A Novel Interleukin-17 Receptor-like Protein Identified in Human Umbilical Vein Endothelial Cells Antagonizes Basic Fibroblast Growth Factor-induced Signaling*

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The abbreviations used are: IL-17, interleukin-17; IL-17R, IL-17 receptor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; EC, endothelial cells; FGF, fibroblast growth factor; bFGF, basic FGF; FGFR, FGF receptor; FiRE, FGF-inducible response element; HUVEC, human umbilical vein endothelial cells; hSEF, human SEF; ECD, extracellular domain; ICD, intracellular domain; FL, full-length.

We have previously utilized a combination of high throughput sequencing and genome-wide microarray profiling analyses to identify novel cell-surface proteins expressed in human umbilical vein endothelial cells. One gene identified by this approach encodes a type I transmembrane receptor that shares sequence homology with the intracellular domain of members of the interleukin-17 (IL-17) receptor family. Real-time quantitative PCR and Northern analyses revealed that this gene is highly expressed in human umbilical vein endothelial cells and in several highly vascularized tissues such as kidney, colon, skeletal muscle, heart, and small intestine. In addition, we also found that it is also highly expressed in the ductal epithelial cells of human salivary glands, seminal vesicles, and the collecting tubules of the kidney by in situ hybridization. This putative receptor, which we have termed human SEF (hSEF), is also expressed in a variety of breast cancer tissues. In co-immunoprecipitation assays, this receptor is capable of forming homomeric complexes and can interact with fibroblast growth factor (FGF) receptor 1. Overexpression of this receptor inhibits FGF induction of an FGF-responsive reporter gene in human 293T cells. This appears to occur as a result of specific inhibition of p42/p44 ERK in the absence of upstream MEK inhibition. This inhibitory effect is dependent upon a functional intracellular domain since deletion mutants missing the intracellular domain lack this inhibitory effect. These findings are consistent with the recent discovery of the zebrafish homologue, Sef (similar expression to fgf genes), which specifically antagonizes FGF signaling when ectopically expressed in zebrafish or Xenopus laevis embryos. Based on sequence and functional similarities, this novel IL-17 receptor homologue represents a potential human SEF and is likely to play critical roles in endothelial or epithelial functions such as proliferation, migration, and angiogenesis.
EXPERIMENTAL PROCEDURES

Reagents—Human basic FGF was from Oncogene Research Products (San Diego, CA). Antibodies against activated-ERK1/2, pan-ERK1/2, activated-p53, and pan-MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Anti-FLAG M2 monoclonal antibody was from Sigma. Anti-Myc 9E10 monoclonal antibody was from Covance (Princeton, NJ). Anti-human FGFR1 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Full-length Cloning and Expression Plasmids—A full-length clone of human basic FGF was identified by searching databases (NCBI) for sequences similar to the IL-17 receptor coding region using the BlastX algorithm (24). Based on gene prediction and public information (GeneBank accession number AF458067), the entire open reading frame of human SEF (hSEF) was identified by sequencing data (nucleotides 206–633) presented in tissue microarray slides. Altogether the various tissue samples analyzed were from more than 50 individuals.

In Situ Hybridization—The details of tissue preparation and in situ hybridization have been described earlier (28–30). Following the manufacturer’s protocol, digoxigenin-labeled antisense and sense riboprobes were synthesized from hSEF cDNA templates (nucleotides 206–633) using reagents supplied by Roche Applied Science. Sectioning, pretreatment of the sections, and hybridization of the probes were done under strict RNase-free conditions. All reagents were prepared using diethyl pyrocarbonate-treated distilled water. Sections of 15-μm-thick were collected on positively charged slides and dried at 55 °C overnight. The sections were deparaffinized, rehydrated in Histosolve (ThermoShandon, Pittsburgh, PA) and ethanol, and rinsed in diethyl pyrocarbonate-treated distilled water. The sections were treated at room temperature with 0.2 × HCL (20 min), 1.5% H2O2 (15 min), 0.3% Triton X-100 (15 min) followed by proteinase K treatment at 37 °C (30 min). The sections were then washed with triethanolamine buffer followed by acetylation with acetic anhydride. After prehybridization in 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) containing 50% formamide at 42 °C overnight, the sections were hybridized with the probes diluted in hybridization solution (2 × SSC, containing 50% formamide, 10× Denhardt’s, 0.001% SDS, 10 mM Tris, pH 7.4, 0.005% sodium pyrophosphate, and 500 μg/ml yeast tRNA) at 55 °C overnight. After hybridization, the sections were washed with 0.1 × SSC (2 × 15 min) and 2 × SSC (2 × 15 min). The sections then were incubated with RNase A at 37 °C for 30 min followed by washes in 2 × SSC at 37 °C (15 min), 0.1 × SSC at 42 °C (40 min), and finally in 0.1 × SSC at room temperature (2 × 15 min). The sections were washed with maleate buffer (30 min) and then blocked with 10 mM Tris buffer, pH 7.6, containing 500 mM NaCl, 4% bovine serum albumin, 0.5% cold-water fish skin gelatin, and 0.05% Tween 20. The sections were then incubated with anti-digoxigenin antibody conjugated to peroxidase (Roche Applied Science) for 1 h. The signal was amplified using TSA-Plus kit (PerkinElmer Life Sciences), and the signal was detected with Vector Blue substrate (Vector Laboratories, Burlingame, CA). After incubation with substrate, the sections were dehydrated in ethanol and xylene, and coverslipped. Hybridization with the sense control probe did not result in detectable signal, indicating the specificity of hybridization.

Microscopy—Slides were observed with an Olympus BX50 microscope (Olympus US, Inc., Melville, NY), using differential interference contrast illumination. The microscope was equipped with a NIKON DXM1200 digital camera (Technical Instruments San Francisco, Burlingame, CA). Digitized images (1280 × 1024-pixel resolution) were acquired using AIT-1 software (Nikon USA, Melville, NY). Images were resized, cropped and assembled using Photoshop version 6.0 (Adobe Systems, San Jose, CA). Apart from equalizing the background intensities, no other digital modifications of the original digital images were carried out.

RESULTS AND DISCUSSION

Identification and Full-length Cloning of hSEF—We have previously utilized a combination of high throughput sequencing and genome-wide microarray profiling analyses to identify novel cell-surface proteins expressed in HUVEC (21). One cDNA identified by this approach encodes a protein sequence homologous to the cytoplasmic domain of human IL-17R. To obtain the full-length cDNA of this gene, the original cDNA fragment was mapped to human genomic sequence (www.ensembl.org) and was found to be localized on chromosome 3p14.3, where a human gene was predicted based on its homology to zebrafish and mouse Sf (similar expression of fgf genes) (22, 23). Multiple oligonucleotides, based on the public sequence, were used to amplify the entire open reading frame.
from a mixture of HUVEC cDNAs. This cDNA contains an open reading frame of 2217 nucleotides and encodes a polypeptide of 739 amino acids (Fig. 1). Hydropathy (31) and protein family (32) analyses predict one 27-residue amino-terminal signal peptide (SP) followed by a 272-residue extracellular domain (ECD), one 20-amino acid membrane-spanning (TM) domain, and a 420-amino acid cytoplasmic domain (ICD) (Fig. 1). The ECD contains seven potential sites for N-linked glycosylation and shares apparent homology with mouse and zebrafish (mSef and zSef) (22, 23) but is otherwise distinct to all other proteins in the data base. Interestingly, the ICD is highly homologous to that of mSef and zSef, and the sequence similarity extends to the ICD of other members of the IL-17R family (15–20% identical) as well (11). It is noteworthy that eight cysteine residues are invariant among the ECD of human, mouse, and zebrafish SEF homologues, suggesting that these residues probably play a critical role in maintaining the secondary structure and/or functions of SEF.

**Cell Type and Tissue Distribution of hSEF Transcript**—To validate the EC origin of this novel receptor, hSEF expression was measured by real-time quantitative PCR (TaqMan) in a panel of cDNAs prepared from a variety of human primary cell types (top) or human tissues (bottom). Normalization was performed using the β2-microglobulin mRNA levels as controls in the same reaction as described under “Experimental Procedures.” b, Northern blot analysis of poly(A)+ mRNA from a variety of human tissues for SEF. One microgram of poly(A)+-enriched mRNA from various human adult tissues was hybridized with hSEF cDNA radiolabeled probe. The hSEF probe identified one major and a minor mRNA species of 8.5 and 4.4 kilobases, respectively. The lower panel shows the same blot hybridized with β-actin probe as a control. PBL, peripheral blood leukocytes; SMC, smooth muscle cells. Sk., skeletal.
sion by TaqMan, hSEF mRNA was expressed in a variety of normal human tissues with highest levels in ovary and breast (Fig. 2a, bottom panel). This expression pattern suggests that hSEF may also be expressed in epithelial cells. To further validate this tissue distribution, a Northern blot containing poly(A)-enriched mRNA (1/2/H9262)g from a variety of human adult tissue was hybridized with a hSEF cDNA radiolabeled probe. The expression level of the hSEF transcripts was highest in kidney followed by heart, skeletal muscle, colon, and small intestine and barely detectable in brain, spleen, liver, placenta, and lung (Fig. 2b), which is broadly consistent with the Taq-Man analysis (Fig. 2a, bottom panel).

Expression of hSEF in several highly vascularized tissues, such as kidney or heart, is consistent with the endothelial origin of hSEF; however, we were unable to confirm the EC expression pattern by in situ hybridization. The mRNA expression level in tissue ECs is below the sensitivity limit of in situ hybridization method we used.

High expression levels of hSEF in human breast prompted us to further examine a variety of epithelial-rich normal and tumor tissues. Intriguingly, we observed that hSEF mRNA expression was localized to ductal epithelial cells in human salivary gland, seminal vesicle, and the collecting tubules of the kidney by in situ hybridization (Fig. 3). hSEF message is uniformly expressed in normal epithelial cells in the intercalated ducts of the salivary glands (both in the parotid and submandibular glands) (Fig. 3a) and the collecting duct in kidney (Fig. 3c). In the seminal vesicle, hSEF is expressed in scattered solitary cells in the connective tissue stroma and in the epithelium (Fig. 3b). The precise identity of those cells is currently unknown.

hSEF Expression in Human Breast Tumors and Breast Carcinoma Cell Lines—We also examined hSEF expression in a number of breast tumor samples with different pathologies by in situ hybridization. As shown in Fig. 4, samples of well differentiated ductal carcinomas of the breast expressed hSEF uniformly in the epithelial cells, whereas the connective tissue stroma was negative in all cases (Fig. 4, a and b). Less differentiated forms of ductal carcinomas expressed hSEF at varying levels (Fig. 4c); several of those cases were negative (data not shown). Variable, non-uniform expression of hSEF was found in other types of breast tumors analyzed, such as fibroadenoma, cribriform carcinoma, and mucus adenocarcinoma (data not shown).

hSEF expression was also studied in three human breast carcinoma cell lines (DAKO HercepTest). These cell lines express the HER-2/neu receptor at various levels scored at 3+/H11001 (SKBR-3), 1+/H11001 (MDA-175), and 0 (MDA-231) (33). hSEF expression, which showed high cell-to-cell variations, correlates well with HER-2/neu oncogene expression in these breast carcinoma cell lines (Fig. 4, d–f). That is, MDA-231 (grade 0, no expression of HER-2/neu) has the weakest hSEF expression (Fig. 4d), whereas SKBR-3 (grade +3 for high expression of HER-2/neu) has the strongest hSEF expression (Fig. 4f). MDA-175 (grade +1 for intermediate expression of HER-2/neu) displayed moderate hSEF expression as compared with SKBR-3 cells (Fig. 4e).

Taken together these expression studies indicate that hSEF is expressed in certain epithelial cell type in vivo and may play
a role in the pathologv of certain tumors of epithelial origin. In addition, reproducible evidence of hSEF expression is seen in primary cultured human endothelial cells.

**hSEF Is Capable of Forming a Homomeric Complex and Interacts with FGFR1**—To elucidate the functions of hSEF, several epitope-tagged expression plasmids were constructed to produce the full-length (FL), ICD-truncated (∆ICD), or ECD-truncated (∆ECD) version of hSEF (Fig. 1b). The FLAG epitope was added at the amino terminus followed by signal peptide cleavage site, whereas the Myc tag was joined at the carboxyl terminus for the detection of the recombinant protein expression. We first examined whether or not hSEF, behaving like a putative receptor, could be expressed and targeted to the cell surface. The FLAG.hSEF-FL, ∆ICD, or ∆ECD proteins were expressed by means of transient expression in human embryonic kidney 293T cells. Forty hours post-transfection, cells were collected and incubated with anti-FLAG M2 antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody, then analyzed by flow cytometry. We found that expression of all three forms of recombinant hSEF proteins resulted in a shift of a population of fluorescein isothiocyanate-labeled cells by fluorescence-activated cell sorter analysis (data not shown), suggesting that hSEF indeed behaves like a receptor and is targeted to the cell surface.

Because families of cytokine receptors or growth factor receptor tyrosine kinases are often capable of forming dimeric or higher ordered structures (34, 35), and because hSEF is an apparent receptor, we hypothesized that oligomeric forms of hSEF may exist. The differential epitope-tagged versions of hSEF were singly or co-transfected in 293T cells, and we then examined their association by co-immunoprecipitation assays (Fig. 5a). Lysates of these cells were immunoprecipitated with anti-Myc antibody, and the precipitates were analyzed by immunoblotting with anti-FLAG antibody. An immunoreactive band of approximately 100 kDa recognized by the anti-FLAG antibody was observed in the anti-Myc immunoprecipitates from cells coexpressing hSEF-FL.Myc and FLAG.hSEF-FL proteins but not from cells coexpressing FLAG.hSEF-∆ICD or ∆ECD proteins in these assays (Fig. 5a). We did not observe an association between hSEF and human IL-1R1, suggesting specificity of the homomeric interactions between hSEF proteins (not shown). These results demonstrate that hSEF proteins are capable of forming oligomeric complexes. However, both the ECD and ICD of hSEF are required to maintain the stable homomeric interactions, at least in overexpressing cells.

Because zebrafish SEF was demonstrated to be co-immunoprecipitated with FGFRs from lysates of overexpressing COS cells (23), we next determined whether or not hSEF associates with human FGFR1 by co-transfection in 293T cells. As shown in Fig. 5b, hSEF-FL as well as deletion constructs of SEF, ∆ICD or ∆ECD, co-immunoprecipitated with human FGFR1. We also found that these associations remained equally efficient either in the absence or presence of FGF stimulation (not shown). These data suggest that the ECD or ICD alone is sufficient to establish the association between hSEF and FGFR1, and this interaction is independent of FGFR1 tyrosine kinase activation.

**hSEF Blocks PGF-mediated Induction of a FGF-responsive Reporter Gene**—The association between hSEF and FGFR1 prompted us to investigate whether or not hSEF can affect FGFR1-mediated signaling. One of the FGF target genes, the syndecan-1 gene, contains an upstream enhancer harboring an FGFRE that has been well characterized and appears to be activated selectively by members of the FGF family but not by other tyrosine kinase receptor-activating growth factors (e.g. epidermal growth factor, platelet-derived growth factor, or insulin-like growth factor) (25). We utilized the FiRE from the syndecan-1 gene to create a luciferase reporter gene that could monitor FGF-activated signaling and gene expression. When transfected into 293T cells, this FiRE reporter manifests an approximate 3-fold increase in expression in the presence of bFGF. This bFGF-mediated stimulation was selectively inhibited by co-expression of hSEF in a dose-dependent manner (Fig. 6). Interestingly, the deletion of the IL-17-like ICD in hSEF-∆ICD markedly reduced this inhibitory effect (Fig. 6). Therefore, overexpressed hSEF appears to suppress FGF-induced signal transduction, and this inhibitory effect is dependent upon a functional intracellular IL-17R-like domain.

**hSEF Is a Negative Feedback Regulator of the FGF-induced ERK Activation**—To further dissect the molecular inhibitory effect of hSEF on FGF-induced ERK signaling, 293T cells were transfected with empty vector or various expression plasmids of hSEF, as shown in Fig. 7. After serum starvation, cells were either left untreated or stimulated with bFGF for 10 min. Activation of ERK1/2 was determined by immunoblotting with a monoclonal antibody that specifically recognizes the activated (diphosphorylated) form of ERK1/2 (p42/p44 MAPK). Expression of hSEF-FL and the ECD truncated hSEF-∆ECD, but not ICD deletion of hSEF (∆ICD), resulted in a reduction in the levels of activated ERK1/2, whereas total ERK1/2 protein...
transfected cells were treated with 50 ng/ml basic FGF for 6 h and FLAG.hSEF-protein expression level of hSEF-FL or hSEF-ICD expression plasmid. After 20 h of serum starvation, transfected cells were treated with 50 ng/ml basic FGF for 6 h and subsequently lysed for the measurement of luciferase activity. Cell lysates were also immunoblotted by FLAG antibody to examine the protein expression level of hSEF-FL or hSEF-ICD (data not shown).

remained unaltered (Fig. 7a). This hSEF-mediated attenuation of FGF-induced ERK activation is consistent with the reduction in FGF-induced reporter gene activity seen previously (Fig. 6). Interestingly, immunoblotting with a monoclonal antibody that recognizes the upstream activated (phosphorylated) forms of MEK1/2 showed that hSEF overexpression had no effect on FGF-induced MEK activation (Fig. 7b). These data indicate that the targeting site of hSEF, at least in 293T cells, is likely to be downstream (or at the level) of MEK1/2 and upstream (or at the level of) of ERK1/2 (Fig. 7, a and b). Overall, our findings are broadly consistent with the molecular action of zebrafish SEF as previously reported (22, 23), although the precise molecular mechanism underlying the hSEF-dependent blocking of FGF signaling is currently unknown.

Recently, a family of negative regulators of receptor tyrosine kinase signaling, Sprouty proteins, have been identified in fly and mammals (36–40). Both epidermal growth factor and FGF up-regulate the expression of Sprouty genes (41, 42). Interestingly, we reproducibly observed that hSEF mRNA expression was induced upon epidermal growth factor and FGF stimulation in HUVEC (data not shown). This supports the hypothesis that hSEF is a feedback-induced antagonist of FGFR1/ERK-mediated FGF signaling. It had been previously demonstrated that membrane translocation of Sprouty proteins is necessary for their phosphorylation, which is essential for their inhibitory activity (43, 44). However, unlike Sprouty, hSEF is already membrane-anchored and interacts with FGFR1. In addition, we did not observe tyrosine phosphorylation of hSEF in response to growth factor stimulation (not shown).

Because members of the IL-17 cytokine family are expanding (11) and because hSEF shares sequence homology with IL-17R, we tested whether or not hSEF can bind to members of IL-17 cytokine family in a binding assay. However, incubation of the recombinant ECD of hSEF-IgG fusion protein with individual recombinant IL-17-like cytokines failed to show an association in a protein A-agarose pull-down assay (data not shown). This study does not exclude a role for hSEF in IL-17R-mediated functions; however, together with data that hSEF can inhibit FGF signaling, these studies suggest that the functions of hSEF in vivo are complex.

Is Human SEF Involved in Tumorigenesis?—Given the negative feedback nature of hSEF on growth factor-receptor tyrosine kinase signaling, it is conceivable that a defect (e.g. loss-of-function mutation) in the hSEF gene might lead to unchecked, malignant cell proliferation. Consistent with this hypothesis, the hSEF gene is located on chromosome region 3p14 (47, 48). This raises the possibility that a loss-of-function mutation in the hSEF gene might lead to a tumor suppressor locus for renal cell carcinoma was physically and functionally mapped within human chromosome region 3p14 (47, 48). This raises the possibility that the hSEF gene may be a viable candidate for this tumor suppressor locus.

In summary, we have identified a novel FGFR negative feedback regulator encoded by a type I transmembrane receptor. Based on sequence and functional similarities, this gene appears to be the human homologue of a recently discovered...
zebrafish SEF and may function as a critical modulator for receptor tyrosine kinase-mediated biology such as cell proliferation, migration, and angiogenesis.

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REFERENCES

1. Lubberts, E., Joosten, L. A., van de Lee, F. A., van den Gersel, L. A., and van den Berg, W. B. (2000) *Arthritis Rheum.* 43, 1300–1306.
2. Chabaud, M., Lubberts, E., Joosten, L., van den Berg, W., and Miossec, P. (2001) *Arthritis Res.* 3, 168–177.
3. Albanesi, C., Scarpone, C., Sebastiani, S., Cavani, A., Federici, M., De Pita, O., Maruoka, M., Mao, W., Foster, J., Kelley, R. F., Pan, G., Gurney, A. L., and Wood, W. I. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 773–778.
4. Lee, J., Ho, W. H., Murasko, M., Corpuz, R. T., Baldwin, D. T., Foster, J. S., Goddard, A. D., Yansura, D. G., Vanden, R. L., Wood, I. W., and Gurney, A. L. (2001) *J. Biol. Chem.* 276, 1660–1664.
5. Hymowitz, S. G., Filvaroff, E. H., Yin, J. P., Lee, J., Cai, L., Risser, P., Maruaska, M., Mao, W., Foster, J., Kelley, R. F., Pan, G., Gurney, A. L., de Vos, A. M., and Starovasnik, M. A. (2001) *EMBO J.* 20, 5332–5341.
6. Starnes, T., Robertson, M. J., Schmid, G., Kuch, S., Nakahata, H., Broxmeyer, H. E., and Hramas, R. (2001) *J. Immunol.* 167, 4137–4140.
7. Cines, D. B., Pollak, E. S., Buck, C. A., Mascia, G., McEve, R. P., Poler, J. S., Wick, T. M., Konkle, B. A., Schwartz, B. S., Bar-Nathan, E. S., McCrane, K. R., Hug, B. A., Schmidt, A. M., and Stern, D. M. (1998) *Blood* 91, 3527–3561.
8. Topper, J. N., and Gimbrone, M. A., Jr. (1999) *Mol. Med. Today* 5, 40–46.
9. Pavletich, N. P., and Chakraburtty, K. (1996) *Science* 273, 588–588.