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Cellular Signalling 19 (2007) 93 – 102
CELLULAR SIGNALLING
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Sef-S, an alternative splice isoform of *sef* gene, inhibits NIH3T3 cell proliferation via a mitogen-activated protein kinases p42 and p44 (ERK1/2)-independent mechanism

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Received 10 May 2006; accepted 29 May 2006
Available online 8 June 2006

Abstract

sef (similar expression to *fgf* genes) was recently identified as a negative regulator of fibroblast growth factor (FGF) signaling in zebrafish, chicken, mouse and human. By repressing events upstream and/or downstream Ras, Sef inhibits FGF-induced ERK activation and cell proliferation. Here we report that Sef-S, an alternative splice isoform of Sef, lacks a signal peptide and is localized in cytosol. Sef-S inhibits FGF-induced NIH3T3 cell proliferation, a similar function to Sef. However, Sef-S represses neither the intensity nor the duration of ERK activation. Moreover, Sef-S does not inhibit Elk1-dependent transcription. Our study revealed that the signal peptide is critical for the different activities between Sef and Sef-S in FGF-Ras-MAPK signaling cascades. Furthermore, we observed that Sef-S associated with FGFR2 in a co-immunoprecipitated complex. These results indicate that Sef-S inhibits FGF-induced NIH3T3 cell proliferation via an ERK-independent mechanism and therefore suggest that alternative splice licenses *sef* gene to inhibit cell proliferation via multiple signaling pathways.

Keywords: Sef-S; Sef; Alternative splice; FGF signaling; ERK; Proliferation

1. Introduction

Growth factor signaling by receptor tyrosine kinases (RTKs) triggers a spectrum of responses in target cells, such as proliferation, differentiation, migration and survival [1–3]. Dysregulation of RTK signaling is causally linked to a variety of diseases, including cancer and other genetic disorders, implying that this signaling must be precisely and tightly regulated spatially and temporally to ensure a physiologically appropriate outcome [1–4]. Among the regulation mechanisms, the negative feedback loop is a classical model that RTK signaling can be attenuated effectively [5,6].

Recent studies have identified several feedback repressors for Ras-MAPK signaling, including members of Sprouty and SPRED (Sprouty-related protein with EVH-1 domain) families [7,8]. Most recently, *sef* (similar expression to *fgf* genes) was identified in zebrafish as a feedback inhibitor of Ras-MAPK-mediated FGF signaling [9,10]. Later, *sef* genes isolated from mouse, human and chicken were found to have similar function [9–14]. These studies indicate that Sef is a conserved inhibitor of FGF signaling in vertebrates, although the detailed mechanisms of the inhibitory role remains quite controversial [9–21]. Based on the amino acid sequence, Sef was predicted to contain a putative signal peptide and a putative transmembrane domain. Therefore, Sef is believed to be a transmembrane protein [9–14,16,17,19,20], and this has been confirmed by the immunostaining results in COS7, Hela and HEK293 cells [19,20]. During embryogenesis of zebrafish, chicken and mouse, *sef* expression was observed to be regulated by FGF signaling and the expression pattern is highly restricted to the locations where *fgf* (*fgf3*, *fgf8*, and *fgf17)* and *sprouty* (*sprouty2* and *sprouty4*) genes are expressed [9–12,16]. Loss of Sef expression was found to be associated with high grade and metastatic prostate cancer [22], in which FGF signaling has been implicated in carcinogenesis. Sef was

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doi:10.1016/j.cellsig.2006.05.033

demonstrated to inhibit FGF signaling [9,10,12,13] upstream Ras or at the receptor level [10,13,14,17,23], possibly by inhibiting FGF-induced phosphorylation of FGF receptors [17]. In contrast, Sef has also been reported to act as a repressor downstream Ras [9,14,20], probably by blocking nuclear translocation of activated ERK [20]. It was also reported that mSef inhibited FGF-induced activation of protein kinase B (PKB/Akt), a key protein in the phosphatidylinositol 3-kinase (PI3-kinase) pathway [17] and that mSef interacted with TAK1 and mediated JNK activation and apoptosis of 293T cells [24]. In addition, an alternative splice isoform of human *sef*, termed hSef-b, has been identified as a cytosolic protein that associates with FGFR1 and inhibits ERK activation [19]. The above studies demonstrated a critical function of Sef in the regulation of FGF signaling, however, *sef* homozygous mutant mice did not show any obvious abnormal phenotype [25].

Almost all the previous studies on Sef were focused on growth factor induced Ras-MAPK signaling cascades and established the correlation between fibroblast proliferation suppression and ERK signaling inhibition. However, it remains unclear whether the other isoform of Sef functions in the same way. In this report, we identified an alternative splice isoform of *sef* gene, Sef-S, which has no signal peptide and is a cytosolic protein. We observed that hSef-S inhibited FGF-induced NIH3T3 cell proliferation via an ERK-independent mechanism, which is quite different from Sef. These findings indicate that *sef* gene is able to inhibit cell proliferation via ERK-dependent and -independent pathways.

2. Materials and methods

2.1. Reagents

Recombinant human basic FGF was purchased from R and D systems. Polyclonal anti-ERK1 (K-23), monoclonal anti-p-ERK (E-4), monoclonal anti-c-Myc (9E10) and monoclonal anti-Bek (FGFR2, C-8) antibodies were from Santa Cruz Biotechnology.

2.2. Plasmid construction

pcDNA3.1/Myc-His/hSef and pcDNA3/6×Myc/hSef-S have been described previously [13]. pcDNA3.1/Myc-His/hSef-S, pcDNA3.1/Myc-His/hSef-b148 and pcDNA3.1/Myc-His/hSef-b187 were constructed by inserting the fragments, indicated in Fig. 1B, and amplified from polymerase chain reaction (PCR), into

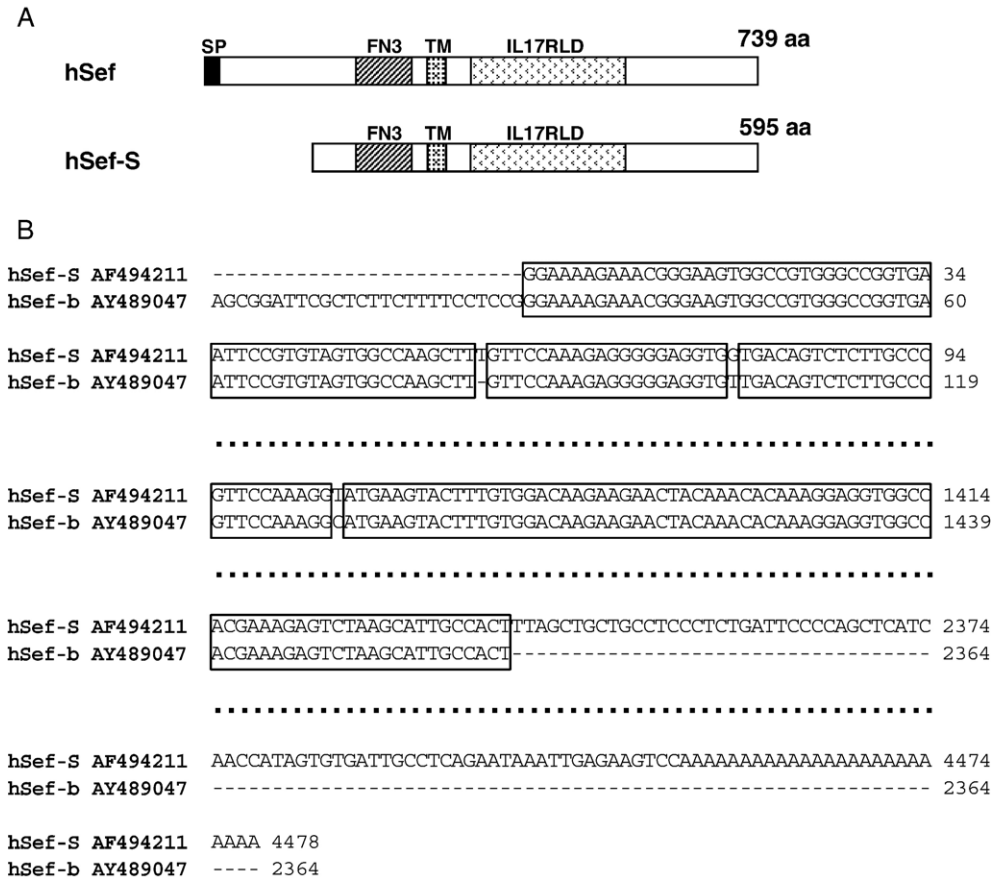

Fig. 1. Sef-S is an alternative splice isoform of *sef* gene lacking a signal peptide. A. Structure of hSef and hSef-S. Shown is the number of amino acids (right). SP, signal peptide; FN3, Fibronectin type III-like domain; TM, transmembrane domain; IL17RLD, IL17 receptor-like domain. B. cDNA sequence alignment between hSef-S and hSef-b. The identical nucleotides are boxed. C. Sequences information flanking the AUG codons of hSef-S and mSef-S. The deduced amino acid sequences are presented under the nucleotide sequences. Arrows indicate the start sites of four constructs, hSef-b148, hSef-b187, hSef-S and mSef-S. Kozak sequences are underlined. D. In vitro translation of hSef-S. The assay was performed as described in the Materials and methods section. Products were analyzed with SDS-PAGE and visualized by phosphoimaging. E. Expression of hSef-S in mammalian cells. 293T cells were transiently transfected with the indicated plasmids for 24 h. Then, the whole cell lysates were subjected to SDS-PAGE and immunoblotted with Myc antibody. F. Subcellular localization of hSef and hSef-S. COS7 cells were transfected with myc-tagged hSef (a) or hSef-S (b). After 36 h, the cells were fixed and stained with Myc antibody. Nuclei were counterstained with DAPI.