTC**ATGGTTCCAAAACTGTTCACT
ECPspG17L-R′	ATAT**GGATCC**TAGGCATGGAGTGAGCCCT
ECPsp1-17-F′	ATAT**GAATTC**ATGGTTCCAAAACTGTTCAC
ECPsp1-17-R′	ATAT**GGATCC**TACCCCAACAGAAGAAGCAG
ECPsp18-27-F′	**GAATTC**ATGCTTATGGGTGTGGAGGGCTCACTCCATGCC
ECPsp18-27-R′	**GGATCC**TAGGCATGGAGTGAGCCCTCCACACCCATAAGCAT

The bolded nucleotides were the restriction sites.

Biological Industries, Israel). HL-60 clone 15 was maintained in RPMI-1640 (Sigma) containing 20% heat-inactivated FCS. All cells were cultured at 37°C in an atmosphere containing 5% CO_2 and 95% air. HL-60 clone 15 cells were differentiated to eosinophil-like cells upon treatment with 0.5 mM butyric acid (BA, Sigma) as previously described [Fischkoff, 1988; Tiffany et al., 1995].

Conditioned Medium Exchange

Equal cell numbers of stably transfected eGFP/A431 and ECPsp-eGFP/A431 were separately cultured with complete medium at 37°C. After 24 h, the conditioned media were collected and stored at 4°C, and the cells were washed and starved with serum-free DMEM. After serum-starvation for 24 h, the collected conditioned media of eGFP/A431 and ECPsp-eGFP/A431 was respectively added to the ECPsp-eGFP/A431 and eGFP/A431 cells, and the cells were subsequently cultured for 24 h at 37°C. The cell lysate of each clone was then prepared for Western blot analysis.

Transfection

TransFast (Promega) was used for introducing the plasmids into A431 cells according to the manufacturer's instruction. A431 cells were plated approximately 80% confluent per 100-mm culture dish. Prior to the transfection, 10 μg plasmid DNA and 60 μl Transfast reagent were mixed in 4 ml serum-free medium. The transfection mixture was vortexed and incubated for 15 min at 37°C to allow formation of transfection complex. The growth medium was removed and subsequently the transfection complex was incubated with the PBS-washed A431 cells. After 1 h, the cells were gently overlaid with 8 ml complete medium and incubated at 37°C. The cells were harvested 48 h after transfection.

For HL-60 clone 15 cells, the plasmids were transfected by electroporation. HL-60 clone 15 cells (10^7 cells/250 μl) were washed once with PBS and resuspended in complete RPMI-1640 without antibiotics. Fifty micrograms of plasmid DNA was added into the cells and incubated on ice. After 15 min, the cell/DNA mixture was transferred to pre-cold electroporation cuvette (4 mm) and electro-pulsed under 275 V and 1,150 μF. The cells were immediately incubated on ice for another 15 min and seeded on 60-mm plate containing 5 ml complete culture medium at 37°C for 48 h for further analysis.

Western Blot

After SDS-PAGE, the proteins were transferred onto a nitrocellular membrane (Pall). The membrane was incubated in 3% BSA/TTBS (20 mM Tris-HCl, pH 7.6, 0.8% NaCl, 1% Tween-20) at 25°C for 1 h and then the diluted antibody (anti-TGF-α mAb, 1:1,000 or anti-EGFR mAb, 1:1,000, Abcam) in 1% BSA/TTBS was added to react with the target proteins with shaking at 4°C for 16 h. The membrane was washed with TTBS three times for 10 min each. The secondary antibody (anti-mouse IgG conjugated with HRP, 1:5,000, Jackson) was diluted in the 1% BSA/TTBS with shaking at 25°C. After 2 h, the membrane was washed with TTBS for three times and the target proteins were visualized by ECL system (Pierce).

Isolation of RNA and Quantitative Real-Time PCR (Q-PCR)

Total RNA of A431 and HL-60 clone 15 cells were isolated by Trizol Reagent (Invitrogen) and the contaminant genomic DNA was further digested by RNase-free DNase I (Promega). Two micrograms of total RNA and 0.5 μg oligo-dT were added in a sterile RNase-free microcentrifuge tube, and heated at 70°C for 5 min. The reverse transcription (RT) mixture containing 5 μl of M-MLV 5× reaction buffer, 1.25 μl of 10 mM dNTP, 1 μl recombinant RNasin (Promega), 1 μl M-MLV reverse transcriptase (Promega) and nuclease-free water was added to a final volume of 25 μl. The RT reaction was performed at 42°C for 1 h. Afterward, 12.5 μl SYBR Green Mix (Applied Biosystems), 10 ng cDNA, 200 nM target gene primers (Table II), and H_2O were mixed to a final volume of 25 μl, and subsequently the Q-PCR was performed at 95°C for 10 min followed by 40 cycle of 95°C for 30 s and 60°C for 30 s with ABI Prism SDS 7000. The result was analyzed with the SDS 7000 software.

Enzyme-Linked Immunosorbent Assay

The concentration of TGF-α was performed using sandwich ELISA kit (R&D systems) according to the manufacturer's instruction. Briefly, 100 μg protein lysates in 50 μl lysis buffer (PBS, pH 7.4, 1% Tween-20 and 1% NP-40) were added into the TGF-α Ab-coated wells

TABLE II. The Primers for Q-PCR

Primer ID	Sequence (5′-3′)
AR-F′	GCACCTGGAAGCAGTAACATGC
AR-R′	TCACAGCAGACATAAAGGCAGC
BTC-F′	AGCGGAAAGGCCACTTCTCTA
BTC-R′	TGCTCCAATGTAGCCTTCATCA
EGF-F′	TGGTTCAAAACGCCGAAGAC
EGF-R′	TGATGGCATAGCCCAATCTGA
EPR-F′	GGCTCCTTCATCGAATGCTAAA
EPR-R′	ACACATGGAGACAAGTCCTGGC
HB-EGF-F′	AAAGCCCAAGGTGCTGATGTC
HB-EGF-R′	AAAGCTACAGGCATGGAAGCC
TGF-α-F′	TTAATGACTGCCCAGATTCCCA
TGF-α-R′	CAACGTACCCAGAATGGCAGA
hSPP-F′	AAAGGCCTCGAAGCAAACAA
hSPP-R′	TCTTCAAGCTGATGTCAAAGCG
actin-F′	CAGTGGTACGACCAGAGGCATA
actin-R′	AGGCCAACCGTGAAAAGATG

containing 100 μl of the kit provided assay buffer and incubated at 25°C for 2 h. After washing for four times, the TGF-α Ab conjugated with horseradish peroxidase was added and incubated for 2 h. The unbound Ab was washed and then the substrates were added into wells and incubated for 30 min before spectrophotometrically monitoring the absorption at 450 nm. The absorption was corrected by subtracting the absorption at 565 nm. The TGF-α concentration in 100 μg lysates was measured by comparison to a standard curve generated from human TGF-α.

Protein Precipitation

One hundred microliters of 100% Trichloroacetic acid (TCA, Sigma) was added to 1 ml culture supernatant, vortexed for 15 s, and then placed on ice for 15 min. After centrifugation at 14,000*g* for 10 min at 4°C, the supernatant was removed, and the pellet was washed twice with 100 μl of acetone. Acetone was removed and the final pellet was allowed to air dry thoroughly for about 60 min at room temperature. One hundred microliters of 1× SDS-PAGE sample buffer was added and the mixture was heated at 95°C for 3 min, and stored at −20°C for further analysis.

RESULTS

EGFR and TGF-α Were Upregulated in A431 Cells Expressing ECPsp-eGFP

The EGFR is a transmembrane glycoprotein that constitutes one of four members of the EGFR family of receptor tyrosine kinase (RTK). Binding of EGFR to its cognate ligands leads to autophosphorylation of RTK and subsequent activation of signal transduction pathways that are involved in regulating cellular proliferation, differentiation, and survival [Yarden and Sliwkowski, 2001]. Moreover, it is reported that the ligands of EGFR generally act over short distances as autocrine or paracrine to stimulate EGFR expression and signal amplification [Tsao et al., 1996; Rusch et al., 1997; Piepkorn et al., 1998; Awwad et al., 1999; Arteaga, 2002]. The mRNA subtraction analysis of eGFP/A431 and ECPsp-eGFP/A431 cells showed that the EGFR was upregulated at the mRNA levels (data not shown). Western blot analysis further showed that the expression of EGFR protein was upregulated in ECPsp-eGFP/A431 cells (Fig. 1A). To investigate the role of ECPsp-eGFP in the regulation of EGFR and EGFR-ligands, the exchange experiments of conditioned media were performed. The A431, eGFP/A431, and ECPsp-eGFP/A431 cells were washed and starved with serum-free DMEM prior to replacement of conditioned media with those collected from different cells for 24 h. The result revealed that EGFR was upregulated in ECPsp-eGFP/A431, A431 cells treated with ECPsp-eGFP/A431-conditioned medium, ECPsp-eGFP/A431 cells treated with A431-conditioned medium, eGFP/A431 cells treated with ECPsp-eGFP/A431-conditioned medium,

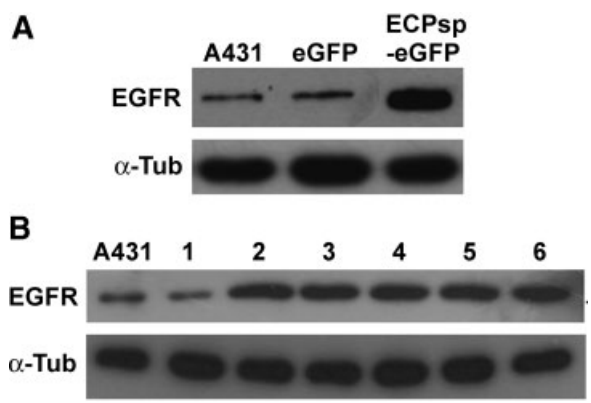

Fig. 1. ECPsp upregulated the expression of EGFR in A431 cells. **A**: The eGFP and ECPsp-eGFP were stably transfected into A431 cells separately. After cell lysis, the EGFR was detected by Western blot employing an anti-EGFR mAb. **B**: The experiments of conditioned medium exchanges were described in the ''Experimental'' section. The EGFR expression in A431, eGFP/A431 (**lane 1**), ECPsp-eGFP/A431 (**lane 2**), A431 cells treated with ECPsp-eGFP/A431-conditioned medium (**lane 3**), ECPsp-eGFP/A431 cells treated with A431-conditioned medium (**lane 4**), eGFP/A431 cells treated with ECPsp-eGFP/A431-conditioned medium (**lane 5**), and ECPsp-eGFP/A431 cells treated with eGFP/A431-conditioned medium (**lane 6**) was analyzed by Western blot. The α-tub (α-tubulin) served as the internal control.

and ECPsp-eGFP/A431 cells treated with eGFP/A431-conditioned medium (Fig. 1B, lanes 2–6). It appeared that some components secreted in the conditioned medium might account for the stimulation of the EGFR expression. Since EGFR-specific ligands including epidermal growth factor (EGF), amphiregulin (AR), transforming growth factor-alpha (TGF-α), betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR) have been reported to possess an EGF-like domain sufficient to confer binding specificity [Olayioye et al., 2000], Q-PCR screening was performed to examine whether any one of the six ligands of EGFR was upregulated during expression of ECPsp-eGFP in A431 cells. Our data indicated that the mRNA levels of AR, BTC, EGF, EPR, and HB-EGF showed no noticeable difference in A431 (lane 1), eGFP/A431 (lane 2), and ECPsp-eGFP/A431 (lane 3) cells as well as the conditioned medium exchange experiments (lanes 4 and 5) (Fig. 2). However, the mRNA level of the TGF-α was upregulated (∼fourfold) in ECPsp-eGFP/A431 as compared to that of eGFP/A431, and both the conditioned medium exchange experiments showed two to threefold upregulation of TGF-α as well (Fig. 2, right bottom panel). It was thus confirmed that the TGF-α was upregulated by the presence of ECPsp-eGFP in A431 cells.

Upregulation of EGFR Was Abolished by Knockdown of hSPP

Our previous study indicated that the ECPsp was a substrate of hSPP and could be cleaved at

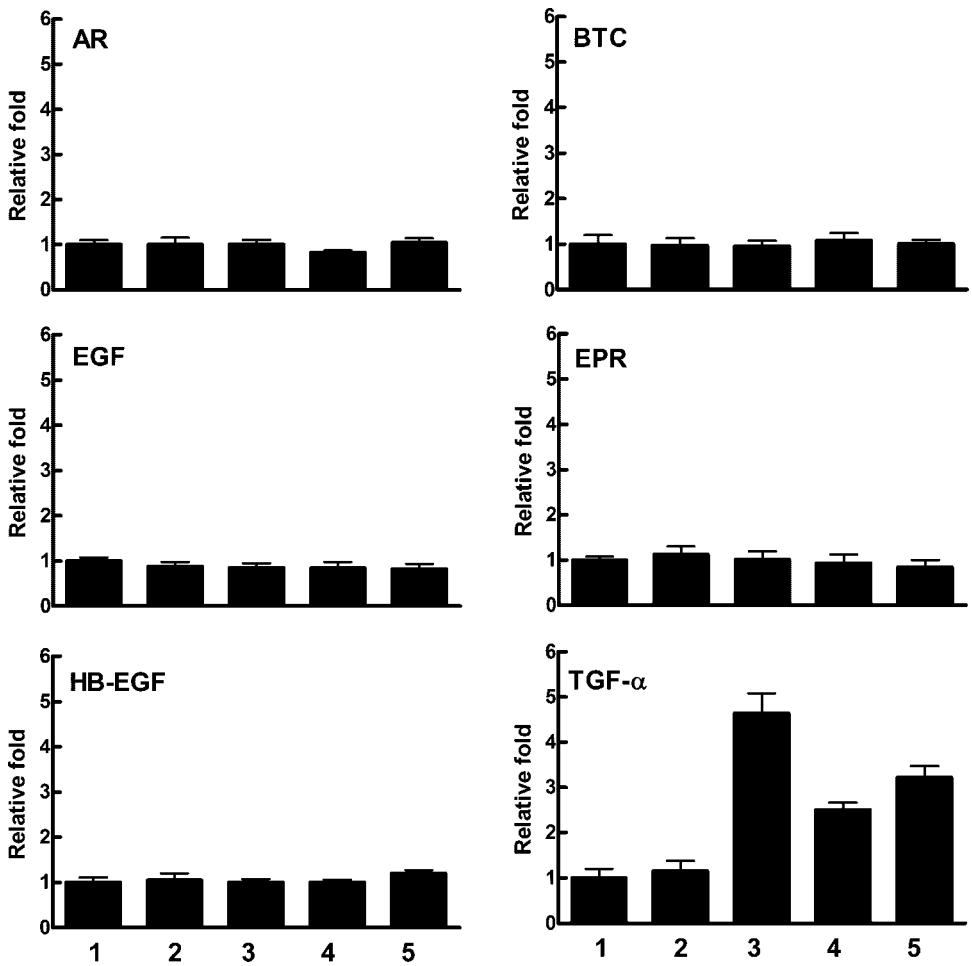

Fig. 2. TGF-α was upregulated in the A431 cells expressing ECPsp. The relative amounts of mRNAs of AR, BTC, EGF, EPR, HB-EGF, and TGF-α, six ligands of EGFR, were detected by Q-PCR. The A431, eGFP/A431, ECPsp-eGFP/A431, eGFP/A431 treated with ECPsp-eGFP/A431-conditioned medium and ECPsp-eGFP/A431 treated with eGFP/A431-conditioned medium were labeled as **lane 1**, **2**, **3**, **4**, and **5**, respectively. The average with standard deviation was derived from three independent experiments and the relative fold was normalized by actin.

glycine-17 by the intramembrane protease to generate two fragments ECPsp1-17 and ECPsp18-27 [Wu and Chang, 2004]. To correlate the upregulation of EGFR with hSPP processed ECPsp, the siRNA205 that targeted to hSPP mRNA was transfected into eGFP/A431 and ECPsp-eGFP/A431 cells separately. As shown in Figure 3A, the mRNA level of hSPP was specifically knocked down 2.7-fold by the presence of siRNA205 in ECPsp-eGFP/A431 cells post-transfection 24 h. Western blot analysis clearly indicated that the inductive production of EGFR was abolished (Fig. 3B);

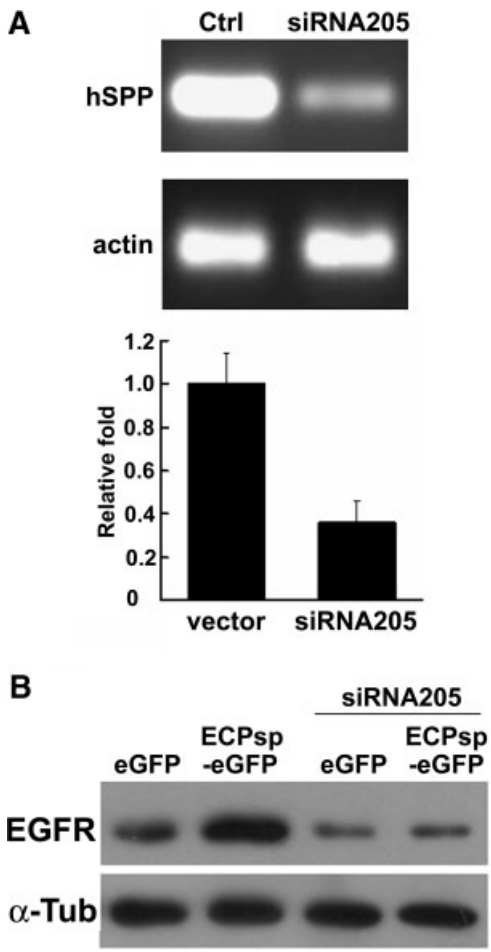

Fig. 3. The induction of EGFR was abolished upon treatment of hSPP siRNA. **A**: After the hSPP siRNA205 was transfected into ECPsp-eGFP stable-transfected A431 cells for 24 h, the mRNA level of hSPP was detected by Q-PCR and the relative fold was calculated using GelPro software. The average with standard deviation was derived from three independent experiments and the relative fold was normalized by actin. **B**: The hSPP siRNA205 silencing plasmid was introduced into eGFP/A431 and ECPsp-eGFP/A431 for 48 h. The EGFR expression was detected by Western blot employing an anti-EGFR mAb. The α-tub was used as the internal control.

presumable due to ECPsp maintained its integrity without cleavage by hSPP.

ECPsp1-17 Upregulated the Expression of TGF-α in A431 Cells

Since it was speculated that one of the two ECPsp fragments generated by hSPP processing might account for the upregulation of TGF-α expression, ECPsp1-17 and ECPsp18-27 fragments and an hSPP resistant mutant, ECPspG17L, were fused to eGFP, separately. The constructs were transfected into A431 cells and the expression of TGF-α was monitored by Q-PCR, Western blot, and ELISA. Figure 4A revealed that the mRNA level of TGF-α was upregulated 5.47- and 5.3-fold in ECPsp1-17-eGFP/A431 and ECPsp-eGFP/A431 cells, respectively. The protein level of TGF-α was also increased 3.52- and 2.96-fold in these two clones (Fig. 4B). In order to monitor the precise concentration of TGF-α in cytoplasm, a sandwich ELISA was performed. The TGF-α concentration in ECPsp-1-17-eGFP/A431 and ECPsp-eGFP/A431 was measured as 806.1 ng and 767.8 ng per 100 μg lysate, respectively, suggesting that a 2.97- and 2.83-fold increase of intracellular TGF-α in these two clones (Fig. 4C). However, the expression level of TGF-α was not affected in the ECPspG17L-eGFP/A431, ECPsp18-27-eGFP/A431, and ECPsp-eGFP/A431/siRNA205 cells, implying no induction of TGF-α expression occurred in these cells. Taken together, the results indicated that the ECPsp1-17 fragment of ECPsp generated by the cleavage of hSPP plays an essential role in the induction of TGF-α in A431 cells.

ECPsp1-17 Induced the Expression and Secretion of TGF-α in HL-60 Cells

HL-60 clone 15 cells were induced and differentiated to eosinophil-like cells using 0.5 mM butyric acid (BA) as previously described [Fischkoff, 1988; Tiffany et al., 1995]. The mRNA levels of both ECP and TGF-α were found to be upregulated after BA treatment (Fig. 5A,B), implying that when HL-60 clone 15 cells were differentiated to eosinophil-like cells, induction of more eosinophil-derived proteins led to higher expression of TGF-α. Q-PCR and ELISA assays showed that in the absence of BA, the TGF-α was upregulated only in ECPsp1-17-eGFP/HL-60, but not in ECPsp18-27-eGFP/HL-60 or

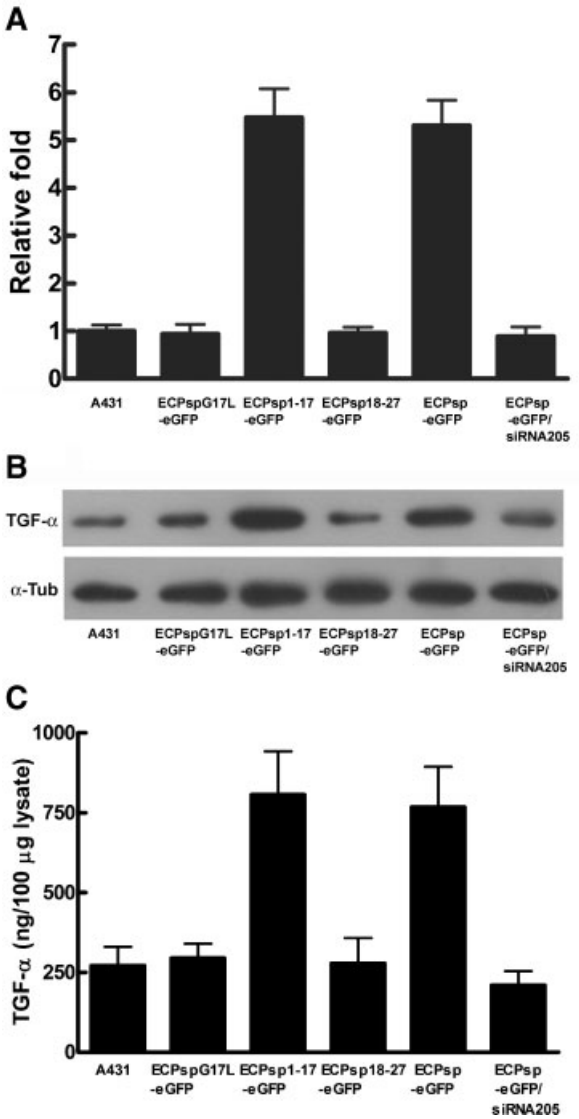

Fig. 4. TGF-α was upregulated by ECPsp-eGFP and ECPsp1-17-eGFP in A431 cells. The plasmids were introduced into A431 for 48 h. The transcriptional and translational levels of TGF-α in A431 were monitored by Q-PCR (**A**), Western blot (**B**), and ELISA (**C**). The relative level was normalized by actin mRNA for Q-PCR and α-tubulin for Western blot. The average with standard deviation was derived from three independent experiments.

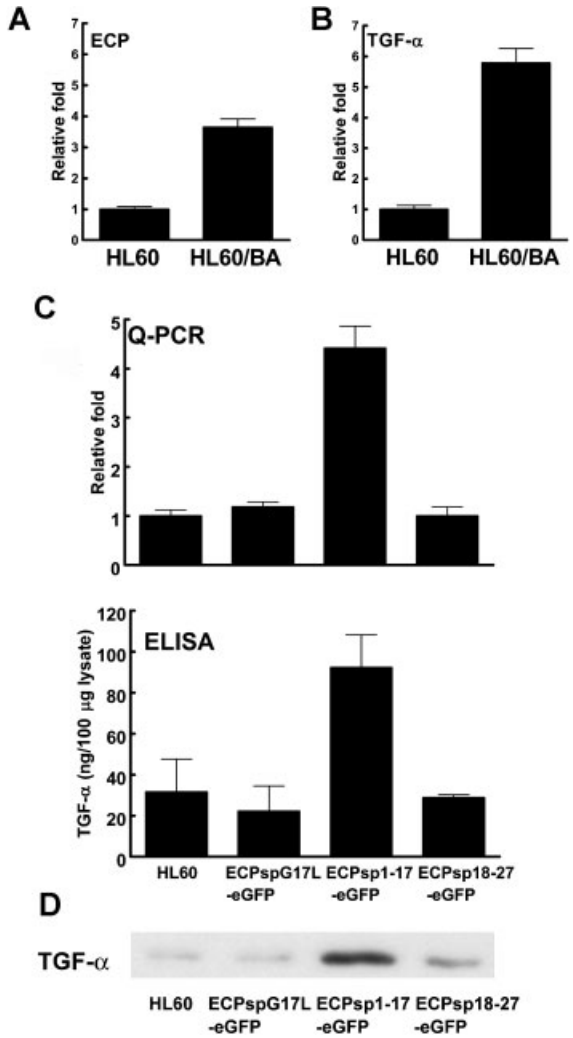

Fig. 5. TGF-α was upregulated by BA and ECPsp1-17-eGFP in HL-60 cells. The HL-60 clone 15 cells were treated with or without 0.5 mM BA. The relative mRNAs of ECP (**A**) and TGF-α (**B**) were monitored by Q-PCR and normalized by actin mRNA. The average with standard deviation was derived from three independent experiments. **C**: The plasmids were transfected into HL-60 clone 15 cells for 48 h. The relative mRNA and protein concentration of TGF-α were detected by Q-PCR (**upper panel**) and ELISA (**lower panel**), respectively. The average with standard deviation was derived from three independent experiments. **D**: The extracellular proteins were collected by TCA precipitation, and the TGF-α was detected by Western blot. Each loading was normalized by cell number.

ECPspG17L-eGFP/HL-60 (Fig. 5C); strongly suggesting that generation of ECPsp1-17 by hSPP was crucial for the consequence of TGF-α expression. TCA precipitation followed by Western blot analysis further revealed that the extracellular level of TGF-α was increased in ECPsp1-17-eGFP/HL-60 medium (Fig. 5D), providing an additional evidence that the ECPsp1-17 fragment was responsible for the induction and secretion of TGF-α in HL-60 clone 15 cells.

DISCUSSION

The hSPP belongs to a protein family of intramembrane-cleaving GXGD aspartic proteinase. The five members of hSPP have been classified into three subfamilies, SPP, SPP-like 2 series (SPPL2a, 2b, and 2c), and SPPL3 [Martoglio and Golde, 2003]. As determined by

microarray and EST database (UniGene, http://www.ncbi.nlm.nih.gov/), the hSPP transcripts are mainly expressed in the tissues such as blood, placenta, and liver [Friedmann et al., 2004]. Based on previous studies, the substrates of hSPP (e.g., ECPsp) contains helix-destabilizing residues in the scissile transmembrane region; the helix stability determines the cleavability by hSPP [Ye et al., 2000; Lemberg and Martoglio, 2002]. Here we provide the first evidence indicating that ECPsp cleaved by hSPP triggers further biological events including the upregulation of TGF-α and EGFR in the human cells.

The remodeled phenotype in asthma may be the consequence of excessive repair processes following repeated airway injury such as increased deposition of several extracellular matrix (ECM) proteins in the reticular basement membrane and bronchial mucosa, as well as increases in airway smooth muscle mass, goblet-cell hyperplasia, and new blood vessel formation [Bousquet et al., 2000]. Many eosinophil-derived proteins including TGF-α [Wong et al., 1990], TGF-β1 [Wong et al., 1991], fibroblast growth factor-2 (FGF-2) [Hoshino et al., 2001], vascular endothelial growth factor (VEGF) [Horiuchi and Weller, 1997], interleukin-13 (IL-13) [Schmid-Grendelmeier et al., 2002], and IL-17 [Molet et al., 2001] have been reported to be involved in airway remodelling. Similarly, the transactivation of EGFR was induced through CCR3 signaling pathway that elicits MAP kinase activation and cytokine production in bronchial epithelial cells such as HCI-H_{292} cell line [Adachi et al., 2004]. Our conditioned medium exchange experiments strongly suggested that the ligand(s) in the conditioned medium acted as an autocrine to induce EGFR expression in A431 cells. Q-PCR screening results revealed that among six potential EGFR ligands, TGF-α was the only upregulated factor induced by ECPsp and ECPsp1-17. The role of TGF-α in airway remodeling mechanism is still not completely unraveled. Asthma, epithelial-cell activation, ECM synthesis, and mucus hypersecretion may have resulted from the release of eosinophil-derived TGF-β, IL-4, IL-13, and TGF-α [Kay et al., 2004]. TGF-α has been reported to stimulate the bronchial epithelial cell proliferation and plays an important role in the repair mechanism in the airway injury [Madtes et al., 1994]. Considering that ECP is a major protein secreted by eosinophil, the central effector cell responsible for airway inflammation, our results have clearly demonstrated that ECPsp1-17 can induce TGF-α and EGFR expression, hence ECPsp may act as an inducer of TGF-α in airway remodeling mechanism when it was processed by hSPP in ER membrane.

Some signal peptides have been reported to possess an extra function besides simply protein targeting [Lemberg et al., 2001; McLauchlan et al., 2002; Martoglio, 2003]. In this study, ECPsp shows another novel biological function in addition to growth inhibition. Furthermore, the N-terminal signal peptide fragments of two hSPP substrates, preprolactin, and HIV gp160, with 20 amino acid residues in length were reported to bind to calmodulin, which in turn triggered the Ca^{2+}/calmodulin-dependent cellular signal transduction pathway [Martoglio et al., 1997]. Calmodulin is a universal Ca^{2+} sensor which interacts with numerous signaling proteins [Meyer et al., 1992], such as metabotropic glutamate receptors subtype 5 and 7 (mGlu5 and mGlu7) [Minakami et al., 1997; Nakajima et al., 1999], μ opioid receptor (MOR) [Wang et al., 1999], D2 dopamine receptor [Bofill-Cardona et al., 2000], and so on. The calmodulin binding domain usually contains a motif with an amphiphilic α-helix at the N-terminus and a C-terminal hydrophobic region [Bogsch et al., 1997]. In addition, the phosphorylation of EGFR and secretion of TGF-α transactivated via calmodulin, Pyk-2, and Src kinase pathway have been reported [McCole et al., 2002]. Interestingly, the ECPsp1-17 fragment seems to possess the specific structural characteristics for its N-terminal MVPKLFTSQIC is predicted to be an amphiphilic α-helix and the C-terminal LLLLLG is predicted to be a hydrophobic α-helix. Therefore, if ECPsp1-17 interacts with calmodulin and influence the downstream cellular signaling are worth of further study. As shown in the transactivation study, the promoter activity of TGF-α could be induced by estradiol, hepatitis B Virus X protein (HBx), and HBV preS1 through both a GC-rich site and an estrogen response element, the AP-2 sites, and a 315-bp response element, respectively [Ono et al., 1998; Vyhlidal et al., 2000; Kim and Rho, 2002]. Furthermore, synthetic peptide ECPsp1-17 was generated in an attempt to investigate the direct assess of the TGF-α expression. However, such peptide was

insoluble in buffer solutions including PBS, Tris, and HEPES, presumably due to the hydrophobic and helical nature. Although organic solvent such as DMSO and methanol could dissolve the peptide, the resulting mixture was still incompatible to transfection reagent and culture medium. Moreover, the synthetic peptide, ECPsp, was also insoluble in buffers since it processed an even longer hydrophobic transmembrane α-helix. Therefore, the detailed molecular mechanisms of TGF-α transactivation and airway remodeling via ECPsp still remain to be investigated. Taken together, our results provide additional evidence that ECPsp, particularly ECPsp1-17, is involved in upregulation of TGF-α and EGFR. This novel function of ECPsp1-17 is discovered and characterized for the first time, indicating that ECPsp1-17 involves in the autocrine system. Our observation on the induction of TGF-α in BA-treated HL-60 cells strongly suggests that as asthma patients are stimulated to generate higher amount of activated eosinophils, and TGF-α is upregulated along with overexpression of mature ECP to our blood. The novel role of ECP has shed a light on the association between allergic inflammatory disease and stimulation of autocrine system.

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