hSef Inhibits PC-12 Cell Differentiation by Interfering with Ras-Mitogen-activated Protein Kinase MAPK Signaling*

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Growth factor signaling by receptor tyrosine kinases regulates several cell fates, such as proliferation and differentiation. Sef was genetically identified as a negative regulator of fibroblast growth factor (FGF) signaling. Using bioinformatic methods and rapid amplification of cDNA ends-PCR, we isolated both the mouse and the human Sef genes, which encoded the Sef protein and Sef-S isoform that was generated through alternative splicing. We provide evidence that the Sef gene products were located mainly on the cell membrane. Co-immunoprecipitation and immunostaining experiments indicate that hSef interacts with FGFR1 and FGFR2 but not FGFR3. Our results demonstrated that stably expressed hSef strongly inhibits FGF2- or nerve growth factor-induced PC-12 cell differentiation. The intracellular domain of hSef is necessary for the inhibitory effect on FGF2-induced PC-12 cell differentiation. Furthermore, our data suggested Sef exerted the negative effect on FGF2-induced PC-12 cell differentiation through the prevention of Ras-mitogen-activated protein kinase signaling, possibly functioning upstream of the Ras molecule. These findings suggest that Sef may play an important role in the regulation of PC-12 cell differentiation.

Cell growth and differentiation mediated by receptor tyrosine kinases are regulated by many extracellular signals, most of which activate the Ras-MAPK1 kinase cascade (1–4). Ras directly interacts with Raf, then activated Raf phosphorylates and activates MEK, which phosphorylates and activates MAPK kinases including ERK1 and ERK2 (5–7). Excessive or inappropriate growth factor signaling by receptor tyrosine kinases has been implicated in the progression of several cancers and disorders of developmental processes. Thus, the strength and duration of this signaling must be controlled strictly in normal biological processes. A negative feedback loop is one of the mechanisms that provides an effective way to terminate, limit, or modulate receptor tyrosine kinase signaling (8, 9). In typical negative feedback regulation, the signaling molecule induces the expression of its own negative regulator such that signaling is inhibited once a threshold is reached (10–12).

Recently, many novel negative regulators of growth factor signaling were described using genetic screening methods. Sprouty was identified in Drosophila melanogaster as an inhibitor of FGF signaling during tracheal development (13). However, how this gene functions remains unknown. During D. melanogaster eye development, Sprouty seems to inhibit the activation of MAPK upstream of Ras (14). In contrast, during wing development, Sprouty was reported to inhibit MAPK downstream of Ras (15). D. melanogaster has only one Sprouty protein, whereas mammals have at least four isoforms (16–19). Sef (similar expression of FGF genes) is another novel negative regulator of receptor tyrosine kinase signaling that was first isolated from a zebrafish embryo library through in situ hybridization (20, 21). Sef was reported to be syn-expressed with FGFR8, FGFR3, Sprouty2, and Sprouty4 in zebrafish and in mouse. Extensive similarities between the expression patterns of these genes indicate that they could be coregulated. In zebrafish, Sef functions as a novel negative regulator of Ras-MAPK-mediated FGF signaling (20, 21). Most recent reports indicated that overexpression of mSef in vitro could inhibit FGF-induced 3T3 cell proliferation associated with a reduction of FGFR1 phosphorylation (22). Those results suggest that Sef could be involved in cell proliferation, differentiation, or other cell fates mediated by FGF signaling. However, the physiological function of mammalian Sef remains unknown. Accordingly, the detailed molecular mechanism that Sef regulates FGF signaling in mammalian cells needed to be clarified.

In this study, we searched GenBank using a conserved domain of the IL-17AR and identified a gene with 31% amino acid identity to IL-17 receptor (23, 24). We then used RACE to isolate full-length cDNAs and identified two isoforms. The long isoform of the sequence was identical to the recently reported mouse and human Sef genes (22, 25, 26). We have named the short isoform Sef-S in this article. More importantly, using PC-12 cell lines as a model, we demonstrate that overexpression of hSef significantly inhibited FGF2- or NGF-induced PC-12 cell differentiation. hSef, as a negative regulator of FGF signaling pathway, exerted an inhibitory effect on PC-12 cell differentiation, possibly through prevention of FGFR-Ras-MAPK signaling on molecules upstream of Ras.

EXPERIMENTAL PROCEDURES

GenBank Data Base Search and RACE PCR—The human high-throughput genomic sequence (HTGS) data base was scanned for "vir-
Fig. 1. Sequence information of Sef. A, nucleotide and deduced amino acid sequence of interleukin-17 receptor-like molecule (hSef). Nested RACE-PCR was used to clone the 5'-fragnent of the Sef cDNA and then the full-length cDNA was assembled by RT-PCR. Translated and untranslated regions of the cDNA are in uppercase and lowercase letters, respectively. Eight conserved Cys residues in the extracellular domain of the gene are in the bold boxes. The predicted signal peptide and transmembrane domain are underlined and doubly underlined, respectively. Putative TIR domain (Toll/IL-1 Receptor domain) in the intracellular domain of the gene (V358-K424) is shown in italics. Nine potential N-linked glycosylation sites are boxed. B, sequence alignment of Sef alternative splicing forms. Both splicing forms of hSef were aligned using ClustalW multiple alignment. hSef-S is shown as a truncated form, which lacks 144 amino acids at the N terminus compared with hSef. C, genomic structure of human Sef. Schematic representation of exons is marked, and spans nucleotides 427,937–498,840 (hSef) and 420,648–506,133 (hSef-S) in NT_005787.8 from human chromosome 3p21. The hSef (long form) has 13 exons and hSef-S (short form) has 14 exons. The start and stop codons are indicated.
rylation sites. To obtain a full-length cDNA, 5′-RACE PCR was performed using mRNA from normal human testis and 293T cells according to the SMART RACE cDNA amplification kit user manual (Clontech). Total RNA was extracted with TRIzol reagent kit (Invitrogen) and reverse-transcribed using an oligo(dT) primer and Superscript II kit (Invitrogen). The 5′-RACE PCR products were then cloned into pT-Adv vector according to the AdvanTage PCR cloning kit instructions and then sequenced.

**Plasmid Construction**—Full-length cDNA of hSef was cloned into pcDNA3.0 and pcDNA3.1/Myc-His by HindIII/XhoI sites. hSef-S and mSef-S were subcloned into the EcoRI/XhoI sites of pcDNA3.0 with a six-repeat Myc tag at the N terminus. The deletions and mutants of hSef Inhibits PC-12 Differentiation 50275

| Plasmid Name | Sequence | Length (aa) |
|--------------|----------|-------------|
| hSef         | MAPWQLQCSVFTNACNLGNSQLAVAAAGSGRARGATCGWRGVPASRNSGGLYNTFKY | 60 |
| hSef-S       | 1        | 1           |
| hSef         | DNCTTYLNPVGKHVIADAQNITISQYACHDQVTVILWSPGALGIEFLKGFVRILBEI | 120 |
| hSef-S       | 1        | 1           |
| hSef         | EGRQCOQLILKPDKQLNSSFRTG | 180 |
| hSef-S       | 1        | 36          |
| hSef         | RTRACDLLLPQDNAACFKPPKRNLRNSQHGSDMQVSPDHAPNFGRPFLHYLHKLHEC | 240 |
| hSef-S       | 37       | 96          |
| hSef         | PFKRKTCEQETQTTETRSCLQNVSPGDIIEELVDDTNTRKVMHYALPKVHSPWAGPIRA | 300 |
| hSef-S       | 97       | 156         |
| hSef         | VAITVPLVISAAPATLPTVMCRKKQHENYSLDEESESSTYTAALPRSLRPRPKVTI | 360 |
| hSef-S       | 157      | 216         |
| hSef         | CYSSKDQNMVQVCFAYFLQDCFGEVALDLWEDFLSCLQREQRWVIKIHIHSQPIV | 420 |
| hSef-S       | 217      | 276         |
| hSef         | VCSKGMKYVFDKNNYKKGCCRGSKGELPLVAISAIAEKLRQARKQSSAALSKFIAVFY | 480 |
| hSef-S       | 277      | 336         |
| hSef         | DYSCBGDVPLGIDSLTRYLMNLPOLCSLHSLRHDGLOEQPQGHTRGSRRNYFSGSR | 540 |
| hSef-S       | 337      | 396         |
| hSef         | SLAYVA1CNMQFDIDEEDPWFKEQVFVPHPPRLREYPVLEFKDSGVLNDVMCPGPESI | 600 |
| hSef-S       | 397      | 456         |
| hSef         | FCLKVEAALVGATPDQSHERSSHGDQDGGEAECAALQLPTHTKAGPSGSDMPR | 660 |
| hSef-S       | 457      | 516         |
| hSef         | DSGIYDSSVPSBELSLPMEGLSTDQETTESETESVVSSGSLGEEEPPALPSKLLSSGSC | 720 |
| hSef-S       | 517      | 576         |
| hSef         | KADLGCROYTDHELHAPVI | 739 |
| hSef-S       | 577      | 595         |

**Fig. 1—continued**
hSef were cloned into pIRESneo expression vector by standard polymerase chain reaction. Mouse FGFR1, -2, and -3 constructs were provided by Dr. D. Onitz. Elk-1 luciferase reporter plasmids and Sprouty4 were the gift from Dr. A. Yoshimura. The constitutive active MEK was donated by Dr. T. Satoh.

**Cell Lines—**293T cells and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Hyclone) and 5% horse serum (Invitrogen). Rat pheochromocytoma PC-12 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 5% horse serum (Invitrogen).

**Antibody Preparation**—For bacterial production of hSef, an open reading frame coding for the peptide in intracellular domain of hSef (M128–157, 138 aa) was amplified by PCR and cloned into pGEX4T-1 vector. The reading frame was sequence-confirmed after cloning. hSef was expressed in the inclusion bodies in Escherichia coli, solubilized with 4x guanidine HCl, and dialyzed against 50 mM sodium acetate buffer, pH 5, containing 0.1 M NaCl. Antiserum were raised in rabbits by standard methods and used for immunoblot analysis after 1,000–10,000-fold dilution.

**Immunofluorescent Staining and Microscopy**—COS-7 cells were cultured on 6-well plates with 8 × 10^4 cells per well (Corning Incorporated, Corning, NY). Cells were cotransfected with mFGFR2 and hSef constructs using Effectene transfection reagent (Qiagen). hSef and mFGFR2 were stained with secondary antibodies conjugated with fluorescein isothiocyanate (green) and Cy3 (red), respectively. The cells were viewed using a Nikon inverted microscope ECLIPSE TE300. The co-localization of the two proteins was shown as a merged figure.

**Generation of Stably Transfected PC-12 Cell Clones**—To establish hSef stably expressed PC-12 cell lines, PC-12 cells were transfected with the above constructs using Transfast transfection reagent (Promega). Forty-eight hours after transfection, cells were plated at several different dilutions in media containing 0.5 mg/ml G418. For the next 2 weeks, the selective media were replaced every 3 to 4 days. Once the distinct “islands” of surviving cells were visualized, the individual clones were transferred into 96-well plates and continued to maintain cultures in selected media. The positive clones were confirmed by immunoblotting.

**Western Blotting and Immunoprecipitation**—The cells were lysed in lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and 1 mM sodium orthovanadate in the presence of protease inhibitors. The immune complexes were captured with protein A or G-Sepharose, washed in lysis buffer, and resolved by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane, and the membrane was blocked with 5% non-fat milk in TBST containing 0.1% deoxycholate. The membrane was incubated with the above constructs using Transfast transfection reagent (Promega). Forty-eight hours after transfection, cells were plated at several different dilutions in media containing 0.5 mg/ml G418. For the next 2 weeks, the selective media were replaced every 3 to 4 days. Once the distinct “islands” of surviving cells were visualized, the individual clones were transferred into 96-well plates and continued to maintain cultures in selected media. The positive clones were confirmed by immunoprecipitation.

**Luciferase Assay**—The Elk-1 luciferase activity assay was performed using trans-reporting constructs, including PFA-Elk1 and PFR-luciferase plasmids (PathDetect in vivo signal transduction pathway transfection system; Stratagene) according to the manufacturer’s instructions. The Elk-1 luciferase activity was measured using a luciferase assay system (Promega). The results were expressed as mean ± S.D. from three independent experiments.

**Differentiation of PC-12 Cells**—PC-12 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 5% horse serum, and 4.5 Glutamax at 37 ºC under 5% CO2. The cells were plated at a subconfluent density on 12-well culture plates containing laminin to improve neurite attachment activity. The next day, cells were transiently transfected with enhanced green fluorescent protein, wild-type hSef, or the mutants for 36 h using Effectene transfection reagent (Qiagen). Cells were stimulated with or without recombinant human FGFR2 or FGFR3 (R&D Systems) for 72 h and then examined by fluorescence microscopy. Cells with processes longer than 1.5 times the diameter of the cell body were considered to be positive for neurite outgrowth. The numbers of undifferentiated and differentiated cultures were counted in three randomly selected fields containing ~200 cells each. Data were expressed as means ± S.D. of three independent counts.

**RESULTS**

**Cloning and Primary Structure of hSef**—To explore for the existence of additional members of the IL-17 receptor gene family, we screened the National Center for Biotechnology Information expressed sequence tag and NR databases using the cytoplasmic domain of the IL-17 receptor with the tBLASTn and Blastp algorithms. We found several expressed sequence tags encoding an unknown protein reported in Gene Bank as the hypothetical human protein DKFZp434N1928 (accession no. AL133097). However, the translated sequence was only a fragment without an N terminus. We performed 5’-RACE PCR using mRNA from human testis tissue and 293T cells and obtained two complete cDNAs that were 4477 and 4478 bp long. The 4477-bp cDNA predicted an open reading frame of 739 amino acids encoding a novel single transmembrane protein (Fig. 1A), whereas the other cDNA encoded a protein of 595 amino acids that lacked the N-terminal 144 amino acids of the longer protein (Fig. 1B). BLAST analysis revealed that the sequences were identical to the Sef gene of human and mouse (20, 21). However, only the longer protein isoform had previously been reported. We had isolated two isoforms of the gene in both human and mouse (GenBank accession numbers AF494208 & AF494211 and AF494210 & AF494209, respectively), and we adopt the name Sef for the long form and Sef-S for the short form in this article. hSef was mapped on human chromosome 3p21.1 with 13 exons and spanned 70,903 base pairs. hSef-S consisted of 14 exons and spanned 85,485 base pairs (Fig. 1C). mSef or mSef-S was located on chromosome 14 with 13 or 12 exons, respectively, and spanned 6,6304 and 13,726 base pairs, respectively. mSef and mSef-S encoded proteins with 738 and 594 amino acids, respectively. hSef is 75% identical to mSef at the amino acid level, whereas hSef-S and mSef-S shared 72% identity.

Computer-assisted analysis suggested that hSef contained a putative signal peptide of 16 amino acids, a 281-amino acid extracellular domain (Cy7–Pron297), a 23-amino acid transmembrane stretch (Ile298–Met320), and a 420-amino acid cytoplasmic tail (Cys323–Leu739). This protein was predicted to be a type I single span transmembrane molecule according to Hartmann membrane topology model and PSORT II server. There were eight cysteine residues and nine potential N-linked glycosylation sites in the extracellular domain, where an immunoglobulin domain and a fibronectin III domain were also predicted. Furthermore, a highly conserved segment (TPPPLRPRKVW) located proximal to the IL-17 receptor transmembrane domain was replaced by the proline-rich segment (PFHPPPLRYREP), a putative SH3 interaction domain, which is a typical feature of transactivation domains for transcription factors. In addition, a putative TIR domain (Val155–Lys254) (Toll/IL-1 Receptor domain) (30) and a putative TRAF6 binding motif (Pro347–Leu361), Pro-X-Glu-X-X (aromatic/acidic residue) (31), were predicted in the intracellular part of hSef.

**Sef Interacted with FGFR**—It has been reported that FGFRs were highly expressed in kidney tissue (32), where hSef was also abundant (data not shown). Based on the suggestion that zSef and mSef might interact with FGFRs (22), we reasoned that hSef could interact with FGFRs and possibly affect FGF signaling. To detect whether a physical interaction of the two receptors occurred, we carried out a co-immunoprecipitation assay by co-expression of the two proteins in COS-7 cells. We successfully precipitated the FGFR1 (Fig. 2A) and FGFR2 (Fig. 2B) using anti-Sef serum, but failed to precipitate FGFR3 (data not shown), suggesting that Sef specifically interacted with FGFR1 and FGFR2 in intact cells. These results are consistent with the report of interactions between zebrafish Sef with Xenopus laevis FGFR1 or FGFR2 (21). It also implied that hSef might elicit an effect on FGF signaling similar to that of Sprad and Sprouty family members, which strongly inhibit FGF signaling (13, 14, 33, 34).

To further examine whether co-expression of the two recep-
tors occurred in mammalian cells, we carried out the immuno-

staining assay with the anti-FGFR2 antibody and anti-hSef

serum. The results demonstrated that overexpressed hSef and

FGFR2 were co-localized in COS-7 cells (Fig. 2C). Interestingly,

we found that hSef was co-localized with FGFR1 in normal

human testis (Fig. 5D), whereas we failed to observe obvious

co-expression of the two proteins in other tissues (data not

shown). Taken together, these data suggested that hSef inter-

acted with FGFR1 and FGFR2 under at least some physiolog-

ical condition.

hSef Strongly Inhibited FGF2 or NGF-induced PC-12 Cell

Differentiation—Although hSef is able to interact with FGFR1

and FGFR2, the biological function of this protein in mamma-

lian cells remains unclear. Next, we examined whether hSef

could affect FGF signaling. We stably expressed hSef in PC-12

cells, a rat pheochromocytoma cell line that could be induced



Fig. 2. Interaction of hSef with mFGFR. A, co-immunoprecipitation of hSef and mFGFR1. COS-7 cells were transiently transfected with

mFGFR1 and hSef constructs as indicated. Whole-cell lysates were immunoprecipitated (IP) with hSef rabbit anti-serum. After electrophoresis,

blots were probed with rabbit polyclonal antibody to mFGFR1(Flg). The expressions of mFGFR1 and hSef were detected by rabbit polyclonal

antibody to mFGFR1 and rabbit polyclonal antibody to hSef using the whole transfected cell lysates. B, co-immunoprecipitation of hSef and

mFGFR2. COS-7 cells were transiently transfected with mFGFR2 and hSef constructs as indicated. The procedure was the same as in A. C,

colocalization of hSef with mFGFR2 in transfected COS-7 cells. COS-7 cells were cotransfected with mFGFR2 and hSef constructs using Effectene

transfection reagent (Qiagen). hSef and mFGFR2 were stained with secondary antibodies conjugated with fluorescein isothiocyanate (green) and

Cy3 (red), respectively. The co-localization of the two proteins is shown as a merged figure. D, endogenous colocalization of Sef with FGFR1 in some

tissues. A triple immunofluorescence staining and confocal analyses for the endogenous colocalization of Sef with FGFR1 in normal human testis

was performed according to the manufacturer’s instructions. Sef staining was performed using rabbit polyclonal anti-Sef antibody followed by

fluorescein isothiocyanate-labeled goat anti-rabbit IgG. FGFR1 staining was performed using mouse anti-FGFR1 antibody followed by Texas-

Red-labeled goat anti-mouse IgG. Nuclear staining was performed using DAPI. One obvious co-localization of Sef and FGFR1 in testis

was observed.
FIG. 3. Effects of hsEf on differentiation of PC-12 cells. A, hsEf inhibits FGF2 and NGF induced PC-12 cell differentiation. Mock-transfected or Sef stably transfected PC-12 clones were screened in selected medium containing 0.5 mg/ml G418. The G418-resistant clones expressing hsEf or empty vector were transiently transfected with enhanced green fluorescent protein plasmid. The PC-12 cells were starved with serum-free...
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into sympathetic neuron-like cells possessing elongated neuritis by basic fibroblast growth factor (FGF-2) or NGF. As showed in Fig. 3A, mock-transfected PC-12 clones were induced into neurite outgrowth in the presence of FGF-2 or NGF as seen in normal PC-12 cells. In contrast, all clones transfected with hSef, remained undifferentiated in response to either FGF2 or NGF, suggesting that hSef elicited an inhibitory effect on the differentiation of PC-12 cells (Fig. 3A). The percentages of cells with neurite outgrowth (differentiated cells) were significantly decreased for cells stably expressing hSef at both dosages of FGF2 or NGF (Fig. 3B). When cells were exposed to FGF2 or NGF for longer periods, the percentage of mock-transfected clones that were induced into neurite outgrowth increased significantly, whereas clones transfected with hSef resisted differentiation in the presence of FGF2 (Fig. 3C) or NGF (Fig. 3D). These results indicate that hSef could significantly inhibit the differentiation of PC-12 cells triggered by FGF2 or NGF, even in conditions of higher dosage or prolonged exposure of growth factor.

To determine which domain of hSef was necessary for inhibition of PC-12 cell differentiation induced by FGF2, we constructed the N-terminal truncated mutant hSefΔ N), which lacks the N-terminal extracellular domain of hSef, the C-terminal truncated mutant hSefΔ C), which lacks C-terminal intracellular domain of hSef, and a mutant hSef(DN), which lacks a motif (Glu327–Leu333) in the intracellular domain containing a putative tyrosine phosphorylation site. We overexpressed these mutants with enhanced green fluorescent protein in PC-12 cells in the presence or absence of FGF2. In control cells and overexpressing hSefΔ C) cells, FGF2 strongly induced cell differentiation. However, in the cells overexpressing hSefWT, hSefΔ N), and hSef(DN), FGF2 failed to induce significant cell differentiation (Fig. 3, E and F), suggesting that the N-terminal-truncated mutant and the (Δ Glu327–Leu333) mutant missing the seven amino acid putative tyrosine phosphorylation site did not affect the inhibitory properties of Sef on FGF2-induced PC-12 cell differentiation. These data indicated that the intracellular domain plays a critical role in the inhibition of PC-12 cell differentiation induced by FGF2. The results also suggest that hSef(DN), which lacks a tyrosine phosphorylation motif (Glu327–Leu333) in the intracellular domain of hSef, does not function as a dominant negative form.

hSef Inhibited Ras-MAPK Signaling Pathway—It has been reported that Ras-MAPK signaling is required for FGF2-induced PC-12 cell differentiation. To investigate the role of hSef in MAPK activation during FGF2-induced PC-12 cell differentiation, we first determined the effects of Sef on Elk-1 mediated luciferase activity. Data showed that overexpression of hSef significantly suppressed FGF2-induced Elk-1 luciferase activity in PC-12 cells (Fig. 4A), which was comparable with the effect of Sprouty4 (14, 33, 34). Compared with hSef, the C-terminal truncated mutant hSefΔ C) and the N-terminal truncated mutant hSefΔ N) had about 23 and 81% of the inhibitory effect, respectively (Fig. 4A, columns 4 and 5). This result was also correlated with the inhibitory effect of hSef on the FGF2-induced PC-12 cell differentiation. Furthermore, our results showed both hSefWT and hSefΔ N) suppressed FGF2-dependent Elk-1 luciferase activity in PC-12 cells in a dose-dependent manner (Fig. 4, B and C).

Earlier studies had shown that activation of ERK1/2 was important for neurite outgrowth in PC-12 cells and that ERK1/2 phosphorylation was strongly but transiently induced by FGF2, with the level of phosphorylation reaching a maximum within 5–10 min and then declining to lower sustained levels (35, 36). With this in mind, we examined whether hSef suppressed endogenous ERK1/2 activation induced by FGF2 in PC-12 cells. As shown in Fig. 4D, overexpression of hSef significantly suppressed endogenous ERK phosphorylation, with a maximum inhibition at 10 min after stimulation. In addition, hSef exhibited an inhibitory effect on ERK activation in a dose-dependent manner (Fig. 4E). These results indicate that hSef could inhibit FGF2-induced PC-12 cell differentiation, possibly through the inhibition of Ras-MAPK signaling.

hSef Inhibited Ras-MAPK Signaling, Possibly by Targeting the Upstream Molecules of Ras—To attempt to identify the signaling component of Ras-MAPK pathway in FGF2-induced PC-12 differentiation that is suppressed by Sef, we used the constitutively active Ras(G12V) or active MEK(MEK1RF) molecules to examine ERK activation and Elk-1 luciferase activity in both PC-12 cells and 293T cells. Both luciferase assays and Western blotting analysis results showed that hSef had no inhibitory effect on the signaling mediated by constitutively active MEK (Fig. 5, A–C) or Ras (Fig. 5, D–F) in both PC-12 and 293T cells. These data suggest that the target signaling molecule for Sef was located upstream of Ras in the FGF2-Ras-MAPK signaling pathway at least in PC-12 and 293T cells.

DISCUSSION

In our attempt to identify novel receptors in the IL-17 family (37–39), we screened GenBank using the intracellular domain of human IL-17AR and found a gene encoding novel single-span transmembrane proteins. Our results show that there are at least two isoforms of this gene in mouse and human. The long form of the sequence was identical to Sef (26), and we adopted the name of SefS for the short form in this article. The specific functions of this gene were unclear. In this report, we provided evidence that hSef has an inhibitory effect on the PC-12 differentiation induced by FGF2 or NGF, zSef and mSef, zebrafish and mouse orthologues of the Sef gene, have recently been reported to be novel modulators of FGF signaling, and zSef might regulate Ras/MAPK signaling triggered by FGF during early zebrafish embryonic development (20, 21). Our data demonstrate that hSef interact and colocalize with FGRFs in some human tissues. This interaction was confirmed by immunoprecipitation assays, which showed that hSef interacted with FGRF1 and FGRF2 but not FGRF3.

We examined the effect of Sef on growth factor-induced neurite outgrowth of PC-12 pheochromocytoma cells, which is de-
pendent upon the Ras-MAPK signaling pathway. Our results show that hSef significantly suppresses FGF2- or NGF-induced PC-12 cell differentiation even in conditions of higher growth factor dosage or elongated exposure. The cytoplasmic domain of hSef is critical for the inhibition of PC-12 cell differentiation induced by FGF-2. We further investigated the molecular mechanism on the effect of Sef on the inhibition of PC-12 cell differentiation. Our results show that the inhibitory effect of

**Fig. 4. Effects of hSef on Ras-MAPK signal pathway during FGF2-induced differentiation of PC-12 cells.**

*Panel A*: inhibitory effects of hSef on Ras-MAPK signal pathway. The Elk-1 luciferase activity assay was performed using trans-reporting constructs, including PPA-Elk-1 and PFR-luciferase plasmids (PathDetect in vivo signal transduction pathway trans-reporting system; Stratagene) according to the manufacturer’s instructions. Equal amounts of the indicated plasmids were transiently co-transfected into PC-12 cells with Elk-1 reporter plasmids by Transfast transfection reagent (Promega). 24 h later, cells were starved with serum-free medium for another 24 h followed by stimulation with 20 ng/ml FGF2 for 6 h as indicated, and then lysed. The Elk-1 luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). Data were normalized by co-transfection with *Renilla reniformis* luciferase reporter vector and expressed as the mean ± S.D. (*n* = 3).

*Panel B*: dose-dependent effects of hSef(WT) and N-terminal-truncated hSef (ΔN) on the Ras-MAPK signal pathway. Luciferase assays were carried out as described above. Data were expressed as the mean ± S.D. (*n* = 3).

*Panel D*: hSef and the equal amount of empty vector were transfected into PC-12 cells for 24 h. After serum starvation for another 24 h, cells were treated with (+) or without (−) 20 ng/ml FGF2 for the indicated times. The endogenous ERK1/2 activation was detected by immunoblotting with anti-p-ERK antibody (pp44 and pp42 form; Santa Cruz Biotechnology). The blots were stripped and re-probed with anti-ERK antibody (pp44 and pp42 form) to verify equal loading. Similar results were obtained in two independent experiments.

*Panel E*: the increasing amounts of hSef plasmid were transiently transfected into PC-12 cells using Transfast transfection reagent (Promega). After serum starvation for 24 h, cells were treated with (+) or without (−) 20 ng/ml FGF2 for 10 min. Whole-cell lysates were transferred to nitrocellulose membrane. The endogenous ERK1/2 activation was detected with anti-p-ERK antibody (Santa Cruz). The increasing expression of hSef was detected with the specific anti-serum of hSef.
**FIG. 5.** hSef interferes with Ras-MAPK signaling by acting on the upstream molecules of Ras. Constitutively active MEK (MEK1RF) (A–C) or constitutively active Ras (Ras G12V) (D–F) constructs were transiently co-transfected into PC-12 cells or 293T cells with Elk-1 luciferase reporter plasmid or increasing amounts of hSef plasmid for 36 h. The luciferase activity was measured as described above. Data were normalized by co-transfection with *R. reniformis* luciferase reporter vector and expressed as the mean ± S.D. (n = 3). Additionally, ERK activation was detected by immunoblotting. The whole lysates of transfected cells with active MEK C, or active Ras F, were immunoblotted with anti-p-ERK, anti-ERK, and anti-Sef rabbit polyclonal serum, respectively.
overexpressed hSef on FGF2-induced PC-12 cell differentiation was significantly correlated with the role of hSef on the prevention of Ras-MAPK activation. Interestingly, two reports showed that zSef was expressed in mid-brain–hindbrain boundary, hindbrain, and forebrain (20, 21). Another group using in situ hybridization demonstrated expression of mSef in the forebrain, mid-brain–hindbrain boundary region and branchial arches in the mouse (25). Recently, a report demonstrated that hSef was detectable in human brain (26), which is consistent with our Northern blot analysis (data not shown). All of those data suggested that the Sef gene is expressed in the brain. In this article, we adopted PC-12 cells as a model to demonstrate that hSef inhibited cell differentiation induced by FGF or NGF. Therefore, we speculate that Sef might play a role in developmental processes in the nervous system.

Opposing results on the point of action of Sef on FGFR-Ras-MAPK signaling have been reported. One group has suggested that Sef acts at the FGFR level (21), whereas another has demonstrated that Sef functions downstream of Ras (20). Like Sprouty, the specific target molecule of Sef interfering with Ras-MAPK signaling is unclear. Our results demonstrated that Sef functions downstream of Ras (20). Like Sprouty, the specific target molecule of Sef interfering with Ras-MAPK activation, possiblyfunctioning upstream of Ras, possibly at the FGFR. We have succeeded in detecting colocalization of endogenous Sef with FGFR1 in the testis and other tissues (data not shown). Sef might also function through other pathways if it can interact with other indirect signaling molecules in FGF signaling. Thus, the detailed molecular mechanism of the physiological regulation of Sef on FGFR signaling remains to be determined.

Taken together, our results show that Sef is an interesting and important molecule that significantly inhibits FGF2-induced PC-12 cell differentiation, probably through prevention of Ras/MAPK signaling. However, the precise functions of Sef in vivo and its splicing isoform remain unclear. The next critical goal is to disrupt the locus in mice for clarifying functions in vivo.

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